The Diagnostic Use of Metabolomics for the Identification of Secondary Infections in Critical Coronavirus Disease 2019

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Abstract

Background

Critically ill patients with coronavirus disease 2019 are at high risk of developing secondary infections, which pose a challenge to identify clinically. Empirical antibiotic usage in this group is therefore high. Identification of novel biomarkers of secondary infections would minimise unnecessary antibiotic usage while ensuring that patients with secondary infections receive appropriate antibiotics as early as possible. This project aimed to investigate whether metabolomics could produce a panel of biomarkers capable of distinguishing critically ill coronavirus disease 2019 patients with and without secondary infections.

Methods

Blood samples were collected from patients in critical care with coronavirus disease 2019, along with a group of healthy volunteer controls. Using high performance liquid chromatography-mass spectrometry, metabolites which showed significant differences in abundance between patients with and without secondary infections were identified. A panel of metabolites capable of distinguishing Gram positive and negative infections was also explored.

Results

A total of 105 patients were recruited to the study, of whom 40 developed a secondary infection during the trial period. The metabolites creatine and 2-hydroxyisovalerylcarnitine were significantly increased in patients with secondary infections, while S-methyl-L-cysteine was significantly reduced. This metabolite panel demonstrated good diagnostic performance with an AUROC of 0.83. The panel of metabolites distinguishing Gram positive and negative infections consisted of betaine, N(6)-methyllysine and four phosphatidylcholines. This panel performed with high accuracy, with an AUROC of 0.88.

Conclusion

Metabolomic profiling may be used to identify biomarkers of secondary infections in critically ill coronavirus disease 2019 patients. Investigation of biomarkers for secondary infections in other critical illnesses should be explored.

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Author's Declaration

I declare that the work outlined in this thesis was performed by me while working as a Clinical Research Fellow in the West of Scotland Academic Unit of Anaesthesia, Critical Care and Perioperative Medicine between, and as a trainee in Anaesthesia and Intensive Care Medicine in the West of Scotland between August 2020 and August 2024. Protocol amendments and application to the ethics committee were carried out by Professor Malcolm Sim, Dr Malcolm Watson and myself. The vast majority of patient screening, recruitment, sample collection and data collection was performed by me, with assistance from local research teams as acknowledged. Metabolite extraction was performed entirely by me in the Roe lab. Metabolomic analyses were performed by Dr Clement Regnault and Dr Gavin Blackburn of the University of Glasgow Polyomics Facility. Statistical analysis and production of machine learning models was performed by Dr Rónán Daly, head of Artificial Intelligence and Machine Learning at the College of Medical, Veterinary and Life Sciences at the University of Glasgow Shared Research Facilities. All other work on this project and writing of this thesis was carried out by me. No part of this thesis has been submitted for any other degree at The University of Glasgow or any other institution.

Dr Gordan McCreath

Research Output

The activity from this research project has produced the following output:

Publications:

McCreath, G., Whitfield, P.D., Roe, A.J. *et al*. A Metabolomics approach for the diagnosis Of SecondAry InfeCtions in COVID-19 (MOSAIC): a study protocol. *BMC Infect Dis* **21**, 1204 (2021). https://doi.org/10.1186/s12879-021-06832-y

Pending Publications:

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Ralston, M.R., **McCreath, G.**, Lees, Z.J., Salt, I.P., Sim, M.A.B., Watson, M.J., Freeman, D.J. Beyond BMI: Exploring the Role of Visceral Adipose Tissue in Intensive Care Unit Outcomes. Submitted to BJA Open

Presentations:

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Abbreviations

1-9	2019-nCoV:	2019 novel coronavirus
A	AAA:	Aromatic amino acid
	ACE:	Angiotensin converting enzyme
	A. *baumannii*:	A*cinetobacter baumannii*
	ACP:	Acyl carrier protein
	ADP:	Adenosine diphosphate
	AGAT:	Arginine:glycine amidinotransferase
	AKI:	Acute kidney injury
	ALI:	Acute lung injury
	ALT:	Alanine aminotransferase
	Ang:	Angiotensin
	AP-1:	Activator protein-1
	APACHE II:	Acute Physiology and Chronic Health Evaluation II
	APC:	Activated protein C
	ARDS:	Acute respiratory distress syndrome
	ARG:	Arginase
	ASL:	Argininosuccinate lyase
	ASS:	Argininosuccinate synthetase
	AST:	Aspartate transaminase
	AT_1:	Angiotensin II receptor 1
	ATP:	Adenosine triphosphate
	AUROC:	Area under the receiver operating characteristic curve
B	BAL:	Broncho-alveolar lavage
	BCAA:	Branched chain amino acid
	BMI:	Body mass index
	BSI:	Bloodstream infection
C	CAP:	Community acquired pneumonia
	Caret:	Classification and regression training
	CD:	Cluster of differentiation
	CK:	Creatine kinase
	CL:	Cardiolipin
	CLABSI:	Central line associated bloodstream infection

	CLR:	C-type lectin receptor
	COPD:	Chronic obstructive pulmonary disease
	CoV:	Coronavirus
	COVID-19:	Coronavirus disease 19
	CPS:	Carbamoyl phosphate synthetase
	CRP:	C-reactive protein
	CSF:	Cerebrospinal fluid
D	DAG:	diacylglycerol
	DAMP:	Damage associated molecular pattern
	DC:	Dendritic cell
	DHEA:	Dehydroepiandrosterone
	DIC:	Disseminated intravascular coagulation
	DNA:	Deoxyribonucleic acid
E	E:	Small envelope
	ECMO:	Extracorporeal membrane oxygenation
	E. coli:	*Escherichia coli*
	ESBL:	Extended-spectrum beta-lactamase
	ETC:	Electron transport chain
F	$FADH_2$:	Flavin adenine dinucleotide
	FID:	Free induction delay
	FiO_2:	Fraction of inspired oxygen
G	GAMT:	Guanidinoacetate N-methyltransferase
	GC:	Gas chromatography
	GCS:	Glasgow coma scale
	GC/MS:	Gas chromatography/mass spectrometry
	GI:	Gastrointestinal
	GLUT:	Glucose transporter
	GM-CSF:	Granulocyte-macrophage colony-stimulating factor
	GPL:	Glycerophospholipid
	GSH:	Reduced glutathione
	GSSG:	Oxidised glutathione
H	*H. influenzae*:	*Haemophilus influenzae*
	HCD:	Higher-energy collisional dissociation

	HCoV:	Human coronavirus
	HDL:	High-density lipoprotein
	HDU:	High dependency unit
	HE:	Hemagglutininesterase
	$HFNO_2$:	High flow nasal oxygen
	HIF-1α:	Hypoxia-inducible factor-1α
	HILIC:	Hydrophilic interaction chromatography
	HIV:	Human immunodeficiency virus
	HMDB:	Human metabolomics database
	HMGB-1:	High-mobility group box-1
	HMG-CoA:	ß-Hydroxy ß-methylglutaryl-CoA
	HPLC:	High performance liquid chromatography
	HPLC/MS:	High performance liquid chromatography/mass spectrometry
	HSP:	Heat shock protein
I	ICAM-1:	Intercellular adhesion molecule 1
	ICU:	Intensive care unit
	IDEOM:	Identification Of Metabolites
	IDO:	Indoleamine 2,3-dioxygenase
	IFN:	Interferon
	IL:	Interleukin
	IL-1RA:	Interleukin-1 receptor antagonist
	IMV:	Invasive mechanical ventilation
	iNOS:	Inducible nitric oxide synthase
	IPA:	Invasive pulmonary aspergillosis
	IQR:	Interquartile range
	IRF:	Interferon regulatory factor
	ISARIC:	International Severe Acute Respiratory and Emerging Infections Consortium
J	JAK:	Janus kinase
K	KEGG:	Kyoto Encyclopaedia of Genes and Genomes
	K. pneumoniae:	*Klebsiella pneumoniae*
L	LC:	Liquid chromatography
	LDL:	Low-density lipoprotein

	LOS:	Length of stay
	LPC:	Lysophosphatidylcholine
	LPS:	Lipopolysaccharide
	LRR:	Leucine-rich repeat
	LTA:	Lipoteichoic acid
M	M:	Membrane
	MAC:	Membrane attack complex
	MAP:	Mean arterial pressure
	MCP-1:	Monocyte chemoattractant protein 1
	MDA5:	Melanoma differentiation-associated protein 5
	MERS:	Middle East respiratory syndrome
	MERS-CoV:	Middle East respiratory syndrome–related coronavirus
	MIF:	Macrophage inhibitory factor
	MODS:	Multiple organ dysfunction syndrome
	MRSA:	Methicillin-resistant *Staphylococcus aureus*
	MS:	Mass spectrometry
	mTOR:	Mammalian target of rapamycin
	m/*z*:	Mass to charge ratio
N	N:	Nucleocapsid
	NAD^+:	Oxidised nicotinamide adenine dinucleotide
	NADH:	Reduced nicotinamide adenine dinucleotide
	NET:	Neutrophil extracellular trap
	NF-κB:	Nuclear factor-κB
	NIV:	Non-invasive ventilation
	NK:	Natural killer
	NLR:	Nucleotide oligomerisation domain-like receptor
	NLRP3:	NOD-, LRR- and pyrin domain-containing protein 3
	NMDA:	N-methyl-D-aspartate
	NMR:	Nuclear magnetic resonance
	NO:	Nitric oxide
	NOD:	Nucleotide oligomerisation domain
	NRF2:	Nuclear factor erythroid 2-related factor 2
	NSP:	Non-structural protein

O	O_2^-	Superoxide
	$ONOO^-$	Peroxynitrite
	OR	Odds ratio
	OTC:	Ornithine transcarbamylase
	OXPHOS:	Oxidative phosphorylation
P	$PaCO_2$:	Partial pressure of carbon dioxide
	P. aeruginosa:	*Pseudomonas aeruginosa*
	PAI-1:	Plasminogen-activator inhibitor type-1
	PAMP:	Pathogen associated molecular pattern
	PaO_2:	Partial pressure of oxygen
	PC:	Phosphatidylcholine
	PC1:	Principal component 1
	PC2:	Principal component 2
	PCA:	Principal component analysis
	PCT:	Procalcitonin
	PD-1:	Programmed death receptor-1
	PE:	Phosphatidylethanolamines
	PEEP:	Positive end-expiratory pressure
	PepG:	Peptidoglycan
	PG:	Phosphatidylglycerol
	PHEIC:	Public health emergency of international concern
	P. mirabilis	*Proteus mirabilis*
	PPAR-α:	Peroxisome-proliferator activated receptor alpha
	PPE:	Personal protective equipment
	PRR:	Pattern recognition receptor
	PS:	Phosphatidylserine
Q	qSOFA:	Quick sequential organ failure assessment
R	RAAS:	Renin-angiotensin-aldosterone system
	RECOVERY:	Randomized Evaluation of COVID-19 Therapy
	REDCap:	Research Electronic Data Capture
	REMAP-CAP:	Randomised, Embedded, Multi-factorial, Adaptive Platform Trial for Community-Acquired Pneumonia
	RF:	Radiofrequency
	RHD:	Rel homology domain

	RIG-1:	Retinoic acid-inducible gene-1
	RLR:	Retinoic acid-inducible gene-1-like receptor
	RNA:	Ribonucleic acid
	RNP:	Ribonucleoprotein
	RNS:	Reactive nitrogen species
	ROS:	Reactive oxygen species
	RT-PCR:	Reverse-transcription polymerase chain reaction
S	S:	Spike
	SAA:	Sulphur containing amino acid
	SAH:	S-adenosylhomocysteine
	SAM:	S-adenosylmethionine
	SARS:	Severe acute respiratory syndrome
	SARS-CoV-1:	Severe acute respiratory syndrome coronavirus 1
	SARS-CoV-2:	Severe acute respiratory syndrome coronavirus 2
	S. aureus:	*Staphylococcus aureus*
	SE:	Standard error
	SIRS:	Systemic inflammatory response syndrome
	SM:	Sphingomyelin
	S. maltophilia:	*Stenotrophomonas maltophilia*
	sn:	Stereochemical numbering
	SOFA:	Sequential organ failure assessment
	SpO_2:	Oxygen saturation
	S. pneumoniae:	*Streptococcus pneumoniae*
	STAT:	Signal transducer and activator of transcription
	sTREM-1:	Soluble triggering receptor expressed on myeloid cells-1
	suPAR:	Soluble urokinase-type plasminogen activator receptor
T	TAG:	Triacylglycerol
	TCA:	Tricarboxylic acid
	TGF-ß:	Transforming growth factor ß
	Th:	T Helper
	TIR:	Toll-IL-1 receptor
	TLR:	Toll-like receptor
	TMPRSS2:	Transmembrane protease, serine 2

	TNF-α:	Tumour necrosis factor alpha
	t-PA:	Tissue plasminogen activator
	T_{reg}:	Regulatory T cell
	TREM-1:	Triggering receptor expressed on myeloid cells-1
U	u-PA:	Urokinase plasminogen activator
	uPAR:	Urokinase-type plasminogen activator receptor
	UTI:	Urinary tract infection
V	VAP:	Ventilator associated pneumonia
	VCAM-1:	Vascular adhesion molecule 1
	VEGF:	Vascular endothelial growth factor
	VILI:	Ventilator-induced lung injury
	vWF:	Von Willebrand factor
W	WCC:	White cell count
	WHO:	World Health Organization
X	XCMS:	eXtensible Computational Mass Spectrometry
Z	ZIC-pHILIC:	Zwitterionic polymeric hydrophilic interaction chromatography

1 Coronaviruses

1.1 Introduction

Coronaviruses (CoVs) are a group of viruses first discovered in America in 1931 as an acute, fatal disease of the respiratory tract in baby chicks (1). As well as avian hosts, CoVs are known to cause respiratory and gastrointestinal illnesses in a wide range of mammals including cows, mice, cats, pigs, camels, bats and civets (2–7). Until recently there had been little research interest in this group of viruses, as the only known human pathogens caused symptoms of the common cold and did not cause severe disease (8).

1.1.1 Etymology and Taxonomy

CoVs belong to the *Nidovirales* order of viruses: a collection of enveloped positive-strand ribonucleic acid (RNA) viruses (9). The taxonomy of *Nidovirales* is depicted in *Figure 1-1*. The *Nidovirales* order is divided into four families: *Coronaviridae*, *Arteriviridae, Roniviridae and Mesoniviridae*. The *Coronaviridae* family consists of two subfamilies: *Coronavirinae* and *Torovirinae* (10).

The genus *Coronavirus* belongs to the *Coronavirinae* subfamily, and comprises four phylogenetic groups: Alpha, Beta, Gamma and Delta. Additionally, there are four separate lineages (A, B, C, and D) within the Beta-CoV genus, which vary by their accessory genes (11).

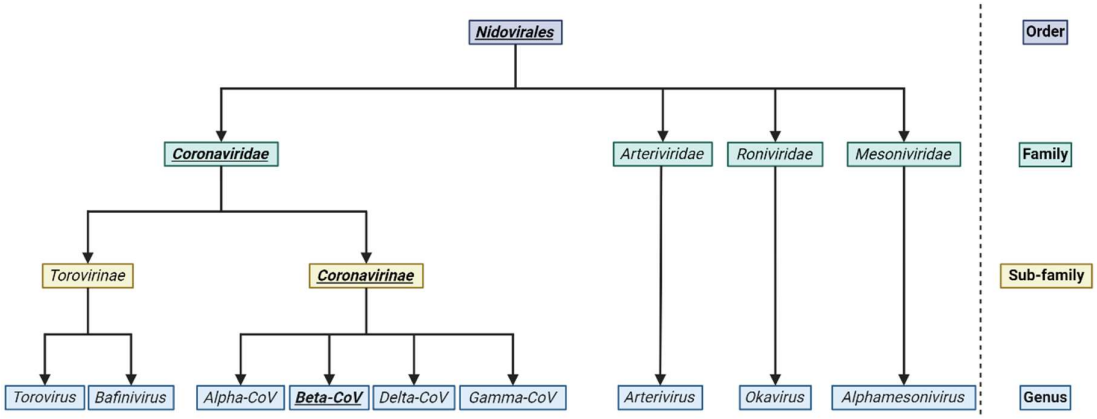

Figure 1-1: The taxonomy of Nidovirales. Adapted from (12) *and* (13).

CoVs have a diverse range of hosts. Gamma and Delta-CoVs predominantly infect birds, but may also infect certain marine mammals (14,15). Alpha and Beta-CoVs typically have mammalian hosts, and bats appear to be the main reservoir for these two groups of CoVs (16,17). Alpha-CoVs may cause a common cold in humans, and can also cause enteritis and respiratory disease in other mammals including pigs, dogs, cats and minks. Beta-CoVs also have a range of mammalian hosts including horses, camels and mice. In humans, Beta-CoVs may cause mild respiratory tract infections; however severe acute respiratory syndrome coronavirus (SARS-CoV-1), Middle East respiratory syndrome–related coronavirus (MERS-CoV) and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) can all cause life threatening respiratory illnesses in humans (18). A list of the most studied CoVs and their hosts can be found in *Appendix A*.

1.1.2 Virion Morphology

CoVs are large sphere shaped, enveloped viruses approximately 120 nm in diameter (19). The name “coronavirus” stems from the distinctive crown shape or “corona” in Latin, produced by surface projections from the virus membrane when observed under electron microscopy (20). The envelope is a thick layer which contains several different types of membrane proteins (21). The structure of a CoV virion is shown in *Figure 1-2*.

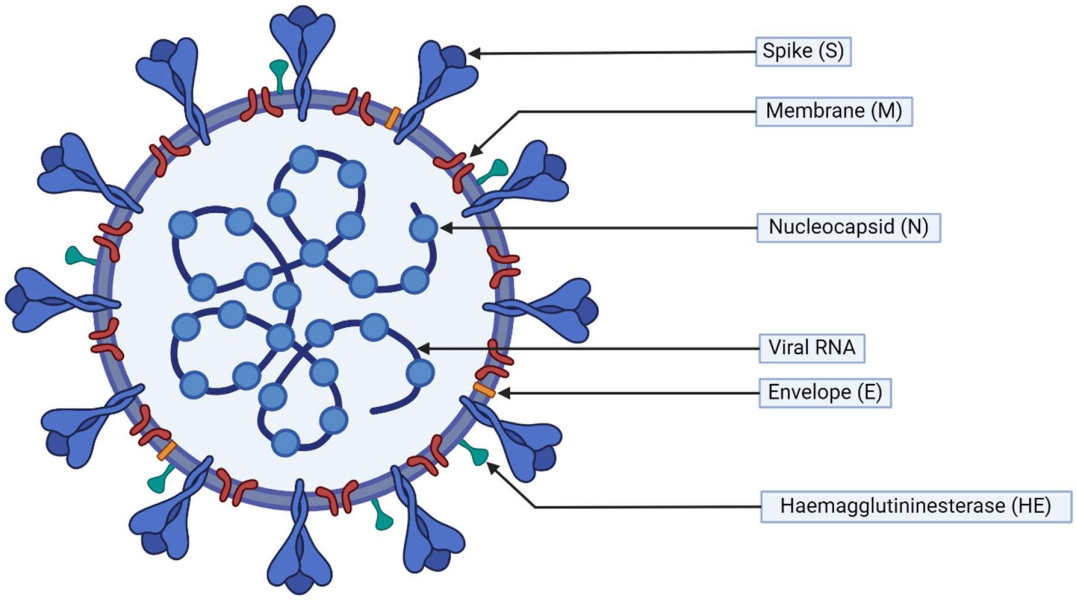

Figure 1-2: CoV virion morphology. Adapted from (22)

Variability exists in the types of accessory and non-structural proteins (NSPs) within different CoV species; however the following structural proteins are highly conserved between CoVs:

1.1.2.1 Membrane Protein

The membrane (M) protein is the most common protein associated with the envelope. It is a structural protein which traverses through the envelope three times (23). Along with providing membrane shape and stability, the M protein is responsible for budding of new viral particles and may also have a role in host cell interactions (19,20).

1.1.2.2 Spike Protein

All CoVs feature a ring of large surface projections made of type I glycoprotein known as spike (S) proteins (20). This ring is responsible for the characteristic corona appearance which inspired the name for the virus. The S protein may be cleaved into two subunits: The S1 subunit is responsible for binding to and interacting with host cell receptors, while the S2 subunit coordinates fusion of the virus and host cell membranes during infection (8,24). The S proteins are therefore the determinants of host cell interactions and tropism. The S protein is the primary target of the immune system (20) and is also the target of CoV vaccines (25,26). Significant sequence variation can occur with S protein expression, which increases viral immune escape potential (20).

1.1.2.3 Haemagglutininesterase Protein

Beta-CoVs of the A lineage also possess an additional smaller surface spike layer composed of haemagglutininesterase (HE) protein. The function of the HE protein has not been fully determined, but viruses expressing HE are considerably more effective at precipitating haemagglutination and haemadsorption. The HE protein is believed to act similarly to the S protein in facilitating viral attachment and entry into cells (27).

1.1.2.4 Envelope Protein

The small envelope protein (E) is a highly hydrophobic protein with several functions. It is a key structural component, and has an important role in assembly of new virus (28). Along with the M protein, it forms new virus-like particles during replication. It can also act as an ion channel during infection, and may also be responsible for induction of host cell apoptosis (8,20,24).

1.1.2.5 Nucleocapsid

Within the envelope, the genomic RNA is bound to a nucleocapsid (N) phosphoprotein which produces a helical ribonucleoprotein (RNP) core (20). The function of the nucleocapsid is to pack in and compress the RNA, as well as being involved in transcription (8,19).

1.1.3 Cell Entry and Replication

The first step in CoV infection is binding of the virus to target cells. The plasma membranes of the virus and host cell bind via interaction between the S proteins and host target receptors (8,20). Once bound, the S protein undergoes a conformational change which permits fusion of virus and host cell membranes, resulting in intracellular release of viral contents (29,30). Replication of the genome then commences via synthesis of an entire negative-strand RNA which acts as a blueprint for new full-length genomic RNA. Viral polypeptides are cleaved into replicase proteins which enable the translation of structural proteins and formation of new viral particles (8,24).

1.1.4 Human Pathogens

In humans, four CoVs have been implicated in minor respiratory infections. Alpha-CoVs Human coronavirus (HCoV)-229E (31) and HCoV-NL63 (32); and Beta-CoVs HCoV-OC43 (33) and HCoV-HKU1 (34) are pathogens which can cause symptoms of a common cold. They tend to cause minor self-limiting illness, although can cause more severe disease in immunocompromised patients (7,20).

An additional three BetaCoVs (SARS-CoV-1, MERS-CoV and SARS-CoV-2) are human pathogens which are considerably more virulent organisms and have each separately been responsible for pandemic diseases (35).

1.1.5 Previous Coronavirus Pandemics

1.1.5.1 Severe Acute Respiratory Syndrome

In November 2002, an outbreak of a highly contagious pneumonia occurred Guangdong Province, China. The significance of this outbreak was not fully inferred until February 2003 when the disease had spread to Hong Kong, at which point international travel spread the illness across the world, predominantly to Asia and North America, as well as some countries in Europe and Australasia (34)

Common initial symptoms included fever, dry cough and sore throat. Subsequent progression to severe pneumonia with alveolar damage, critical illness and death could occur (36). Consequently, the name Severe Acute Respiratory Syndrome (SARS) was coined, and the novel CoV SARS-CoV was cultured from SARS patients (36). The name was subsequently changed to SARS-CoV-1 to distinguish the virus from SARS-CoV-2.

Throughout 2002-2003, more than 8000 individuals were infected worldwide. Of those infected, over 900 died, resulting in a case fatality rate of around 10% (37). Elderly patients were particularly susceptible to severe disease (38). The SARS pandemic was deemed to have ended in July 2003. Strict isolation of infected patients helped to control disease spread (39), and no new cases have been reported since 2004 (40).

The emergence of SARS-CoV-1 appears to have originated from bats, which serve as the natural viral reservoir (39). Masked palm civets are theorised to have acted as an intermediate host (41), with subsequent spillover to humans as the terminal host (7).

SARS-CoV-1 was the first virus to be classified within the species *Severe acute respiratory syndrome-related coronavirus*, also known as *Betacoronavirus pandemicum,* which belongs to the *Sarbecovirus* subgenus of Beta-CoVs. Other newly discovered viruses have also been assigned to this species, including numerous SARS-like Beta-CoVs isolated from bats (7).

1.1.5.2 Middle East Respiratory Syndrome

MERS-CoV was first identified approximately 1 decade after the emergence of SARS-CoV. The virus was first detected in Jeddah, Saudi Arabia in 2012 (42), and resulted in 2494 confirmed infections and 858 deaths from a severe respiratory disease which became known as Middle East respiratory syndrome (MERS). The case-fatality rate of MERS-CoV is approximately 35%, making it a considerably more lethal virus than SARS-CoV, and one of the most deadly viruses capable of infecting humans (7,38).

Patients infected with MERS-CoV may initially experience mild symptoms including fever, myalgia, vomiting, diarrhoea, sore throat, and cough. In severe cases, dyspnoea and pneumonia may quickly develop, followed by acute respiratory distress syndrome (ARDS), renal failure, septic shock and death (43). Severe disease is more common in patients with obesity and diabetes mellitus, and in those with pre-existing cardiac and lung disease (44).

Like SARS-CoV, bats appear to be the original source of MERS-CoV. However, the intermediate host is the dromedary camel. Middle Eastern dromedary camels have a high seropositivity rate for MERS-CoV, and as a result the virus now endemic in the Arabian Peninsula (45). Numerous outbreaks of MERS have occurred; all associated with travel to the Middle East. For instance, a superspreading event occurred in South Korea in 2015 which resulted in 186 cases and 38 deaths (46).

1.2 SARS-CoV-2

The virus SARS-CoV-2 is the pathogen responsible for the most recent CoV pandemic. This virus has had a devastating effect worldwide and is considered the greatest global health crisis since the 1918 influenza pandemic (47,48).

Shortly after its discovery, the World Health Organisation (WHO) provided a temporary identifier for the virus as 2019 novel CoV (2019-nCoV) (49). Given the high sequence homology to the SARS-CoV-1 virus, the name was later changed to SARS-CoV-2 (49). SARS-CoV-2 is not a direct descendent of SARS-CoV-1 (50), but was classified as a sister virus of SARS-CoV, and was also placed within the *Severe acute respiratory syndrome-related coronavirus* species (51). The two viruses share many genetic and structural similarities including highly conserved regions in the nsp1 and ORF8 genes, as well as structurally similar S proteins, especially in the receptor-binding domain (7,52). Moreover, both viruses can cause severe respiratory disease in humans, further justifying the decision to name the virus SARS-CoV-2.

1.2.1 Origin and Spread

On the 12th of December 2019 a cluster of atypical pneumonia cases were detected in Wuhan, Hubei province, China. Several patients developed severe respiratory disease requiring hospitalisation (52). The pneumonia cases were alerted to the WHO on the 31st of December 2019. Subsequent investigation revealed that many of the initial cases has an association with an animal and seafood wet market in Wuhan (52). The market was closed for disinfection on the 1st of January 2020 (53).

By the 3rd of January 2020, 44 patients had developed pneumonia, a quarter of whom were severely ill. Broncho-alveolar lavage (BAL) samples were taken from hospitalised patients, and reverse-transcription polymerase chain reaction (RT-PCR) identified a novel CoV as the causative organism on the 7th of January (54).

On the 30th of January 2020 the WHO declared the outbreak a Public Health Emergency of International Concern (PHEIC). The PHEIC declaration brought with it a focus on identifying the origin of the outbreak, further clarifying the spectrum of disease severity, methods of transmission, effectiveness of prevention measures to attempt to contain the spread globally including vaccine production (55). Despite these efforts, the virus spread throughout Asia and then globally. The WHO confirmed that the virus had reached pandemic status on the 11th of March 2020, as case numbers climbed to over 118,000 across 114 countries, with over 4000 deaths attributed to the virus. Many countries enacted strict lockdown regulations and travel restrictions (56).

By the 4nd of April 2020 over 1 million infections had been confirmed (57), and the number of deaths attributed to SARS-CoV-2 surpassed 1 million on the 28th of September 2020 (58). Vaccination rollout began in December 2020, by which point more than 50 million cases had been confirmed. Vaccination led to a major reduction in risk of infection and protected against severe disease when breakthrough infections did occur. New SARS-CoV-2 variants appeared throughout 2021 and into 2022 with increased infectivity, however the number of deaths declined throughout 2022, and on the 3rd December 2022 the WHO indicated that over 90% of the population has some degree of immunity against SARS-CoV-2. As of the 5th of May 2023, the WHO announced that COVID-19 represents an established and ongoing health issue, but no longer represents a PHEIC (58,59).

1.2.1.1 Zoonotic origin

Similarly to other Beta-CoVs, bats are the most likely natural reservoir for SARS-CoV-2. Indeed, there is approximately 89% genomic sequence similarity between SARS-CoV-2 and two bat CoVs bat-SL-CoVZC45 and bat-SL-CoVZXC21, and 96.2% similarity with the bat CoV RaTG13 (7,52,60). However, there were no reports of bats being sold at the Wuhan wet market (52), and the majority of bat species were in hibernation when SARS-CoV-2 was first detected (7). Therefore, as with both SARS-CoV-1 and MERS-CoV, an intermediate host between bats and humans is speculated to have facilitated the spillover into humans (54), with pangolins or farmed minks as potential candidates (61,62).

An alternative hypothesis suggests that the SARS-CoV-2 virus was inadvertently released from the Wuhan Institute of Virology, a research laboratory in close proximity to the wet market. However, no evidence exists to suggest that a virus similar to SARS-CoV-2 was being studied in the lab at this time. Moreover, the earliest known cases of SARS-CoV-2 infection occurred in the wet market, not in the Institute of Virology. Signs of genetic engineering such as codon usage bias or deletion of the S protein furin cleavage site, were not evident in the ancestral SARS-CoV-2 isolates. As such, the lab leak theory has generally been discredited by the wider scientific community (63,64).

1.2.2 Transmission

SARS-CoV-2 is not as lethal as SARS-CoV-1 but is more transmissible. The primary mode of viral transmission is via respiratory droplets from an infected person talking, breathing, coughing or sneezing. This prompted guidance to reduce transmission by maintaining social distancing and wearing personal protective equipment (PPE) including face masks when indoors (65). Indoor gatherings were restricted to minimise potential airborne transmission through aerosolization, and throughout the world lockdown measures were put in place to prevent non-essential face-to-face contact. Moreover, potentially aerosol generating healthcare procedures such as endotracheal intubation were avoided when possible or performed using full PPE when deemed necessary (54,55,66,67). Other lesser forms of transmission included via fomites such as contaminated surfaces, and via the faecal-oral route. As such, campaigns promoting regular handwashing were implemented (68).

1.2.3 Viral Entry & ACE2

Angiotensin converting enzyme (ACE) 2 was identified as the target receptor for SARS-CoV-2 (69). ACE2 is an intramembrane enzyme which plays an important role in regulation of the renin-angiotensin-aldosterone system (RAAS) by opposing the effects of ACE. Angiotensin I (Ang I) is converted to Ang II by ACE, which can then bind to the Ang II receptor 1 (AT_1) causing vasoconstriction and retention of sodium and water. ACE2 counterbalances this pathway by converting Ang I and II into Ang (1-9) and (1-7) respectively. Ang (1-7) exerts vasodilatory, anti-inflammatory and cardioprotective effects (70).

SARS-CoV-2 utilises the S protein for cell entry. The S1 subunit initiates binding with ACE2, which triggers viral internalization. Before the S protein can initiate viral entry, it requires priming which is achieved by cleavage through the actions of transmembrane protease, serine 2 (TMPRSS2) (69). Once primed, the S protein can initiate membrane fusion via the S2 subunit, permitting release of viral components intracellularly in preparation for new virion assembly (71) (See *Figure 1-3*).

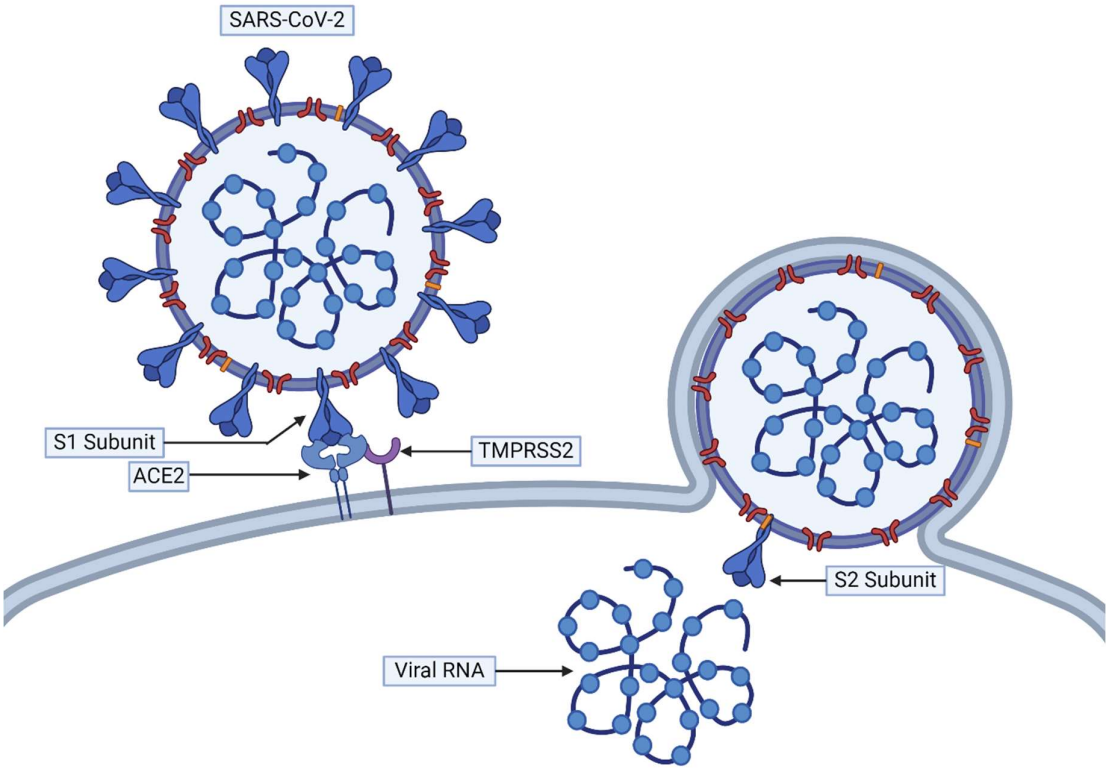

Figure 1-3: SARS-CoV-2 viral infection facilitated by ACE2 and TMPRSS2. Adapted from (72).

The respiratory tract contains cells which express high levels of ACE2, particularly type II pneumocytes. As such, this is the primary site of SARS-CoV-2 infection. However ACE2 is also expressed throughout the body in other tissues including the gastrointestinal (GI) tract, kidneys, endothelium and heart (73). Accordingly, post mortem studies have shown detectable SARS-CoV-2 virus in the kidneys, liver, heart and brain (74), suggesting cells outside of the respiratory tract are also vulnerable to infection.

1.3 COVID-19

The disease caused by SARS-CoV-2 is called Coronavirus disease 19 (COVID-19). The name was chosen to avoid reference to a particular location or group of people which could cause stigma (75). COVID-19 has had a catastrophic effect globally, putting significant strain on healthcare systems and causing massive economic disruption (76).

1.3.1 Epidemiology

The current number of COVID-19 cases reported to the WHO stands at over 773,000,000 with nearly 7 million deaths, with the highest number of deaths occurring in the United States of America, Brazil and India. The United Kingdom has the 6th highest death count in the world (77).

1.3.2 Symptoms

Up to a third of infected individuals may have no symptoms whatsoever (78). Fever, dry cough, fatigue and malaise are common initial symptoms (54,79). Other less common presenting symptoms include productive cough, shortness of breath, sore throat, myalgia, headache, nausea and vomiting, and diarrhoea (54,80). Pneumonia can develop in severe cases with profound dyspnoea. The severity of respiratory symptoms appears to be less marked than in cases of SARS and MERS (81).

1.3.3 Severity Classification

The WHO classified disease severity according to *Table 1-1*. Most infected individuals experience a mild self-limiting disease and will make a full recovery. Pre-vaccination, approximately 14%-23% of cases developed severe disease characterised by respiratory distress, hypoxia and pulmonary infiltrates on chest imaging. Around 11% of patients required admission to the intensive care unit (ICU), 7% required mechanical ventilation and 6% of patients became critically unwell, with severe respiratory failure, septic shock, and/or multiple organ dysfunction syndrome (MODS) (79). Widespread vaccination uptake and recovery from prior infection has led to a high rate of immunity to SARS-CoV-2 within the general population, and therefore severe or critical disease is now much rarer than observed during the early stages of the pandemic (82).

Severity Classification	Features
Mild	Symptomatic patients without evidence of pneumonia or hypoxia
Moderate	Clinical signs of pneumonia (fever, cough, dyspnoea, tachypnoea). No signs of severe pneumonia. SpO2 ≥ 90% on room air.
Severe	Clinical signs of pneumonia as above, plus one of the following: • Respiratory rate > 30 breath per minute • Respiratory distress • SpO2 < 90% on room air.
Critical: ARDS	Onset within 1 week of clinical insult or associated with worsening respiratory symptoms. Chest imaging demonstrating bilateral pulmonary infiltrates not fully explained by volume overload, lobar or lung collapse, or nodules. ARDS severity: • Mild ARDS: PaO_2/FiO_2 ratio ≤ 300 mmHg • Moderate ARDS: PaO_2/FiO_2 ratio ≤ 200 mmHg • Severe ARDS: PaO_2/FiO_2 ratio ≤ 100 mmHg
Critical: Sepsis	Acute life-threatening organ dysfunction in response to suspected or proven infection. Presence of altered mental status, tachypnoea, dyspnoea, hypoxia, oliguria, tachycardia, hypotension, skin mottling, coagulopathy, thrombocytopenia, acidosis, raised lactate and/or raised bilirubin.

Table 1-1: COVID-19 Severity classification. Adapted from (88). FiO_2: Fraction of inspired oxygen, PaO_2: Partial pressure of oxygen, SpO_2: Oxygen saturation.

1.3.3.1 Long COVID

Approximately 10 % of infected patients will go on to develop post-acute sequelae of COVID-19, otherwise known as long COVID. Features may include heart failure, impaired gas exchange, chronic fatigue, cognitive impairment and dysautonomia (83).

1.3.4 Risk Factors

Several factors were identified which increased the risk of progressing to severe COVID-19:

1.3.4.1 Age

Increasing age is associated with increased disease severity, ICU admission, need for invasive mechanical ventilation (IMV) and death (80). Elderly patients are particularly vulnerable to severe disease, and every increase in age by 10 years increases the risk of mortality by 50% (84). Infection in children tends to be mild, with only 0.2% of critical disease occurring in the paediatric population (54).

1.3.4.2 Gender

There is an increased COVID-19 incidence, severity and mortality rate in males compared to females (85). This may be related to environmental factors such as increased smoking and alcohol use amongst males (86). Alternatively, males may be at greater risk of infection due to increased TMPRSS2 expression in response to androgens (87).

1.3.4.3 Ethnicity

African American patients experienced an increased risk of mortality from COVID-19 during the early days of the pandemic. This may be partly due to increased comorbidities in this group, as well as social factors such as living conditions and working environments which made social distancing difficult (88).

1.3.4.4 Comorbidities

Patients with hypertension, diabetes, coronary heart disease and chronic obstructive pulmonary disease (COPD) have an increased risk of developing severe COVID-19 (80,89). Patients who smoke and those with COPD have increased ACE2 expression, possibly increasing their susceptibility to infection (90).

1.3.5 Organ Dysfunction

While SARS-CoV-2 predominantly infects cells of the respiratory tract, the ACE2 receptor can be found throughout the body, and therefore multisystem effects are observed. Injuries to the vasculature, myocardium, nervous system, GI tract, and kidneys have been reported (67).

1.3.5.1 Respiratory

Respiratory failure is the primary cause of organ dysfunction in severe COVID-19. The initial stages of pulmonary COVID-19 are associated with oedema and epithelial damage. Viral attachment causes degradation of ACE2 leading to an overall reduction in ACE2 on cell surfaces (91). This leads to a reduction in Ang II cleavage to Ang (1-7), leading to accumulation of Ang II which promotes pulmonary vascular constriction, increasing hydrostatic pressure and causing pulmonary oedema (91,92). Ang II accumulation increases activation of the AT_1 receptor, increasing inflammation and oxidative stress, exacerbating acute lung injuries (ALIs) (91).

Release of inflammatory mediators from immune cells within the lungs promotes neutrophil migration (93). Inflammation causes epithelial injury, diffuse alveolar damage, and leads to hyperplasia of pneumocytes. Desquamation of macrophages is observed contributing to consolidation (94–96). Dilution of surfactant reduces surface tension and leads to alveolar collapse. ARDS develops, compromising gas exchange and causing profound hypoxia (97). Interestingly, the bizarre phenomenon of “happy hypoxaemia” may occur, where patients may appear unperturbed despite experiencing critical hypoxaemia. These patients can deteriorate extremely quickly, so timely assessment and admission to critical care is essential (98).

Patients with severe respiratory failure who remain hypoxic despite supplemental oxygen delivery may require intubation and mechanical ventilation to support gas exchange (98). Patients who survive the ARDS insult may subsequently develop lung fibrosis causing a persistent, chronic decline in lung function (99).

1.3.5.2 Coagulation

Venous, arterial and microvascular thrombotic phenomenon occur frequently in COVID-19, especially in severe disease (100). Approximately one third of critically ill COVID-19 patients will experience a thrombotic complication (101). Around 90% of hospitalised patients have a raised D-dimer level in keeping with activation of coagulation cascades (89).

Endothelial cells express ACE2, therefore SARS-CoV-2 can directly infect endothelial cells leading to their dysfunction (94,102). Increased circulating Ang II acts to increase serum plasminogen activator inhibitor-1, impairing fibrinolysis through inhibition of tissue plasminogen activator (tPA) (100). Clotting factor depletion can precipitate disseminated intravascular coagulation (DIC) and result in microthrombotic phenomena, contributing to end organ damage (97). Increased Ang II promotes tissue factor release, which triggers the extrinsic coagulation cascade and promotes endothelial dysfunction and microvascular thrombosis (103). Ang II increases production of platelet-derived growth factor, which promotes platelet aggregation and increases arterial thromboembolism risk (104).

Anticoagulation may reduce the risk of thrombotic events in hospitalised COVID-19 patients, but the evidence for the use of anticoagulation in critically ill patients is less promising. Moreover, anticoagulation may be detrimental to this patient group due to an increased risk of major bleeding (105).

1.3.5.3 Cardiovascular

Cardiovascular manifestations are common in COVID-19 and likely multifactorial. Approximately 30% of patients with severe disease will suffer a cardiac injury (106) as demonstrated by raised high-sensitivity troponin I (89). Acute coronary syndromes, arrhythmias, congestive heart failure, myocarditis and pericardial effusions have all been observed in COVID-19 patients (106). Those with pre-existing cardiac disease are more likely to suffer additional cardiac injuries, and have a higher risk of death (107).

ACE2 is present on cardiac myocytes, and so the myocardium is vulnerable to damage via direct infiltration of the SARS-CoV-2 virus (96,108), leading to myocarditis (109). Moreover, pro-inflammatory cytokines and high levels of circulating catecholamines may exert a cardiotoxic effect on the myocardium (109). Myocardial injury can cause a Takotsubo cardiomyopathy, resulting in impaired left ventricular systolic dysfunction and cardiogenic shock (110). Thrombotic complications also increase the risk of developing right ventricular dysfunction, which carries a mortality rate in COVID-19 of 86% (111). Vascular endothelial damage, hypercoagulation and microthrombi formation can compromise coronary artery blood supply, leading to ischaemic injury (94). This may be exacerbated by hypoxaemia and hypotension in severe disease further reducing the delivery of O_2 (97).

1.3.5.4 Neurological

Neurological phenomena may occur during a SARS-CoV-2 infection. Common symptoms may include anosmia, fatigue, vertigo, headache and changes in cognition (112). A proportion of patients may experience persistent symptoms. Chronic fatigue and cognitive impairment can cause ongoing debilitation long after the initial infection has resolved (113). Other severe and life-threatening presentations include stroke and transient ischaemic attack, intracerebral haemorrhage, encephalitis and Guillain–Barre syndrome (112,114).

It is unclear whether SARS-CoV-2 can directly infect brain tissue. ACE2 receptors are present within the brain and have been found in high abundance in the olfactory bulb, which may explain why anosmia is such a commonly reported early symptom (97). Moreover, SARS-CoV-2 virus may invade endothelial cells leading to disruption and failure of the blood-brain barrier (115). Neurological disorders in COVID-19 have been associated with raised fibrinogen, an acute phase protein which is also a marker of hypercoagulability. Fibrinogen may cross the disrupted blood-brain barrier and act locally to encourage cerebral microthrombosis and ischaemic injury (115). Severe disease can further exacerbate cognitive impairment as respiratory failure causes profound hypoxemia resulting in hypoxic brain injuries (116).

Inflammation secondary to the cytokine storm may lead to oxidative damage to neurones as demonstrated by raised pro-inflammatory cytokines detected within the cerebrospinal fluid (CSF) of COVID-19 patients. Viral particles are less likely to be detected in CSF, suggesting that the inflammatory response may play a larger role in neurological symptoms rather than direct viral infection of the nervous system (117,118).

1.3.5.5 Gastrointestinal

Although observed less frequently than respiratory symptoms, GI manifestations of COVID-19 are seen commonly as early symptoms of the disease. Anorexia, nausea, vomiting and diarrhoea all may occur (119,120). Viral entry to cells of the GI tract may damage the intestinal mucosa causing diarrhoea (119). This suggests that the faecal-oral route is a viable mode of transmission for SARS-CoV-2 (121). Indeed, viral RNA has been detected in stool samples of SARS-CoV-2 positive patients (120). Liver injuries may occur, particularly in severe disease with corresponding increases in liver enzymes aspartate transaminase (AST), alanine aminotransferase (ALT) and bilirubin (122,123). Liver damage likely occurs as a combination of direct hepatocyte infection, hypoxic ischaemic injury and through cytotoxic injury via the cytokine response (120).

1.3.5.6 Renal

Acute kidney injury (AKI) is a common sequela of severe COVID-19. In critically ill patients the incidence is as high as 29% (124,125). Acute tubular necrosis is the most common renal pathology observed in COVID-19 (126). The cause is likely to be multifactorial, with direct viral cytotoxicity; microvascular injury; hypoperfusion; RAAS imbalance and pro-inflammatory cytokine associated damage all likely contributors. The administration of nephrotoxic drugs and mechanical ventilation with high positive end-expiratory pressure (PEEP) may further contribute to renal injuries through iatrogenic means (124–127). Diuretics used as part of the management of ARDS may contribute to volume depletion and hypoperfusion (128). Hypertensive patients, who are already at an increased risk of severe disease, may be treated with antihypertensive medications including ACE inhibitors, which may also impair renal blood flow (89,124).

1.3.6 Treatment

Early management of hospitalised patients with COVID-19 is centred on general supportive measures (80). Most patients hospitalised with SARS-CoV-2 infection require supplemental oxygen. As disease severity increases, more advanced respiratory support strategies may be required including high flow nasal oxygen ($HFNO_2$), non-invasive ventilation (NIV), IMV or extracorporeal membrane oxygenation (ECMO) (84).

Numerous drugs were investigated as potential treatment options. Several breakthrough discoveries were made, while many other drugs failed to show benefit.

1.3.6.1 Antiviral Agents

Multiple antiviral agents aimed at virus elimination were investigated. One of the first to be considered was remdesivir, a broad-spectrum antiviral drug which acts to inhibit viral RNA polymerase. Remdesivir had previously demonstrated efficacy against SARS-CoV-1 and MERS-CoV (129), and showed promising results against SARS-CoV-2 *in vitro* (130). An early pandemic study, the Adaptive Covid-19 Treatment Trial, demonstrated a significantly shorter time to recovery in hospitalised COVID-19 patients treated with remdesivir compared to placebo (131). However, the Solidarity trial found that remdesivir did not show benefit in patients already on mechanical ventilation, and only had a small effect against death or progression to ventilation in other hospitalised patients (132). Remdesivir appears to be most beneficial when administered as early as possible in the disease course, and is currently only recommended in these circumstances (133).

Other antiviral agents include molnupiravir: a broad spectrum oral antiviral drug which demonstrated a significant reduction in hospitalisation and death for mild to moderate COVID-19 cases compared to placebo (134). Ritonavir-boosted nirmatrelvir (Paxlovid), a combination of two oral antiviral agents, also showed a significant reduction in hospital admission and mortality compared to placebo when given early in COVID-19 onset (135). Both molnupiravir and Paxlovid are approved for treatment of outpatients at high risk of disease progression (133).

Lopinavir/ritonavir is a combination antiviral used in the management of human immunodeficiency virus (HIV). The use of this therapy was investigated as a repurposed drug for the treatment of COVID-19. The addition of lopinavir/ritonavir to standard care does not convey a benefit in terms of mortality, viral clearance or adverse events (136).

Several other drugs were also investigated for their potential to be repurposed as antiviral agents. These included the anti-malarial drug chloroquine, the anti-rheumatic drug hydroxychloroquine, the antibiotic azithromycin and the anti-parasitic drug ivermectin. Despite widespread publicity promoting these drugs globally, randomised controlled trials consistently failed to demonstrate benefit in COVID-19 treatment or prophylaxis, and in fact could cause harm (137–139).

1.3.6.2 Neutralizing Antibodies

Studies were conducted investigating the effects of administering antibodies which would target and neutralise the SARS-CoV-2 virus. Convalescent plasma donated from patients who had recovered from a recent SARS-CoV-2 infection was investigated early in the pandemic as a therapy. Unfortunately, studies failed to demonstrate efficacy as a COVID-19 treatment, and its use is not recommended (140).

Several monoclonal antibodies including sotrovimab and casirivimab/imdevimab (Ronapreve) initially showed promising results in randomised controlled trials, however guidance on their use was subsequently reviewed. These drugs were released before the Omicron subvariant became the dominant SARS-CoV-2 strain, and thus their effectiveness against subsequent variants was significantly reduced (141,142). As such, neutralising monoclonal antibodies are no longer indicated for COVID-19 management in hospitalised patients (133).

1.3.6.3 Immunomodulatory Therapies

Various drugs exerting immunosuppressant and immunomodulatory effects were investigated. The first major breakthrough in management of COVID-19 came from the use of dexamethasone. The Randomized Evaluation of COVID-19 Therapy (RECOVERY) trial found that dexamethasone reduced 28 day mortality in ventilated patients by one third, and those requiring oxygen by one fifth, and as such dexamethasone became part of treatment protocols for hospitalised COVID-19 patients (143).

Interferon (INF) ß-1a is an interferon used in the treatment of multiple sclerosis. Inhaled INFß-1a was investigated for its potential antiviral properties. While some studies were encouraging and suggested a reduction in hospital length of stay and reduction in respiratory symptom severity with INFß-1a treatment (144), other studies showed no benefit and in fact found INFß-1a had a potentially detrimental impact (145,146).

Several cytokine blocking drugs were also studied. The interleukin (IL)-1 receptor blocker anakinra, and IL-6 antagonists tocilizumab and sarilumab have previously been used in the management of rheumatological disorders. The hypothesis was that these drugs could interrupt the cytokine storm seen in severe COVID-19 disease. Unfortunately anakinra failed to demonstrate clinical effectiveness in the Randomised, Embedded, Multi-factorial, Adaptive Platform Trial for Community-Acquired Pneumonia (REMAP-CAP) trial (147); however REMAP-CAP did demonstrate that both tocilizumab and sarilumab led to an increase in survival and a reduction in organ support duration in severe COVID-19. Additionally, the RECOVERY trial found that in severe COVID-19, patients who received tocilizumab had greater survival rates, shorter length of hospital stays and lower requirement for IMV (148).

Baricitinib is an oral immunomodulatory drug, which block the Janus kinase (JAK)/ signal transducer and activator of transcription (STAT) pathway through inhibition of JAK1 and JAK2. Inhibition of the JAK/STAT pathway is an alternative method of interrupting the cytokine storm in severe COVID-19. The RECOVERY trial demonstrated a statistically significant mortality reduction in patients with severe COVID-19 who received baricitinib (149).

1.4 Secondary Infections

A secondary infection in COVID-19 occurs when an additional pathogenic organism is recovered in addition to the primary SARS-CoV-2 virus. This may occur as a co-infection, where a second pathogen is detected simultaneously with the initial SARS-CoV-2 infection. These infections are often community acquired. Alternatively, a superinfection develops subsequent to the onset of the primary viral infection. A superinfection typically occurs more than 48 hours after the initial infection, and therefore will generally represent nosocomial infection. (150–152). Secondary infections have important implications in terms of the patient's clinical course, with significant impact on morbidity and mortality. Moreover, there is a substantial financial cost associated with secondary infections, with an estimated £2.1 billion spent on the management of hospital acquired infections in NHS England annually (153). The development of a secondary infection increases a patient's hospital length of stay by a mean of 9 days. Hospital acquired infections were responsible for 7.1 million hospital bed days in England between 2016-2017, which accounts for 21% of the total inpatient bed occupancy throughout NHS hospitals in England (153).

1.4.1 Incidence

Co-infections have a low incidence in the general COVID-19 population, affecting approximately 5-7% of hospital admissions (154,155). Two meta-analyses performed by Langford *et al*. found the co-infection rate to be 3.5% (95% CI 0.4–6.7%) and 5.3% (95% CI 3.8–7.4) (156,157).

The rate of secondary bacterial superinfection is considerably higher than that of co-infection with an overall hospital incidence of 15-16% (158,159). The meta-analysis by Langford and colleagues reported a secondary infection rate of 18.4% (95% CI 14·0–23·7) (157). The ICU incidence of secondary infection in COVID-19 is even greater, with a rate of 25-58% (154,158,160–170). Conversely, The odds of COVID-19 patients with secondary infection requiring ICU admission is over 8 times higher than those without (171). Secondary infection incidence was highest during the first wave of the pandemic and reduced over time, which may have reflected trends in reducing broad spectrum antibiotic usage (172). The high rates of ICU acquired infection in COVID-19 highlight the significant burden this condition causes within the ICU.

1.4.2 Risk factors

Multiple factors likely play a role in the increased incidence of secondary infections in ICU. Overwhelming numbers of patient admissions to ICU during the first wave of the pandemic with limited space led to overcrowding, increasing the risk of spread of nosocomial infections. A surge in hospital admissions reduced staff, equipment and PPE availability, likely contributing to suboptimal infection control measures being practiced (163). Other patient related factors are outlined below:

1.4.2.1 Severity

Although the overall rate of co-infection and superinfection in patients with COVID-19 is low, the rate is positively correlated with disease severity, with critically ill patients at the greatest risk of developing secondary infections (150,173). Patients with severe COVID-19 are more vulnerable to infection during the immunosuppressive phase of the illness, characterised by lymphopenia (89), immature neutrophil release (174) and reduced IFN production (175), which all may increase the risk of secondary infection occurrence.

1.4.2.2 Sex

Males have a higher rate of COVID-19 infection, severity and mortality compared to females (85). A multicentre study by Grasselli *et al*. found over three quarters of patients admitted to ICU with COVID-19 were male (176). This suggests an increased susceptibility to COVID-19 infection in males, and may possibly be linked to immunomodulatory properties of sex hormones (177). The review by Langford *et al*. suggested a reduced rate of secondary infections in female COVID-19 patients compared to males (adjusted odds ratio 0.73, 95% CI 0.55–0.97) (158), however various observational studies found no difference between the rates of secondary infections in men or women (176,178–180).

1.4.2.3 Age

Age is a known risk factor for COVID-19 severity, with increasing age being associated with an increased risk of ICU admission and death (80). The multi-centre study by Grasselli *et al*. found the median age at ICU admission was 62 years old (176). Age may also be a risk factor for developing secondary infections in COVID-19. A retrospective cohort study by Iacovelli and colleagues found a significant difference in age. The median age of COVID-19 patients in the high dependency setting with a superinfection was 77, whereas the median age of those without superinfection was 66 (181). A large study by Murray *et al*. found an association with increasing age over 40 and secondary infection prevalence. Patients over 70 years old were at a 3.76 times greater risk of developing a secondary infection than patients age 20-29 (182). Furthermore, Lv and colleagues found that 75% of patients with secondary infections were over 50 years old (168).

Interestingly, Karaca *et al*. did not find a significant difference between age and incidence of secondary infections in COVID-19 (183). Likewise, Wu and colleagues did not find an overall difference between age and rates of secondary infections, but did find that older patients were more predisposed to developing gram-negative infections compared to younger patients (180).

1.4.2.4 Comorbidities

Patients with chronic heart and lung disease, diabetes mellitus and hypertension are at increased risk of severe COVID-19 (89). Similarly, several studies have suggested certain comorbidities may increase the risk of developing secondary infections, including immunosuppression, diabetes, ischaemic heart disease, atrial fibrillation, chronic renal disease and malignancy (181,183–185). The risk of developing an infection is up to four times higher in diabetic patients, and outcomes including mortality are worse in this population. Diabetes and hyperglycaemia causes a chronic inflammatory state resulting in immune dysfunction, predisposing to the development of a bacterial infection results in a poorer immune response (186)

1.4.2.5 Device Related Infections

Up to 39% of COVID-19 ICU patients will develop a device related infection secondary to mechanical ventilation, invasive lines and/or urinary catheters (163). COVID-19 patients with severe respiratory failure may require prolonged periods of IMV, which increases the risk of ventilator associated pneumonia (VAP). The use of invasive lines for arterial blood pressure monitoring and central venous access increases the risk of central line associated blood stream infections (CLABSIs) (187). The longer these lines remain *in situ*, the greater the risk of causing a secondary infection (163,188).

1.4.2.6 Immunomodulation

As mentioned, drugs aimed at diminishing the immune response to COVID-19 became the standard of care for critically ill patients. Corticosteroids, IL-6 inhibitors and JAK inhibitors demonstrated survival benefit when administered to hospitalised COVID-19 patients. While immunomodulatory drugs may help to attenuate the deleterious pro-inflammatory cytokine storm seen in severe COVID-19, there is a risk that excessive inhibition could prevent an effective immune response from being mounted against a secondary infection.

Several small studies have suggested an increased risk of secondary infections with dexamethasone use (189,190), and Saade *et al*. found patients who received dexamethasone were three times more likely to develop secondary infections (166). Conversely, several other studies found no association between immunomodulatory drug usage and rates of secondary infection (158,176,180,191). While the RECOVERY trial found that neither tocilizumab nor baricitinib led to an increase in secondary infections (148,149), they did not specifically address this potential complication when examining dexamethasone (143).

Tocilizumab does not appear to predispose patients to developing secondary infections, and some evidence suggests that it may in fact reduce the risk. IL-6 inhibitors may halt the progression of disease severity therefore reducing the infection risk. Alternatively, their immunosuppressive effects may reduce the release of traditional markers of infection, making detection of a secondary infection more challenging (192).

1.4.3 Mortality

Critically ill COVID-19 patients with secondary infections have a significantly increased mortality rate compared to those without secondary infections (169). The mortality rate associated with secondary infections is high, with 40-50% of patients dying (89,181,193). This is even higher for patients in ICU with secondary infections, where the figure rises up to 65% (194). A small study found the mortality rate of secondary infections in ICU was as high as 95% (195). COVID-19 patients with secondary bacterial infections may develop refractory septic shock and multi organ failure at higher rates than those without secondary infections (196).

Karaca and colleagues did not detect a mortality difference within the first week of developing a secondary infection in ICU, but did find a significantly increased rate of mortality at 21 days (183). The International Severe Acute Respiratory and Emerging Infections Consortium (ISARIC) WHO Clinical Characterisation Protocol UK study did not demonstrate an increase in mortality amongst those with secondary infections in ICU, which they speculated may be due to a high mortality rate early in patient's hospital stay, so many patients may have died in this study before a secondary infection had time to develop (159).

1.4.4 Infection Sites

1.4.4.1 Pneumonia

The most common site of secondary infection in COVID-19 is the respiratory tract (183). This is likely due to a combination of factors including impaired respiratory epithelial function; localised immune dysfunction; prolonged intubation and mechanical ventilation; increased risk of ARDS; and micro-aspiration from prone positioning (150,163,194).

The proportion of secondary infections which occurs in the respiratory tract is between 23-40% (165,169,181,183), with one study by Li *et al.* finding that 86% of secondary infections were of pulmonary origin (194). Ventilator associated pneumonia (VAP) is a common occurrence among intubated COVID-19 patients, with 24-51% of infections attributed to VAP. Hospital acquired pneumonia not attributed to VAP is rare, and only occurs in 2-3% of secondary infections (163,176,178).

While sputum or tracheal aspirates may be used to culture respiratory pathogens, BAL provides the best yield. Due to risks of producing aerosols leading to viral spread, BAL was less frequently performed during the peak of the pandemic, which may have resulted in an underestimation of the number of secondary respiratory tract infections (197).

Gram negative organisms predominate in secondary pneumonias, particularly Gram negative bacilli (173,194). They are most commonly nosocomial superinfections caused by lengthy ICU stays and use of broad-spectrum antimicrobials (154). The most common Gram negative pathogens in secondary pulmonary infections are A*cinetobacter baumannii* (*A. baumannii), Haemophilus influenzae (H. influenzae), Pseudomonas aeruginosa (P. aeruginosa), Enterobacter* species, *Klebsiella pneumoniae (K. pneumoniae), Escherichia coli (E. coli) and Stenotrophomonas maltophilia (S. maltophilia)* (150,154,169,194,198–201).

Gram-positive cocci are also common culprits (200), with *Staphylococcus aureus* (*S. aureus)* responsible for 10% of secondary chest infections (195,198,201). *S. aureus* is a common cause of community acquired pneumonia (CAP) in COVID-19 and as such tends to cause infections early in the ICU stay (197,199). Despite being a common cause of CAP in the general population, *Streptococcus pneumoniae (S. pneumoniae)* is not a common cause of secondary infections in COVID-19 (159).

1.4.4.2 Bloodstream Infections

Bloodstream infections (BSIs) are also common in critically ill COVID-19 patients. The proportion of BSIs in ICU is 10-40% of all secondary infections (158,163,165,169,176,178,181,183,194). BSIs tend to appear later, and are more common with increased ICU length of stay (158,169).

BSIs frequently occur concomitantly with respiratory infections, and these are often caused by the same organism (194). In 16.3% of bloodstream infections, the initial infection has spread from the respiratory tract (158). Organisms such as *K. pneumoniae* and *A. baumannii* may cause an initial pneumonia, then translocate to the bloodstream causing a simultaneous bacteraemia (194).

In severe COVID-19, most BSIs are caused by Gram negative organisms. *A. baumannii, K. pneumoniae, P. aeruginosa and E. coli* are the most common Gram negative organisms implicated in causing BSI (165,173,202). Gram positive organisms also commonly causes BSIs, however the high proportion of coagulase-negative S*taphylococci* may be related to blood culture contamination (173,183). Other common causes of Gram positive BSI are *S. aureus* and *Enterococcus* species (165,169,173,202).

CLABSI is common with up to 25% of secondary infections originating from a central line (165,176,181). Approximately half of all secondary BSIs are associated with central lines (178), and up to 80% of Gram positive BSIs occur in patients with central venous catheters.

1.4.4.3 Urinary Tract Infection

Urinary tract infections (UTIs) are less common causes of secondary infection than bloodstream or respiratory infections, however the proportion of UTIs is variable and ranged from 2%-28.5% of secondary infections (165,169,176,178,181,183,194). Prolonged urinary catheter use and trauma caused by prone positioning may increase the risk of UTI in COVID-19 (163). Between 1.6-22% of UTIs are associated with urinary catheters (163,183). The most common organism is *E. coli* (194), but *Enterococcus* species are also frequently implicated (165).

1.4.4.4 Others:

Other causes of secondary infections are rare, and include skin and soft tissue infections (1.5-2.7%) and intra-abdominal infections 1.5%-1.8 (178,181). These have been studied less closely than VAP, BSI and UTI, but show increased incidence in the COVID-19 patient population (192).

1.4.5 Antimicrobial Usage

A major discrepancy exists between the incidence of secondary infection and the rate of antibiotic usage. Up to 77% of hospitalised COVID-19 patients receive antibiotics, despite a much lower incidence of secondary infection in this group. Antimicrobial therapy is frequently commenced empirically and tends to have broad-spectrum coverage (150,158).

Interestingly, patients who have received antibiotics early in their admission are significantly more likely to develop a secondary infection. This is likely due to disruption of normal flora, permitting subsequent proliferation of pathogens (203). Iacovelli and colleagues found antibiotic exposure in the previous 30 days was an independent risk factor for developing secondary infections, with an odds ratio (OR) of 4.82 (181). Wu *et al*. determined previous antibiotic exposure is an even greater risk for development of a secondary infection, with an OR of 17.23. Exposure to the third-generation cephalosporins ceftriaxone and cefotaxime significantly increased the risk of developing Gram positive secondary infections (180). These findings highlight the importance of antimicrobial stewardship and cautious use of broad-spectrum antibiotics.

Antibiotics are often administered by clinicians when there is evidence of an inflammatory response, and a CRP of greater than 100 is associated with increased rates of antibiotic usage (159). CRP is not specific to secondary infections, and a raised CRP may simply be indicative of worsening COVID-19. Empiric antibiotics are often commenced due to manifestations of sepsis, but it is very challenging to differentiate a viral sepsis from a secondary bacterial sepsis (204). Early in the pandemic, antibiotics such as azithromycin were repurposed as COVID-19 treatments, exacerbating the problem of excessive antimicrobial usage (150).

A worrying development is the increasing proportion of secondary nosocomial infections in hospitalised COVID-19 patients caused by multi-drug resistant organisms (167,169). Bardi *et al*. reported that 31% of all secondary infections were sustained by multi-drug resistant organisms (165). Resistant pathogens including Methicillin-resistant *Staphylococcus aureus* (MRSA), extended-spectrum beta-lactamase (ESBL) producing *K. pneumoniae* and *E. coli*, carbapenem-resistant *K. pneumoniae*, and multi-drug resistant *P. aeruginosa* have all been cultured in critically ill COVID-19 patients (165,198). Grasselli *et al*. determined that over half of *S. aureus* isolates were methicillin resistant (205), while the study by Li and colleagues found that all cultures of *S. aureus* and coagulase negative *Staphylococci* were resistant to methicillin, and three quarters of *E. coli* isolates were ESBL producing organisms (194). Furthermore, over 40% of Gram negative organisms exhibited carbapenem resistance, with particularly high levels of resistance in *A. baumanni* and *K. pneumoniae* secondary infections at 91.7% and 76.6% respectively (205). Over half of patients with secondary infections in d'Humières' study were culture positive beyond seven days from their initial culture positivity, which may suggest high rates of treatment failure due to antimicrobial resistance (169). This is a highly concerning trend, as increasing rates of antimicrobial resistance limit the treatment options for managing infections. It is therefore crucial to be able to accurately identify secondary infections to ensure patients are receiving the correct treatment when they need it, while avoiding the potential harms of unnecessary antibiotic exposure.

1.5 Conclusion

CoVs historically have been responsible for minor, self-limiting infections in humans. Within the past three decades, three novel CoVs have been responsible for pandemic diseases. SARS-CoV-2, the most recent to emerge, was the organism responsible for the illness COVID-19 which resulted in one of the deadliest pandemics in modern history. The majority of infected patients have a minor, self-limiting illness, however a subset of patients may develop severe disease associated with organ failure and death. Patients who become critically ill with COVID-19 are at risk of developing a secondary infection, which significantly increases morbidity and mortality. Secondary infections are difficult to identify, therefore antibiotics are often used in excess in COVID-19 patients. New diagnostic techniques which would allow for rapid identification of secondary infections in critically ill patients with COVID-19 would help to ensure that appropriate antimicrobial therapy is provided to the correct patients at the earliest possible opportunity.

2 Sepsis in the Intensive Care Unit

2.1 Introduction

Severe COVID-19 is associated with immune dysfunction and an increased incidence of secondary infections. The clinical features of severe COVID-19 and a secondary infection with sepsis are similar and therefore difficult to differentiate. A high index of suspicion for the development of a secondary infection and sepsis is required by clinicians when critically ill patients with COVID-19 demonstrate a clinical deterioration.

2.2 Definition

Sepsis is a medical emergency clinical syndrome caused by an infective source, causing widespread physiological, biochemical and metabolic derangements (206). It can quickly result in severe organ damage and is often fatal without swift intervention. It is therefore one of the leading causes of mortality globally (207).

The word “sepsis” originates from ancient Greece, where it was used to represent the decomposition and rotting of animal and vegetable material (208). In modern times, the term describes the dysregulated and exaggerated inflammatory host response to an infection. Multiple attempts have been made to provide a clear definition for the syndrome in recent years; however, the variable clinical presentations of sepsis have made it challenging to find an appropriate descriptor. Three iterative consensus definitions have been produced since the 1990s named Sepsis 1, Sepsis 2 and Sepsis 3:

2.2.1 Sepsis 1

In 1991, a task force held jointly by the American College of Chest Physicians and the Society of Critical Care Medicine provided the first consensus definition of sepsis (See *Table 2-1*). This definition was the first to introduce the concept of the systemic inflammatory response syndrome (SIRS): a host inflammatory response which occurs in a variety of clinical conditions such as trauma, burns or pancreatitis. Sepsis was defined as the presence of SIRS specifically in response to infection. Progression to severe sepsis occurred when there was evidence of organ dysfunction, and septic shock was defined as persistent hypotension despite adequate fluid resuscitation (209).

Term	Definition
SIRS	A systemic inflammatory response resulting from various insults, with the following criteria: • Temperature >38°C or <36°C • Heart rate > 90 beats per minute • Respiratory rate > 20 breaths per min or $PaCO_2$ < 32 mmHg • WCC >12,000/mm^3, <4,000/mm^3, or >10% band neutrophils
Sepsis	A systemic response, confirmed by the presence of two or more of the SIRS criteria, as a result of infection.
Severe sepsis	Sepsis resulting in organ dysfunction, hypoperfusion (e.g. raised lactate, oliguria or reduced GCS), or hypotension
Septic shock	Sepsis leading to hypotension despite adequate fluid resuscitation.

Table 2-1:The Sepsis 1 definition. Adapted from (209). *$PaCO_2$: Partial pressure of oxygen, GCS: Glasgow coma scale, WCC: White cell count.*

2.2.2 Sepsis 2

A decade later a new task force convened and re-explored the definitions produced by Bone *et al*. Due to concerns that the SIRS criteria were too sensitive and non-specific, the taskforce sought to expand the accepted signs and symptoms of sepsis for diagnostic purposes. However, there had been very little progress in terms of new evidence for sepsis diagnosis in this time, and so the definitions essentially remained the same as per the Sepsis 1 criteria (210).

2.2.3 Sepsis 3

In 2016 a third international task force convened to discuss the definition of sepsis. This was again prompted by concerns with the over-sensitivity of SIRS. The aim was to update the definition to distinguish sepsis from otherwise uncomplicated infections. The presence of organ dysfunction was deemed to be central to sepsis pathophysiology, thus the definition of sepsis was amended to a "life-threatening organ dysfunction caused by a dysregulated host response to infection". The SIRS criteria were scrapped, and instead the presence of organ dysfunction was defined as an increase in the sequential organ failure assessment (SOFA) score of 2 or more points (211) (See *Table 2-2*). As organ dysfunction was now considered essential for the diagnosis of sepsis, the term "severe sepsis" became obsolete. Septic shock was kept as a term, and was deemed present if there was a vasopressor requirement to maintain a mean arterial pressure (MAP) of 65 mmHg or greater and serum lactate level greater than 2 mmol/L in the absence of hypovolemia (206).

Organ System	Units	Score				
		+0	+1	+2	+3	+4
Respiration	PaO_2/FIO_2, mmHg (kPa)	≥400 (53.3)	<400 (53.3)	<300 (40)	<200 (26.7) with respiratory support	<100 (13.3) with respiratory support
Coagulation	Platelets ($\times 10^3/\mu L$)	≥150	<150	<100	<50	<20
Liver	Bilirubin, mg/dL (µmol/L)	<1.2 (20)	1.2-1.9 (20-32)	2.0-5.9 (33-101)	6.0-11.9 (102-204)	>12.0 (204)
Cardiovascular	MAP or catecholamine dose (µg/kg/min)	MAP ≥70 mm Hg	MAP <70 mm Hg	Dopamine <5 or dobutamine (any dose)	Dopamine 5.1-15 or epinephrine ≤0.1 or norepinephrine ≤0.1	Dopamine >15 or epinephrine >0.1 or norepinephrine >0.1
Central Nervous System	Glasgow Coma Scale	15	13-14	10-12	6-9	<6
Renal	Creatinine (µmol/L) (or urine output)	<110	110-170	171-299	300-440 (or <500 ml/day)	> 440 (or <200 ml/day)

Table 2-2: The Sequential Organ Failure Assessment Score. Adapted from (211).

Since SOFA is a complex tool requiring multiple biochemical tests to ascertain a score, a more clinically focused tool was also devised, named the quick SOFA (qSOFA), which uses three simple clinical measures (respiratory rate, systolic blood pressure and GCS) to enable rapid identification of septic patients (See *Table 2-3*). Patients outside of ICU with a qSOFA score of two or more have a 3 to 14 fold increase in hospital mortality (206).

Criteria	Score
Respiratory Rate ≥ 22 breaths per minute	1
Altered Mental State	1
Systolic BP ≤ 100mmHg	1

Table 2-3: The qSOFA score criteria. Adapted from (206).

2.3 Epidemiology

2.3.1 Incidence

Sepsis is a very common condition. While the true incidence is unknown, a recent Global Burden of Diseases study determined that there are at least 48.9 million incident cases annually worldwide according to the Sepsis 3 definition. The incidence is higher in low and middle-income countries (212). Increasing age is a major risk factor for the development of sepsis, with the majority of cases occurring in patients aged 65 or older (213). Sepsis is a major burden in ICUs, with 30% of patients having a diagnosis of sepsis (214). The incidence of septic shock in ICU was estimated at 10.4% (215).

2.3.2 Mortality

Sepsis accounts for one of the largest causes of mortality worldwide. The global study by Rudd *et al*. found that sepsis was responsible for 11 million deaths in 2017, which accounted for 20% of the total deaths worldwide (212). A diagnosis of sepsis carries with it an in-hospital mortality rate of over 10%. This climbs to over 40% when septic shock is present (206). The mortality rate of septic patients in ICU is double that of non-septic ICU patients at 26% (214).

2.3.3 Economics

Sepsis is an expensive condition. A report in 2013 found that sepsis was associated with the highest aggregate hospital costs at more than $23 billion. This single condition accounted for 6.2% of the total US hospital healthcare expenditure (216).

2.4 Aetiology

Any infection has the potential to cause sepsis, however the most common sources are the lungs (64%), intraabdominal cavity (20%), bloodstream (15%) and genitourinary tract (14%) (217). These figures sum to greater than 100% as patients may present with infections involving more than one site. Hospital inpatients are also at risk of iatrogenic causes, for example secondary to central venous catheters or from surgical site infections.

Sepsis can be caused by all types of microorganism including bacteria, fungi, viruses and parasites; however bacterial sepsis is the most common cause. A study by Vincent and colleagues found that 62% of positive microbiological cultures in ICU are from gram-negative organisms, 47% from gram-positive, and 19% are caused by fungi. Polymicrobial infections are common, hence why these figures again sum to greater than 100%. *S. aureus* is the most common cause of gram positive infections in ICU, whereas *Pseudomonas* species and *E. coli* are the most common gram negative causes (217).

2.5 Management

The initial management of sepsis includes intravenous antibiotics, fluid resuscitation and vasoactive cardiovascular support. Resuscitation with at least 30ml/kg of intravenous fluid should be given within the first three hours of resuscitation. If fluid therapy fails to maintain a MAP greater than 65mmHg then vasopressor support should be commenced, which will typically be noradrenaline. If hypotension persists then adding second line agents such as vasopressin and administration of intravenous corticosteroids may be considered. Source control is crucial to prevent ongoing infection, and this often includes surgical intervention. Microbiological cultures should be gathered to help identify organisms, but this should not delay antimicrobial therapy. Administration of antibiotics within one hour of recognition of septic shock is associated with reduced mortality (218). There have been no breakthrough treatments for sepsis for several decades. Human recombinant activated protein C (APC) had previously shown promise as a treatment for sepsis in phase III trials (219), but was subsequently withdrawn from the market as it failed to demonstrate clinical efficacy (220).

2.6 Sepsis Induced Organ Dysfunction

The exact pathophysiology of sepsis has not been fully delineated; however a key facet is the development of a mismatch between oxygen supply and demand. Excessive inflammation consumes oxygen at an increased rate, while hypoperfusion reduces oxygen delivery to cells leading to tissue hypoxia (221). Endothelial damage activates the coagulation cascade resulting in thrombi formation; as well as release of vasoactive substances including nitric oxide (NO) and arachidonic acid, contributing to vasodilation and reducing blood flow to organs. The injured endothelium has increased permeability to leukocytes and fluid, leading to inflammation and oedema within tissues, causing intravascular fluid depletion (222).

Dysfunctional mitochondria decrease adenosine triphosphate (ATP) yield and increase damaging reactive oxygen species (ROS) production. Apoptosis is triggered when ATP production falls to critically low levels, leading to cell death and organ damage (223). As sepsis progresses, ongoing hypoperfusion and tissue inflammation worsens organ injury (223). MODS occurs when two or more organs are affected, and carries a very poor prognosis (224). The organ dysfunction commonly seen in sepsis is outlined below:

2.6.1 Neurological

Altered mentation is common in initial sepsis presentation and suggests central nervous system failure. Early symptoms of sepsis may include delirium and confusion (225). Sepsis associated encephalopathy may cause severe delirium. This may be reversible with successful treatment of the underlying septic injury (226), however it may also precipitate a chronic cognitive and functional decline, particularly in elderly patients (227). Polyneuropathy due to axonal degeneration may develop later in the illness course due to prolonged periods of mechanical ventilation (226). A experimental model suggests that the cytokine tumour necrosis factor alpha (TNF-α) may be neurotoxic and therefore excessive cytokine release in sepsis may contribute to neuronal damage (228). Post mortem examination of septic patients has demonstrated high incidence of cerebral haemorrhages, ischaemia and micro-abscesses (229).

2.6.2 Respiratory

Sepsis may present with respiratory failure caused by a primary pneumonia. Alternatively, respiratory dysfunction may occur as sepsis progresses, with development of ARDS due to the action of proinflammatory cytokines within the pulmonary circulation causing alveolar inflammation and damaging type I pneumocytes (230). Lung endothelial and epithelial barriers are injured which increases permeability, leading to pulmonary oedema (231). Alveolar fluid impairs gas exchange resulting in hypoxaemia and hypercapnia. Damage to type II pneumocytes reduces surfactant production, decreasing lung compliance and causing atelectasis (232). Platelet and neutrophil accumulation worsens inflammation and consolidation (233). Respiratory failure may necessitate tracheal intubation and mechanical ventilation, however ventilator- induced lung injury (VILI) can exacerbate ARDS (225). Patients who survive the initial injury may develop pulmonary fibrosis, causing a chronic lung disease (232).

2.6.3 Cardiovascular

Up to 50% of patients with septic shock will develop impaired cardiac function. Cytokines such as TNF-α and IL-8 may have a direct cardiotoxic effect resulting in myocardial depression and decreased ejection fraction (234). A decrease in cardiac output will reduce coronary artery blood flow leading to ischaemia. This will further increase myocardial injury and impair heart function (235).

Fluid leakage and pyrexia may lead to hypovolaemia and decreased left ventricular filling pressures (236). Fluid resuscitation may help to correct this, however septic shock results in a significant drop in afterload, reducing cardiac output. Hypotension is caused by inflammatory mediators and cytokines, which increase the activity of inducible nitric oxide synthase (iNOS), leading to increased NO production and reduced vascular tone (237). High dose inotropic and vasopressor support may be required to combat vasoplegia, however there is downregulation of ß-adrenergic receptors in response to NO and proinflammatory cytokines, which may reduce the effectiveness of cardiovascular support (235).

2.6.4 Renal

The pathophysiology of sepsis induced acute kidney injury is not fully understood. Reduced renal blood flow secondary to septic shock resulting in hypoperfusion injury is thought to play an important role. However, an animal study has demonstrated AKI in the presence of hyperdynamic sepsis with increased renal blood flow, suggesting there are other mechanisms at play (238). Peritubular inflammation causing oxidative stress may contribute to microvascular dysfunction and tubular injury (239). Microthrombi formation secondary to clotting cascade activation may contribute to ischaemic damage (240). Other contributing mechanisms may involve RAAS dysfunction and use of nephrotoxic drugs such as such as aminoglycoside antibiotics (241).

2.6.5 Hepatic

The liver has an important protective role during sepsis, acting to detoxify bacteraemic blood and to produce acute phase inflammatory proteins. (242). Hepatic impairment is a less common cause of organ dysfunction in sepsis. It tends to be a late sequela and carries a poor prognosis (243).

Kupffer cells act to remove pathogens from the portal circulation through release of pro-inflammatory cytokines and ROS. During sepsis this response is heightened, and these compounds may cause damage to hepatocytes and sinusoidal endothelial cells. Inflammatory mediators may subsequently spill out into the systemic circulation, causing widespread injuries (244).

Sepsis may reduce hepatocyte bile acid and bilirubin transport capacity leading to cholestasis (245). Bile acid accumulation may be an earlier indicator of hepatic injury than elevated bilirubin and is a predictor of poor outcome. Moreover, drug metabolism may be impaired leading to toxic accumulation of certain compounds, which could further exacerbate liver injuries (243).

2.7 Biomarkers

Sepsis results in widespread inflammation and dysregulation of physiological, metabolic and immune processes, resulting in release of pro-inflammatory mediators including cytokines and acute phase proteins. These compounds may signal a developing septic response, and therefore have the potential to be used as sepsis biomarkers. A biomarker is any molecule which can be used to either diagnose a condition and/or predict the disease outcome (246). Currently 258 potential biomarkers have been considered for sepsis (247). The vast majority of these have examined sepsis prognosis, with few having utility for diagnosis (248). The following compounds have shown the greatest potential as biomarkers of sepsis:

2.7.1 Lactate

Serum lactate is the most commonly utilised biomarker in sepsis (249). This marker forms part of the diagnostic criteria for septic shock (206) and can be used in goal directed therapy to monitor responses to treatment (250). Widespread pro-inflammatory processes in sepsis increase oxygen requirement, while there is a simultaneous reduction in oxygen delivery to tissues. Combined with reduced hepatic clearance and increased ß-adrenergic activity, hyperlactataemia is a common feature of sepsis. However it is a non-specific finding which may be present in any condition which results in increased lactate production or impaired clearance such as haemorrhage or cardiac failure, and therefore has limited value in the initial diagnosis of sepsis (251).

2.7.2 Procalcitonin

Calcitonin is a hormone involved in calcium metabolism by reducing circulating calcium concentration (252). The precursor PCT is normally released by neuroendocrine cells in the thyroid gland and lung; however during sepsis PCT acts as acute phase protein and can be released by essentially all tissue types in the body (253), increasing its concentration up to 10000 times the normal value (254). The exact function of PCT in sepsis is unclear, but it is believed to play a role in chemoattraction and in release of pro-inflammatory cytokines (253,255).

The use of PCT in sepsis has been extensively studied, and there is evidence for utilising PCT as a marker for antimicrobial stewardship. A study by Bouadma and colleagues suggests using a serum PCT level below <0.5 ng/mL as a cutoff for discontinuing antibiotics in sepsis (256). While PCT is elevated in bacterial sepsis, it remains low in other infections. For instance, PCT is elevated in paediatric bacterial meningitis, but remains low in viral causes of meningitis (257). Unfortunately PCT it is also released in response to other non-infectious conditions such as trauma, malignancy and after surgery (253), so is not a sepsis-specific biomarker and its role in sepsis diagnosis remains unclear (258).

2.7.3 C-Reactive Protein

CRP is another acute phase protein produced by the liver in response to inflammation. It was named after the reaction which occurred between this protein and the capsular polysaccharide of pneumococcal bacteria (259). It is a member of the short pentraxin group of proteins and has an important role in initiation of the complement cascade during an inflammatory response (260). Clinically it is widely used as a marker of infection. Normal values for CRP are <10mg/L in healthy individuals, but can climb to over 300mg/L within 4 hours of a severe infection (261). CRP has pro-inflammatory effects such as promoting release of cytokines IL-6 and TNF-α (262), but in high concentrations may also exhibit some anti-inflammatory properties (263).

Studies have suggested that CRP is an effective marker for predicting improvement in sepsis (261). While CRP is a good indicator of systemic inflammation, it is not as sensitive as PCT for identifying bacterial infections (264). CRP is a useful marker for risk of mortality in sepsis (265), but is less useful for sepsis diagnosis. While CRP is often elevated in acute infection, it is a non-specific indicator of inflammation. Thus while it may be used to guide decision making, it does not demonstrate the specificity or sensitivity to be used for diagnostic purposes in sepsis (266).

2.7.4 Cytokines

Cytokines are small molecules less than 25kDa in size released as part of the inflammatory response. They have immunomodulatory properties, with different cytokines exhibiting pro and anti-inflammatory effects. Cytokine release can have both local and systemic effects (267).

Pro-inflammatory cytokines such as TNF-α and IL-6 are increased early on in a septic insult and are responsible for promoting the ongoing inflammatory response. TNF-α is increased by a factor of 10 in septic patients compared to healthy controls, with a sensitivity of 82.6%, a specificity of 91.7% (268). Raised serum TNF-α is associated with poor prognosis and increased 28-day mortality. As such, it is a potential diagnostic and prognostic biomarker (268).

IL-6 levels have been found to be higher in sepsis non-survivors compared with those who survived their illness, suggesting a prognostic role (269). IL-6 levels rapidly rise and fall in the first 6 hours of sepsis, making it a possible marker of the early stages of sepsis (270). A meta-analysis found that in septic adults, IL-6 had a diagnostic sensitivity of 85.0% and specificity of 62.0% (271).

2.7.5 Monocyte Chemoattractant Protein-1

Monocyte chemoattractant protein-1 (MCP-1) is a chemokine: a type of cytokine which activates and encourages movement of leucocytes towards sites of inflammation (272). MCP-1 has shown promise as a prognostic biomarker in sepsis, with several studies linking raised MCP-1 levels with sepsis severity and mortality (273). Zhu and colleagues found that MCP-1 was a better predictor of mortality in sepsis compared with PCT, Acute Physiology and Chronic Health Evaluation II (APACHE II) score and SOFA score (274). A recent meta-analysis also suggested promising results for MCP-1 as a diagnostic biomarker, with a sensitivity of 0.84 and specificity of 0.82 (275). Wang and colleagues found that MCP-1 was a good predictor of sepsis in trauma patients, with an area under the receiver operating characteristic curve (AUROC) of 0.82 (276). Further studies looking into utilising MCP-1 as a diagnostic sepsis biomarker are warranted.

2.7.6 Programmed Death Receptor-1

Programmed death receptor-1 (PD-1) is present on the surface of activated T cells, B cells and monocytes (277). Activation of the receptor inhibits cellular proliferation and cytokine production, and promotes apoptosis, thus PD-1 exerts an anti-inflammatory effect (278). Sepsis resulted in upregulated expression of PD-1 on B cells, cluster of differentiation (CD)4+ and CD8+ T cells in a mouse model 48 hours after caecal ligation and puncture (279). This suggests PD-1 may be a promising diagnostic sepsis biomarker, however human studies are limited. Nivolumab, a PD-1 inhibitor, is currently being investigated as a treatment for sepsis, with successful phase I/II studies (280). Additional research will help elucidate the potential for the PD-1 pathway to be used for diagnosis and/or treatment of sepsis.

2.7.7 Soluble Triggering Receptor Expressed on Myeloid Cells-1

Triggering receptor expressed on myeloid cells-1 (TREM-1) is a receptor present on innate immune cell surfaces which acts to potentiate pro-inflammatory responses during infection. A soluble form (sTREM-1) also exists freely in the plasma, and has been investigated as a sepsis biomarker (281).

Numerous studies have examined sTREM-1 as a prognostic indicator in sepsis with promising results (282–284). Several studies have also shown exciting results for utilising sTREM-1 as a diagnostic sepsis biomarker, with impressive AUROCs of 0.935 (285), 0.925 (286) and 1.0 (287). However other studies have been less encouraging, with AUROCs of 0.62 (288), 0.733 (289) and 0.78 (290); and a sensitivity of 70% and specificity 60% (291). A meta-analysis from 2012 suggested sTREM-1 had moderate performance for diagnosing sepsis, with a pooled sensitivity and specificity of 79% and 80% respectively (292). A recent meta-analysis had more favourable results with a sensitivity of 85% and specificity of 79% (293). As such, the evidence for using sTREM-1 as a diagnostic biomarker in sepsis is inconclusive.

2.7.8 Soluble Urokinase-type Plasminogen Activator Receptor

Urokinase-type plasminogen activator receptor (uPAR) is a glycoprotein receptor found on the surfaces of immune and endothelial cells. It is activated by the serine protease urokinase plasminogen activator (u-PA), promoting proteolytic activity which converts plasminogen to active plasmin causing fibrinolysis. Other actions relate to inflammation and include chemotaxis, cell adhesion and apoptosis (294). Binding of urokinase leads to cleavage of uPAR releasing a soluble form (suPAR) into the plasma. The process of suPAR cleavage is increased during acute inflammation (295).

Plasma suPAR levels have been shown to exhibit promising correlation with sepsis prognosis. A systematic review from 2012 has suggested that suPAR levels are consistently higher in sepsis non-survivors compared with survivors, with an AUROC of 0.80, sensitivity of 89 % and specificity of 63 %. This meta-analysis found that suPAR outperformed CRP, PCT and sTREM-1 as a prognostic biomarker. However suPAR performed poorly as a diagnostic marker of sepsis (296).

Two other more recent meta-analyses have shown better results for sepsis diagnosis using suPAR, with a sensitivity, specificity and AUROC of 0.73, 0.79, and 0.82 (297); and 0.76, 0.78 and 0.83 (298) respectively.

2.8 Conclusion

Sepsis is a life-threatening organ dysfunction in response to infection. Mortality is high, and treatment relies on source control and supportive measures. Definitions of sepsis currently rely on clinical markers of organ dysfunction, therefore a degree of organ damage will have already occurred by the time sepsis is identified. Biomarkers which could help to identify sepsis early and therefore initiate treatment before the organ damage had occurred would likely help to reduce the morbidity and mortality associated with sepsis. Unfortunately, there are no biomarkers currently sufficiently capable of early identification of sepsis.

The situation is even more challenging in conditions like COVID-19, where the clinical features of the primary critical illness present very similarly to a secondary infection. Clinical signs, imaging and routine biochemical tests often show similar trends in primary COVID-19 and secondary infections, making it very difficult to differentiate the two conditions. Examination of the underpinning metabolic perturbations may help to yield new biomarkers capable of distinguishing patients with sepsis due to a secondary infection from those with severe COVID-19.

3 Mechanisms of Sepsis and COVID-19

3.1 Introduction

The exact pathophysiological mechanisms of sepsis are not fully understood; however immune dysfunction plays a central role in disease progression. During a localised infection, there is a balance between pro and anti-inflammatory mediators which leads to a controlled and coordinated immune response (232). During sepsis, this coordination is lost and a surge in release of inflammatory mediators into the systemic circulation results in a disordered and damaging immune response distant to the local site of infection (299). This response is uncontrolled, with increased release of catecholamines and cortisol; activation of complement cascades; and release of adhesion molecules and cytokines. The resultant distributive shock, tissue hypoxia and mitochondrial dysfunction ultimately causes a failure of ATP production (299,300).

Microorganisms exist everywhere. Most are harmless; however some can cause illnesses. The immune system must eliminate pathogenic organisms which could disrupt normal physiology, while avoiding damage to the host. There are two categories of the immune system: the innate and adaptive immune responses.

3.2 Immune Response

The innate immune system is an ancient pattern recognition immune response present in almost all living organisms (301). It provides an immediate, non-specific first line of defence against infections without having to recognise the specific identities of each invading pathogen (302). The innate immune system is composed of barriers such as the skin, endothelium and gastric acid; myeloid phagocytes including granulocytes, macrophages and dendritic cells (DCs); and the complement system (303,304).

The adaptive immune system uses B and T lymphocytes to produce coordinated antigen binding responses against specific microbes. This takes longer to achieve than an innate response since a smaller number of cells are involved (272). The adaptive immune response is able to generate an immune memory, whereby cells which have previously encountered a pathogen persist, ready to mount a quicker and more efficient response the next time the same pathogen is encountered (302,303).

The innate immune system is responsible for initial detection of pathogens, and as such it key to the immune dysregulation seen in sepsis.

3.2.1 Pathogen Detection

An infection occurs when a pathogen accesses a normally sterile part of the body by breaching defensive barriers. This immediately triggers an innate response via a series of inflammatory pathways. To initiate the innate response, the pathogen needs to first be detected (301).

A group of receptors expressed on leukocytes called pattern recognition receptors (PRRs) are responsible for initiating the innate immune response. They detect various molecules known as pathogen associated molecular patterns (PAMPs), which are conserved components shared by many types of microorganisms (305). PAMPs include lipids, proteins, lipoproteins, and nucleic acids. Structural cell wall components such as lipopolysaccharide (LPS) in Gram negative species; and lipoteichoic acid (LTA) and peptidoglycan (PepG) in Gram positive organisms are important PAMPs in bacterial sepsis (306).

Another group of compounds called damage associated molecular patterns (DAMPs) or alarmins can also activate PRRs. These are endogenous molecules which are released from injured host cells such as high-mobility group box-1 (HMGB-1) protein, heat shock proteins (HSPs), fibrinogen, and mitochondrial deoxyribonucleic acid (DNA) (307,308). This allows an inflammatory response to be initiated in response to host tissue damage (309). Therefore, a combination of exogenous PAMPs from invading pathogens and endogenous DAMPs from injured cells act on PRRs to initiate and amplify the innate immune response.

Four categories of PRR have been identified in vertebrates (310). Toll-like receptors (TLRs) have a wide range of activity and detect PAMPs from bacteria, fungi, viruses and protozoa. Nucleotide oligomerisation domain (NOD)-like receptors (NLRs) can detect bacterial fragment PAMPs. Retinoic acid-inducible gene-1 (RIG-1)-like receptors (RLRs) bind PAMPs from viruses. C-type lectin receptors (CLRs) recognise PAMPs associated with *Leishmania*, viruses, and fungi (310–312). TLRs were the first to be identified and have been most thoroughly investigated. Activation of PRRs initiates various signalling pathways which result in upregulation of expression of genes involved in the inflammatory response (307).

3.2.2 Toll-like Receptors

The Toll protein, subsequently named Toll-1 (313), was first identified in *Drosophila*, where it was found to have a role in dorsoventral orientation of embryos (314). Subsequently, Toll-1 was found to have a protective role against fungal infections in adult *Drosophila* (315). It is now known that an entire family of dimerized Toll proteins exist as TLRs, which are expressed on many cells of the immune system, primarily on the membranes of macrophages and DCs (302).

As shown in *Figure 3-1*, TLRs contain an exterior domain composed of a horseshoe structure of leucine-rich repeats (LRRs) which are responsible for recognition of PAMPs. They also have a transmembrane region, and a cytosolic Toll-IL-1 receptor (TIR) domain responsible for initiating secondary messenger signalling pathways (316). Thirteen different TLRs have been identified in mice, however only the first ten of these have been detected in humans (317) (See *Table 3-1*).

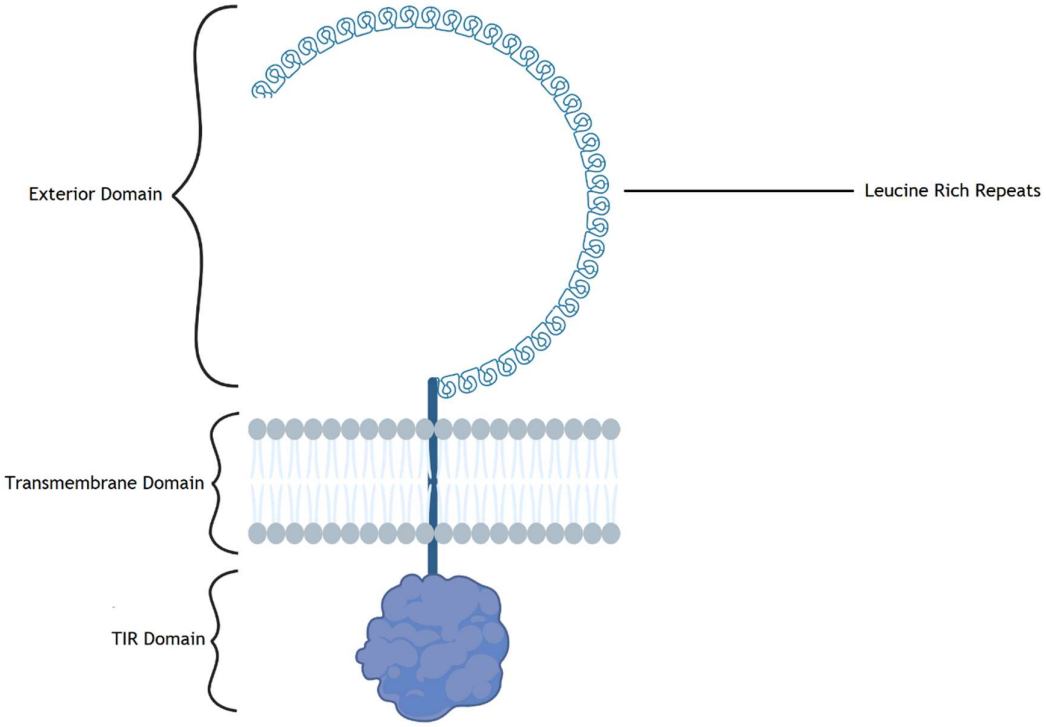

Figure 3-1: Structure of the Toll-like receptor. Adapted from (318).

Receptor	Ligands
TLR1	Triacyl lipoproteins
TLR2	Lipoproteins, lipopeptides, PepG, LTA, glycoproteins, glycolipids, β-glucan, mannan, HSP70
TLR3	Double-stranded RNA
TLR4	LPS, HSP60, HSP70, fibrinogen
TLR5	Flagellin
TLR6	Diacyl lipoproteins, LTA, β-glucan
TLR7	Single-stranded RNA
TLR8	Single-stranded RNA, imidazoquinolines
TLR9	Bacterial DNA, haemozoin
TLR10	Unknown

Table 3-1: Human Toll-like receptors and their ligands. Adapted from (272,319). *RNA: ribonucleic acid*

TLRs can be expressed on cell surfaces or intracellularly. TLRs 1, 2, 4, 5, 6, and 10 are cell surface TLRs, and are primarily responsible for detecting pathogen membrane components. Of note, TLR 4 binds LPS, and TLR2 in combination with TLR 1 or 6 binds PepG and LTA among other compounds, making these receptors key early responders in bacterial infections. TLRs 3,7,8, and 9 are intracellular receptors which mainly detect bacterial and viral nucleic acids (320).

Activation of TLRs triggers multiple intracellular signal transduction pathways (321). The end result of these signalling cascades is the liberation and activation of several transcription factors such as nuclear factor-κB (NF-κB), activator protein-1 (AP-1) and interferon regulatory factor (IRF) 3 which translocate to the nucleus and upregulate expression of pro-inflammatory genes (321–324). This stimulates the production and release of cytokines, chemokines and IFNs (320). These compounds further amplify the innate immune response, leading to additional pro-inflammatory cytokine release, as well as triggering cells of the adaptive immune system (302).

3.2.3 Nuclear Factor-κB

NF-κB is a group of proteins which can alter transcription of genes controlling a range of processes including immune regulation, ROS production, cytokine release and cell survival. NF-κB is comprised of subunits which contain Rel homology domains (RHDs) which are capable of binding to DNA at specific κB sites (325). Upon activation, NF-κB translocates to the nucleus and the RHDs bind to the κB sites, increasing transcription of early activation genes, resulting in increased production of cytokines including TNF-α, IL-1, IL-6, IL-12, IL-18 and interferons, as well as inflammasome precursors including pro-IL-1β (307). These in turn promote release of additional cytokines as well as complement and coagulation pathway activation (232,307).

3.2.4 The Inflammasome

The inflammasome is a structure formed from several different protein subunits which conjoin after PRR signalling. Once assembled, the inflammasome is capable of cleaving and activating important pro-inflammatory molecules. For instance, inflammasome cleavage of pro-caspase 1 produces the activated caspase 1, which can subsequently cleave the inactive pro-IL-1ß and pro-IL-18 into pro-inflammatory cytokines IL-1ß and IL-18. Additionally, inflammasome activation can in turn increase NF-κB activation in neighbouring cells, creating a self-perpetuating inflammatory response (326).

Inflammasome assembly can trigger a type of cell death called pyroptosis which causes cytosol contents to be released from dying cells. This helps control the number of immune cells participating in the inflammatory response, while increasing local production of DAMPs and pro-inflammatory cytokines (327).

3.3 Cytokines

PRR signalling leads to an increased inflammatory response which is mediated via cytokines. Several different categories exist, including interleukins, chemokines, interferons, and growth factors (267).

Proinflammatory cytokines include interleukins IL-1α IL-1ß, IL-6, IL-8, IL-12, and IL-17, along with TNF-α, macrophage inhibitory factor (MIF) and IFN-γ (307,328). These cytokines promote pyrexia, stimulate release of additional pro-inflammatory cytokines and acute phase proteins, and act as chemoattractants (328). While this contributes to destruction of pathogens, excessive cytokine release may result in host tissue damage (299).

Anti-inflammatory cytokines include IL-1, IL-6, IL-10, and transforming growth factor ß (TGF-ß) (307). They reduce the immune response and ultimately help to terminate it once pathogens have been destroyed (329).

Several key pro-inflammatory cytokines are discussed in more detail below:

3.3.1 Interleukin-1

IL-1 is a subgroup of interleukins released by monocytes and macrophages. IL-1α and IL-1ß are the most widely studied IL-1 cytokines (329). IL-1ß plays an important role in the inflammatory response, and is activated by caspase-1 in response to inflammasome formation (328). Binding of IL-1ß to the IL-1 receptor increases pro-inflammatory signalling and results in additional cytokine production via the AP-1 transcription factor. As a result, IL-1 leads to a large increase in the release of pro-inflammatory cytokines, growth factors and colony-stimulating factors. Systemic effects can include fever, neutrophilia, tachycardia, and hypotension through increased circulating NO (330). Increased plasma levels of IL-1ß have been shown to correlate with increased mortality in sepsis (331).

3.3.2 Interleukin-6

IL-6 is a cytokine produced by macrophages and T-cells which can exhibit both pro- and anti-inflammatory effects (307). It is produced in response to many different conditions including trauma, burns and cardiovascular disease as well as sepsis (328). It has several actions including promotion of differentiation of B cells, release of acute phase proteins, and haematopoiesis regulation (332). IL-6 can activate the JAK/STAT3 pathway, which upregulates genes controlling the production of IL-8, MCP-1 and vascular endothelial growth factor (VEGF). Moreover, additional IL-6 is released, creating a positive feedback loop (333). IL-6 will also activate AP-1, further increasing pro-inflammatory cytokine release (334). IL-6 promotes B lymphocyte activation, but also encourages activation of innate immune cells including neutrophils, macrophages and natural killer (NK) cells (335). There is an association with IL-6 levels and disease severity in sepsis, as persistently raised plasma IL-6 is associated with poorer outcomes (336).

3.3.3 IL-12

The cytokine IL-12 is released from immune cells such as dendritic cells, macrophages and B-lymphocytes. The main function of IL-12 is to facilitate the differentiation of naïve T-cells into mature T helper$_1$ (Th_1) cells. Additionally, IL-12 leads to stimulation of release of large quantities of TNF-α and IFN-γ from T-cells and NK cells (328). IL-12 levels have been shown to be persistently raised in septic patients who died compared with sepsis survivors (331)

3.3.4 IL-17

IL-17 is a family of potent pro-inflammatory cytokines primarily released from Th_{17} CD4+ memory T cells, but may also be produced by cytotoxic T cells and NK cells (337,338). IL-17 is released upon stimulation of TLR-5 by bacterial flagellin protein (339). IL-17 has an important role in linking the innate and adaptive immune response. Several autoimmune and inflammatory disorders including Crohn's disease are thought to have impaired IL-17 signalling involved in their pathogenesis (340). IL-17 acts upon many different cell types such as monocytes, macrophages, epithelial cells and fibroblasts. IL-17 signalling upregulates release of a broad range of pro-inflammatory cytokines including interleukins, colony-stimulating factors and chemokines (337).

3.3.5 TNF-α

TNF-α is a member of the TNF family, first identified as a compound with anti-cancer activity when it was observed that tumours could undergo haemorrhagic necrosis following certain bacterial infections (341). TNF-α is released from many different cell types including neutrophils, macrophages, eosinophils, NK cells and CD4+ T cells (307). TNF-α activates both the AP-1 and the NF-κB pathways, resulting in pro-inflammatory cytokine release. Moreover, TNF-α also induces apoptosis, releasing cell contents which further exacerbate the local inflammatory response (342). TNF-α also acts as an adipokine and is associated with obesity-linked insulin resistance and type 2 diabetes mellitus (343). TNF-α levels have repeatedly been shown to be greatly elevated during sepsis (328).

3.3.6 Interferons

IFNs are a group of proteins which exhibit both antiviral and antibacterial effects (344). They were named after their ability to interfere with viral replication (345). Other effects include activation of NK cells and macrophages, and assistance with antigen presentation to lymphocytes (344). PAMP binding with TLR-3 can trigger nuclear translocation of IRF 3 and 7, which in turn increases production of type I interferon (321,344).

Three groups of IFNs exist. Type I IFN includes IFN-α and IFN-β which are the most studied interferons in this class. Practically all cell types can produce type I IFN, and the type I IFN receptor is expressed on almost all cells in the human body, hence this type of interferon has a broad range of actions (346). They bind to receptors predominantly on T lymphocytes, macrophages and monocytes, promoting antiviral and antibacterial action (344).

There is only one member of the type II IFN group: IFN-γ. This compound is released by CD4+ and CD8+ T cells and NK cells in response to activation by cytokines such as IL-12, IL-15, IL-18, and type I IFN (346). IFN-γ increases release of pro-inflammatory cytokines IL-2, IL-6 and TNF-α (344). Additional actions include increasing lymphocyte activity, blocking viral replication and anti-cancer effects by limiting cell proliferation (344). IFN-γ increases TLR4 expression, further promoting pro-inflammatory signalling (347). IFN-γ expression has been shown to be persistently enhanced in patients who died of sepsis (331).

Type III IFN is comprised of four IFN-λ subtypes. Receptors for type III IFN are predominantly located on epithelial cell surfaces, and as such they form a key part of the first line of defence in viral infections (346). After binding to its receptor, IFN-γ signals via JAK1, JAK2, STAT1 and AP-1 pathways (272,344).

3.3.7 Chemokines

Chemokines are signalling molecules which play a role in recruitment and activation of different cells of the immune system (272). They are prefixed depending on whether they contain two adjacent cysteine residues (CC) or have a different amino acid between these residues (CXC) (307). Chemokines have a degree of specificity in terms of which leukocytes they recruit. CX3CL1 recruit monocytes, CXCL1 and CXCL2 recruit neutrophils, and CXCR3 recruits T lymphocytes. Chemokines aid in the coordination of an immune response by directing leukocytes towards the site of infection and stimulating the release of cells from the bone marrow and spleen (272). IL-8 is an important chemokine released by macrophages and endothelial cells. It is also known as neutrophil chemotactic factor due to its ability to promote chemotaxis of neutrophils (328).

3.3.8 Growth Factors

Growth factors are mediators which can affect the proliferation and activity of a variety of cells. An important example is the granulocyte macrophage-colony stimulating factor (GM-CSF) which is a haemopoietic growth factor responsible for stimulating proliferation of bone marrow progenitors (348). GM-CSF is released by several cell types including T-cells, macrophages, mast cells and endothelial cells (328). It increases signalling via multiple pathways leading to activation of NF-κB, JAK2/STAT5, and AP-1, and results in the maturation of neutrophils, monocytes and basophils (328,348).

3.3.9 Anti-Inflammatory Cytokines

Anti-inflammatory cytokines act to diminish the inflammatory response, reducing potential damaging effects of uncontrolled inflammation. Important anti-inflammatory cytokines are IL-1 receptor antagonist (IL-1RA), IL-4, and IL-10.

IL-1RA is an endogenous blocker of the IL-1 receptor, and thus prevents the pro-inflammatory effects of IL-1α and IL-1β (349). IL-4 promotes lymphocyte proliferation and halts programmed cell death. IL-10 is a crucial anti-inflammatory cytokine and suppresses the release of many different pro-inflammatory cytokines (309,328,350).

3.4 The Cytokine Storm

In health, the effects of pro-and anti-inflammatory cytokines are well balanced to enable clearance of pathogens while minimising collateral damage to the host. During sepsis, this balance is lost and a hyperinflammatory state may occur (351). NF-κB induced expression of pro-inflammatory cytokines includes IL-1, IL-12, IL-18, TNF-α and IFNs. These cytokines stimulate the release of additional cytokines such as IFN-y, IL-6 and IL-8 (232). In sepsis, the increased production of cytokines can trigger a cytokine storm: a self-perpetuating cascade of massive cytokine release which triggers systemic complement and coagulation cascades and widespread inflammation leading to tissue damage (352). Pro-inflammatory cytokines IL-1, IL-6 and TNF-α are most significant players in the development of the cytokine storm. They encourage the pro-coagulant state, stimulate production of NO, and release of ROS (328,353).

3.5 Complement

The complement cascade refers to a group of soluble plasma proteins that are preformed by the liver. These proteins act as early activators of the innate immune response (354). Infection or tissue damage triggers activation of a series of around 30 complement proteins in a stepwise manner. This leads to a plethora of activated complement proteins at the site of infection. Complement has an important role in promoting chemotaxis and increasing vascular permeability. They are activated by pathogens coated in antibodies (the classical pathway), by spontaneous hydrolysis of C3 (the alternative pathway), or by pathogens coated in carbohydrate binding proteins (the lectin pathway) (355).

The classical complement pathway is triggered by antibody activation of C1 protein, and thus is a result of the acquired immune system. However, complement activation is also achieved by the alternative pathway which does not require C1 activation. Here, microbial membrane carbohydrates trigger the activation of C3b, leading to opsonisation and initiation of the cascade (302).

Complement activation results in the C3 convertase enzyme breaking the C3 protein into 3a and 3b components. The C3b component acts to opsonise the pathogen, identifying it as harmful and encouraging phagocytosis. C3b also forms a subunit of the membrane attack complex (MAC) which forms a pore in pathogen membranes, causing influx of water and ions, ultimately resulting in pathogen destruction. C3a acts as a chemoattractant and encourages migration of additional phagocytes towards the pathogen (354,355).

Complement undergoes widespread activation during a septic insult (356). Proteins C3a and C5a, known as the anaphylatoxins are key players. Component C5a is a chemoattractant and promotes neutrophil migration into the microcirculation causing tissue damage. Additionally, C5a causes activation of coagulation cascades through tissue factor. Concurrently, thrombin activation leads to further production of C5a and C5b from C5, creating a spiral of increased complement, coagulation and fibrinolytic pathway activation resulting in microcirculatory dysfunction (307). Excessive activation of the complement cascade leads to caspase 11 pathway activation which promotes apoptosis, worsening sepsis severity (357,358).

3.6 The Endothelium and Coagulation

Inflammation and coagulation are intrinsically linked, and both play a central role in sepsis pathogenesis. Coagulation can initiate inflammatory cascades and vice versa. As such, coagulopathy commonly occurs sepsis (359).

The endothelium forms the innermost lining of blood vessels. In health, endothelial cells have anticoagulant properties through expression of thrombomodulin and heparin which help to maintain control over the coagulation cascade. Endothelial cells express PRRs and thus form a component of the innate immune system. PAMPs released into the bloodstream and DAMPs such as pro-inflammatory cytokines can trigger a response. Endothelial cell activation increases expression of adhesion molecules such as intercellular adhesion molecule 1 (ICAM1) and vascular adhesion molecule 1 (VCAM1) which allow for leukocytes to bind to the walls of the vasculature and migrate into target tissues. Endothelial cells also produce chemokines, encouraging additional leukocytes to migrate out of the vasculature, as well as production of additional procoagulant factors (272).

Endothelial dysfunction occurs in sepsis. Breaching of the endothelium exposes collagen and tissue factor. Collagen binds to von Willebrand factor (vWF) which can subsequently bind platelet glycoprotein 1β, resulting in platelet activation and clot formation. Activated platelets can encourage additional inflammatory responses by releasing thrombin and activating DCs (353).

Increased tissue factor expression triggers the coagulation cascade via the extrinsic pathway, as tissue factor complexes with factor VII, which can then convert factor X to factor Xa. This results in increased thrombin generation, endothelial fibrin deposition, complement activation and clot formation. Paradoxically, excessive coagulation cascade activation combined with a decrease in protein synthesis and increased protein catabolism in sepsis can result in depletion of clotting factors, increasing the risk of bleeding (360).

During an inflammatory response the anticoagulant effect is diminished as endothelial cell surface thrombomodulin and heparin expression is reduced. As a result, activation of the coagulation cascade occurs more readily. Thrombin acts on endothelial cells to increase permeability. Moreover, thrombin causes further release of cytokines and adhesion molecules from endothelial cells, encouraging additional inflammation (361). Antithrombin, APC and heparin production is reduced, decreasing anticoagulant capacity (362). Additionally, there is an increase in production of plasminogen-activator inhibitor type-1 (PAI-1), which decreases fibrinolysis through inhibition of tissue plasminogen activator (t-PA) and u-PA. Excessive coagulation can cause DIC, resulting in microthrombi formation, leading to microcirculatory occlusion and reduced organ perfusion. Subsequent secondary haemorrhage may then occur as platelets and coagulation factors are consumed (353).

3.7 Mitochondrial Dysfunction

Mitochondria are cellular organelles which perform several important functions including lipid and steroid production, calcium signalling, regulation of metabolism, and triggering apoptosis. However, one of their most important functions is energy generation, as they are the site of ATP production during aerobic respiration via oxidative phosphorylation (OXPHOS).

Sepsis leads to stagnant hypoxia, reducing oxygen delivery to mitochondria. This causes incomplete oxidation within mitochondria, leading to increased free radical formation. ROS depletes endogenous antioxidant stores leading to a state of oxidative stress (300). Pro-inflammatory cytokines increase iNOS activity, increasing NO production. NO can react with superoxide (O_2^-) to form the reactive nitrogen species (RNS) peroxynitrite ion ($ONOO^-$). This is a highly reactive molecule which can cause massive mitochondrial damage, inhibiting the electron transport chain (ETC) and disrupting respiration, thereby generating even more ROS. This results in a cycle of ROS induced ROS release, causing overwhelming mitochondrial damage, triggering inflammasome mediated apoptosis (223,224). Mitochondrial dysfunction is central to the development of MODS in sepsis (223).

3.8 Anti-inflammatory Phase

Septic patients who survive the initial systemic inflammatory response may then enter an immunosuppression phase, with a switch towards anti-inflammatory mechanisms to counterbalance the initial pro-inflammatory response. Release of anti-inflammatory cytokines such as IL-1RA and IL-10 dampen down inflammatory cascades (349,350,363). Increased secretion of glucocorticoids and reduced metabolism of cortisol also helps to reduce the inflammatory response (364). Additional anti-inflammatory mechanisms are highlighted below:

3.8.1 Lymphopenia

A reduced lymphocyte count is commonly observed in septic patients (365). Specifically, a reduction in B and CD4+ lymphocytes has been observed (366). Depletion of lymphocytes likely occurs through caspase-3 triggered apoptosis (367). In sepsis, there is increased expression of PD-1 and other inhibitory immune checkpoint mediators on T cells and antigen presenting cells (368). A depletion of circulating B lymphocytes and associated impairment in IgM production is associated with increased mortality in sepsis (369).

Circulating CD4+ and CD8+ T cell numbers are significantly reduced during sepsis (370), and while numbers return to normal values for most patients, failure for lymphocyte count to return to the normal range is associated with a poor prognosis (371). T-cell depletion with an associated increase in regulatory T (T_{reg}) cells has been seen in septic patients who have experienced reactivation of latent viruses, suggesting that sepsis associated immunosuppression predisposes patients to secondary infections (372,373).

3.8.2 Antigen Presenting Cells

Sepsis may also induce apoptosis of other immune cells, including antigen presenting cells such as monocytes, DCs and macrophages. Septic shock patients have significantly lower circulating DCs than healthy controls which can contribute to immunosuppression through impaired B and T cell function (374,375). Surviving DCs and monocytes have reduced cell surface monocyte leukocyte antigen-DR expression, which is associated with reduced monocyte pro-inflammatory responses and a failure to promote T-cell responses to antigens (376).

A reduction in total circulating macrophages is seen in sepsis, with a transition from pro-inflammatory M1 phenotypes to anti-inflammatory M2 macrophages predominating. This results in a reduction in pro-inflammatory cytokine production, and an associated increased release of anti-inflammatory mediators including IL-10 and TGF-β (377).

3.8.3 Immature Neutrophils

In sepsis there is increased release of myeloid cells from the bone marrow. Immature cells are released to quickly increase white cell numbers. These immature neutrophils have impaired phagocytic ability (378) and produce fewer neutrophil extracellular traps (NETs) which act to ensnare and immobilise pathogens (379). Immature myeloid cells release increased quantities of IL-10 and TGF-ß, promoting anti-inflammatory mechanisms (380). Additionally, immature cells can also trigger CD4+ T cell apoptosis, further suppressing lymphocyte activity (381).

These mechanisms of immunosuppression initially act to dampen down the excessive damaging inflammatory response. However, the persistence of anti-inflammatory mechanisms can lead to immunoparalysis, whereby sufferers are unable to mount an effective immune response against pathogens. This makes them vulnerable to developing secondary nosocomial infections and/or reactivation of latent infections (352,382).

3.9 COVID-19 Pathophysiology

Many of the inflammatory pathways involved in bacterial sepsis are also activated in COVID-19. SARS-CoV-2 RNA acts as a PAMP and activates TLRs 3, 4, 7 and 8, promoting NF-κB and IRF3/7 inflammatory pathways, increasing the expression of IL-1, IL-6, TNF-α and IFNs (383). Furthermore, viral nucleic acids activate cytoplasmic PRRs including RIG-I and melanoma differentiation-associated protein 5 (MDA5) which also promote translocation of NF-κB and IRF3 into the nucleus, increasing expression of type I and III interferons and pro-inflammatory cytokines (384,385). Another intracellular receptor known as NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) is activated in response to SARS-CoV-2 infection (386). This leads to the assembly of the NLRP3 inflammasome which activates caspase-1, stimulating the release of IL-1β and IL-18, and promoting apoptosis (387).

This pro-inflammatory response blocks viral replication, encourages phagocytosis of infected cells, and recruits the adaptive immune system (388). In the majority of SARS-CoV-2 infections, this response is sufficient to lead to viral clearance and resolution of illness. However, a subset of patients experience a persistent and exaggerated inflammatory response resulting in severe disease. Similarly to bacterial sepsis, a cytokines storm is observed, with high circulating levels of TNF-α, IL-1, IL-6 and GM-CSF (24). IL-6 signalling triggers JAK/STAT signalling pathways, promoting lymphocyte activation, as well as further neutrophil, macrophage and NK cell activation. Massive cytokine release includes IL-8, VEGF and MCP-1, as well as additional IL-6, creating a positive feedback loop (333).

An important result of the innate immune response in viral infections is release of INFs. These play a key role in viral destruction, and in mild disease this is sufficient to clear the virus leading to disease resolution (389). In severe COVID-19, inhibition of INF responses is seen through dendritic cell dysfunction (390). Lymphopenia is frequently observed in severe SARS-CoV-2 infection through apoptotic lymphocytes (24,391) secondary to impaired activation of T cells by damaged antigen presenting cells (333). Reduction in CD4+ and CD8+ T cells further decreases production of IFN-γ (333,391). Reduced INF production enables viral replication, resulting in additional pro-inflammatory cytokine release (390,392). Ultimately, excessive inflammatory mediator release can precipitate a cytokine storm much in the same way as in severe sepsis, resulting in increased vascular permeability, hypotension, shock, pulmonary oedema, ARDS and multi organ failure.

3.10 Conclusion

Bacterial sepsis and COVID-19 both share common immune signalling pathways and produce many of the same inflammatory mediators. It can therefore be extremely challenging to identify secondary infections in COVID-19 patients, as the inflammatory response is similar in both conditions. Commonly used markers of infection such as WCC, CRP and PCT are often elevated in severe COVID-19 regardless of the presence of a secondary infection. A metabolomics study may be able to identify signals capable of differentiating the inflammatory response seen in a secondary infection from that of severe COVID-19.

4 Metabolomics

4.1 Introduction

The “omics” disciplines are high-throughput fields of research within systems biology which aim to identify and quantify all biomolecules within a particular set. The four fundamental omics disciplines are genomics, transcriptomics, proteomics and metabolomics, studying the entire complement of genes, RNA, proteins and metabolites respectively. As such, omics studies can generate a huge amount of data. Rather than focussing on a specific biological pathway, an omics study permits the investigation of how multiple pathways interact with each other (393).

4.2 The Metabolome

The metabolome consists of the entire complement of metabolites within a given biofluid (394). Metabolites are small molecules of size 1500 kDa or less, and include endogenously produced compounds such as amino acids, lipids, hormones, carbohydrates, nucleic acids and volatile organic compounds, as well as exogenous compounds and xenobiotics such as drug metabolites and foreign molecules produced by pathogens (393,395). The metabolome does not exist in a fixed state, and its composition will vary depending on the physiological state of the host. Age, diet, stress, drug effects and disease will all alter the metabolome. Thus metabolomics studies aim to investigate the metabolome under a specific set of circumstances, providing a snapshot of the metabolome at a given point in time (393). Strictly speaking, the term “metabonomics” is used to describe studies which investigate the metabolic response to a specific biological change, whereas “metabolomics” refers to the description and quantification of all metabolites within a set, and how they interact with each other. In practice the terms are used interchangeably (395).

A metabolomics study uses a "top-down" approach, whereby the results of metabolic perturbations are used to generate hypotheses regarding the wider network structure of upstream regulatory mechanisms (396). Indeed, upstream genomic, transcriptomic or proteomic perturbations from environmental or disease processes are represented and magnified in changes to the metabolome, therefore metabolomics provides the closest representation of the host phenotype of all the omics disciplines (397,398). This makes metabolomics an ideal modality for novel biomarker discovery (399) (See *Figure 4-1*).

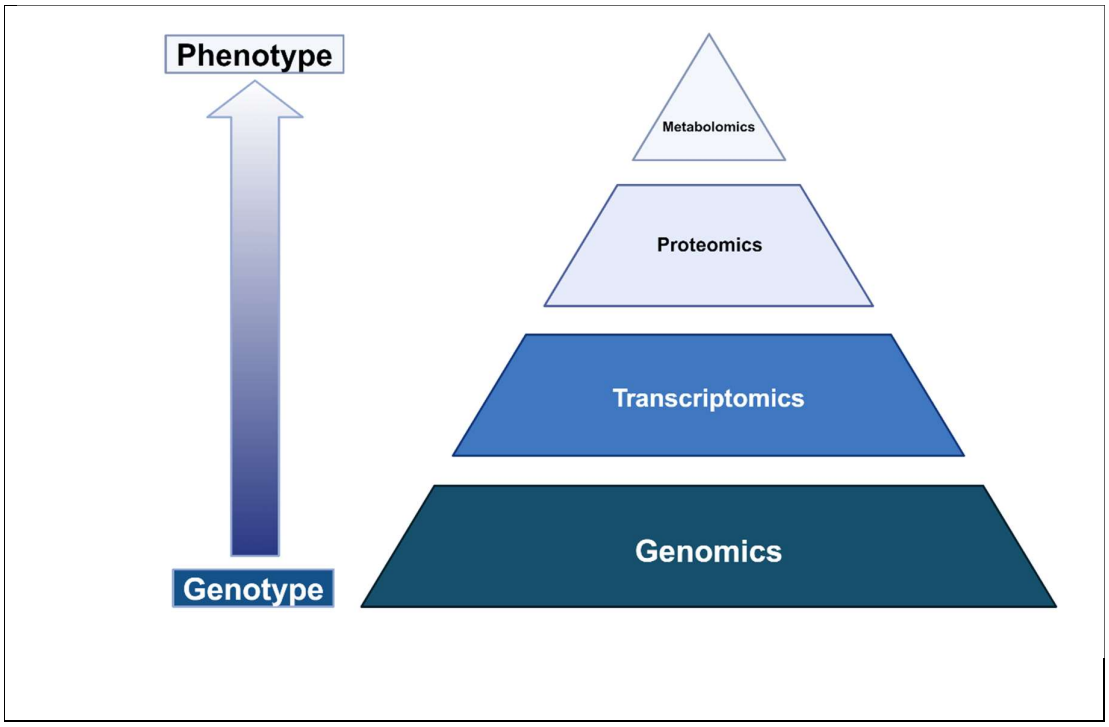

Figure 4-1: The omics hierarchy. Adapted from (400)

4.3 Sampling and Metabolite Extraction

Metabolomics studies typically start by identifying an illness of interest, and recruiting cohorts of affected and unaffected individuals (352). Biological samples are collected from participants, which are most commonly blood (serum or plasma), but any biofluid is possible for analysis including saliva, urine, and cerebrospinal fluid, as well as solid tissues (401).

Once the samples are collected, they need to be ‘quenched’. This is the process of terminating enzymatic activity within the sample to prevent ongoing metabolism from occurring. This can be achieved by rapidly freezing the sample to a temperature of −80 °C. Care should be taken to collect and process all samples as uniformly and consistently as possible, as even small changes such as variation in the background temperature may add in uncertainty and error into the analysis (402).

Once the samples have been collected and frozen, metabolites must be separated. This may occur through ultrafiltration or liquid-liquid extraction using a solvent such as methanol-chloroform. The advantage of solvent extraction is that lipid metabolites will be extracted, whereas these will be lost during the ultrafiltration process (403,404).

4.4 Metabolomic Analyses

Various analytical techniques may be used for metabolomics studies such as capillary electrophoresis, infrared spectroscopy, and Raman spectroscopy; however the most commonly used tools are nuclear magnetic resonance (NMR) spectroscopy, gas chromatography/mass spectrometry (GC/MS), or high performance liquid chromatography/mass spectrometry (HPLC/MS) (399).

4.4.1 Nuclear Magnetic Resonance Spectroscopy

NMR spectroscopy utilises powerful magnets to manipulate the spin of nuclei within a sample. Numerous types of nuclei may be utilised in NMR spectroscopy including ^{13}C, ^{15}N and ^{31}P, however proton (^{1}H) is most commonly used in metabolomics due to the ubiquitous presence of hydrogen within metabolites (405). *Figure 4-2* outlines the basic principles of NMR spectroscopy. In summary, when a sample is placed within an electromagnetic field, the spin of the protons can be made to either align with the magnetic field (a low energy state) or opposed to it (a high energy state). A second pulse of electromagnetic radiation termed the radiofrequency (RF) pulse can then be applied to the sample. The protons will absorb the RF pulse, and in doing so will enter the high energy state of aligning in opposition to the magnetic field. Once the RF pulse stops, the protons return to the low energy state, releasing the absorbed energy in a process known as free induction decay (FID). A RF receiver can be used to detect these signals, and using Fourier transformation, an NMR spectrum can be produced from the FID by plotting peaks based on the chemical shift in parts per million against the signal intensity. The resultant spectrum is different for each metabolite and acts as a unique identifier, and the area under the peak is proportional to metabolite concentration. As such, NMR spectroscopy can rapidly provide both identity and quantification of metabolites (406,407).

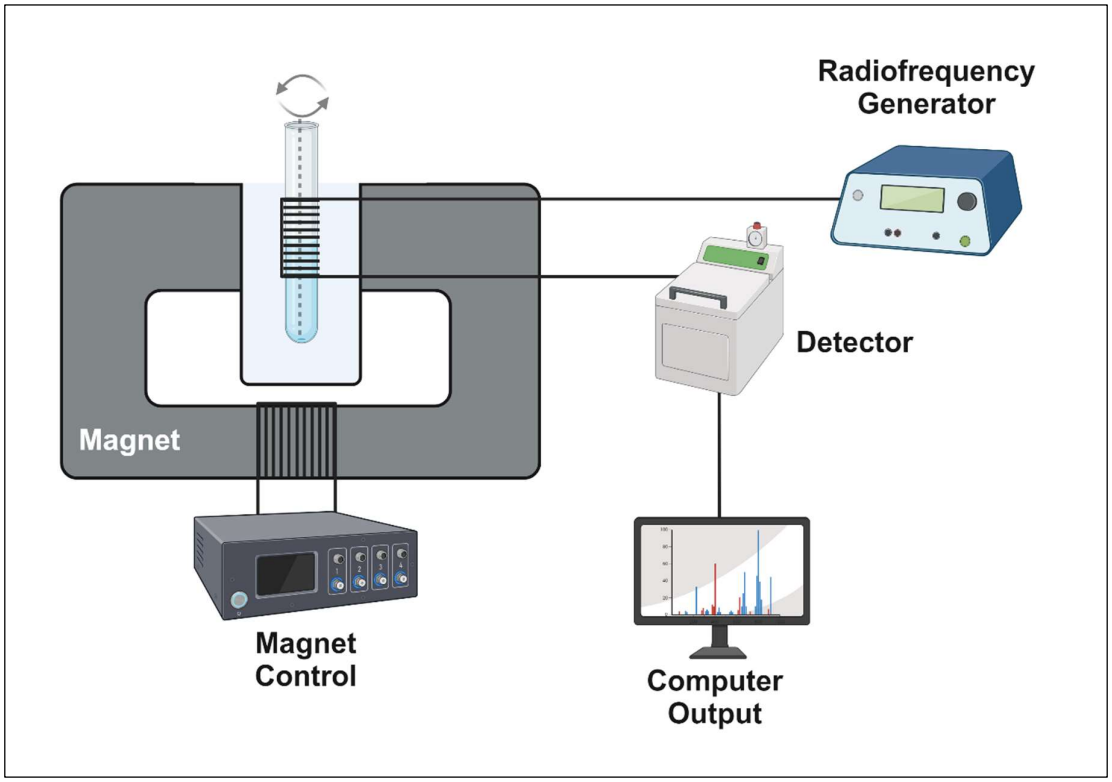

Figure 4-2: Basic overview of NMR spectroscopy. Adapted from (373).

NMR spectroscopy has several advantages over other metabolomics modalities. It is a highly reproducible technique which requires little sample preparation prior to analysis and can be performed rapidly compared to other techniques (407). It is not restricted to analysis of fluids, and intact tissues may be analysed (408). It is performed as a single assay and does not require the use of columns for sample separation. NMR is non-destructive, and so samples may be able to be recovered after analysis for subsequent retesting (409). Finally, NMR excels in detection of polar metabolites and small compounds less than 100 Da (403).

Drawbacks with NMR include a requirement for a larger sample volume, reduced sensitivity and limited coverage of the metabolome compared to other techniques. Compounds with wide spectral peaks such as lipids may overlap with smaller metabolites, making it difficult to distinguish each metabolite (397,407,410). Similarly, the presence of water in a sample may obscure other metabolites with overlapping peaks (403).

4.4.2 Liquid Chromatography-Mass Spectrometry

The other main metabolomics analysis tool is mass spectrometry (MS). This is typically twinned with a technique aimed at separating the sample into constituent metabolites, such as high-performance liquid chromatography (HPLC) or gas chromatography (GC). HPLC is the most commonly used configuration for metabolomics studies, however GC may be better placed for detection of volatile compounds (411).

Figure 4-3 outlines the process of liquid chromatography. HPLC utilises a stationary phase compound such as silica gel within a column which aims to slow down molecules as they pass through, and a mobile phase liquid containing the sample. As the mobile phase passes through the stationary phase, metabolites will separate out at different rates based on features such as size, polarity and charge (412). The retention time is the length of time it takes a metabolite to traverse the column. Several different types of chromatography columns can be used in LC. Normal phase and reverse phase columns can be used to separate non-polar and polar compounds respectively (407). Hydrophilic interaction chromatography (HILIC) columns composed of hydrated silica are commonly used, as they too have improved capabilities at detection of polar metabolites (409).

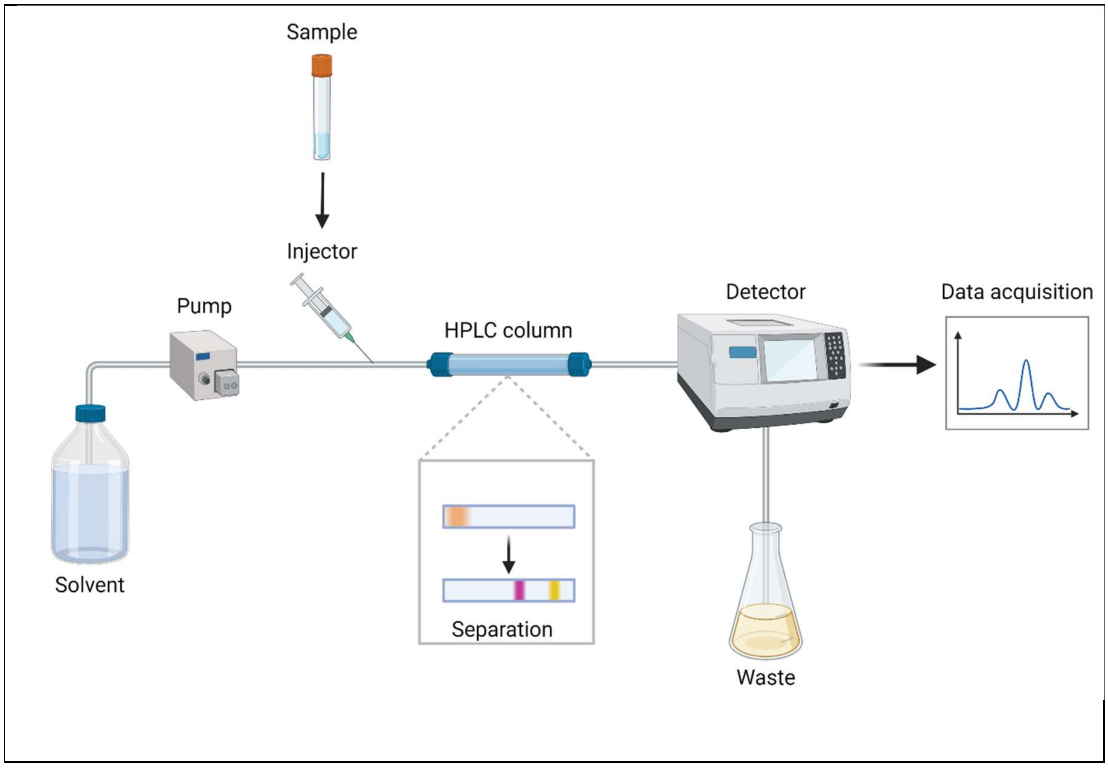

Figure 4-3: Overview of high performance liquid chromatography.

Once the chromatography step is complete, metabolites are fed into the mass spectrometer. The initial MS analysis, sometimes referred to as MS1, requires the sample to be ionised using a technique such as electrospray ionisation, where metabolites are nebulised into an aerosol through a droplet spray and given a positive or negative charge (413). Ions pass through a vacuum before being directed into the analyser (404). Analysis can be performed twice in both positive and negative ionisation mode to increase the breadth of detectable metabolites (413). For each ion, a corresponding mass to charge ratio (m/z) and associated intensity is provided, which is used to produce a mass spectrum. The mass spectrum can be combined with the chromatographic retention time to provide initial clues to the identity of each ion (404). Tandem mass spectrometry, also known as MS/MS or MS2, is a second MS analysis where selected ions from the MS1 spectra are fragmented and reanalysed, providing a fragmentation spectrum. This spectrum is unique for each ion, thus it serves as additional evidence for metabolite identification (413,414).

Orbitrap mass spectrometers such as the Q-Exactive mass spectrometer are commonly used for metabolomic analyses for enhanced sensitivity and resolution. *Figure 4-4* shows a typical Orbitrap mass spectrometer. In this technique, the *m*/*z* of ions is determined by measuring their oscillatory motion within an electrostatic field. After ionisation, the ions are passed through an S-lens which focuses and guides them into a vacuum. Ions then enter the quadrupole – an arrangement of four rods which have oscillating RF and direct current voltages. The quadrupole can be used to filter out unwanted *m*/*z* ranges. Ions are then fed into and gathered in the C-trap, which helps to control the number of ions injected into the analyser. Ions enter the analyser via the higher-energy collisional dissociation (HCD) cell if fragmentation is required for analysis. Once in the analyser, ions become electrostatically fixed between a central spindle-shaped electrode which is encircled by two other electrodes which form a barrel-shape. When an electric field is applied, ions will rotate around the electrode, while also oscillating axially. Ion oscillation induces a current on the detection electrodes as a time-domain signal. Fourier transformation can be used to convert the signal into a frequency domain spectrum. The *m*/*z* of each ion can then be calculated, as the frequency of oscillation is inversely proportional to the square root of the *m*/*z* (415,416).

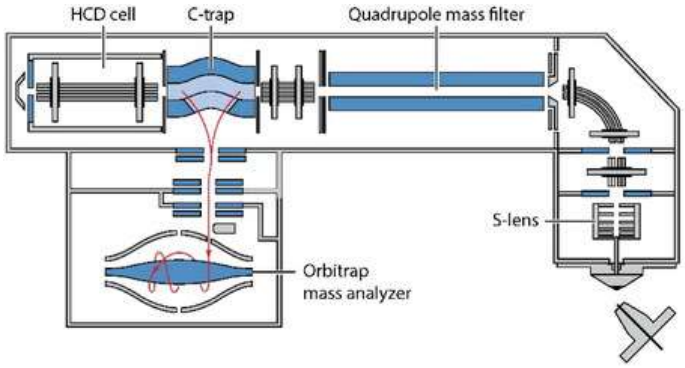

Figure 4-4: Schematic diagram of an Orbitrap mass spectrometer. Taken from (416)

Putative metabolite identification and data analysis can be performed using a range of software packages such as MetaboAnalyst (417). The resulting output will provide peaks with *m/z,* retention times and relative abundance from the peak area. Centring and scaling of the output makes the data considerably easier to interpret. Using logarithmic transformation such as $\log_2$ transformation of the relative abundance provides symmetry around zero. A $\log_2$ fold change of 1 represents a doubling of relative abundance, while a $\log_2$ fold change of –1 represents a halving. Moreover, there may be very wide ranges in different metabolite concentrations within data sets which do not necessarily reflect the biological relevance of these metabolites. Logarithmic transformation permits compression of data ranges, which decreases the impact of extreme values (418).

Isomerism can lead to multiple metabolite identities being provided for one peak. Definitive metabolite identification requires matching of spectra with those of confirmed metabolites, either through online databases or by a further MS analysis on known authentic standards. The mass spectra of the standards can be compared with those from the samples, and a match confirms the IDs of the metabolites (404).

The main benefit of LC-MS over NMR spectroscopy is the exquisite sensitivity of MS: with smaller sample volumes, MS is capable of detecting many thousands of metabolites at much lower concentrations compared to NMR spectroscopy (403). Drawbacks include the need for additional sample preparation steps, the requirement of chromatography and additional quantification assays, making it a more time-consuming process compared to NMR (419). Variability with measurement techniques means consistency and reproducibility of results between centres is more challenging to achieve than with NMR (403). Finally, MS is a destructive method of analysis, meaning samples will be unrecoverable after testing (397).

4.5 Untargeted vs Targeted Metabolomics

Two analysis strategies can be utilised in a metabolomics study: untargeted or targeted approaches. Untargeted (also known as global) approaches do not rely on *a priori* knowledge, and therefore analysis is aimed at the detecting the widest possible range of metabolites. This gives the best chance at biomarker discovery, giving an unbiased view of the metabolome, potentially allowing changes in unexpected or unknown metabolites to be detected (414). A targeted analysis uses prior information to detect specific metabolites of interest. It has a more narrow approach, and is therefore useful for providing validation of an untargeted study (397).

4.6 Data Analysis

The data obtained from a metabolomics study is often complicated and multidimensional. Online analytical tools such as eXtensible Computational Mass Spectrometry (XCMS) (420) and mzMatch (421) are available to automate the complex data processing required. Analysis may begin with a dimension reduction technique such as principal component analysis (PCA). PCA simplifies data sets by permitting multidimensional data to be displayed on a two or three-dimensional plot. Principal components are used, which are linear combinations of the variables within the data set. Principle component 1 (PC1) is displayed on the x-axis, and depicts the direction of greatest variance within the dataset. Likewise, PC2 accounts for the second-most variance in the dataset and is displayed on the y-axis. PC2 is always displayed orthogonally ay 90^0 to PC1 to ensure there is no covariance between the two principal components (407). PCA is a useful tool for identifying groups which cluster together within the dataset, and to make initial investigation into which variables are responsible for separating the different groups (422).

4.7 Biomarker Identification

Detection and quantification of metabolites is important, but of limited value without identification. Databases such as the human metabolomics database (HMDB) compile comprehensive information relating to metabolite identities and their associated biological and physiological properties (423). Other databases such as the Kyoto Encyclopaedia of Genes and Genomes (KEGG) can be used for pathway analysis whereby identified metabolites are mapped to biochemical pathways. This provides a biological context for metabolic perturbations, helping to understand why metabolite concentrations may be altered and potentially helping to identify potential future therapeutic targets (424).

4.8 Conclusion

Metabolomics is a modern discipline which permits the detection of thousands of metabolites within a given biofluid. HPLC/MS can identify biomarkers in a wide range of pathological conditions. Metabolomics may therefore be capable of identifying novel biomarkers in secondary infections.

5 Metabolomics in Sepsis: A Literature Review

5.1 Introduction

Septic patients enter a catabolic state characterised by increased protein, lipid and carbohydrate breakdown, which leads to significant alterations in plasma metabolite concentrations. Additionally, compounds produced by infectious microorganisms may also contribute to metabolite perturbations. Metabolomics studies may therefore aid in identification of sepsis biomarkers.

A review of the literature was conducted to find studies focused on using metabolomics to diagnose sepsis. The Medline and Embase databases were searched for all studies conducted prior to June 2022. Studies which featured metabolomic analyses of patients with sepsis and focused on metabolites capable of sepsis diagnosis were eligible for inclusion. The search strategy used can be found in *Appendix B*.

From this, 36 studies were examined (419,425–459), and the metabolites found to be significantly different between patients with and without sepsis were gathered. The metabolic perturbations seen in septic patients commonly involve energy metabolism, lipid metabolism and protein catabolism. These pathways are discussed in detail below.

5.2 Energy Metabolism

5.2.1 Glycolysis

Glycolysis is an ancient metabolic pathway present in both prokaryotic and eukaryotic organisms. A molecule of glucose is split into two molecules of pyruvate through a series of 10 cytosolic reactions (*Figure 5-1*). This process consumes two molecules of ATP, but generates 4, giving a net yield of 2 ATP molecules per glucose (460).

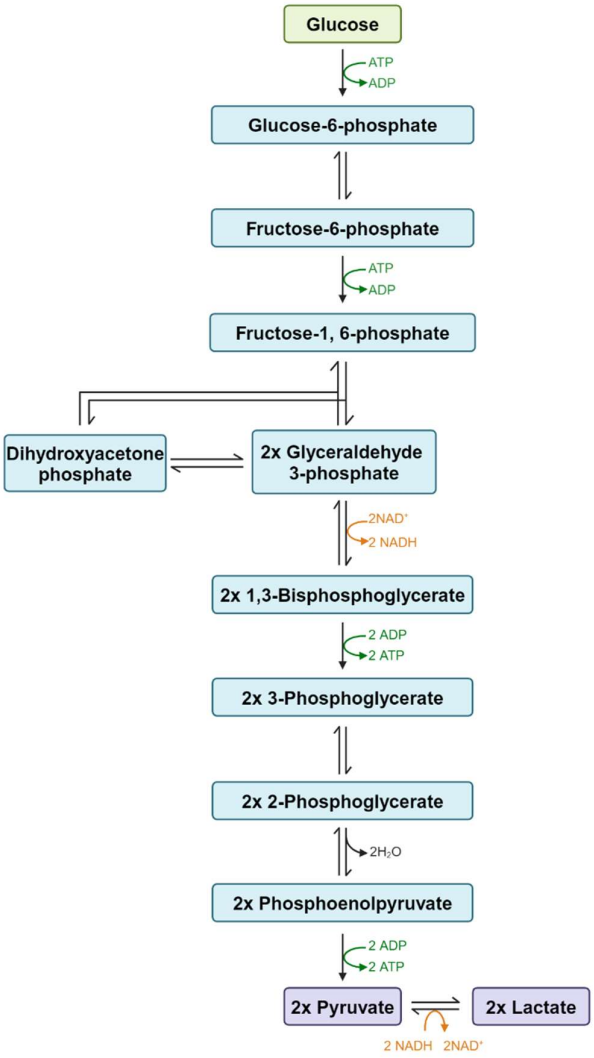

Figure 5-1: The glycolysis pathway. ADP: Adenosine diphosphate, NAD^+: Oxidised nicotinamide adenine dinucleotide; NADH: Reduced nicotinamide adenine dinucleotide.

Glycolysis is an anaerobic process, meaning that O_2 is not required for this pathway to occur. In situations where oxygen is readily available, pyruvate is metabolised through the tricarboxylic acid (TCA) cycle followed by OXPHOS. This is considerably more efficient than glycolysis, and generates up to 36 ATP molecules per molecule of glucose (461).

During hypoxic conditions, mitochondrial respiration is limited. A reduction in ATP production from OXPHOS results in increased flux through the glycolysis pathway (462). Despite the relative inefficiency of glycolysis compared to OXPHOS, this pathway can be upregulated quickly in hypoxic conditions through the actions of the transcription factor hypoxia-inducible factor 1-α (HIF-1α) to partially compensate for the decreased ATP production by OXPHOS. In established sepsis, a metabolic switch away from OXPHOS towards glycolysis may occur even when oxygenation is adequate. This phenomenon of aerobic glycolysis is known as the Warburg effect. Several biochemical changes occur within mitochondria which can maintain the Warburg effect including inhibition of the pyruvate dehydrogenase enzyme, and accumulation of succinate. These changes upregulate genes which lead to inhibition of OXPHOS, and also stabilise HIF-1α, further upregulating pro-glycolytic gene expression (461).

5.2.1.1 Lactate and Pyruvate

If pyruvate is unable to progress with aerobic respiration, it is instead converted to lactate by the enzyme lactate dehydrogenase. This reaction is essential as it regenerates oxidised NAD+ from reduced NADH, which is then used to facilitate additional glycolysis (463). Reconversion of lactate to glucose can occur in the liver via the Cori cycle when adequate oxygenation occurs, however prolonged hypoxia can lead to accumulation of lactate causing a lactic acidosis (460). Moreover, increased catecholamine production further drives glycolysis, and increases the release of lactate from skeletal muscle (464). As a result, increased concentrations of both lactate and pyruvate may be observed in sepsis. Indeed, lactate has become a widely used sepsis biomarker, and forms part of the definition of septic shock (206). Metabolomics studies have confirmed that lactate is consistently raised in sepsis (431,437,441,456). The L-lactate stereoisomer is produced in eukaryotes, whereas prokaryotes utilise D-lactate preferentially. Therefore the presence of serum D-lactate from invading organisms may be a more specific marker of sepsis (454). Pyruvate and lactate tend to increase simultaneously (465). Accordingly, two metabolomics studies also found an increase in pyruvate concentration in patients with sepsis (433,437).

5.2.1.2 Glucose

A mild to moderate hyperglycaemia is frequently observed in critical illness, as increased serum glucose helps to maintain a supply for vital organ functioning (466). However, stress hyperglycaemia is common in sepsis and severe hyperglycaemia is associated with increased mortality (467). Several interlinked pathways contribute to hyperglycaemia in sepsis. Massive release of acute phase hormones including glucagon, catecholamines, growth hormone and cortisol all act to promote hyperglycaemia (468). Pancreatic insulin secretion in critical illness is suppressed (469), and cytokines such as TNF-α inhibit insulin signalling, increasing insulin resistance with downregulation of glucose transporter (GLUT)-4 (470–472). Furthermore, increased hepatic gluconeogenesis occurs via conversion of lactate to glucose through the Cori cycle in addition to increased utilisation of amino acids produced through protein catabolism (473). Interestingly, hyperglycaemia can promote pro-inflammatory responses, with increased cytokine, inflammatory mediator and ROS release (474). This can result in a cycle of hyperglycaemia exacerbating inflammation which promotes further hyperglycaemia (475). In metabolomics studies, glucose was found to be increased in sepsis by Jaurila and colleagues (432), but decreased in the two studies by Mickiewicz *et al*. (441,442). This reduction in glucose may be explained by increased glucose utilisation, particularly with an increased drive towards glycolysis, thus potentially depleting available glucose.

5.2.1.3 Other Carbohydrates

Concentrations of several other carbohydrates have been found to change in sepsis. An increase in several disaccharide carbohydrates has been observed, including lactose (432), maltose (432) and sucrose (441). Reduced hydrolysis of disaccharides may possibly occur due to sepsis induced liver failure (441).

Mannose, a monosaccharide, was found to be decreased in septic shock (441). Mannose can be converted to fructose-6-phosphate, which can then enter the glycolytic pathway, thus a drive for increased glycolysis in sepsis may result in a reduction in mannose.

Wang and colleagues found an increase in D-galactose in septic patients. (452). Galactose can be reduced to the compound galactitol, which is osmotically active, and accumulation can lead to cellular membrane rupture. Galactitol inhibits antioxidant enzymes, thus leading to increased formation of free radicals, encouraging oxidative injury (476). A rat model of sepsis suggests that D-galactose may impair glucose and lipid metabolism, increase oxidative stress and promote apoptosis, resulting in immune dysregulation and increased mortality (477).

5.2.2 The Tricarboxylic Acid Cycle

The TCA cycle, also known as the Krebs cycle or citric acid cycle, is an essential part of aerobic respiration. The cycle involves eight enzymes which process intermediate compounds sequentially, releasing electrons which are transported via cofactors NADH and flavin adenine dinucleotide ($FADH_2$) to the mitochondrial membrane to generate ATP during OXPHOS (*Figure 5-2*).

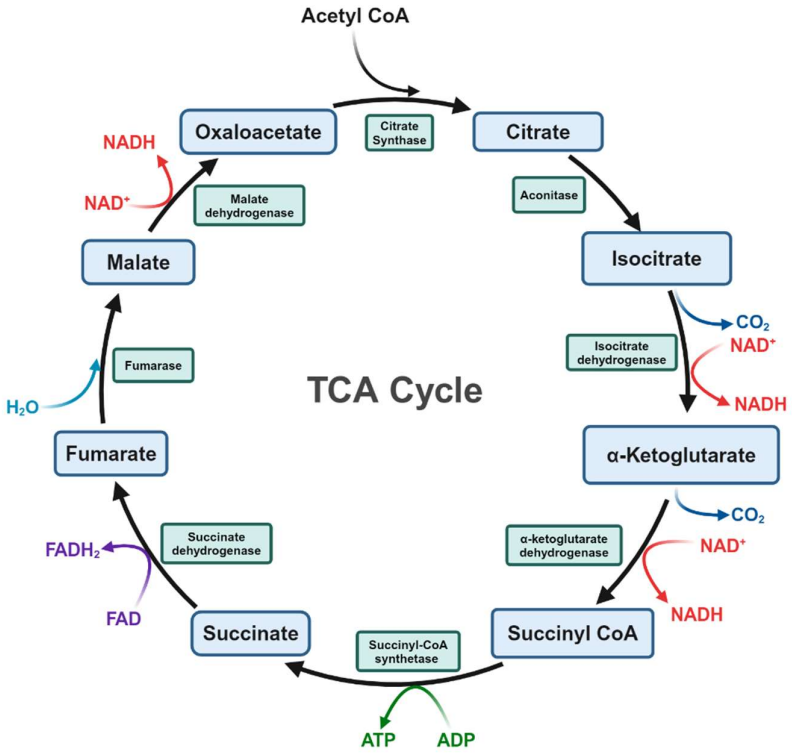

Figure 5-2 The tricarboxylic acid cycle

In summary, the process begins with the movement of pyruvate and/or free fatty acids formed in the cytosol into the mitochondrial matrix where they are converted to acetyl-CoA. The acetyl-CoA combines with oxaloacetate and water to form citrate and CoA, catalysed by the enzyme citrate synthase. The enzyme aconitase isomerises citrate to isocitrate, which is then decarboxylated by isocitrate dehydrogenase to produce α-ketoglutarate, CO_2 and NADH. Succinyl-CoA is then formed via decarboxylation by the enzyme α-ketoglutarate dehydrogenase, yielding another molecule of CO_2 and NADH. Succinyl-CoA synthetase then converts the succinyl-CoA to succinate leaving behind a CoA molecule and regenerating a molecule of ATP from ADP. Succinate is oxidised by succinate dehydrogenase to form fumarate and a molecule of $FADH_2$, which is then hydrated by fumarase to yield malate. Finally, malate is converted by malate dehydrogenase via oxidation to produce oxaloacetate and NADH. The oxaloacetate is then free to bind another acetyl-CoA to restart the cycle again (478).

5.2.3 Tricarboxylic Acid Cycle Intermediates

Mitochondrial dysfunction occurs in sepsis, impairing aerobic metabolism and reducing ATP production (479). Levels of intermediate compounds of the TCA cycle are subsequently deranged due to failure to maintain OXPHOS. There may be a reduction in TCA cycle intermediates as there is an increased drive to maintain aerobic catabolism, increasing the utility of these substrates. Conversely, a shift towards aerobic glycolysis may reduce the consumption of TCA cycle compounds leading to their accumulation. Indeed, a failure to utilise these metabolites may suggest an increased severity of sepsis, and could potentially indicate a poor prognosis (480,481).

5.2.3.1 Itaconate and Succinate

The metabolite itaconate does not directly form a part of the TCA cycle, however it may be produced through aconitate (an intermediate compound formed in the conversion of citrate to isocitrate) being diverted away from the TCA cycle in response to inflammatory stimuli such as LPS (455,482). Itaconate has been shown to possess anti-inflammatory and antibacterial properties. It can interfere with bacterial growth (483), and activates the transcription factor nuclear factor erythroid 2-related factor 2 (NRF2), (484) a master regulator of cellular antioxidant and detoxification responses (485). Additionally, itaconate inhibits the enzyme succinate dehydrogenase which is utilised both during the TCA cycle and OXPHOS. Inhibition of succinate dehydrogenase interrupts mitochondrial ROS production, as well as upregulating antioxidant production (482,486). Conversely, inhibition of succinate dehydrogenase leads to the accumulation of the TCA metabolite succinate which has pro-inflammatory effects. Succinate increases the release of cytokines such as IL-1ß and promotes the activation of HIF-1α, encouraging Warburg metabolism (455,461). Thus, increased itaconate will have mixed inflammatory results in sepsis.

The study by Beloborodova *et al.* found that itaconate was elevated in early sepsis but was completely undetectable in healthy individuals or in the later stages of sepsis. This may make itaconate an effective acute sepsis biomarker (454). On the other hand, itaconate was found to be reduced in septic patients in the study by Lin *et al.* (437).

Succinate levels have been shown to increase in critical illness, including in trauma patients with haemorrhagic shock (487). In sepsis metabolomics, succinate was increased in studies by Lin *et al.*, Mickiewicz *et al.*, and Pan *et al.* (437,441,446), but decreased in the study by Beloborodova *et al.* (454). Additionally, succinate was increased in late sepsis in another study by Beloborodova *et al.* (455). This may be due to an adaptive response where mitochondria enter a state of hibernation and reduce their activity during the early stages of sepsis in an attempt to reduce further ROS production and therefore prevent damage caused by oxidative stress (488).

5.2.3.2 Other TCA cycle intermediates

Other compounds from the TCA cycle such as citrate, fumarate and malate are generally increased in sepsis, suggesting an overall increase in TCA activity to utilise excess pyruvate produced during glycolysis. These metabolites are further increased in sepsis non-survivors, suggesting that increased attempts to generate energy via the TCA cycle are unsuccessful and worsen mitochondrial dysfunction (489).

Citrate has several metabolic regulatory functions including promotion of gluconeogenesis and lipid synthesis, and inhibition of glycolysis through negative feedback (432). Additionally, citrate inhibits pyruvate dehydrogenase and succinate dehydrogenase, reducing TCA cycle activity (490). Citrate also exerts pro-inflammatory effects, increasing prostaglandin, NO and ROS production (490). Metabolomics studies show that citrate may be increased (438,453) or decreased (432,433,437) in sepsis. During acute illnesses, increased rates of citrate may be taken up by the liver, reducing its availability (432). Conversely, citrate is excreted by the kidneys, so sepsis induced AKI could lead to an accumulation of serum citrate (432).

In sepsis, an increase in α-ketoglutarate and fumarate may occur. This is likely due both to increased production of α-ketoglutarate from the amino acid glutamate, along with inhibition of TCA cycle dehydrogenases (455).

5.3 Amino Acids

The hypermetabolic state seen in sepsis causes an increase in catabolism to maintain substrates for energy production. While carbohydrates and lipids are more typically used as energy substrates, proteins may also be used, particularly under conditions of stress such as sepsis. Amino acids liberated into the bloodstream can then be used by the liver for gluconeogenesis (427,472). Loss of protein occurs early in septic patients, with significant skeletal muscle bulk loss in the first 10 days of illness (491). Up to 260g of protein can be catabolised daily in sepsis, which equates to roughly 1kg loss of muscle bulk daily. This has implications for recovery, ventilator weaning and physiotherapy for septic patients in ICU. (492).

Amino acids are proteinogenic or non-proteinogenic. There are twenty-two proteinogenic amino acids which form the building blocks for protein synthesis. However, there are a number of other non-proteinogenic amino acids which despite not having a role in protein synthesis have other biological functions such as neurotransmitters, antimicrobial compounds and toxins (425,493). The levels of most amino acids are altered in sepsis (450).

5.3.1 Branched Chain Amino Acids

There are three proteinogenic branched chain amino acids (BCAAs): valine, leucine and isoleucine. BCAAs increase the activity of the mammalian target of rapamycin (mTOR), a serine/threonine kinase which acts as a master regulator of cellular metabolism. Activation of mTOR increases signalling via pathways which control protein, lipid and nucleotide anabolism, as well as regulation of the cell cycle, inhibition of autophagy and increasing the inflammatory response via NF-κB (494,495).

While some have reported an overall decline in BCAAs during sepsis (496), metabolomics studies are inconsistent. Reductions in valine (437,441,442), leucine (441) and isoleucine (433,441) have been observed, while other metabolomics studies have found an increase in valine (443,453), leucine (437,443,444) and isoleucine (443,444,453).

5.3.2 Aromatic Amino Acids

The aromatic amino acids (AAAs) include phenylalanine, tryptophan and tyrosine. These are essential amino acids which act as precursors for several important hormones. Tyrosine is the precursor molecule for the production of the catecholamines dopamine, noradrenaline and adrenaline (See *Figure 3*) (497), while tryptophan is a precursor for the neurotransmitter serotonin and the hormone melatonin (498).

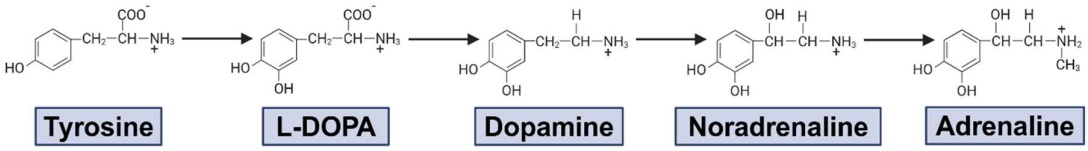

Figure 5-3: Catecholamine synthesis pathway. Adapted from (497).

Multiple sepsis metabolomics studies report an increase in phenylalanine (419,425,437,441,442,444,445) and tyrosine (443,444,453). Raised phenylalanine is predictive of sepsis induced AKI (437), and is also associated with increased mortality (425). The catecholamine surge observed in sepsis acts via negative feedback to downregulate the activity of phenylalanine hydroxylase, thus leading to phenylalanine accumulation and a decrease in tyrosine production (419,425). The ratio of phenylalanine:tyrosine is an indicator of phenylalanine hydroxylase activity, and a reduced ratio may be a potential sepsis marker (443). Interestingly, the ratio of BCAAs:AAAs may also be used as a diagnostic biomarker, as this ratio has been shown to decline in sepsis (450).

5.3.3 Tryptophan and Kynurenine

Tryptophan is an essential AAA which is degraded into the compound kynurenine through the action of the enzyme indoleamine 2,3-dioxygenase (IDO). Kynurenine is an endothelium derived relaxing factor which promotes arterial relaxation resulting in hypotension (499). Kynurenine is also a toxic compound, and persistently elevated concentrations have been associated with Alzheimer's dementia (500) and amyotrophic lateral sclerosis (501).

The activity of IDO is increased in APCs and endothelial cells in response to pro-inflammatory cytokines, particularly IFN-γ (425). This increased activity may have several physiological benefits. Endogenous stores of tryptophan are low and can rapidly become depleted. As a result, there is a reduced availability of tryptophan for invading microorganisms to facilitate their replication during an infection (502). Conversion of tryptophan to kynurenine initially encourages additional pro-inflammatory responses. Kynurenine has bactericidal properties, so increasing IDO activity may act as a defence mechanism during an infective insult (503).

Increasing IDO activity in early sepsis may have damaging consequences for the host, worsening septic shock and promoting organ dysfunction (502,504). Prolonged IDO activation eventually leads to anti-inflammatory effects, as tryptophan deficiency promotes release of anti-inflammatory cytokines such as IL-10 from APCs, while kynurenine inhibits cytotoxic T-cells and NK cells. This may help prevent an excessive inflammatory response from developing, but prolonged immunosuppressive effects may increase the risk of developing secondary infections (502,504,505).

While Lu *et al*. and Zheng *et al*. reported an increase in tryptophan in sepsis (438,453), most sepsis metabolomics studies detected a decrease in tryptophan (425,427,430,437). Studies consistently confirm raised kynurenine in sepsis (425,426,438,445). As such, the ratio of tryptophan:kynureinine as an indicator of IDO activity may be a useful sepsis biomarker.

5.3.4 Sulphur Containing Amino Acids

Sulphur containing amino acids (SAAs) include cystine, cysteine, taurine and methionine. SAA level have been shown to decline in sepsis and correlate with disease severity (450).

Cystine is formed by the conjoining of two cysteine molecules. Cystine and cysteine are converted interchangeably in vivo (449). Cysteine is used in the production of glutathione and taurine, which both have detoxifying properties (506) Administration of cysteine may reduce the inflammatory response and promote survival in sepsis (507).The compound N-acetylcysteine, which is commonly administered in the treatment of paracetamol toxicity, has antioxidant, anti-inflammatory and vasodilatory properties, and may be able to limit damage from oxidative stress in sepsis (508). Lin and colleagues found that cysteine was increased in septic patients (437), while Pan et al. and Su et al. found a reduction in sepsis (446,450).

Taurine decreases in sepsis (478,509,510) and is further reduced with increasing sepsis severity (450). Taurine has antioxidant and anti-inflammatory properties, thus oxidative stress in sepsis may deplete taurine levels through excess consumption leading to worsening metabolic and cardiorespiratory indices (511). Nutritional supplementation of pharmaconutrients including taurine may aid recovery in sepsis (450,512).

Methionine levels may be increased in sepsis (437), and further increased in septic non-responders (513). Methionine is an essential amino acid with several functions including acting as a precursor for the production of polyamines, creatine and cysteine (506). Methionine also has an important role in methylation. Methionine is converted to S-adenosylmethionine (SAM) which acts as a methyl doner in various reactions, converting SAM to S-adenosylhomocysteine (SAH) in the process. SAH acts via negative feedback to inhibit transmethylation, so the ratio of SAM:SAH is an indicator of methylation capacity (514). The SAM:SAH ratio is decreased in sepsis, suggesting impaired methylation ability (514,515).

5.3.5 The Urea Cycle

The process of excreting waste ammonia is achieved via the urea cycle. Ammonium ions produced from amino acid catabolism are converted to urea in the liver in a stepwise process (*Figure 5-4*).

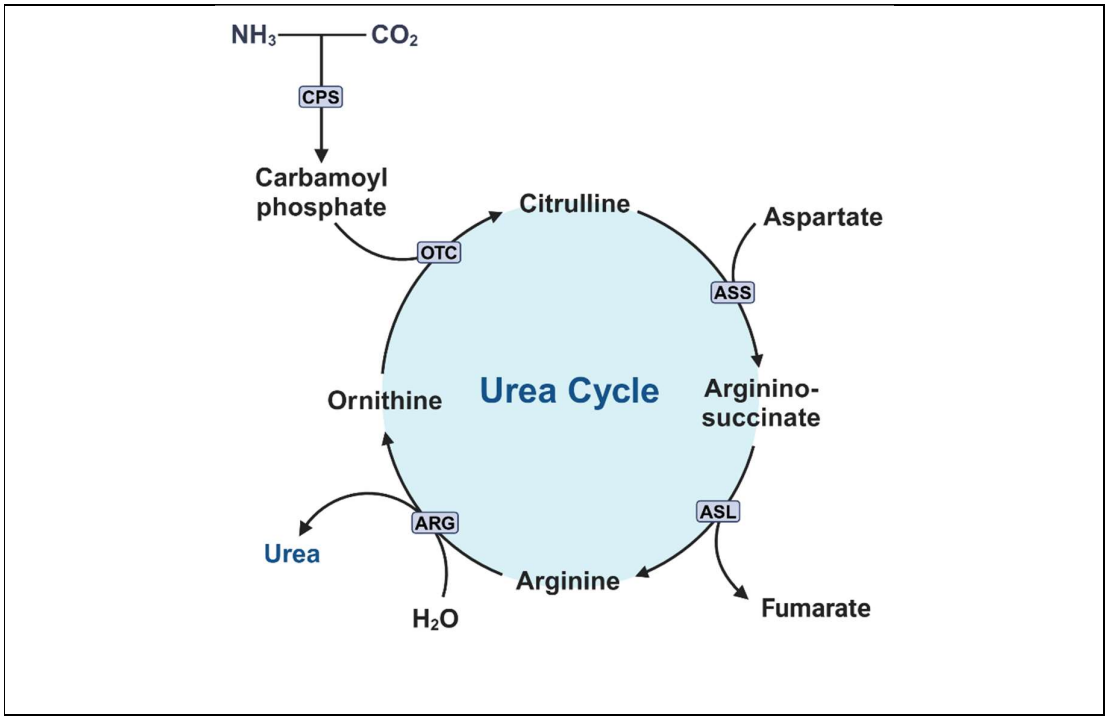

Figure 5-4: The urea cycle. Adapted from (516). *ARG: arginase, ASL: argininosuccinate lyase, ASS: argininosuccinate synthetase, CPS: Carbamoyl phosphate synthetase, OTC: ornithine transcarbamylase*

The cycle begins with ammonia combining with carbon dioxide and phosphate from a molecule of ATP under the action of the enzyme carbamoyl phosphate synthetase to produce carbamoyl phosphate. Next, ornithine transcarbamylase moves the carbamoyl group from carbamoyl phosphate onto a molecule of ornithine to form citrulline. The enzyme argininosuccinate synthetase then catalyses the binding of a molecule of aspartate to the citrulline to form the compound argininosuccinate, which is then broken down into arginine and fumarate by argininosuccinate lyase. Finally, hydrolysis of arginine occurs via the enzyme arginase, producing urea and ornithine. The urea can then diffuse to the systemic circulation in preparation for excretion, while the ornithine is free to form another citrulline molecule, repeating the cycle (516,517). Of note, the fumarate produced from the lysis of argininosuccinate can then enter the TCA cycle, demonstrating a connection between these two cycles (518).

5.3.5.1 Arginine

Along with its role in the urea cycle, arginine is involved in wound healing, cellular regeneration and immune function, and is used in the synthesis of polyamines and creatine (510). The enzyme iNOS produces NO from arginine, which has potent vasodilatory properties. Profound hypotension in septic shock is thought to be due to vasoplegia in part through excessive release of NO (519).

Mierzchala-Pasierb *et al*. found increased arginine in sepsis, with a further increase linked to disease severity (443). However this appears to be an outlier, as many other studies report a decline in arginine during sepsis (425–427,441,442). A decline in arginine may be seen in sepsis as more arginine is utilised by iNOS (425). Moreover, arginine may preferentially be utilised by arginase through the urea cycle in sepsis in an attempt to reduce additional production of NO (427,443). Low arginine in sepsis may impair NO production leading to microvascular compromise (520). Thus, maintaining a correct balance of arginine may be important in sepsis to maintain normal haemodynamic parameters.

5.3.5.2 Citrulline

Citrulline is a non-essential amino acid intermediate of the urea cycle, and may also be produced in the intestine from glutamine (510). As such gastrointestinal dysfunction in sepsis can lead to a reduction in citrulline production (450,521). Low citrulline is associated with increased mortality in septic shock (522). Indeed, the metabolomics study by Mierzchała-Pasierb and colleagues demonstrated a reduction in urinary citrulline in sepsis patients compared to healthy controls (444).

Citrulline is also produced as a byproduct in the conversion of arginine to NO, and can also be used to regenerate arginine in the kidneys, endothelium and in macrophages (510,523). Sepsis induced AKI may therefore limit the capacity of the kidneys to regenerate arginine, leading to an accumulation of citrulline (521). The study by Zheng *et al*. found an increase in plasma citrulline in sepsis patients compared to those with SIRS (453). Citrulline may therefore act as a potential biomarker of sepsis induced acute intestinal and/or renal failure (521).

5.3.5.3 Ornithine

Ornithine is another urea cycle amino acid with several other functions including a role in wound healing (524) and in polyamine synthesis (517). The two studies by Mierzchała-Pasierb *et al*. both reported an increase in ornithine in septic patients (443,444). Ornithine reduces the uptake of arginine by endothelial cells, which reduces its conversion to NO by iNOS (525). Increased ornithine may therefore reduce NO induced vasodilation.

5.3.6 Other Amino Acids

5.3.6.1 Glycine

Glycine is the smallest amino acid, and one of the most abundant in the body, accounting for 11.5% of total body amino acids. Along with being a proteinogenic amino acid, glycine acts as an antioxidant, regulates immune function and facilitates bile acid conjugation (526). Glycine was found to be increased in sepsis (432,443,444), but decreased in septic shock (441). Increased glycine is associated with worse SOFA scores in sepsis (444).

5.3.6.2 Glutamine

Glutamine is the most abundant free amino acid in the human body (527). Glutamine has several functions, including maintenance of redox balance via regulation of glutathione production (528); and transport of nitrogen between organs. Glutamine also acts as a supply of carbon and nitrogen for cellular processes, and may aid in generation of ATP through conversion to α-ketoglutarate as a fuel source for the TCA cycle (444).

Numerous studies have reported a reduction in glutamine in sepsis (419,425–427,433,437,441,446) which may be a result of excessive consumption due to oxidative stress (510). However, several studies found an increased concentration of glutamine in sepsis (438,443,444,453). Glutamine is metabolised in the liver, thus increased glutamine levels in sepsis may be linked to sepsis induced liver injury (444).

5.3.6.3 Glutamate

Glutamate is another protective amino acid with antioxidant effects. Glutamate is used in the synthesis of glutathione. Reduced glutathione (GSH) is an important scavenger of ROS and is converted to oxidised glutathione (GSSG) in the process. As such, the ratio of GSH:GSSG denotes cellular redox potential and may be used as a marker of oxidative stress (529). Depletion of GSH allows accumulation of ROS resulting on oxidative stress, thus maintenance of GSH stocks through glutamate is necessary to maintain cellular health (530). There are conflicting results with glutamate levels in sepsis, with several studies finding increased glutathione concentrations (425,437,453), with others reporting a decrease in sepsis (441,442).

5.3.6.4 Aspartate

Aspartate has several roles including pyrimidine nucleotide synthesis and promoting cell proliferation (531). Synthesis of aspartate occurs during the malate–aspartate shuttle, which is used for transportation of electrons produced during glycolysis across the mitochondrial membrane for use during OXPHOS. Malate carries electrons into the mitochondrion, where it is converted first to oxaloacetate, and then aspartate by the enzyme aspartate aminotransferase, which is then free to exit the mitochondrion (532). This process is reliant on a functioning mitochondrial ETC. Disruption of the ETC, as observed in sepsis, reduces cell proliferation which is thought to be partly due to the decrease in aspartate production (532).

A preprint study using a rat model of sepsis found a 50% decline in aspartate levels in septic rats compared to controls, and supplementation with aspartate increased TNF-α production, improved bacterial clearance rates, reduced the incidence of AKI and improved overall survival (533). Metabolomic studies reported inconsistent findings, with several showing an increase in sepsis (425,437,445), while Ding *et al.* reported a reduction (427).

5.3.6.5 Alanine

Liver uptake of alanine results in conversion via alanine transaminase to pyruvate and glutamate, with subsequent conversion of pyruvate to glucose (427,534). As such, alanine levels have been shown to decrease in sepsis (419,441), likely as a result of upregulated gluconeogenesis in an attempt to maintain glucose availability for glycolysis.

5.3.6.6 Lysine

Lysine, an essential amino acid found in eggs, fish and red meat, appears to have roles in both iron and zinc uptake (535). Lysine has inhibitory effects on the uptake of arginine by endothelial cells, decreasing NO synthesis (525,536). Supplementation of septic rats with lysine led to an improvement in haemodynamic indices and reduced lactate (536), suggesting a potential therapeutic role in septic shock. Additionally, lysine supplementation resulted in reduced ROS production and pro-inflammatory cytokines, increased antioxidant availability and improved lung architecture in septic mice, suggesting it may have a protective role against sepsis induced ALI (537). There are mixed findings in sepsis, with several studies finding a decline in lysine (433,441,450), while others report an increase (443,444,453).

5.3.6.7 Proline

Proline has functions including control of cellular redox state, regulation of lymphocyte proliferation and protection against apoptosis. Proline is a major constituent of collagen and thus plays an important role in wound healing (528). Proline also acts as a neurotransmitter via activation of N-methyl-D-aspartate (NMDA) receptors (538). Proline metabolism produces O_2^- free radicals and therefore contributes to oxidative stress (539). Numerous studies report an increase in proline levels in sepsis (441–443,453).

5.3.6.8 Threonine

Threonine is essential for maintaining gastrointestinal barrier integrity. There may be an increased threonine requirement to maintain normal intestinal functioning, leading to a depletion in sepsis (540). Other functions include prevention of apoptosis, promotion of cell growth and stimulation of antibody production (528). While Kauppi *et al.* and Mickiewicz *et al.* found a decrease in threonine concentration in sepsis (433,441,442), the studies by Mierzchała-Pasierb *et al.* reported an increase (443,444).

5.3.6.9 Serine

Serine is a gluconeogenic amino acid, and is also involved in the synthesis of glycine, SAAs, and phospholipids (541). Metabolomics studies have inconsistent findings for serine in sepsis, with several showing an increase (443,445,453) and others showing a reduction (433,437,441,446).

5.3.6.10 Histidine

Histidine is an essential amino acid with several unique functions. Its structure contains an imidazole ring which allows it to participate in proton buffering, metal ion chelation and ROS and RNS detoxification (542). As a result, histidine deficiency has been associated with increased oxidative stress, inflammation and increased mortality in patients with chronic kidney disease (543). Histidine levels are negatively correlated with severity of organ dysfunction (432), while increased histidine is associated with sepsis non-survivors (513). Multiple metabolomics studies revealed a reduction in serum histidine in septic patients (427,432,444,446). Mierzchala-Pasierb and colleagues reported an increase in serum histidine, but a decline in urine histidine in sepsis (443).

5.4 Lipids

Lipids are a broad class of hydrophobic molecules with a diverse range of functions. Their main roles are as energy storage compounds, as structural components of plasma membranes, and as signalling molecules (544). Lipids form precursors for other important biomolecules including hormones, vitamins and bile salts (545,546). The three main classifications of lipids within humans are glycerolipids, sphingolipids and sterols (545). Fatty acids are key constituents of lipids and are the main providers of the hydrophobic properties.

5.4.1 Lipid Synthesis

Synthesis of lipids begins with the production of fatty acids, primarily occurring within the cytosol. Pyruvate produced from glycolysis is decarboxylated and oxidised by the enzyme pyruvate dehydrogenase to form acetyl-CoA. Malonyl-CoA is then formed from a reaction between acetyl-CoA and bicarbonate. This compound has a key role in both the initiation of fatty acid synthesis, as well as promoting their subsequent elongation. Fatty acids are elongated by converting acetyl-CoA and malonyl-CoA to acetyl- acyl carrier protein (ACP) and malonyl-ACP respectively, which are used by the enzyme fatty acid synthase to sequentially add acetyl- CoA groups, increasing the chain length by two carbons at a time. This is achieved through a series of repeating reactions in the order condensation, reduction, dehydration and reduction again. Palmitate is the first fatty acid to be synthesised, which can then undergo modifications such as elongation or desaturation to produce other types of fatty acids (545–547).

Synthesised fatty acids can bind with glycerol 3-phosphate to produce a triacylglycerol (TAG) molecule; however several other intermediate molecules are produced during this process. The three carbons within a glycerol molecule are often denoted as stereochemical numbering (*sn*)- 1, *sn*-2 and *sn*-3 (545). The addition of the first fatty acid to *sn*-1 of glycerol 3-phosphate creates 1-acyl-*sn*-glycerol 3-phosphate, also called lysophosphatidic acid. Addition of a second fatty acid to *sn*-2 will form phosphatidic acid, which is the basic precursor for phospholipid synthesis. The enzyme phosphatidic acid phosphatase converts phosphatidic acid into a diacylglycerol (DAG). This DAG can be used to produce several important glycerophospholipid molecules, or alternatively can bind with a third fatty acid to produce a TAG molecule (546,548).

5.4.2 Catabolism

While lipid anabolism primarily occurs in the cytosol and endoplasmic reticulum, catabolism occurs mainly in the mitochondrion (546). Lipid catabolism provides an alternative feedstock for ATP production in situations where glucose availability is low. TAGs are metabolised through hydrolysis to form fatty acids and glycerol. The glycerol molecule can be converted into dihydroxyacetone phosphate, which is a glycolytic intermediate compound (472).

Fatty acids are metabolised within the mitochondrial matrix through a stepwise process called ß-oxidation. This is initiated by converting cytosolic fatty acids to fatty acyl-CoA, which can then be transported into the mitochondrion. This transportation is achieved via the carnitine shuttle. Here, the fatty acyl-CoA molecule has its CoA component replaced with a molecule of carnitine to form a fatty acylcarnitine, which can then be transported across the mitochondrial membrane. This is achieved using an antiporter, which exchanges one fatty acylcarnitine molecule for a carnitine molecule, thus maintaining the availability of carnitine outside of the mitochondrion. Once the fatty acylcarnitine is within the mitochondrial matrix, it is reconverted to fatty acyl-CoA, liberating the carnitine which is shuttled back out across the mitochondrial membrane ready to bind another fatty acyl-CoA (472). Once the fatty acyl-CoA is inside the mitochondrial matrix, ß-oxidation begins with the stepwise breakdown of the long-chain fatty acids into acetyl-CoA molecules (546).

During a septic insult, energy requirements will increase. Lipid catabolism is accelerated to accommodate increased requirements. While some lipid species may increase, the general trend in sepsis is of lipid catabolism and active transport of lipids intracellularly, resulting in an overall decrease in plasma lipid concentration (430,539).

5.4.3 Triacylglycerols

TAGs, also known as triglycerides, are used as energy storage molecules. A TAG molecule is composed of three fatty acid molecules of varying length and saturation bound to a molecule of glycerol (546). In sepsis there is an increase in endocrine activity, leading to release of catecholamines and glucocorticoids. This antagonises the effects of insulin and leads to increased adipose tissue lipolysis and decreased oxidation in the liver, kidneys and skeletal muscle. As a result, there is an elevation of plasma TAGs, which are subsequently hydrolysed and leads to an accumulation of free fatty acids and glycerol, along with diacylglyceride and monoacylglyceride intermediate compounds. Lipid oxidation is the most efficient method of providing ATP to cells, so impaired ß-oxidation greatly reduces ATP production, contributing to organ dysfunction in sepsis (549,550).

5.4.4 Glycerol and Fatty Acids

In early sepsis, lipolysis increases in adipose tissue. Acute phase hormones such as catecholamines, glucagon, cortisol, and growth hormone stimulate lipolysis. Furthermore, sepsis is associated with insulin resistance, which further promotes lipolysis. (472). The nuclear receptor peroxisome-proliferator activated receptor alpha (PPAR-α) has several crucial functions, including control of cell proliferation, inflammation and apoptosis. It also acts as a key metabolic regulator, particularly regarding lipid metabolism (551). In sepsis, PPAR-α activity is reduced, causing an accumulation of fatty acids and glycerol, with an associated decrease in survival (551,552). Drugs such as fibrates which increase PPAR-α activity may have a beneficial effect in sepsis (553).

Glycerol is increased in septic patients according to Zheng *et al.*, which may be explained by increased lipid catabolism leading to glycerol accumulation (453). However, the study by Wang *et al.* found that glycerol levels decreased in sepsis (452). Glycerol can be quickly metabolised via glycolysis, thus a reduction in glycerol in sepsis may reflect upregulated glycolytic activity (472).

There are mixed metabolomic results with changes in fatty acids during sepsis. Studies reported an increase in oleic acid, corticrocin, myristic acid, suberic acid, adipic acid and palmitoleic acid among others (430,433,437,439,446). Concomitantly, a decrease in fatty acids such as pelargonic acid, azelaic acid, palmitic acid, docosahexaenoic acid, and linoleic acid has been observed (433,437,440). Lin and colleagues found heptadecanoic acid was increased in septic patients compared to healthy controls (437), but Lu *et al.* reported a decrease in the same fatty acid in septic patients compared to those with SIRS (438). Likewise, stearic acid was increased in sepsis in the study by Pan *et al.* (446), but decreased in the study by Lin *et al.* (437). Stearic acid depletion has been associated with worse outcomes in sepsis (513).

Myristic acid, a long chain fatty acid, was raised in the studies by Kauppi *et al.* (433) and Beloborodova and Osipov (447), but decreased in the study by Ding and colleagues (427). Accumulation of myristic acid may increase the risk of adverse health effects including steatosis and cardiovascular events (554).

Linolenic acid has been shown to possess anti-inflammatory properties. In a murine model of sepsis, supplementation with linolenic acid reduced the severity of sepsis induced ALI by reducing the activation of NF-κB, decreasing pulmonary leukocyte infiltration, reducing TNF-α, IL-6 and IL-1β secretion and increasing antioxidant capacity (555). Interestingly, Ding *et al.* found that raised linolenic acid was associated with a worse prognosis in sepsis (427). Increased linolenic acid may help to reduce initial pro-inflammatory responses, but excessive accumulation may contribute to immunosuppression and risk of secondary infections (427).

During acute inflammation, membrane phospholipids are converted to arachidonic acid by the enzyme phospholipase A2. Arachidonic acid is then converted to eicosanoids such as prostaglandins and leukotrienes under the action of cyclooxygenase and lipoxygenase enzymes respectively (427). Increased levels of circulating phospholipase A2 has been detected in sepsis (439), so one might expect a corresponding increase in arachidonic acid. However, several studies found a reduction in arachidonic acid during sepsis (437,440). This may be due to an increased conversion of arachidonic acid to leukotrienes and prostaglandins. Lu *et al*. found an increase in leukotriene D4 in sepsis (438), and Mecatti and colleagues reported an increase in leukotriene F4 and prostaglandin E2 in septic patients compared to those with SIRS (440). These leukotrienes possess bronchoconstrictor and vasoconstrictor activity; promoting eosinophil and dendritic cell migration, increasing release of pro-inflammatory cytokines from lymphoid cells and stimulating mast cell degranulation. Moreover, leukotriene D4 is an important mediator in the pathogenesis of asthma (556,557). On the other hand, prostaglandin E2 has vasodilatory and bronchodilatory effects, is involved in wound healing, and is used in obstetrics for its oxytocic effects. Prostaglandin E2 exhibits immunomodulatory effects, suppressing innate immune responses and potentially increasing susceptibility to developing secondary infections in critical illness (558).

5.4.5 Acylcarnitines

In sepsis, lipids are utilised as an energy source. This requires movement of fatty acids into the mitochondrion using the carnitine shuttle before ß-oxidation can occur (472). Sepsis induced mitochondrial dysfunction leads to a failure of ß-oxidation and elevation of acylcarnitines unable to be metabolised (553). Excess acylcarnitines are transported back out of the mitochondrion and into the plasma, therefore elevated plasma acylcarnitines may be an indicator of sepsis induced mitochondrial dysfunction (440). The normal concentration of carnitine is considerably greater than acylcarnitines, and so an increase in the ratio of acylcarnitine:carnitines above 0.4 is considered abnormal and represents failure of mitochondrial metabolism (559), and is associated with increased organ damage and mortality in sepsis (505,560,561). Kelly and colleagues reported a reduction in carnitine in sepsis (434). Supplementation of carnitine has recently been investigated in septic patients with promising results (562).

Several studies found an increase in acylcarnitines in septic patients, including adipoyl-L-carnitine (458), octanoylcarnitine (440,448), gamma-linolenyl carnitine (440), L-acetylcarnitine (441,442), L-palmitoylcarnitine (440) hexanoylcarnitine (445,448), valerylcarnitine (448) and propionylcarnitine (448). However, multiple studies have also reported a decline in acylcarnitine levels in sepsis, with dodecanoylcarnitine and L-palmitoylcarnitine both being found to be significantly reduced by several studies (427,430,434,445). Mecatti *et al*. found that L-octanoylcarnitine and L-palmitoylcarnitine, along with gamma-linolenyl carnitine and linoleyl carnitine were raised in sepsis, and L-octanoylcarnitine demonstrated particularly promising results as a sepsis diagnostic biomarker, with an AUC of 0.89 (440).

5.4.6 Ketone Bodies

The ketone bodies are three small compounds produced when the liver metabolises fatty acids. They include acetone, acetoacetate and ß-hydroxybutyrate. Ketone bodies may be used as an energy source during starvation or prolonged exercise, particularly by tissues with a high metabolic demand such as the brain or skeletal muscles. Ketone bodies are taken up by target tissues and then converted to acetyl-CoA for entry into the TCA cycle (563). Increased quantities of ketone bodies may be seen in sepsis secondary to increased lipolysis (432), although a reduction may instead occur due to increased utilisation by peripheral tissues (564).

Increased ß-hydroxybutyrate has been seen in sepsis patients (432,458), as well as sepsis non-survivors (432). However a decline in ß-hydroxybutyrate in sepsis may also be observed. ß-hydroxybutyrate has anti-inflammatory effects via inhibition of the NLRP3 inflammasome formation, as well as promoting autophagy. Levels of ß-hydroxybutyrate were reduced in septic patients who died in a study by Acar (509), therefore low ß-hydroxybutyrate may be associated with more severe disease due to diminished anti-inflammatory and autophagy responses (509).

5.4.7 Phospholipids

Phospholipids are molecules containing a phosphate head bound to a glycerol backbone and two additional fatty acid molecules (546). Glycerophospholipids are a subgroup of phospholipids in which the phosphate group binds with an additional small molecule headgroup, for example choline. A molecule of DAG can have a hydroxyl group added to *sn*-3 to produce phosphatidic acid, the most basic phospholipid. Other glycerophospholipids may be made by adding different types of headgroups to the phosphatidic acid. For instance, addition of choline, ethanolamine, serine, inositol or glycerol to phosphatidic acid produce molecules of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylglycerol (PG) respectively.

5.4.7.1 Phosphatidylcholines

PCs are the most common phospholipids in mammals and are the key phospholipids found within cell membranes, (545,565). PCs may exert a protective effect in sepsis, as they may bind to and neutralise PAMPs such as LPS, thereby helping to reduce the inflammatory response (448).

Metabolomics studies show a variable response in PC levels during sepsis. Schmerler *et al*. found increased PCs in sepsis compared to SIRS patients, in particular PCs of carbon chain length C32–C36 (448). Mecatti *et al*. also reported an increase in several PCs of length 33-36, such as PC(16:0/18:1(9Z)) and PC(15:0/18:2(9Z,12Z)); while also finding a reduction in two others of length C36: PC(16:0/20:3(8Z,11Z,14Z)) and PC(16:0/20:1(11Z)) (439). The study by Arshad and colleagues showed globally reduced PCs in CAP patients, while Neugebauer *et al*. demonstrated a general decline in most PCs in sepsis, including PCaaC(32:2), (PCaaC36:6) and (PCaaC42:6) (436,445). Cambiaghi *et al*. found sepsis non-responders had a reduction in a wide range of PCs, suggesting that depletion of PCs in sepsis may prevent their protective effects, leading to worse outcomes (566).

5.4.7.2 Lysophosphatidylcholines

Lysophosphatidylcholines (LPC) are formed by hydrolysis of PCs by phospholipase A2. LPCs exert pro-inflammatory effects through increasing adhesion molecule expression by endothelial cells, and promotion of monocyte chemotaxis (428). Injured cells have ruptured membranes leading to release of LPCs which can act as DAMPs, further promoting the innate immune response. Moreover, LPCs have a role in T lymphocyte response modulation. Active T cells increase LPC production, further leading to paracrine and autocrine upregulation of neighbouring T cell responses (567,568).

Local inflammation appears to trigger a rise in LPCs, but a systemic response leads to a decline (439). Multiple metabolomics studies confirm a reduction in LPCs in sepsis (426–428,430,435,436,439,445,458), with LysoPC(18:2/0:0), LysoPC(18:0/0:0), LysoPC(16:0/0:0), LysoPC(18:1/0:0) and lysoPC (20:3) consistently showing reductions in septic patients. Moreover, the overall ratio of LPCs/PCs is reduced in sepsis (428). The reduction in LPCs in septic patients may be due to increased conversion to lysophosphatidic acid (569), which promotes pro-inflammatory cytokine and chemokine production (570). In contrast, Zheng *et al*. found an increase in LPCs LysoPC(16:1/0:0), LysoPC(16:0/0:0), and LysoPC(24:0/0:0), which they speculate may be due to mitochondrial dysfunction causing a reduction in lipid catabolism, leading to LPC accumulation (453).

Reduction in LPCs is associated with an increased mortality in sepsis (513,539). Interestingly, an animal and in vitro model of sepsis showed that LPC supplementation could have a protective effect in sepsis through increasing bacterial clearance and enhancing the oxidative burst in neutrophils (571).

5.4.7.3 Phosphatidylethanolamines

PEs are the second most common phospholipids in human membranes (565). PEs have a conical shape, which makes them useful structural components as this feature helps to add curvature to phospholipid membranes (545). Aside from their structural role, PEs have several other functions. They are involved in regulation of inflammation (568), and they promote cell autophagy which increases longevity (572). They increase cell proliferation (573) and have cardioprotective effects via activation of STAT-3 (574). PEs may reduce respiration in dysfunctional mitochondria which may limit oxidative damage in sepsis (575). The study by Guan *et al*. found differences in PE levels in sepsis patients compared to non-septic individuals. They found that PE(20:4(5Z,8Z,11Z,14Z)/P-18:0) was increased in septic patients, while PE(P-18:0/0:0) was reduced (430).

5.4.7.4 Phosphatidylserines

PSs are negatively charged lipids contained within the inner bilayer of cell membranes (545). They act as signalling molecules, for instance through activation of protein kinase C, promoting intracellular downstream signal transduction (576). They also are important initiators of apoptosis. Cell injury damages the inner membrane, exposing PS to the extracellular environment which acts as a DAMP and promotes phagocytosis by macrophages (577).

Mecatti *et al*. reported an increase in PS(14:0/15:0) and PS(18:0/22:1(13Z)) in septic patients compared to healthy controls (439), and also found an increase in PS(16:0/16:0) and PS(18:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z)) in patients with sepsis compared to those with non-septic SIRS (440).

5.4.7.5 Phosphatidylglycerols

PGs are found in low abundance in human lipid membranes compared to other species of phospholipids. PGs do however form a major constituent of most bacterial membranes (578), so an increase in serum PGs could be due to destruction of bacteria during sepsis. Moreover, production of PGs can be induced in the presence of LPS (579). Mecatti *et al*. reported a significant increase in serum PG (O−32:0) in patients with sepsis compared to those with non-infectious SIRS (440).

5.4.7.6 Cardiolipin

Cardiolipins (CL) are important mitochondrial lipids. Due to their distinctive shape, the presence of CL molecules adds significant curvature to plasma membranes. This is useful for the inner mitochondrial membrane which has multiple cristae to increase membrane surface area. When mitochondria are damaged, release of CL from the inner membrane acts as a signal to encourage mitophagy (545). Therefore, increased mitochondrial damage in severe sepsis may result in increased CL. Indeed, Mecatti reported an increase in cardiolipin CL(1'-[18:0/18:2]/3'-[20:0/20:0]) in the serum and in erythrocytes of septic patients compared to healthy controls (439).

5.4.8 Sphingolipids

Sphingolipids are a group of lipids which contain a sphingosine backbone. Sphingosine is made by the condensation of palmitoyl CoA and serine. The basic building block of sphingolipids is ceramide, formed from a long-chain fatty acid connected to a sphingosine base by an amide bond. Ceramide can then be converted to several other compounds, including sphingomyelins (SM) through addition of a phosphocholine group, or glycosphingolipids by adding sugars such as glucose or galactose (545).

5.4.8.1 Ceramides and Sphingomyelins

Ceramides have a protective role in forming a normal component of the skin barrier. Additionally, they play a role in immunomodulation, stimulating a pro-inflammatory response. Ceramides share a similar structure to LPS, and so are capable of binding to and activating the CD14 receptor, promoting NF-κB mediated inflammatory responses (580,581). Ceramides also are involved in cell cycle control, and regulate cell death through promotion of apoptosis and autophagy (428,440,545).

SMs are the most common sphingolipids and are components of the external leaflet of the plasma membrane. They interact with and recruit other proteins to the plasma membrane, increasing rigidity (568,582). SMs readily form hydrogen bonds and so may bind to various molecules, particularly cholesterol (583). Certain pathogens such as ebolavirus bind with SM to facilitate infection (584).

Increased sphingomyelinase activity occurs in sepsis, resulting in an increase in ceramides and corresponding decrease in SMs (445,585). A decreased quantity of SM in membranes reduces their stability and exposes PS, promoting apoptosis (439). An increase in the ceramide:SM ratio is associated with increased mortality in sepsis (428). Inhibition of sphingomyelinase may have a protective effect, as a murine model of sepsis revealed reduced organ damage with administration of sphingomyelinase inhibitors, suggesting this may be a potential therapeutic target for sepsis (586,587).

Naugebauer *et al*. found a single SM 16:1 to be raised in sepsis, however other metabolomics studies show a definitive decline in SM levels in patients with sepsis, CAP and cerebral malaria (426,434,436,439,445) Correspondingly, ceramides such as Cer(d18:1/16:0) and Cer(d18:1/18:0) are consistently elevated in sepsis (428,436,440). However, Drobnik et al reported a reduction in ceramides Cer(d18:1/23:0) and Cer(d18:1/24:0) in patients with sepsis (428).

5.4.9 Sterols

Cholesterol is the main sterol in humans. It forms an essential component of cell membranes, and is the precursor for steroid hormones and bile acids (545). Most cholesterol is synthesised in the liver, but all cells are capable of producing it. Additionally, cholesterol may be obtained by dietary intake. The structure of cholesterol comprises four aromatic rings bound to a hydroxyl group and a hydrophobic chain. Binding of a fatty acid to the hydroxyl group of cholesterol forms a cholesterol ester (588). Synthesis of cholesterol involves several steps, with intermediate isoprenoids being formed in the process. ß-Hydroxy ß-methylglutaryl-CoA (HMG-CoA) reductase is a crucial enzyme involved in cholesterol synthesis, and is the target for the statin class of drugs (589).

Due to its hydrophobic nature, cholesterol tends to be confined to lipid membranes or bound with lipoproteins or albumin, which allows its transport in the plasma. Low-density lipoproteins (LDLs) are responsible for transporting cholesterol from the liver to the peripheries, while high-density lipoproteins (HDLs) return cholesterol back to the liver, where it is metabolised or converted to bile acids (588). Along with cholesterol transport, HDLs have anti-inflammatory and anti-oxidant effects, and can bind with and clear bacterial PAMPs such as LPS and LTA (590,591).

Levels of HDLs and LDLs decline in sepsis (539). Decreased HDL in early sepsis may act as a prognostic indicator for the development of organ dysfunction (590), and so supplementation of HDL in septic patients may have positive therapeutic effects (591). Total cholesterol has also been shown to be reduced in sepsis, and is associated with worse prognosis (592). Interestingly, the metabolomics study by Lu *et al*. reported an increase in total cholesterol in septic patients compared to those with SIRS. This is a finding that has been replicated in a mouse model of sepsis, where the activity of HMG-CoA reductase was found to be increased by LPS (593).

5.4.9.1 Steroids

All steroid hormones use cholesterol as their precursor. Cholesterol is initially oxidised to produce pregnenolone, and further oxidation yields progesterone. Modifications in the endoplasmic reticulum can then yield various steroid hormones including sex hormones, glucocorticoids and mineralocorticoids (589,594).

Sex steroids appear to have immunomodulatory properties. Oestrogens may have a protective role in sepsis, with a rat model suggesting that oestrogen may prevent sepsis induced liver injury through prevention of oxidative stress mediated pyroptosis (595). Moreover, a murine model of sepsis determined that administration of 17β-oestradiol led to a reduction in TNF-α expression upon LPS administration in mice (596). In metabolomics studies, Kelly *et al*. found that septic patients had an increase in estrone sulphate: the most abundant oestrogen in non-pregnant females.

Lu and colleagues found decreased testosterone, methyltestosterone and dehydroepiandrosterone (DHEA) in sepsis patients (438). Androgens appear to have a suppressive effect on the immune system, and this may partly explain the increased mortality in males with sepsis (597). Testosterone receptor blockade may help to prevent androgen induced immunosuppression in sepsis (177).

Cortisol levels usually increase with sepsis acutely through activation of the hypothalamic-pituitary-adrenal axis (598), and through peripheral release of free cortisol from carrier proteins (599). However, patients with septic shock may exhibit impaired adrenocortical reserve and a relative adrenal insufficiency with poor responses to endogenous glucocorticoids (600). Indeed, the study by Zheng *et al*. found reduced cortisol in septic patients (453). Administration of low dose corticosteroids in sepsis aims to dampen down the excessive pro-inflammatory response while still maintaining a sufficient innate immune response to fight off infection (601), while also reducing the incidence of vasoplegia by limiting NO production, thus reducing noradrenaline requirements (601).

5.4.10 Bile acids

Liver dysfunction is common in critical illness (602). Sepsis induced liver dysfunction may lead to cholestasis, whereby bile production fails and bile acids accumulate (603). In sepsis, sinusoidal dilatation and Kupffer cell hyperplasia is observed, along with leukocyte infiltration and platelet aggregation. This leads to luminal obstruction, preventing bile flow. Moreover, sepsis induced hypoxia impairs contractile activity of bile ducts, further impairing flow (604).

Several metabolomics studies reported increased bile acids in sepsis including cholic acid, taurocholic acid, taurodeoxycholic acid, 3-Sulphodeoxycholic acid and glycocholic acid (427,438,440). Cholestasis may occur early in sepsis, and bile acids may be an earlier biomarker of sepsis induced liver dysfunction than traditionally used liver function tests (243).

5.5 Conclusion

Sepsis causes massive derangement in physiological processes, leading to significant metabolite perturbations. Disruption in energy metabolism commonly leads to elevated lactate and pyruvate, along with TCA cycle intermediates succinate and itaconate. Protein catabolism leads to significant derangement in amino acid levels. Enhanced lipolysis in sepsis affects many lipid species, particularly LPCs and acylcarnitines.

Many metabolomic studies have sought to identify sepsis biomarkers both for diagnostic and prognostic purposes. However, there is little research available investigating biomarkers for secondary infections in critically ill patients, and it remains to be seen if secondary infections would produce similar metabolic derangements as seen in primary sepsis. There is likely to be difficulty in identifying a single biomarker capable of identifying all secondary infections due to the heterogeneity in terms of infection site, underlying pathogen species, and host response and underlying comorbidities. The use of metabolomics to identify a panel of biomarkers may provide more success for diagnosis of secondary infections in COVID-19 compared to single biomarkers.

6 Aims and Methods

6.1 Introduction

Critically ill patients with COVID-19 are at an increased risk of developing a secondary infection, which carries a much higher risk of morbidity and mortality compared to those without secondary infections. Current biochemical and radiographic diagnostic tools are not sufficiently specific or sensitive to confirm or rule out the presence of an infection, and the gold standard method of culture and sensitivity is a slow process. Identification of novel biomarkers which could permit the early diagnosis of secondary infections would help to ensure that antibiotics are given as early in the disease process as possible, while preventing unnecessary empirical administration of broad-spectrum antibiotics.

6.2 Objectives

The objectives of this project were:

1. To identify and quantify metabolites significantly different between critically ill COVID-19 patients with and without secondary infections using metabolomics.

2. To determine the diagnostic capability of this metabolomic panel for identifying secondary infections.

3. To identify metabolites capable of differentiating Gram positive from Gram negative infections.

6.3 Recruitment

6.3.1 Study Design

This was a prospective, diagnostic, observational study which was open for recruitment from November 2020 until October 2021.

6.3.2 Location

Patients were recruited from high dependency units (HDUs) and ICUs of three hospitals within the NHS Greater Glasgow and Clyde health board: the Queen Elizabeth University Hospital, Glasgow; Glasgow Royal Infirmary, Glasgow; and the Royal Alexandra Hospital, Paisley. Sample processing and metabolite extraction occurred in the Roe Laboratory at the University of Glasgow. Metabolomic analysis took place at the Polyomics facility at the University of Glasgow Garscube campus.

6.3.3 Inclusion Criteria

Daily screening took place to identify potential patients for recruitment to the study. Patients were eligible for recruitment if they met each of the following inclusion criteria:

- They were 18 years of age or over
- They were a current hospital inpatient within medical HDU or ICU requiring level 2 care, defined as organ support and/or continuous monitoring beyond what is typically delivered on a ward setting.
- They had a positive SARS-CoV-2 RT-PCR or point of care test within the preceding seven days.

6.3.4 Exclusion

Patients were excluded from participation if they met any of the following criteria:

- Patients who were currently pregnant
- Patients who were not expected to survive their illness.

6.3.5 Healthy Volunteers

A separate group of healthy volunteers were also recruited to the study as controls. These volunteers were eligible for recruitment if they met the following criteria:

- They had no chronic health conditions
- They were not taking any current medication
- They had never been infected with SARS-CoV-2
- They had not yet received a vaccine against SARS-CoV-2

6.3.6 Consent

Eligible patients and/or their family members were provided with a patient information sheet which fully outlined the rationale for the study, requirements for participation, and any associated risks. Informed, written consent was sought for all patients in the first instance where possible. Patients who were incapacitated due to their current illness were not able to provide consent and therefore consent from their nearest relative was sought. Consent forms were signed face to face where possible, but telephone consent was gained when restrictions on hospital visitation prevented family members from attending. Patients or family members were free to withdraw their consent at any time. Patient information sheets and consent forms can be found in Appendices C-H.

6.3.7 Ethics

This study was presented to the Scotland Research Ethics Committee and gained approval (reference number 17/SS/0062). Recruitment to the study was carried out in adherence with the Declaration of Helsinki and in accordance with good clinical practice guidelines.

6.4 Data Collection

6.4.1 Paper Forms

All paper forms including signed consent forms and delegation logs were kept locally at participating sites in a secure site file in a locked cabinet. Participating sites utilised a research office which was locked out of hours.

6.4.2 Clinical Data

Upon recruitment, patient details were entered into the EDGE: a secure online clinical research management system. Each patient was allocated a unique study number, which was the only identifier used for patients outside of the EDGE system. All collected data was anonymised using the study numbers.

Patient data was extracted from paper case notes and electronic clinical records. A password protected electronic Excel spreadsheet kept on a secures server was used to compile data. The following clinical data was collected for each patient:

- Age, gender and ethnicity
- Body mass index
- Comorbidities
- Date of SARS-CoV-2 infection
- Hospital and critical care length of stay
- Survival outcome
- Organ support requirements
- SOFA score
- Inflammatory markers
- Microbiological culture results
- Drug infusions, antibiotics and immunomodulating drugs.

To check for potential manual data entry errors, 10% of the data was randomly subjected to a repeat retrospective collection. An online random number generator was used to provide eleven patients for repeat data collection (605). This check did not reveal any major systematic errors in the data collection process. See *Appendix I* for the results of this data check.

6.4.3 Secondary Infection Definitions

Secondary infections were confirmed according to the Centers for Disease Control and Prevention National Healthcare Safety Network definitions (606–609) (See *Table 6-1*). A repeated growth of the same organism was considered the same infection. Consultant clinical microbiologist Professor Alistair Leanord acted as an independent adjudicator for occasions of diagnostic uncertainty, as outlined in *Appendix J*.

Infection Type	Criteria
Bloodstream	1) Single growth of a pathological organism OR 2) Two or more cultures of a skin contaminant on two consecutive days.
Respiratory Tract	1) Growth of an organism from a BAL OR 2) Growth of an organism from an upper respiratory tract sample AND Evidence of an inflammatory response (WCC >12, Temp < 36 or > 38) AND CXR changes AND O_2 requirement AND Purulent secretions
Urinary Tract	Growth of an organism with ≥ 10^5 CFU/ml AND Pyrexia and/or symptoms of urinary tract infection
Gastroenteritis	Acute onset diarrhoea >12 hours with no clear non-infectious cause OR At least two of the following: nausea, vomiting, abdominal pain, pyrexia or headache AND At least one of the following: a) Positive identification of enteric pathogen from stool or rectal swab; b) Positive identification of enteric pathogen by microscopy; c) Diagnostic single antibody titre (IgM) or 4-fold increase in paired sera (IgG) for organism.

Table 6-1: Secondary infection diagnostic criteria.

6.5 Sample Collection and Processing

6.5.1 Blood Sampling

Serial blood samples were gathered in a red topped serum clot activator tube from patients at three time points: on the day of recruitment (day 0), day 3 and day 10. An additional blood sample was collected in any patient who demonstrated evidence of developing a secondary infection by way of yielding positive microbiological cultures or through a clinical deterioration felt likely to be due to an infective cause. This permitted a maximum of four blood samples to be collected per patient across a 10-day period. Sampling was stopped before day 10 if patients were discharged from hospital or withdrew their consent for ongoing participation. Blood samples were collected via venepuncture or by sampling from a pre-existing arterial or central venous catheter. Healthy volunteers had a single blood sample collected on the day of recruitment.

6.5.2 Sample Processing

Collected blood samples were allowed to fully clot for one hour. Once clotted, blood tubes were spun down in a centrifuge at 2000G for a period of 15 minutes. The supernatant was then pipetted and stored in a -80^{0}C freezer. Samples were transported to the University of Glasgow Roe Laboratory in bulk using dry ice containers.

6.5.3 Metabolite Extraction

A metabolite extraction solvent was prepared by combining chloroform, methanol, and water in a 1:3:1 ratio (v/v/v). The solvent was bottled and kept refrigerated at 4^{0}C. While in use, the bottle was placed in a container of ice to maintain this temperature. Extraction of metabolites was performed by first allowing samples to fully defrost for 1 hour. A 25µl measure of each sample was added to 1ml solvent in an Eppendorf tube, then each tube was vortexed for 1 minute. The Eppendorf tubes were placed in a centrifuge and were spun down at 13000G for 3 minutes at a temperature of 4^{0}C. A 200µl aliquot of each sample was then collected in a screw-top vial. The vials were frozen in a -80^{0}C freezer and transported to the Polyomics laboratory in containers filled with dry ice.

6.6 Metabolomic Analysis

6.6.1 High Performance Liquid Chromatography-Mass Spectrometry

Metabolomic analysis of samples was performed by Dr Clement Regnault and Dr Gavin Blackburn of the University of Glasgow Polyomics Facility, supervised by Professor Phil Whitfield. HILIC was carried out on a Dionex UltiMate 3000 Rapid Separation LC platform (Thermo Fisher Scientific, Hemel Hempstead, UK) using a Merck SeQuant zwitterionic polymeric hydrophilic interaction chromatography (ZIC-pHILIC) 150 mm × 4.6 mm, 5 µm column kept at a temperature of 25ºC.

Samples were eluted over 26 minutes with a flow rate of 0.3 ml/min using a mobile phase linear gradient comprising (A): 20mM ammonium carbonate in water; and (B): acetonitrile as outlined in *Table 6-2*.

Time (minutes)	%(A)	%(B)
0	20	80
15	80	20
15	95	5
17	95	5
17	20	80
26	20	80

Table 6-2: Liquid chromatography mobile phase linear gradient

6.6.2 Mass Spectrometry

MS analysis was performed using a Thermo Orbitrap QExactive (Thermo Fisher Scientific) system. Samples were maintained at 5°C, and a sample volume of 10µl was injected into the system. Analysis was conducted with a *m/z* range of 70-1050, resolution of 70,000, and an automatic gain control setting of 1e6. Polarity switching was used, with spray voltages for positive and negative ionisation modes of +3.8 kV and -3.8kV respectively. Sheath gas pressure was 40 units, auxiliary gas pressure was 5 units and sweep gas pressure was 1 unit. The probe temperature was maintained 150°C and the capillary temperature was set to 320°C.

6.6.3 Metabolomic Analysis

Metabolomic analyses and production of chromatograms and mass spectra were overseen by Dr Clement Regnault and Dr Gavin Blackburn. The two processes of LC-MS (separation and detection) produce complicated data sets that require extensive processing before statistical analysis can take place (610). Raw data was initially processed using opensource software including the XCMS (420) and mzMatch (421) computational tools. XCMS was used to extract relevant peaks, align retention times and produce chromatograms. The 'mzMatch' pipeline was used to filter background noise, combine duplicate peaks based on their *m/z* ratio and retention times, and provide relative abundance. Putative metabolite identification was achieved by comparison of *m/z* ratios of peaks with database values using the excel macro software Identification Of Metabolites (IDEOM) software (611).

6.6.4 Metabolite Identity Confirmation

Confirmation of metabolite identities was achieved first by looking for a match between the *m/z* and retention times of putatively identified peaks and authentic standards. If this did not provide a confirmatory identification, tandem mass spectrometry was utilised, and fragmentation spectra of peaks was compared with authentic standards. Where available, the KEGG database was searched to identify relevant biological pathways that each identified metabolite is involved in.

6.7 Bioinformatics

6.7.1 Abundance of Metabolites

Statistical analysis and production of plots was undertaken by Dr Rónán Daly, head of Artificial Intelligence and Machine Learning at the College of Medical, Veterinary and Life Sciences at the University of Glasgow Shared Research Facilities. All analysis was performed using R (612). Metabolites significantly different in abundance were identified using the limma software package (613). This tool uses parametric empirical Bayes statistical methods which permitted initial exploration of data. The data was log2 transformed, and potential batch effects and confounders was explored. Initial multivariate analysis was conducted using PCA, which permitted assessment of clustering of data. Significant differences in metabolite concentrations were determined using t-tests. As multiple comparisons were conducted, the R qvalue package (614) was used to provide q-values from the resulting p-values. A q-value is the minimum false discovery rate where a test may still be considered significant (615). The cut-off threshold for significance was determined *post-hoc*.

6.7.2 Predictive Models

Logistic regression predictive models were created for identification of secondary infection using the metabolomics data in isolation, and in combination with SOFA score and inflammatory marker data. An additional predictive model investigating differentiation of gram staining was also produced. These models were created using the R classification and regression training (caret) (616) and arm (617) packages. The final model used a 10-fold cross-validation procedure, repeated 100 times to gauge validated performance. Graphs depicting test performance were produced by plotting specificity against sensitivity.

6.8 Conclusion

Metabolomics is a modern field of analytical science which allows many thousands of metabolites to be measured simultaneously. Metabolic phenotyping using HPLC/MS has been shown to be possible in sepsis. This study aimed to apply the same principle to identify patients who were critically ill with COVID-19 to identify metabolic phenotypes for patients who developed secondary infections. Moreover, the study aims to differentiate between patients with Gram positive and negative infections.

7 Results: Patient Population

7.1 Introduction

This chapter provides the background demographics and details about the hospital stay for the patients recruited to the study, as well as the number and characteristics of the infective organisms identified.

7.2 Screening and Recruitment

Screening for recruitment occurred daily from November 2020 to October 2021. *Figure 7-1* outlines patient recruitment. A total of 158 patients were screened within this time, of which 53 were excluded. The most common reason for exclusion was patient refusal of additional blood sampling. The remaining 105 patients were recruited to the study. Of these, 40 (38%) went on to develop a secondary infection, and 65 (62%) remained without a secondary infection. In addition, 49 healthy volunteers agreed to participate in the study.

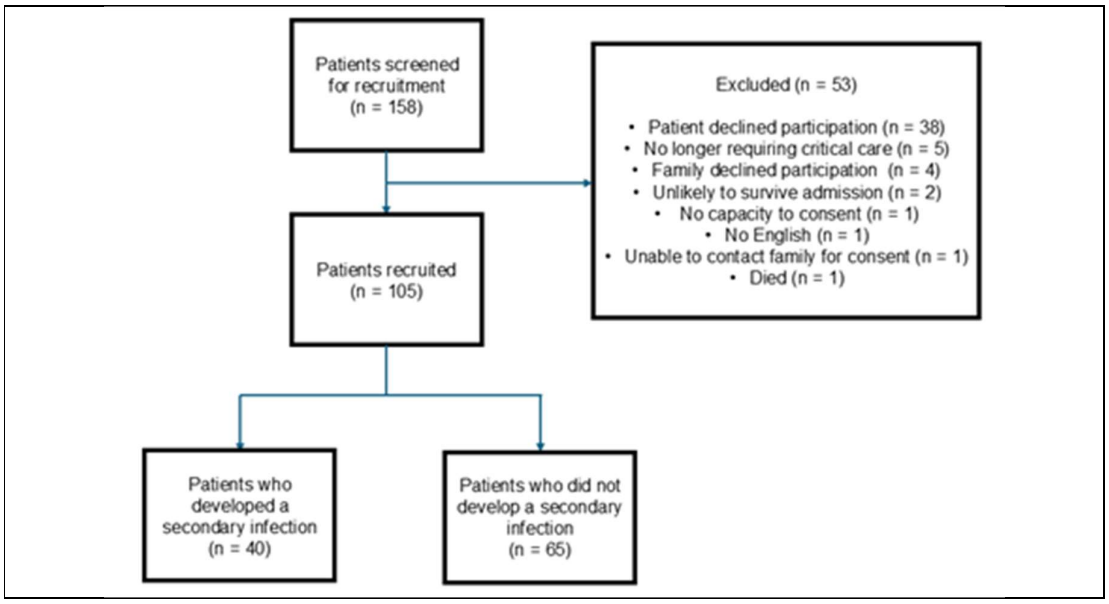

Figure 7-1: CONSORT diagram depicting patient participation

A total of 299 blood samples were collected from patients. Of these, 103 samples were gathered from patients with a secondary infection, while 196 were from patients without secondary infections. Combined with the 49 samples from healthy volunteers, this resulted in a grand total of 348 blood samples.

7.3 Demographics

The background demographics for recruited patients are outlined in the table below.

	Secondary Infection 40 (38.1%)	No Secondary Infection 65 (61.9%)
Age, years (Median ± IQR)	59 ± 13.75	57 ± 11
Sex n (%)		
Female	22 (55%)	22 (33.8%)
Male	18 (45%)	43 (66.2%)
Ethnicity n (%)		
White	34 (85%)	55 (84.6%)
Asian, Asian Scottish or Asian British	3 (7.5%)	4 (6.2%)
Black, Caribbean or African	1 (2.5%)	3 (4.6%)
Multiple Ethnicities	1 (2.5%)	0 (0%)
Other Ethnic Group	1 (2.5%)	0 (0%)
Not recorded	0 (0%)	3 (4.6%)
BMI (Median ± IQR)	32 ± 8.15	32 ± 9.1
Comorbidities n (%)		
Hypertension	13 (32.5%)	18 (27.7%)
Asthma	10 (25%)	8 (12.3%)
Diabetes Mellitus	9 (22.5%)	11 (16.9%)
Immunosuppression	9 (22.5%)	9 (13.8%)
Ischaemic Heart Disease	3 (7.5%)	4 (6.15%)
Active Cancer	2 (5%)	5 (7.7%)
COPD	1 (2.5%)	5 (7.7%)

Table 7-1: Comparison of demographics between patients with and without secondary infections. BMI: Body mass index, COPD: Chronic obstructive pulmonary disease, IQR: Interquartile range.

A greater proportion of males were recruited than females, however secondary infections were more common in female patients. The most common comorbidities were hypertension, asthma and diabetes. Recruited patients had a median BMI of 32, which is categorised as obese. BMI was the same between those with and without secondary infections.

7.4 Hospital Stay and Therapies

Table 7-2 depicts the patient lengths of hospital stay and some of the critical care therapies received.

	Secondary Infection 40 (38.1%)	No Secondary Infection 65 (61.9%)
Days From SARS-CoV-2 Infection to Hospital Admission (Median ± IQR)	5 ± 7.25	3 ± 6.5
Days from Hospital Admission to Critical Care Admission (Median ± IQR)	1 ± 2.25	1 ± 2
Critical care LoS (Median ± IQR)	18 ± 15	8 ± 12.5
Hospital LoS (Median ± IQR)	29 ± 30.5	13 ± 17
Outcome n (%) Survived Died	 23 (57.5%) 17 (42.5%)	 46 (70.8%) 19 (29.2%)
Mechanical Ventilation n (%)	37 (92.5%)	23 (35.4%)
Renal Replacement Therapy n (%)	4 (10%)	7 (10.8%)
Antimicrobials n (%)	39 (97.5%)	36 (55%)
Corticosteroids n (%)	40 (100%)	65 (100%)
IL-6 Inhibitors n (%)	33 (82.5%)	45 (69.2%)
JAK Inhibitor n (%)	1 (2.5%)	8 (12.3%)

Table 7-2: Comparison of lengths of stay and therapies between patients with and without secondary infections. LoS: Length of stay.

Patients with secondary infections had both a longer critical care and hospital stay than those without. The mortality rate was higher in patients with secondary infections. The majority of secondary infections occurred in patients who were mechanically ventilated. While almost all patients with a secondary infection appropriately received antibiotics (97.5%), over half of those without a secondary infection also received antimicrobial therapy unnecessarily.

7.5 Secondary Infections

During the trial period 213 microbiological samples grew an organism. There were 62 positive blood cultures, 130 positive respiratory tract samples, 20 positive urine cultures and a single positive stool sample. The full list of positive microbiology can be found in *Appendix K*. Any positive sample which did not meet the definition of infection as per *Table 6-1* was excluded. Common skin commensal organisms including coagulase-negative staphylococci, *Propionibacterium* species and diphtheroids were frequently excluded from blood cultures, as well as organisms commonly found as part of the normal flora of the oropharynx and upper respiratory tract, such as *Candida* and *S. aureus*. Advice from a clinical microbiologist was sought for cases in which the presence of a secondary infection was difficult to determine based solely on the diagnostic criteria. The results of these discussions are available in *Appendix J*.

After exclusion of positive samples which did not meet the infection criteria, there were 70 confirmed infections in 40 patients. *Table 7-3* shows the secondary infections detected in blood, urine, respiratory cultures and GI tract.

Infection	Incidence
BSI (n=1)	
Staphylococcus aureus	1 (100%)
CLABSI (n=6)	
Candida	1 (16.6%)
Staphylococcus aureus	1 (16.6%)
Pediococcus acidilactici	1 (16.6%)
Staphylococcus hominis	1 (16.6%)
Escherichia coli	1 (16.6%)
Enterococcus faecalis	1 (16.6%)
Urine (n=10)	
Escherichia coli	8 (80%)
Klebsiella pneumoniae	1 (10%)
Proteus mirabilis	1 (10%)
Chest (n=52)	
Staphylococcus aureus	11 (21.15%)
Haemophilus influenzae	6 (11.53%)
Escherichia coli	4 (7.69%)
Klebsiella pneumoniae	4 (7.69%)
Aspergillus fumigatus	4 (7.69%)
Streptococcus pneumoniae	3 (5.77%)
Pseudomonas aeruginosa	3 (5.77%)
Proteus mirabilis	3 (5.77%)
Candida	3 (5.77%)
Klebsiella aerogenes	2 (3.85%)
Raoultella ornithinolytica	2 (3.85%)
Enterobacter cloacae	2 (3.85%)
Raoultella planticola	1 (1.92%)
Streptococcus agalactiae	1 (1.92%)
Klebsiella oxytoca	1 (1.92%)
Stenotrophomonas maltophilia	1 (1.92%)
Morganella morganii	1 (1.92%)
Gastrointestinal tract (n=1)	
Campylobacter	1 (100%)
Single infection	21 (52.5%)
Polymicrobial	19 (47.5%)

Table 7-3: Secondary infections cultured from blood, urine, respiratory tract and gastrointestinal tract. BSI: Bloodstream infection, CLABSI: Central line associated bloodstream infection

Most secondary infections were cultured from the respiratory tract. *S. aureus*, *Klebsiella* species and *H. influenzae* were the most frequent respiratory pathogens. Just under half of all infections were polymicrobial in nature.

7.6 Discussion

7.6.1 Incidence

The incidence of secondary infection in this ICU patient cohort was 38.1% This is in keeping with the incidence of secondary infection reported in other studies of 25-58% (154,158,160–170). Recruitment to this study began after the first wave of the pandemic had concluded. Given that the first wave was associated with the most frequent usage of broad spectrum antibiotics in the COVID-19 population (172), it is possible that the true incidence of secondary infection may have been even greater during that period. Conversely, there was an increased use of immunomodulatory drugs beyond the first wave, which may have contributed to an increased incidence of secondary infection in ICU.

7.6.2 Mortality

The overall mortality rate in this study was 34%. The mortality rate for patients without a secondary infection was 29.2%, which climbed to 42.5% for patients who did develop a secondary infection, which again is similar to the mortality rate in other studies (89,181,193). The rise in mortality associated with secondary infections highlights the deadly consequence of this complication within the COVID-19 population, demonstrating the importance of early recognition and treatment.

7.6.3 Comorbidities

Several comorbidities are associated with an increased risk of developing a secondary infection in COVID-19 such as diabetes mellitus, atrial fibrillation, ischaemic heart disease, and malignancy (181,183,184). In the present study, hypertension, diabetes and asthma were the most common comorbidities. Asthma was more common in patients with secondary infections. Asthma is characterised by chronic airway inflammation leading to epithelial injury and remodelling, with metaplasia and increased mucus production contributing to increased rates of bacterial colonisation and adherence, particularly *S. pneumoniae* (618). Furthermore, long term inhaled corticosteroid usage may be associated with increased rates of pneumonia among the asthmatic population (619). As a result, patients with asthma are at an increased risk of developing pneumonia (620).

7.6.4 Immunosuppression

While the number of patients admitted with COVID-19 to critical care has decreased in recent times, patients who are immunocompromised may still suffer severe disease. Over 20% of patients with secondary infections in this study were receiving immunosuppressant drugs prior to admission. In theory, the use of immunosuppressive drugs in organ transplant patients could further mitigate the inflammatory cytokine storm seen in COVID-19, perhaps reducing the risk of severe disease (621). However this does not appear to be the case in clinical practice, with immunosuppressed patients having an increased risk of severe disease and worse outcomes (622).

Rituximab is an anti-CD20 chimeric antibody used to deplete B-cells in various haematological and rheumatological conditions. Rituximab administration is implicated in reduced hormonal response after SARS-CoV-2 vaccination, reducing its effectiveness and therefore putting these patients at increased risk of severe disease (623). Moreover, hypogammaglobluninaemia is a recognised side effect of rituximab (624) and can increase the risk of developing severe infections (625). Other drugs which have been similarly suggested to reduce seroconversion include mycophenolate, methotrexate and abatacept (626). Patients with rheumatological disease who take immunosuppressant drugs may be more likely to develop severe COVID-19, and as a result have increased rates of ICU admissions and mechanical ventilation, increasing their risk of secondary infection (627).

Patients with solid organ transplants are at a greater risk of developing a secondary infection. The study by Rinaldi *et al*. found that transplant patients with COVID-19 had a rate of superinfection of 50%, while it was only 15% in non-transplant patients (628). Immunosuppressed patients with solid organ transplants appear to be at risk of prolonged viral shedding and increased disease severity (629). Compared to other transplants, patients who have had lung transplants are at the highest risk of severe disease and have the highest mortality rate, likely in part due to increased immunosuppression requirements for lung transplants (630). In an American cohort study by Aversa *et al.*, of 32 lung transplant recipients who were hospitalised with COVID-19, just under 50% developed a secondary infection (631). Ventilated immunocompromised patients appear to be particularly at risk of opportunistic infections such as pulmonary aspergillosis (632).

7.6.5 Infection Site

7.6.5.1 Bloodstream Infection

Invasive line infection is common in critically ill COVID-19 patients, and the longer the duration of indwelling lines, the greater the risk of infection (163,188). In the present study, there were six CLABSI cases and a further case of primary BSI. These seven BSIs equated to 10% of all secondary infections in this cohort. This is towards the lower end of the incidence of bloodstream infections in COVID-19 ICU patients quoted in other studies, where the incidence is reported as 10-40% of all secondary infections (158,163,165,169,176,178,181,183,194).

The majority of the BSIs in this study were caused by Gram positive organisms. This is in keeping with the study by Grasselli *et al.* who found that Gram negative infections took longer to culture than Gram positive and fungal infections. The median time to positivity for gram negative infections was 15 days from ICU admission, whereas gram positive and fungal infections only took 10 days (176).

The short observation period at the beginning of the patients' critical care stay employed during this study may account for the lower incidence of BSIs and smaller proportion of Gram negative infections. The patients in this study were recruited early in their critical care admission and followed up for a 10-day period only. It is possible that a longer observation period may have yielded more positive cultures.

7.6.5.2 Mechanical Ventilation and Ventilator Associated Pneumonia

Progression of respiratory failure is often the key factor in COVID-19 patients being admitted to ICU. Subsequent intubation and mechanical ventilation was required in a large number of patients, particularly early on in the pandemic (205). A meta-analysis found that IMV prevalence in patients admitted to ICU with COVID-19 was 69% (633). Rates varied geographically, with 85% of ICU patients receiving IMV in a New York study (634) compared with 29% in a Chinese study (635).

In the present study, 92.5% of secondary infections occurred in mechanically ventilated patients. Critically ill COVID-19 patients who receive IMV are more likely to develop a secondary infection (166,198). This may be related to the severity of their disease, as critically ill patients are at the highest risk of developing a secondary infection (150,173). Alternatively, the increased use of IMV in this population may contribute to increased rates of VAP.

The meta-analysis by Langford et al found that mechanically ventilated COVID-19 patients were at an increased risk of both co-infection and secondary infection, with an odds ratio of 1.24 and 1.44 respectively (158). The longer the duration of IMV, the greater the risk of developing a secondary infection (176). Saade *et al.* found the incidence of secondary infections to be 48% in mechanically ventilated COVID-19 patients, compared to 2.2% in non-ventilated patients (166). The use of endotracheal tubes which bypass upper airway defences, as well as an increased incidence of ARDS, prone positioning, and use of immunomodulatory drugs may all increase the risk of developing VAP in critical COVID-19 (636).

7.6.6 Pathogens

7.6.6.1 Gram Negative Infections

In patients with COVID-19, secondary infections caused by Gram negative organisms tend to be nosocomial and often occur later in the ICU stay (154). Gram negative bacteria were responsible for 30% of secondary infections in ICU in the study by Bardi *et al.* (165), but were responsible for over 85% in the study by Li *et al.* (194). In the present study, the incidence of Gram negative respiratory tract infections was twice that of Gram positive infections, at 30 and 15 respectively.

The most common Gram negative organisms responsible for secondary infections in COVID-19 are *Enterobacterales* (18-45%) (163,178); *Klebsiella* species (154,158,183), especially *K. pneumoniae* (11-30%) (178,194); A*cinetobacter species* (29.5-48%) (163,173,183,194); *E. coli* (21-25%) (154,173,183,637); *H. influenzae (12%)* (155,158); and *P. aeruginosa* (11-12%) (155,178,183).

Several studies report that *A. baumannii* is a very common cause of secondary infection in COVID-19 (173). Indeed, Karaca and colleagues found that *Acinetobacter spp.* was the most common pathogen, responsible for 48% of infections (183), and a study in Iran found *A. baumannii* was responsible for 90% of secondary pneumonias (195). However Falcone *et al.* found it to be uncommon, with only 2.75% of infections caused by *A. baumannii* (178). This organism was not responsible for any infections in the current study, which may represent epidemiological differences secondary to geography. *A. baumannii* is a pathogen which can cause opportunistic infections in ICU where it tends to appear in outbreaks, and often exhibits multi-drug resistance. Hospitals in Eastern and Southeastern Europe experience frequent outbreaks of *A. baumannii* infections, where carbapenem resistance is high. Countries in the Middle east are also commonly affected. The rate of positive clinical samples for *Acinetobacter* spp. is low in the UK at 1.21% (638).

7.6.6.2 Gram Positive Infections

Gram positive infections were the most common infection type according to Bardi *et al.*, accounting for 55% of secondary infections in this study (165). Community acquired infections are more likely to be Gram-positive compared to nosocomial infections (183). The most common Gram positive pathogens are *S. aureus (4.6-16%)* (158,178,183); Coagulase negative *Staphylococci* (16%) (158,173); and *Enterococcus* species (5.5-18%) (178,183).

MRSA infections were common in the study by Karaca *et al.*, *with* 7% of infections caused by this organism. Moreover, Methicillin-resistant *Staphylococcus epidermidis* was found to be responsible for 10% of secondary infections (183). Coagulase negative Staphylococci are common contaminants of cultures, and so higher rates of culture of these organisms may simply reflect blood culture contamination rather than true infection (158,183).

In the present study, the most common causative organism for secondary bacterial pneumonia was *S. aureus*. Studies have demonstrated increased rates of nosocomial *S. aureus* infection in COVID-19, particularly MRSA (639). This was the most common Gram positive organism linked to VAP in the systematic review by Fumagalli and colleagues (640). Likewise, *S. aureus* was the most common cause of secondary pneumonia in the studies by Petty *et al*. and Park *et al*. (184,641). Cavitating pneumonia may result from *S. aureus* infection particularly when toxin producing organisms are involved. Panton-Valentine leucocidin-producing *S. aureus* can cause severe necrotising pneumonia which can be rapidly fatal, requiring prompt recognition and management (642).

S. aureus forms part of the normal flora of the skin and mucous membranes, and may be present as a coloniser in the oropharynx and nasopharynx. A positive growth of *S. aureus* from a sputum sample does not necessarily signify an infection, and it can therefore be challenging to determine if a positive *S. aureus* culture represents harmless colonisation or a genuine infection.

7.6.6.3 Fungal Infections

Fungal infections are a rare cause of secondary pneumonia in COVID-19, and can occasionally cause UTI or line infections (155,197). Critically ill COVID-19 patients appear to be at an increased risk of invasive pulmonary aspergillosis (IPA) (643), and the risk increases with duration of ICU stay and is particularly high in patients receiving high dose immunosuppression (201,644). Concerningly, a French cohort study putatively identified IPA in nearly one third of their mechanically ventilated COVID-19 patients (645), while another reported a rate of 20% (200). In the present study, there were 4 cases of pulmonary *Aspergillus* and 3 instances of pulmonary *Candida,* with an additional case of *Candida* CLABSI.

Aspergillus and *Candida* species are the most common fungal secondary infections, particularly *Aspergillus fumigatus, Candida albicans* and *Candida parapsilosos* (154,155,165,173,198,205). *Candida* secondary infections are most frequently linked to urinary catheters or lines used for parenteral nutrition (644). *Candida* and unspecified yeast growth often occurs in sputum samples, but may simply represent oral thrush or commensal organisms rather than genuine fungal respiratory tract infection (197). The presence of galactomannan in a BAL sample is a better technique for identifying fungal infection, but due to the risk of aerosol generation, BAL was performed less frequently during the pandemic, which may have caused an underestimation in the number of fungal infections (155).

7.6.7 Antibiotic Usage

While almost all patients with a secondary infection appropriately received antibiotics, 55% of patients without a secondary infection also received antimicrobial therapy when it was not indicated. This is a concerning finding, as unnecessary antimicrobial administration is associated with an increased incidence of secondary infections (181). Within the ICU setting, increased exposure to antimicrobials cultivates resistant organisms, making future treatment of infections challenging. This again highlights the importance of identifying new methods for determining the presence of secondary infections, to avoid unnecessary antibiotic usage.

7.7 Conclusion

Bacterial and fungal secondary infections complicate COVID-19, and critically ill patients are at an increased risk of developing them. Mortality is increased with secondary infections, so prompt identification and appropriate treatment is paramount. Paradoxically, high rates of antibiotic usage in critically ill patients with COVID-19 increase the risk of developing secondary infections. These are often multidrug-resistant pathogens which present a challenge to treat. Antimicrobial stewardship is essential to ensure appropriate antibiotics are administered to patients who will benefit from them. The clinical manifestations of COVID-19 make it difficult to diagnose secondary infections. Traditional biomarkers for infection are non-specific and will frequently be abnormal in COVID-19 regardless of the presence of a secondary infection. Novel biomarkers of secondary infections would help to make this distinction.

8 Using Metabolomics to Identify Secondary Infections in Critically Ill Patients With COVID-19

8.1 Introduction

Metabolomics has rapidly gained traction over the past two decades as an invaluable tool within systems biology. LC-MS metabolomics studies have shown promise in identifying novel sepsis metabolite biomarkers, as well as characterising metabolic pathways which show derangement in sepsis, highlighting potential therapeutic targets. Unfortunately, there are at present no biomarkers specific for identifying secondary infections in COVID-19. This chapter therefore explores the use of LC-MS metabolomics to identify potential biomarkers of secondary infections in COVID-19.

8.2 Principal Component Analysis

As discussed in Chapter 7, 103 samples were gathered from patients with a secondary infection, along with 196 samples from patients without secondary infections and an additional 49 healthy volunteer samples. The PCA plots are depicted in *Figure 8-1*.

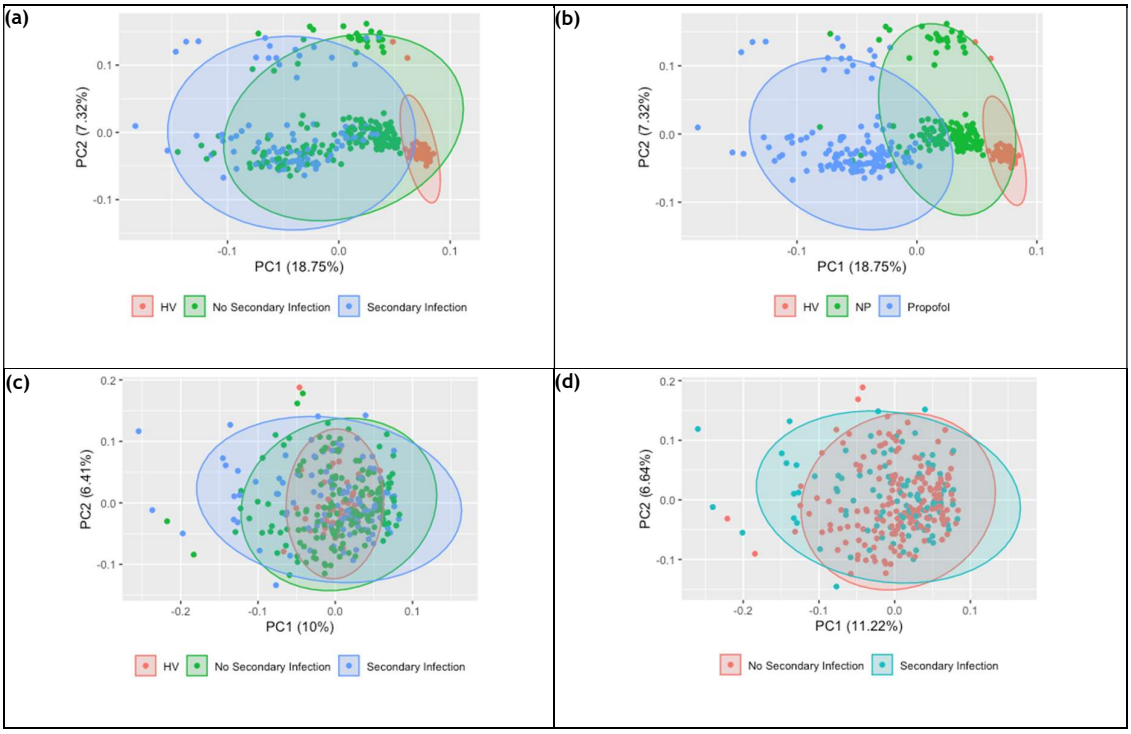

Figure 8-1: Principal component analysis. Plots defined by principal components PC1 and PC2 for: (a) full data set labelled according to secondary infection status (b) full data set labelled according to use of propofol infusion, (c) after filtering, (d) after filtering with exclusion of healthy volunteers (HV). NP: No propofol.

Figure 8-1 (a) shows the complete data set. Clustering of the healthy volunteer samples is apparent, along with a large proportion of the non-secondary infection samples. There is then a mix of secondary infection and non-secondary infection samples spreading across the x-axis. On further investigation, these samples were predominantly from patients receiving level 3 care. *Figure 8-1 (b)* shows the same plot, colour coded for patients receiving a propofol infusion. The similarities between *Figure 8-1 (a)* and *(b)* suggests that a proportion of the separation was likely due to metabolite differences caused by patients being admitted to the intensive care unit. Drug infusions such as propofol and atracurium will likely have been major contributors to the observed differences. Additionally, there is a clear separation along the y-axis, with two horizonal bands of discrete clusters occurring. This top band represents the last batch of samples to undergo metabolite extraction in the laboratory. These samples were all processed after a different brand of pipette tips had been acquired for the laboratory. As such, this separation is likely due to differences in the metabolites extracted from the plastic pipette tips.

To account for this confounding, the data was filtered to minimise background signals, and a correction was applied to account for covariates including extraction date, location, sedation, and paralysis use. *Figure 8-1 (c)* and *(d)* shows the PCA plots after this filtration and correction was applied, with and without the presence of the healthy volunteer samples. Applying this correction has successfully removed the separation caused by the confounders.

8.3 Putative Identification of Metabolites

From the initial LC-MS analysis, 32 peaks were detected which differed significantly between patients with and without secondary infections. Signals which were of poor quality were excluded, along with isotope peaks and non-annotated peaks. This left seven peaks which corresponded to four compounds, putatively identified as creatine, S-methyl-L-cysteine, 2-hydroxyisovalerylcarnitine and L-prolinylglycine. The chromatograms for these four peaks are shown in *Figure 8-2*.

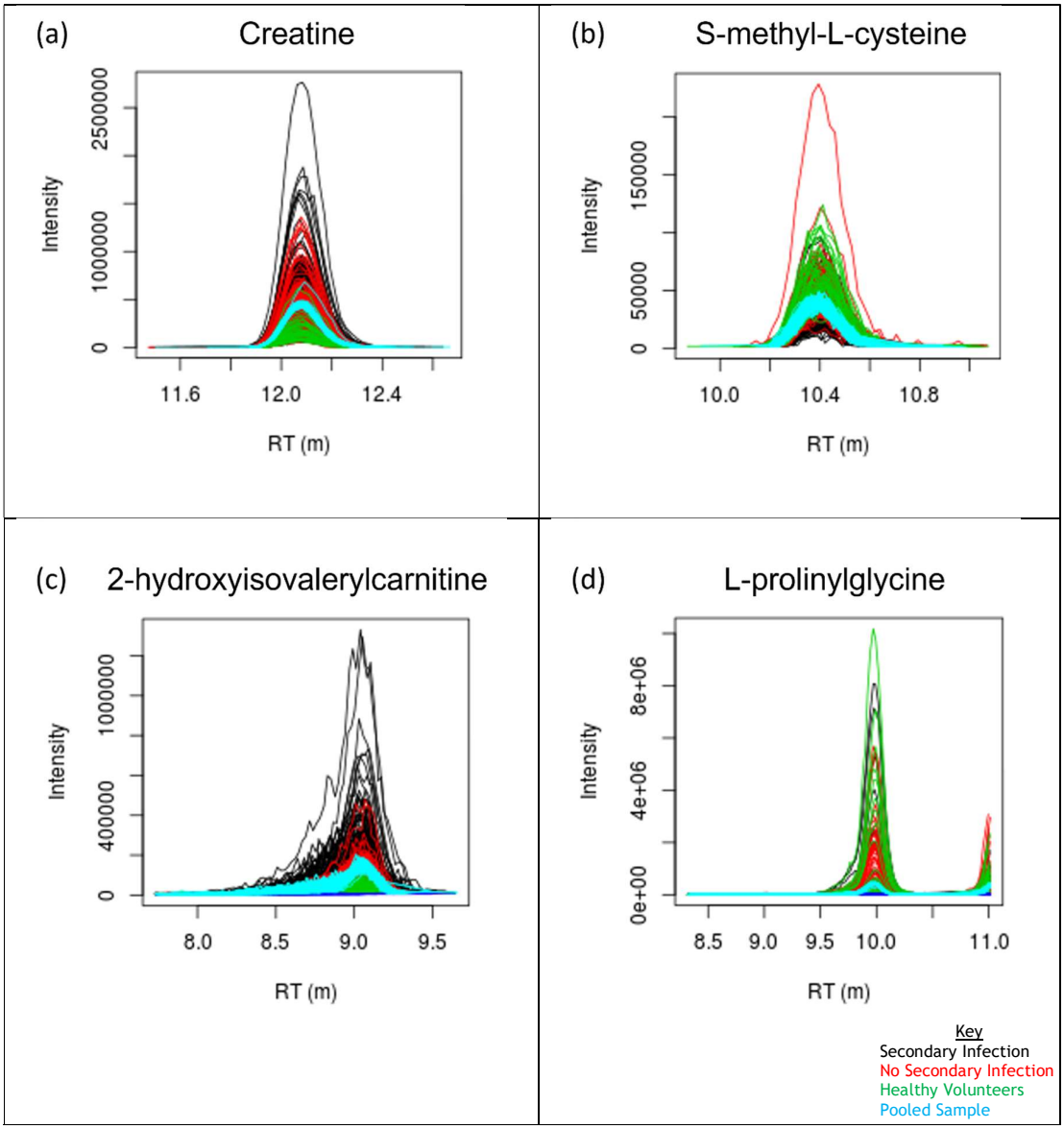

Figure 8-2: Chromatograms for putatively identified peaks (a) creatine, (b) S-methyl-L-cysteine, (c) 2-hydroxyisovalerylcarnitine, (d) L-prolinylglycine.

8.4 Confirmation of Peak Identities

8.4.1 Creatine and S-methyl-L-cysteine

To confirm the identities of the detected peaks, an injection of pooled sample was analysed alongside authentic standards for creatine and S-methyl-L-cysteine. The resultant mass and retention times between the standards and the peaks demonstrated an exact match, as shown in *Figure 8-3* and *Figure 8-4*.

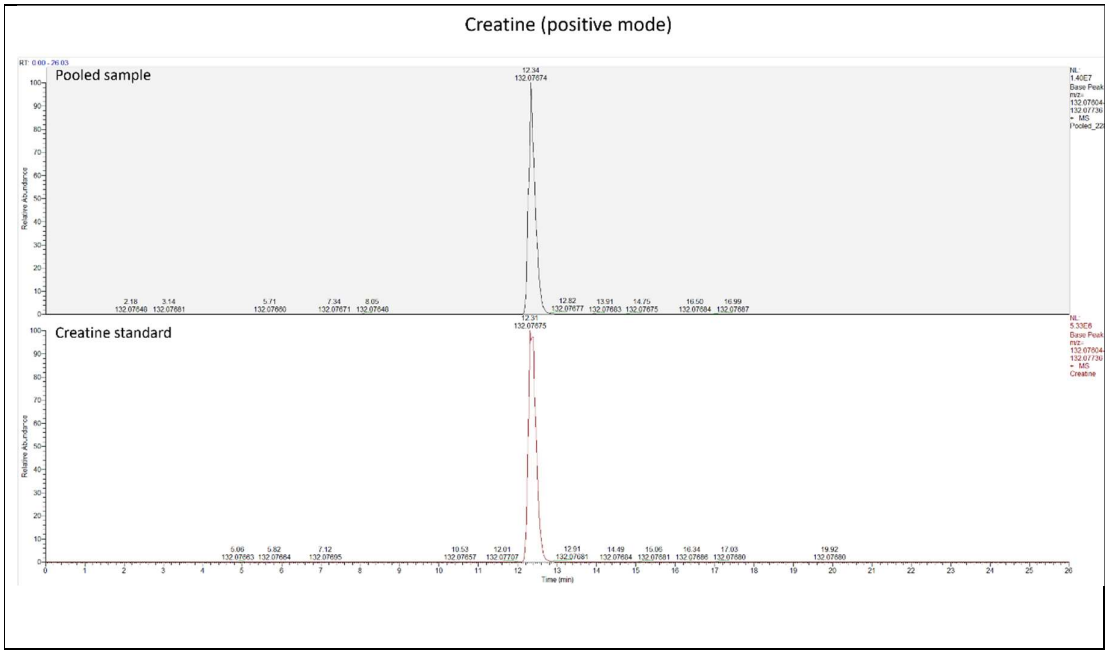

Figure 8-3: Chromatograms for creatine in sample (top) and in standard (bottom).

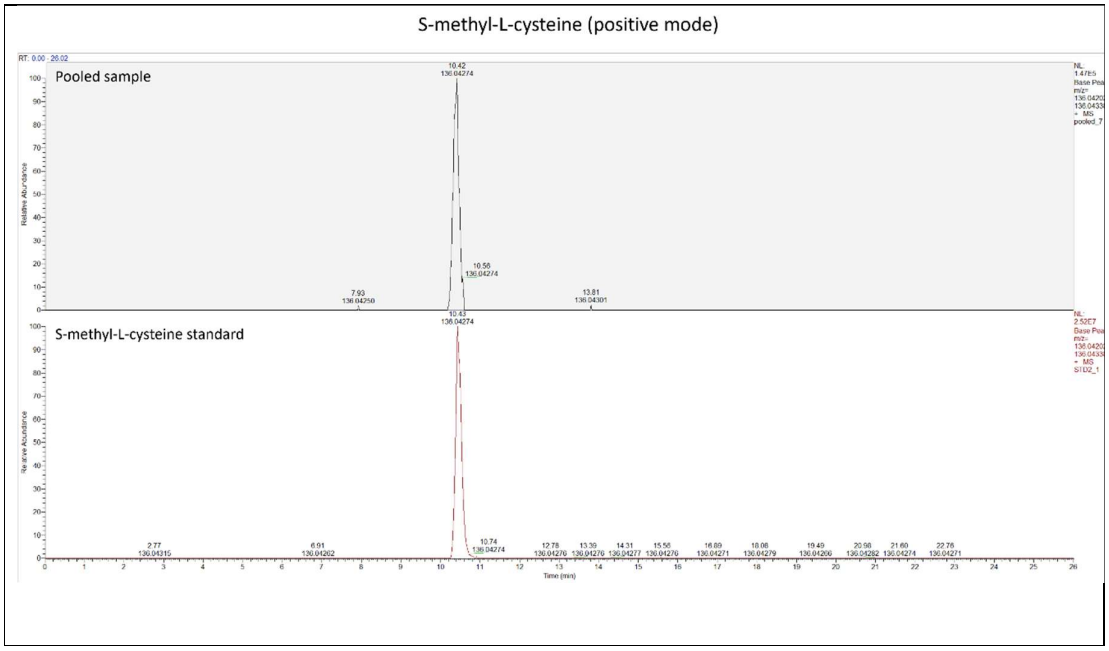

Figure 8-4: Chromatograms for S-methyl-L-cysteine in sample (top) and in standard (bottom).

Additionally, fragmentation data was captured in positive ionisation mode via MS2. *Figure 8-5* and *Figure 8-6* show the MS2 spectra for the creatine and S-methyl-L-cysteine peaks demonstrating the same fragmentation spectrum as their respective standards, confirming the identities of the metabolites.

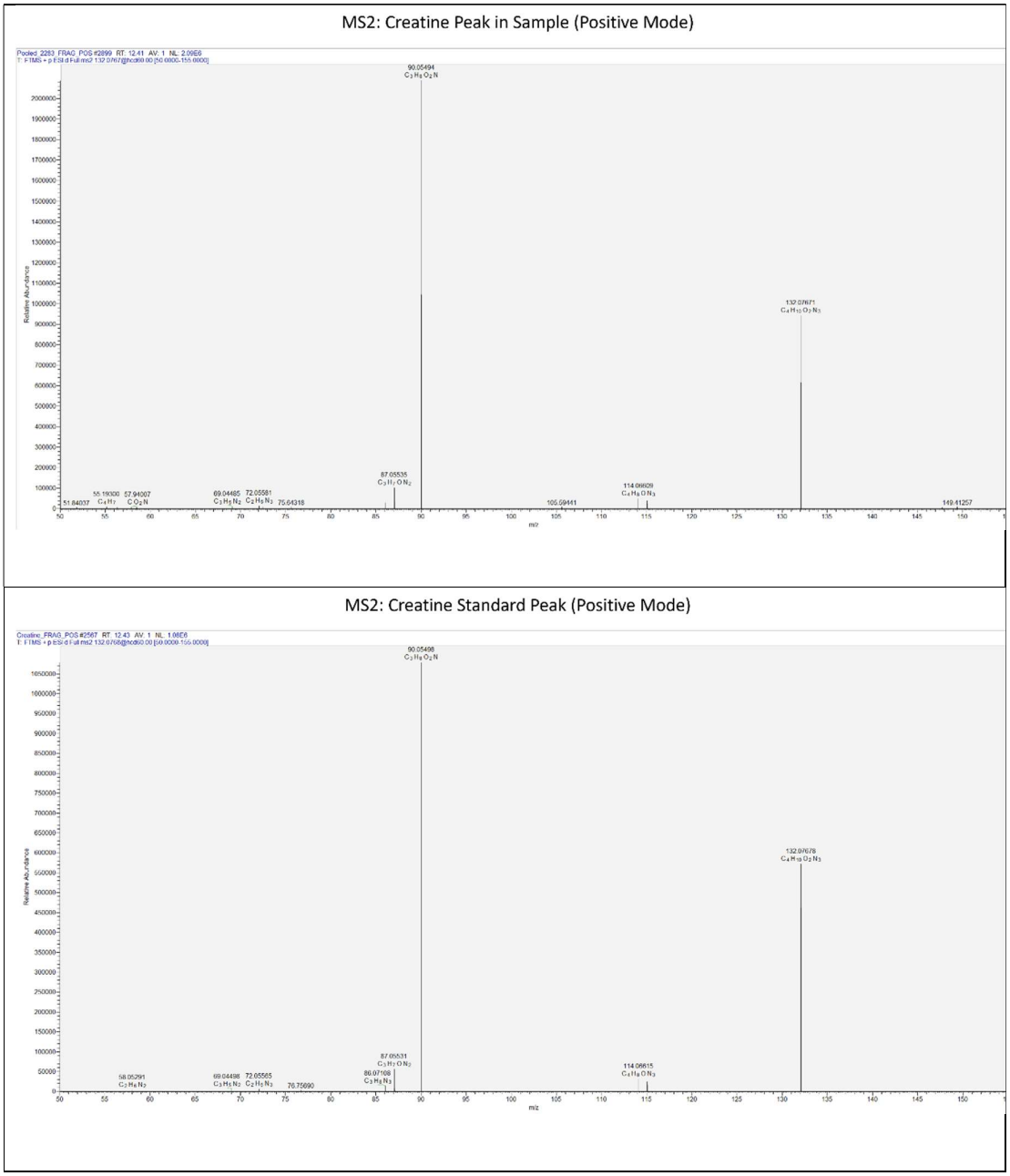

Figure 8-5: MS2 fragmentation spectra for creatine in pooled sample (top) and in standard (bottom).

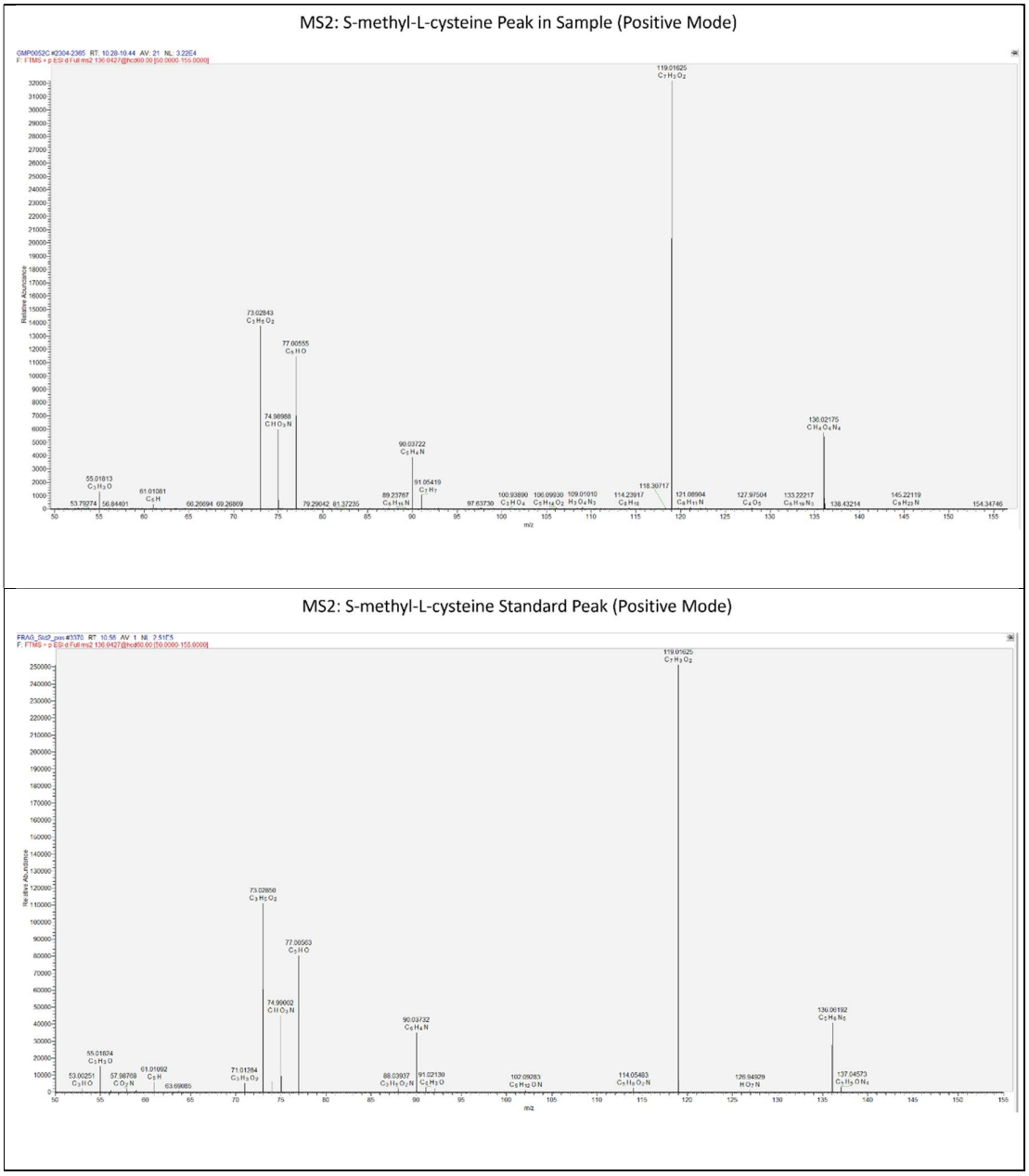

Figure 8-6: MS2 fragmentation spectra for S-methyl-L-cysteine in pooled sample (top) and in standard (bottom).

8.4.2 2-hydroxyisovalerylcarnitine and L-prolinylglycine

Authentic standards were unavailable for 2-hydorxyisovalerylcarnitine and L-prolinylglycine. However, fragmentation data was gathered for both peaks. The spectrum for 2-hydroxyisovalerylcarnitine demonstrated a fragment with a *m/z* of 85, which has been shown to be characteristic of acylcarnitines (646) Additionally, a fragment with an *m/z* of 145.05 was detected, which has previously been reported as a constituent of the metabolite 3-hydroxyisovalerylcarnitine in the paper by Maeda and colleagues (647). *Figure 8-7* and *Figure 8-8* show the MS2 fragmentation spectrum and the HMDB predicted LC-MS/MS spectrum for 2-hydroxyisovalerylcarnitine respectively, both demonstrating *m/z* peaks at 85 and 262. As such, confidence in the identity of this metabolite is high.

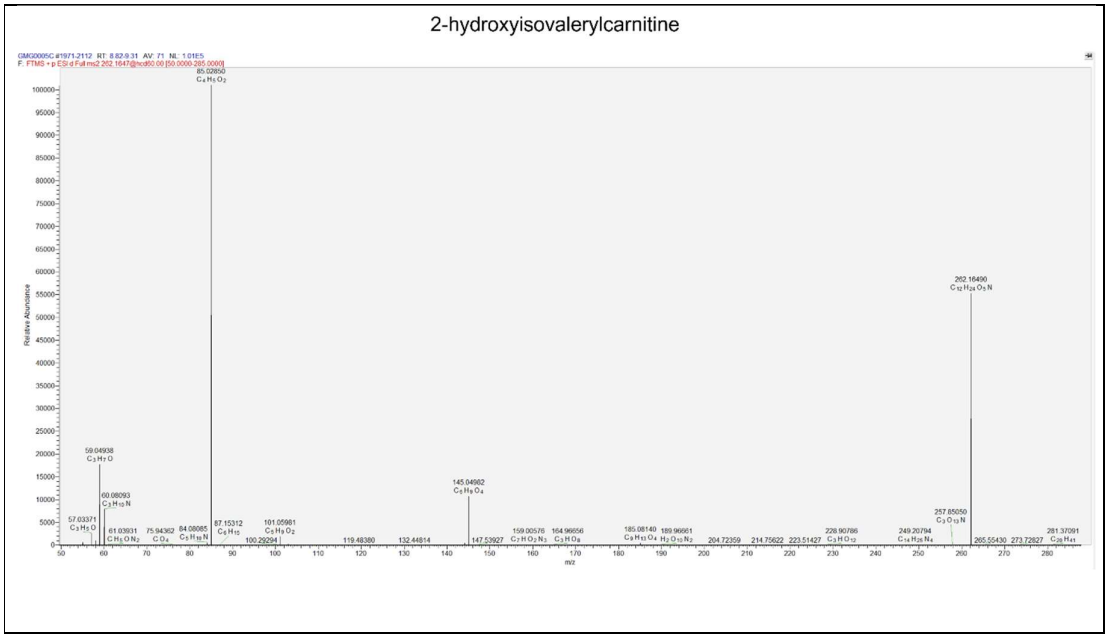

Figure 8-7: MS2 fragmentation spectrum for 2-hydroxyisovalerylcarnitine.

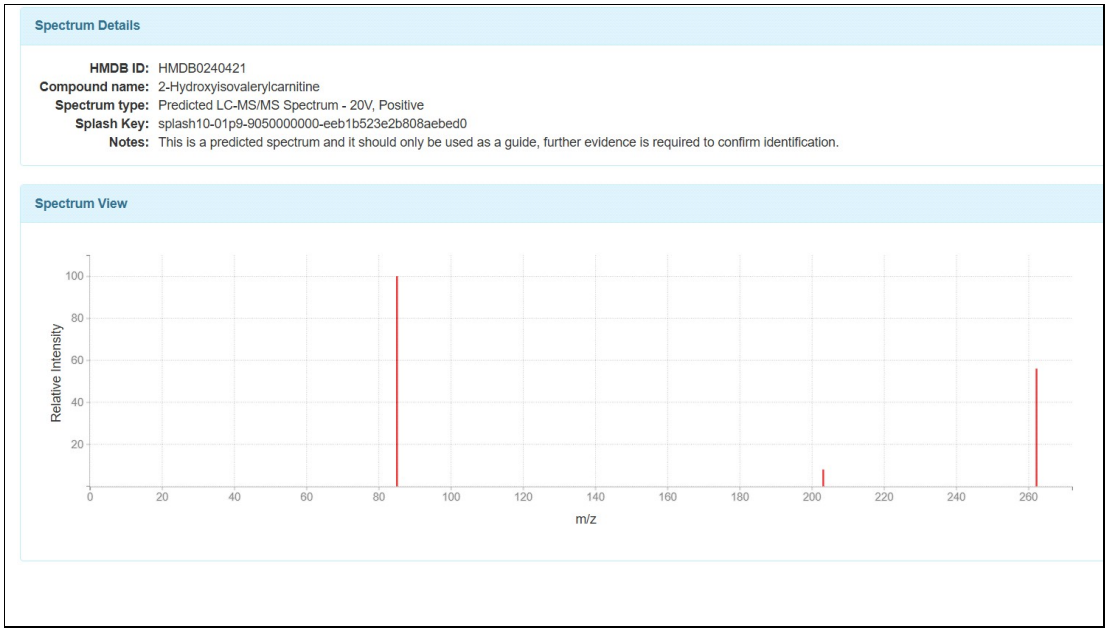

Figure 8-8: HMDB predicted peak for 2-hydroxyisovalerylcarnitine (648).

Unfortunately, the fragmentation data for the putatively identified metabolite L-prolinylglycine did not show a database match. Furthermore, there is poor correlation between our fragmentation spectrum and the predicted HMDB spectrum as shown in *Figure 8-9* and *Figure 8-10*. Since we could not confirm the identity with an authentic standard, we did not have sufficient confidence to confirm the identity of this metabolite. This peak was therefore excluded from the final model.

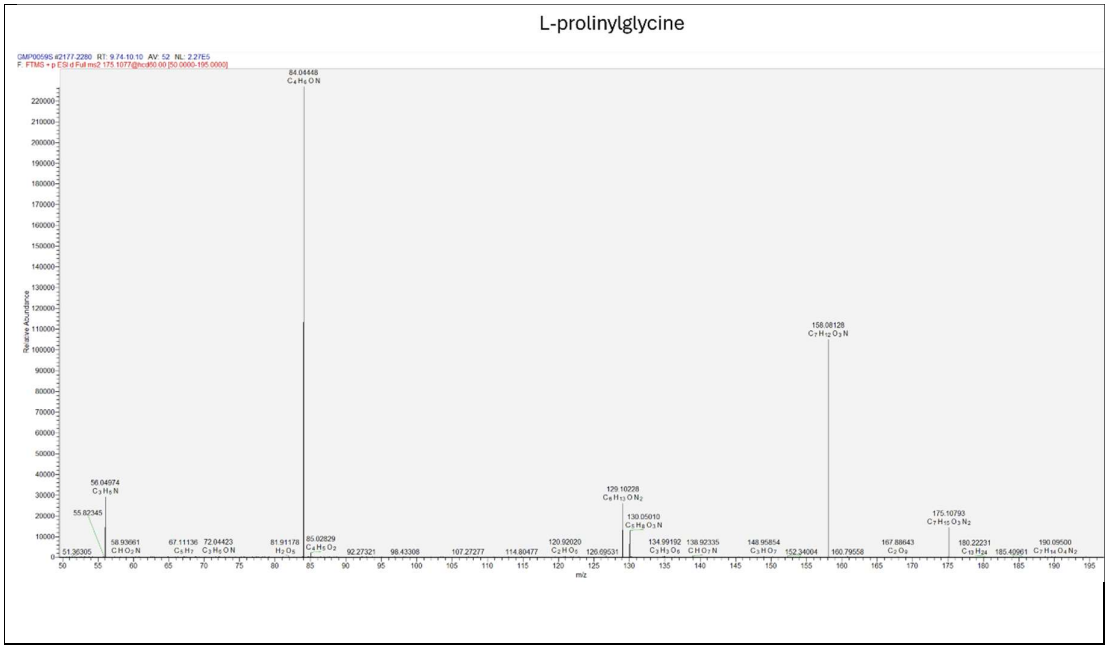

Figure 8-9: MS2 fragmentation spectrum for L-prolinylglycine.

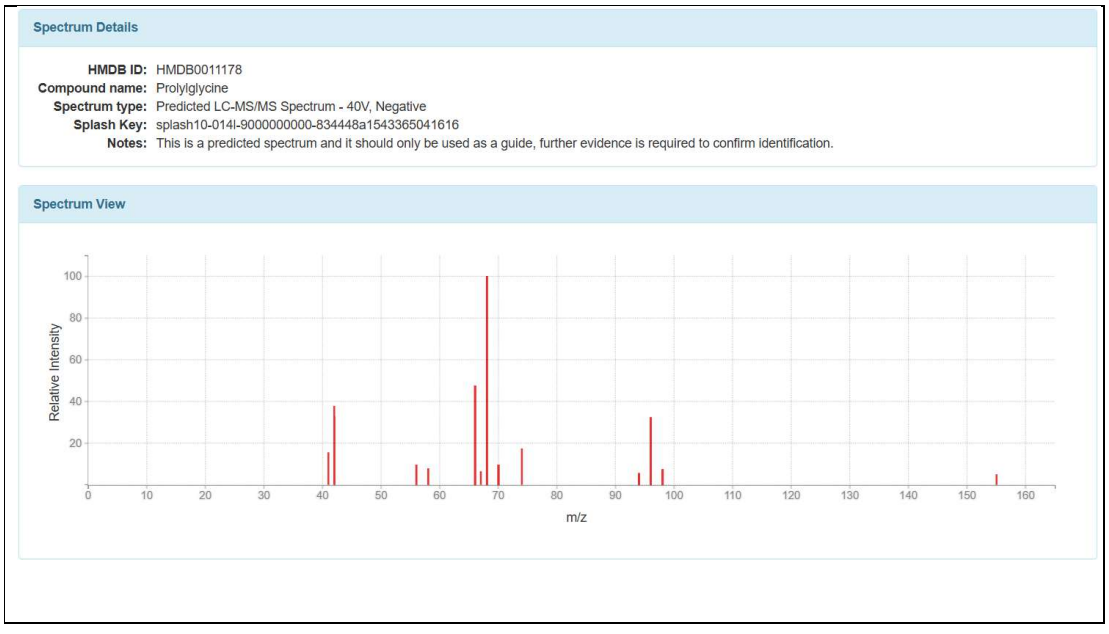

Figure 8-10: HMDB predicted peak for L-prolinylglycine (649).

8.5 Final Metabolite Identities

Exclusion of L-prolinylglycine left three metabolites which were significantly different in concentration between patients with and without secondary infections as shown in *Table 8-1* and *Figure 8-11*. The metabolites creatine and 2-hydroxyisovalerylcarnitine were found to be significantly increased in patients with secondary infections, while S-methyl-L-cysteine was significantly lower.

Metabolite	Direct Parent	Molecular Formula	*m/z*	Retention Time (s)	Relative Intensity (mean ± SE)		Log2 Fold Change	q-value
					Secondary Infection	No Secondary Infection		
Creatine	Alpha amino acids and derivatives	$C_4H_9N_3O_2$	130.0622	724	19.075 ± 0.0068	18.348 ± 0.0775	0.056	0.0573
S-methyl-L-cysteine	Cysteine and derivatives	$C_4H_9NO_2S$	134.0282	623	15.119 ± 0.004	15.541 ± 0.036	-0.04	0.0573
2-hydroxyisovalerylcarnitine	Acyl carnitines	$C_{12}H_{23}NO_5$	262.1647	541	17.734 ± 0.0059	17.069 ± 0.0535	0.055	0.0859

Table 8-1: Metabolites identifying secondary infection. A q-value < 0.1 indicates statistical significance. SE: standard error.

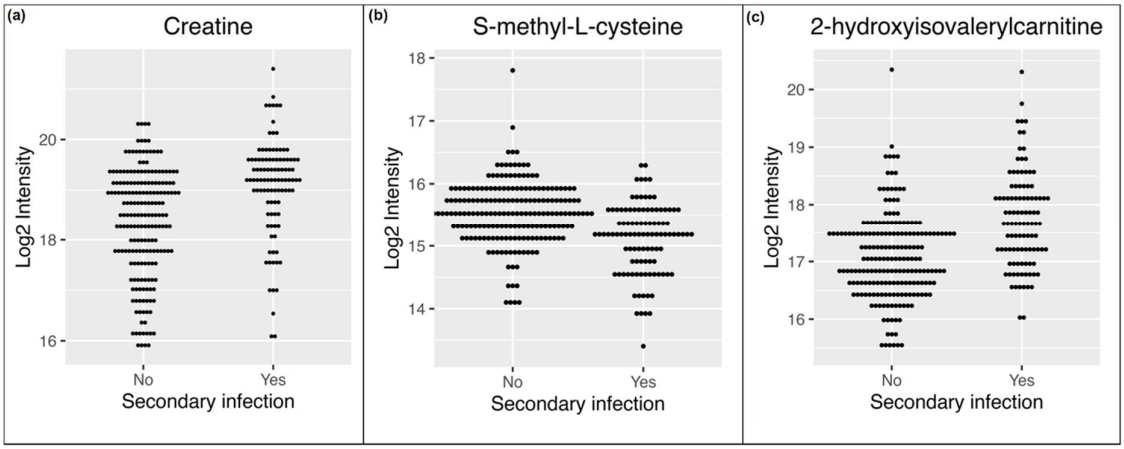

Figure 8-11: Dot plots depicting significantly different metabolites with and without secondary infections. (a) creatine, (b) S-methyl-L-cysteine, (c) 2-hydroxyisovalerylcarnitine.

8.6 Model Performance

Figure 8-12 shows the receiver operating characteristic curve depicting the sensitivity and specificity of the metabolomic panel for diagnosis of secondary infections, with an AUROC of 0.83 ± 0.074.

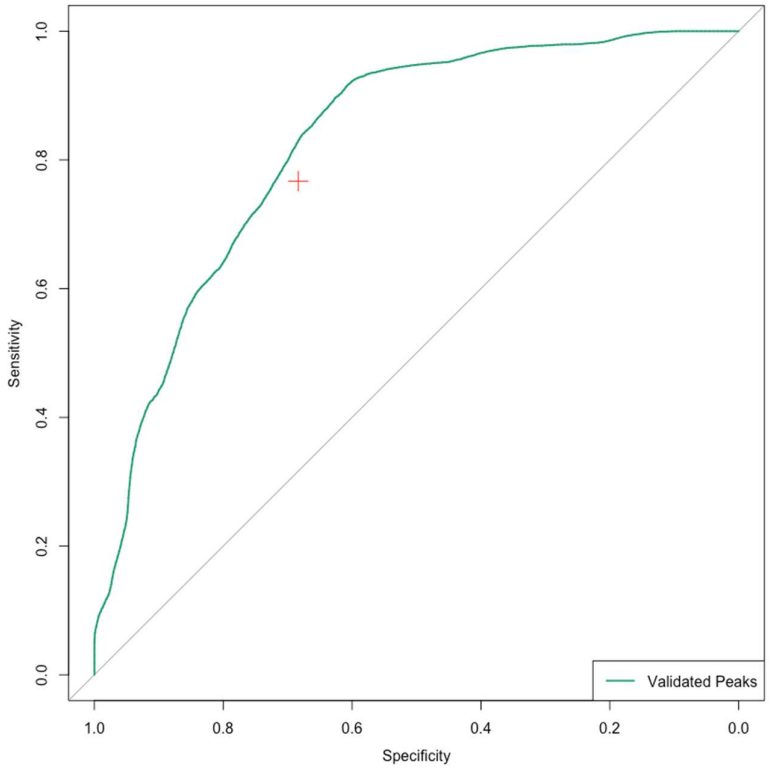

Figure 8-12: Receiver operating characteristic curve demonstrating test performance of metabolomic panel of the three validated peaks for identification of secondary infections. Red plus represents sensitivity and specificity of clinician diagnosis of secondary infection based on antibiotic usage.

8.7 Modelling with Inflammatory Markers and SOFA score

The model was re-run with inclusion of clinical data consisting of inflammatory markers and SOFA scores, to assess if this would improve the predictive capability. The WCC, CRP and PCT were gathered from the day of each blood sampling ± 24 hours if routine bloods had not been collected on the day of blood sampling. Some values for PCT were missing as this blood test was not always routinely collected from all patients, particularly from patients receiving level 2 care in high dependency. Individual components of the SOFA score were also calculated for each blood sample day. Many patients were continuously proned and paralysed in ICU for extended periods without a sedation break, therefore it was often impossible to get a true assessment of GCS. As such the neurological component was not included in this model. The receiver operating characteristic curves for the new model with and without the clinical data are shown in *Figure 8-13*. The AUROC for the metabolite panel alone and in combination with the clinical data was 0.828 and 0.827 respectively, showing that the addition of the inflammatory marker and modified SOFA score made no difference to the predictive capability of the model. The AUROC for the clinical data in isolation was 0.746, showing that the metabolite panel outperformed the clinical data.

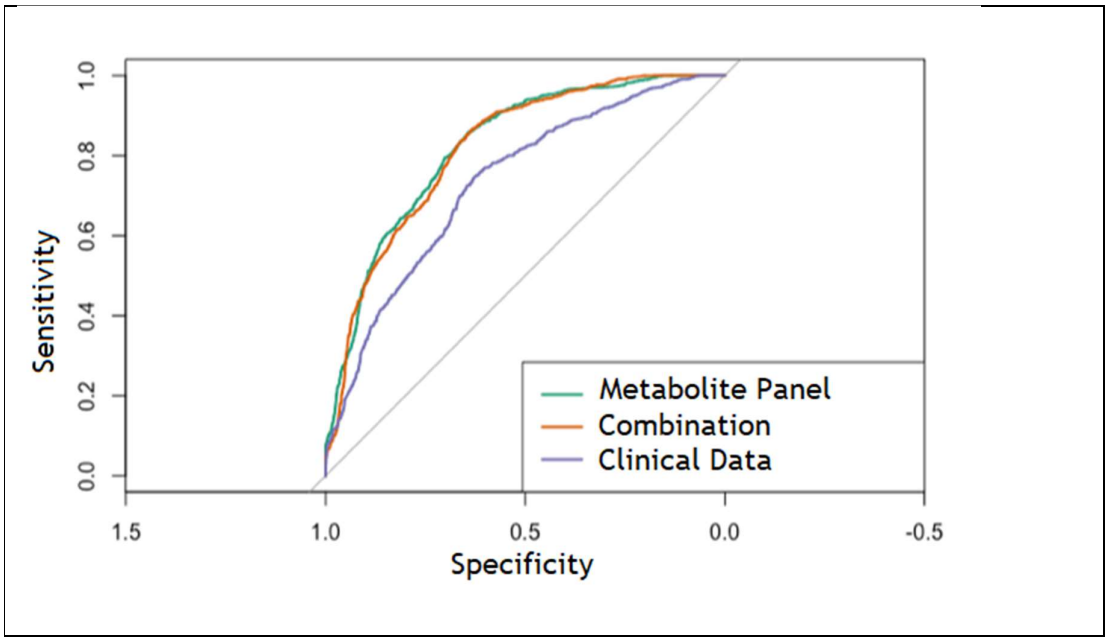

Figure 8-13: Receiver operating characteristic curves for the metabolite panel; clinical data consisting of inflammatory markers and SOFA score components; and the two data sets combined.

An additional model was produced examining the performance of the metabolite panel compared to the individual performances of WCC, CRP and PCT, as shown in *Figure 8-14*. The metabolite panel was superior to each of these markers for identifying secondary infections (AUROC 0.83; 95% CI 0.68-0.97; compared to total WCC (AUROC 0.59; 95% CI 0.37-0.80), CRP (AUROC 0.48; 95% CI 0.25-0.70) and PCT (AUROC 0.50; 95% CI 0.28-0.72).

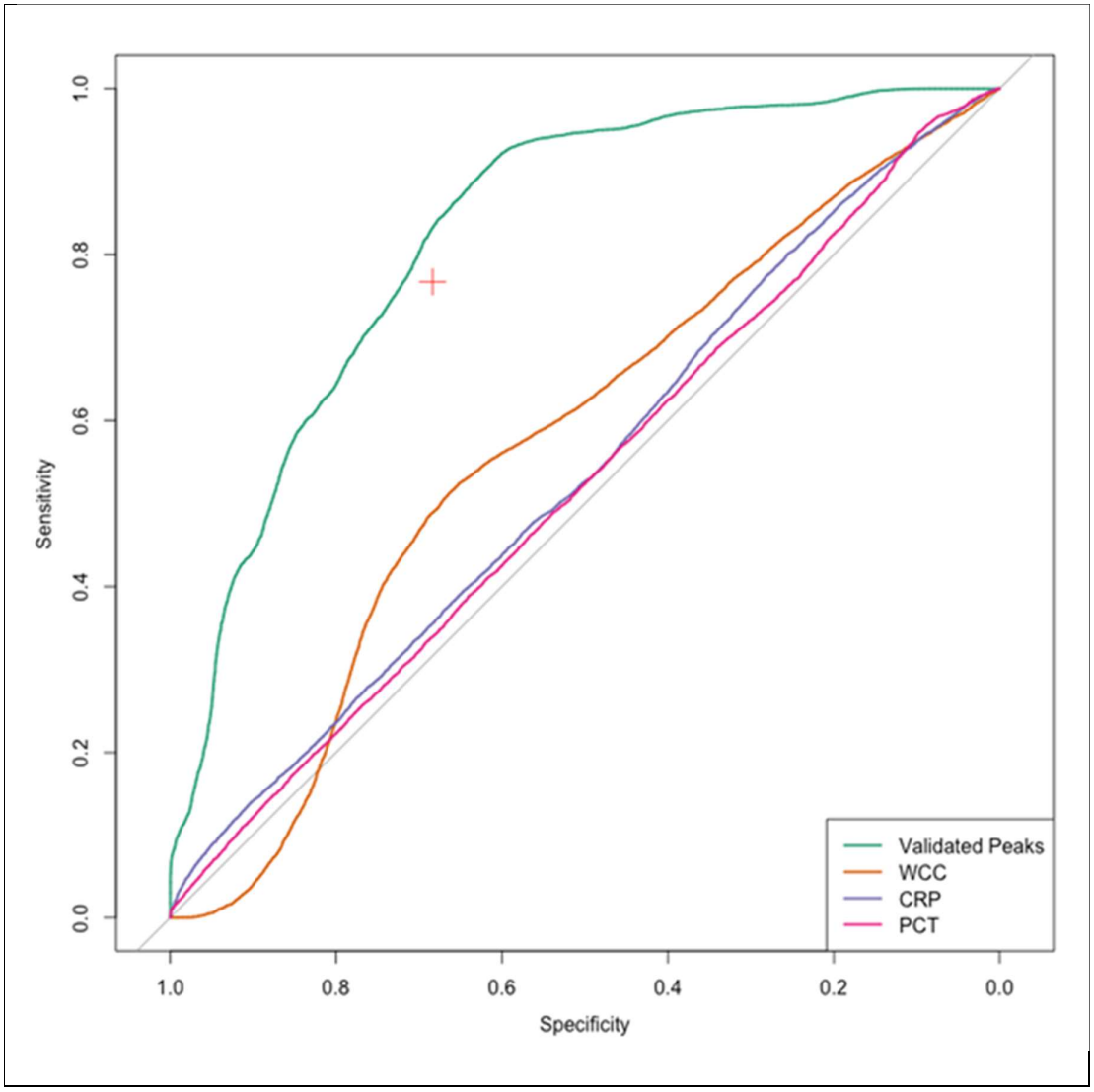

Figure 8-14: Receiver operating characteristic curve demonstrating test performance of metabolomic panel and routine inflammatory markers. Red plus represents sensitivity and specificity of clinician diagnosis of secondary infection based on antibiotic usage.

8.8 Discussion

HPLC/MS metabolomic analysis of samples from critically ill COVID-19 patients has produced a panel of three biomarkers with strong predictive capability for identifying the presence of a secondary infection. The panel consisted of the amino acid derivatives creatine and S-methyl-L-cysteine, and the acylcarnitine 2-hydroxyisovalerylcarnitine.

8.8.1 Creatine

Creatine is an organic compound with an important role in transportation of energy substrates to metabolically active tissues. It is commonly taken as a supplement to increase muscle mass and performance (650). Creatine formation is linked to the urea cycle (See F*igure 8-11*). The first step in creatine synthesis involves the binding of arginine and glycine under the action of the enzyme arginine:glycine amidinotransferase (AGAT) to produce ornithine and guanidinoacetate. Ornithine can continue round the urea cycle, while guanidinoacetate is methylated by the enzyme guanidinoacetate N-methyltransferase (GAMT) to form creatine (651).

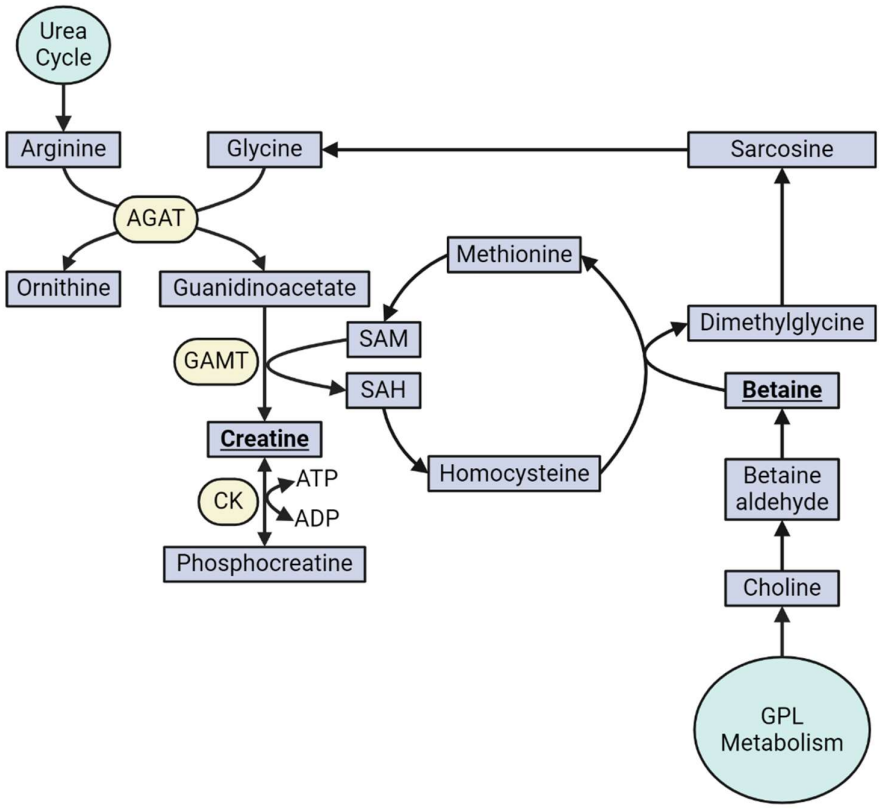

Figure 8-15: Arginine and glycine metabolism pathways. Adapted from Andrade (652). *ADP: Adenosine diphosphate; AGAT: Arginine:glycine amidinotransferase; ATP: Adenosine triphosphate; CK: Creatine kinase; GAMT: Guanidinoacetate N-methyltransferase; GPL: Glycerophospholipid; SAH: S-adenosylhomocysteine; SAM: S-adenosylmethionine.*

Creatine plays a crucial role in the creatine kinase (CK)/phosphocreatine/creatine system, an important mechanism of ensuring additional rapid delivery of high energy substrates to metabolically active tissues such as skeletal muscle, myocardium, spermatozoa and brain tissue, rather than simply relying on diffusion of mitochondrial ATP (651). Inborn errors of creatine synthesis such as the rare condition arginine glycine amidinotransferase deficiency causes intellectual disability and language impairment, highlighting the importance of creatine for cognitive functioning (646).

CK enzymes act as a shuttle to deliver high energy phosphate molecules to target sites. Mitochondrial CK enzymes within the intermembrane space take the γ-phosphate group from ATP and combine it with creatine, yielding phosphocreatine and adenosine diphosphate (ADP). This ADP can then re-enter the mitochondrial matrix where it can bind with another phosphate to regenerate ATP. The phosphocreatine is free to travel out of the mitochondrion to target sites, where it binds with a cytosolic CK molecule, regenerating ATP and reforming a creatine molecule. The creatine can then move back inside the mitochondrion where it can once again form another phosphocreatine molecule, continuing the cycle (651).

In tissues with a high energy demand such as fast twitch muscle fibres, phosphocreatine can accumulate under relaxed conditions and act as an energy store. Rapid hydrolysis of phosphocreatine can then occur under conditions of increased tissue activity, providing a surge of ATP (651).

Several rat studies (653,654) as well as a human metabolomics study (441) have shown creatine to be increased in sepsis. Increased utilisation of phosphocreatine, with a reduced capacity for rephosphorylation in sepsis increases creatine concentration (653,655). Creatine is metabolised to creatinine predominantly by the liver. Sepsis induced liver hypoperfusion can decrease the conversion of creatine to creatinine, promoting creatine accumulation. Furthermore, creatine can be converted to creatinine in a non-enzymatic temperature dependent process. Sepsis induced hypothermia could therefore further reduce creatine conversion to creatinine (656).

8.8.1.1 Creatine and Mitochondrial Dysfunction

As previously mentioned, sepsis often induces a state of stress hyperglycaemia. Increased cellular metabolism promotes mitochondrial ROS production from the ETC. Excessive ROS production leads to oxidative stress, resulting in mitochondrial dysfunction. Mitochondrial CK enzymes are strong promoters of OXPHOS (657). Increased OXPHOS acts to reduce the mitochondrial membrane potential, thereby decreasing the production of ROS (658). As such, mitochondrial CK acts in an anti-inflammatory and anti-oxidant capacity to reduce oxidative injury. Accumulation of glucose-6-phosphate, a product of pyruvate oxidation, inhibits mitochondrial CK activity (658). Therefore, during a secondary infection, a sepsis induced hypermetabolic state would decrease CK activity, promoting ROS formation and mitochondrial dysfunction while concomitantly increasing creatine as less is converted to phosphocreatine (659). The activity of mitochondrial CK could therefore be a useful marker of mitochondrial dysfunction.

8.8.1.2 Protective Effects of Creatine

Creatine may have protective effects during a secondary infection. Increased ATP concentration inhibits glycolysis through negative feedback. Therefore, increasing creatine will allow CK to convert ATP to phosphocreatine, preventing feedback inhibition of glycolysis. This is important during sepsis where hypoxic conditions reduce mitochondrial ATP generation, and metabolism relies much more heavily on glycolysis (650).

Creatine can increase neutrophil antibacterial activity through promoting the respiratory burst and increasing cytokine production. A mouse model of sepsis found that this enhancement of neutrophils by creatine resulted in improved survival (660).

Creatine concentration may be increased through *de novo* synthesis, or though reduced renal excretion (661). Reducing excretion to increase creatine would reduce de novo synthesis, allowing precursor amino acids to instead be used for gluconeogenesis (651). Alternatively, an increase in creatine availability may represent an attempt to shuttle what little ATP is available to energy deplete tissues (651).

8.8.2 S-methyl-L-cysteine

S-methyl-L-cysteine is a naturally occurring a thioether analogue of methionine (662,663). It is an important substrate for enzymes involved in cysteine and methionine metabolism. S-methyl-L-cysteine is a xenobiotic i.e. it is not produced in humans. However it is obtained from the diet, and is found in many types of vegetable including garlic, cabbage, turnip, broccoli, cauliflower and kale, as well as several types of beans (662).

S-methyl-L-cysteine exhibits various beneficial health effects, including antioxidant and anti-lipid actions (663–665). In an animal study, antioxidant responses were increased by S-methyl-L-cysteine supplementation. The antioxidant enzymes glutathione peroxidase and catalase demonstrated increased activity in response to S-methyl-L-cysteine, which decreased oxidative stress and reduced the incidence of metabolic syndrome (666). In a mouse model of *cryptosporidium* infection, treatment with S-methyl-L-cysteine decreased faecal oocyte numbers, reduced enteritis and decreased markers of hepatic injury. Furthermore, release of pro-inflammatory cytokines TNF-α, IL-6, and IFN-γ were reduced, and glutathione and superoxide dismutase antioxidants were increased (667).

S-methyl-L-cysteine may also reduce vasodilation observed in septic shock. *S*-nitrosothiols are a collection of compounds formed when an NO group reacts with a thiol group. NO has a very short half-life, however it may be prolonged through conversion to an *S*-nitrosothiol which acts as an NO donor (668). Furthermore, *S*-nitrosothiols activate downstream signal transduction independently from NO, thereby further increasing vasodilatory responses (669). S-methyl-L-cysteine is capable of inhibiting S-nitrosothiol induced vasodilation, and could therefore help to prevent the progression to septic shock during an infection (670).

S-methyl-L-cysteine may be oxidised to the compound S-methyl-L-cysteine sulphoxide, which may have antimicrobial effects. The presence of S-methyl-L-cysteine sulphoxide has been shown to inhibit the growth of several bacterial species including Staphlococci (671).

S-methyl-L-cysteine has been detected in several different species of bacteria. Organisms such as *E. coli* and *Proteus mirabilis* (*P. mirabilis)* can use S-methyl-L-cysteine as a source of sulphur to promote their growth and replication. Thus depletion of S-methyl-L-cysteine during a secondary infection may be due to increased bacterial uptake (672).

Reduction of S-methyl-L-cysteine may represent an indication of disease severity. Depletion could occur in severe sepsis, where oxidative stress occurs as antioxidant responses are overwhelmed. Alternatively, reduced S-methyl-L-cysteine may simply represent decreased dietary intake due to critical illness reducing intake of green vegetables. Interestingly, an animal study found that injected radiolabelled S-methyl-L-cysteine is metabolised and then incorporated into molecules of choline and creatine (673). It is possible that the increase in creatine observed in secondary infections is in part mediated by metabolism and depletion of S-methyl-L-cysteine.

8.8.3 2-hydroxyisovalerylcarnitine

As outlined in chapter 4, The carnitine shuttle is responsible for transferring fatty acids inside the mitochondrial matrix in preparation for ß-oxidation. Sepsis has been shown to disrupt ß-oxidation, leading to an accumulation of acylcarnitines (438,440,441,445,448) which can subsequently leak out into the plasma (559).

Literature regarding 2-hydroxyisovalerylcarnitine is sparse. However the similar compound 3-hydroxyisovalerylcarnitine has been shown to be elevated in certain congenital disorders of carnitine metabolism (674). Interestingly, a paediatric metabolomics study examined children with confirmed bacterial vs viral infection. The fatty acid 2-hydroxyisovaleric acid was found to be elevated in viral infections but reduced in bacterial infections (675). Similarly, a study examining CAP found a significant reduction in 2-hydroxyisovaleric acid in young adults with CAP compared to controls (676). It is possible a reduction in 2-hydroxyisovaleric acid in bacterial infections could be due to accumulation of 2-hydroxyisovalerylcarnitine.

8.8.4 Inclusion of SOFA and inflammatory markers

An attempt to further increase the predictive capability of the model was made through the addition of inflammatory markers and SOFA scores. However, this addition did not demonstrate an improvement to the predictive capability of the model.

The study by Karaca and colleagues found that early SOFA scores on admission to ICU could not predict development of a secondary infection, but at day 3 SOFA scores in patients with secondary infections were significantly higher (183). As a measure of organ dysfunction, SOFA scores may better reflect disease progression and severity, and thus may be better for prognostic models rather than diagnostic models.

The study by Guo *et al.* suggested that secondary infections were associated with hepatic failure, but this was demonstrated by reductions in levels of cholinesterase, pre-albumin, and albumin. These markers of liver function may be better at identifying secondary infections than bilirubin (677).

When examining the individual performances of WCC, CRP and PCT, each of the three markers of infection performed substantially worse than the metabolomic panel for identifying secondary infections. This is in keeping with the study by Pintea-Simon and colleagues, who found that WCC, CRP, PCT and ferritin were all poorly associated with the presence of a secondary infection in COVID-19 (196).

CRP and WCC are routinely measured markers of inflammation and infection. Several studies confirm an increase in CRP in the presence of superinfections (168,173,178,180). As a non-specific marker of inflammation, CRP will rise in response to many different insults including a primary COVID-19 infection, limiting its utility as a secondary infection biomarker. Furthermore, use of corticosteroids and other immunomodulatory drugs may suppress synthesis of acute phase compounds such as CRP, blunting the inflammatory response which could potentially mask the presence of a secondary infection (678). making them less While Lv *et al.* found a rise in total WCC with secondary infections (168), several other studies did not replicate this finding (173,180,199). An increase in neutrophil count does appear to correlate better with the presence of a secondary infection,

with several studies demonstrating an increase in neutrophils with secondary infections (168,180,183). Three studies reported that a reduction in lymphocyte count was indicative of a superinfection (173,178,180), and Karaca *et al*. determined that the neutrophil:lymphocyte ratio was increased in secondary infections (183). The study by Ripa and colleagues found that the severity of lymphopenia was a predictive factor for the development of a secondary infection (173). Similarly, Falcone and colleagues found that lymphopenia was associated with secondary infections (178). In the present study, the model used WCC rather than lymphocyte count. It may be worth assessing whether lymphocyte count or the ratio of lymphocytes to neutrophils has a better predictive result than WCC in a future model.

There are mixed results regarding PCT as a biomarker for secondary infection in COVID-19. Several studies report a significant rise in PCT with secondary infection (168,178,183), while others found no difference (173,180,199). PCT may remain within the normal range during the early stages of an infection, as PCT synthesis is inhibited during a viral infection by IFN-γ production (679). Ongoing circulation of IFN-γ during a viral infection could limit PCT production even in the presence of a bacterial secondary infection, which would greatly decrease the utility of this biomarker in COVID-19 patients (180). PCT may therefore be a more useful secondary infection biomarker in the latter stages of COVID-19.

Other biomarkers which may have use in identifying secondary infections include interleukins IL-6, IL-10, IL-1β, IL-2R, IL-6, and TNF-α (168,180). Other miscellaneous markers found to be increased in secondary infections included D-dimer (168), ferritin (173,180), and lactate dehydrogenase (173). While some of these markers may assist in the identification of a secondary infections, their diagnostic value is likely to be limited as most have been shown to increase in severe COVID-19 regardless of the presence of a secondary infection (80).

The failure of the inflammatory markers to aid in identification of secondary infections in COVID-19 may reflect the difficulty with separating genuine infections from colonisers. This highlights a challenge faced by clinicians within the fields of clinical microbiology and infectious diseases. The clinical signs and symptoms, imaging abnormalities and pattern of changes of inflammatory markers can be very similar with both primary COVID-19 and secondary infection, making

it difficult to delineate the two conditions. In this study, many positive cultures were excluded as they represented contamination from commensal organisms or growth of colonisers. Strict application of the definitions of infection with assistance from a clinical microbiologist will have minimised false positives in this study. However, it is still possible that some cultures were misclassified as a positive infection when they in fact could have represented a non-infectious culture. Conversely, genuine pathogens may have been missed, especially if cultures were taken after administration of antibiotics. This challenge highlights the need for novel biochemical markers which are appropriately specific for the identification of secondary infections.

8.9 Conclusion

HPLC/MS metabolomic analysis of serum samples from critically ill COVID-19 patients produced a panel of three metabolites capable of identifying patients with secondary infections. This panel was superior at identifying secondary infections compared to inflammatory marker and SOFA score data, and the addition of this data to the metabolite panel made no difference to the predictive ability of the model.

9 Differentiation of Gram Positive and Gram Negative Secondary Infections in Critically Ill Patients With COVID-19

9.1 Introduction

The early identification of a secondary infection is key to ensuring that antibiotic therapy is only provided to patients who will benefit from it. However, if additional biomarkers could be detected which would give more information regarding the nature of the secondary infection, this would help target antimicrobial therapy. The ability to differentiate between Gram positive and Gram negative infections would enable the spectrum of coverage for antimicrobial therapy to be significantly narrowed. This would reduce the frequency of use of broad-spectrum antimicrobial agents such a carbapenems and decrease the proliferation of multidrug-resistant organisms. This chapter examines the use of LC-MS to identify metabolite biomarkers which show differences between Gram positive and negative infective organisms.

9.2 Putative Identification of Metabolites

After HPLC/MS analysis with subsequent exclusion of poor quality and isotope peaks, seven peaks were obtained which were significantly different between patients with Gram positive and Gram negative infections. Seven peaks were found which corresponded to six compounds putatively identified as betaine and N(6)-methyllysine, as well as four phosphatidylcholines (PC(36:4), PC(38:4), PC(38:6) and PC(40:6)). *Figure 9-1* displays the chromatograms for the six putatively identified compounds

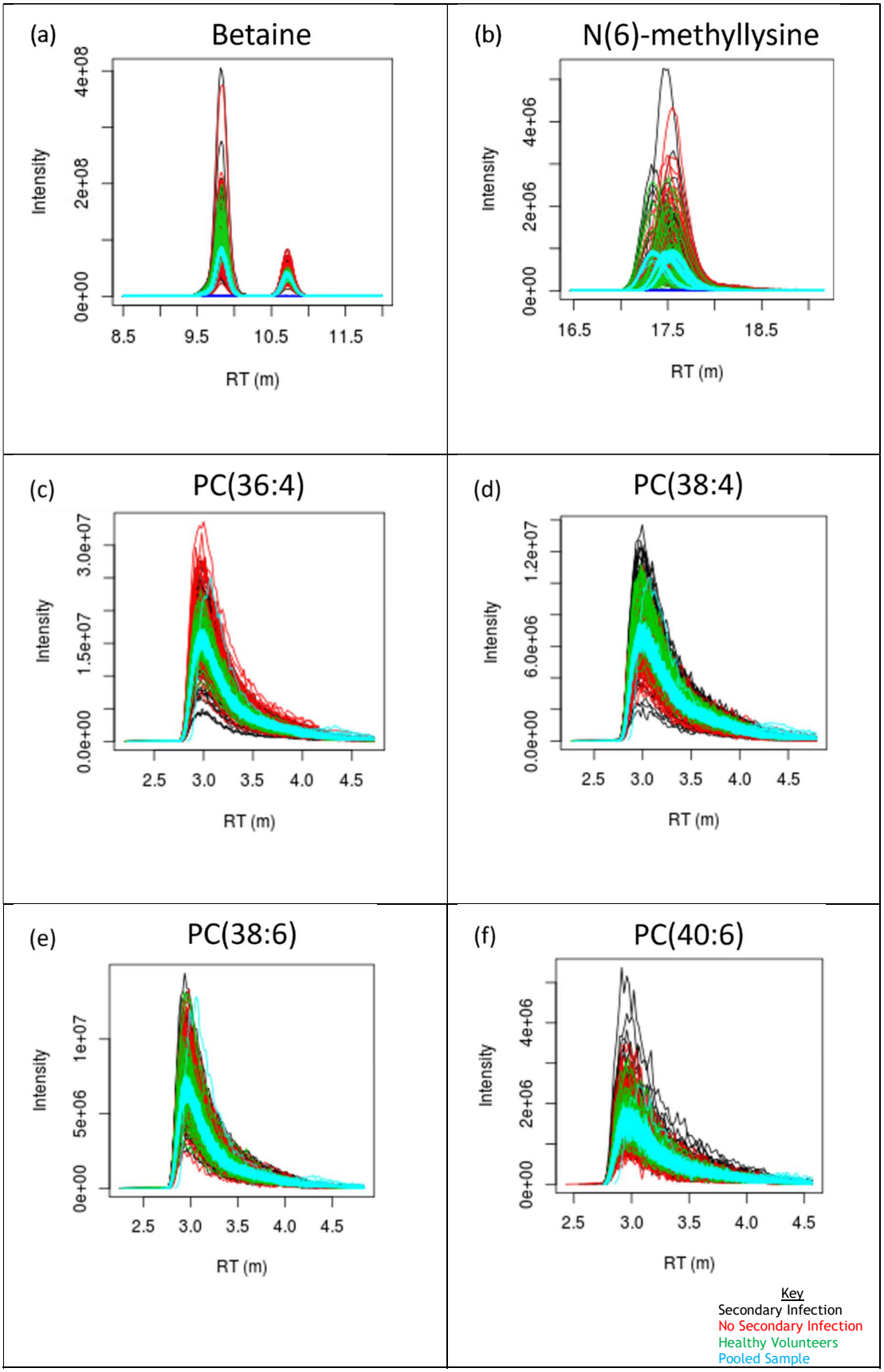

Figure 9-1: Chromatograms for putatively identified peaks (a) betaine, (b) N(6)-methyllysine, (c) PC(36:4), (d) PC(38:4), (e) PC(38:6), (f) PC(40:6)

9.3 Confirmation of Peak Identities

9.3.1 Betaine and N(6)-methyllysine

Identity confirmation was achieved for betaine and N(6)-methyllysine by running an injection of pooled sample with authentic standards. *Figure 9-2* and *Figure 9-3* show chromatograms for betaine and(6)-methyllysine respectively, highlighting a match in mass and retention time.

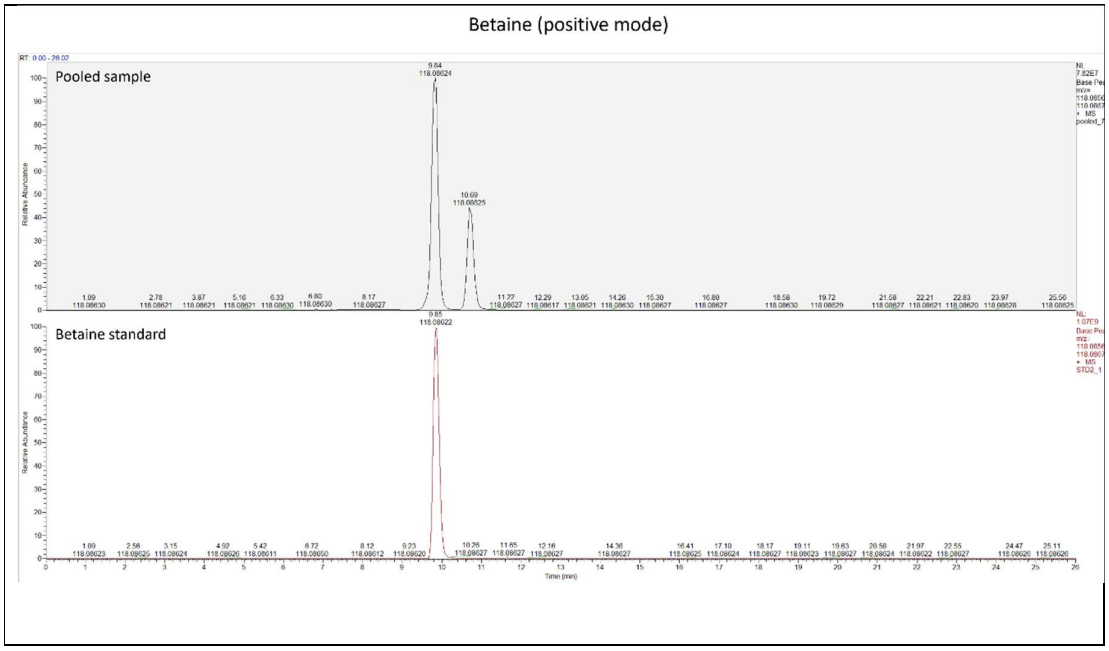

Figure 9-2: Chromatograms for betaine in sample (top) and in standard (bottom).

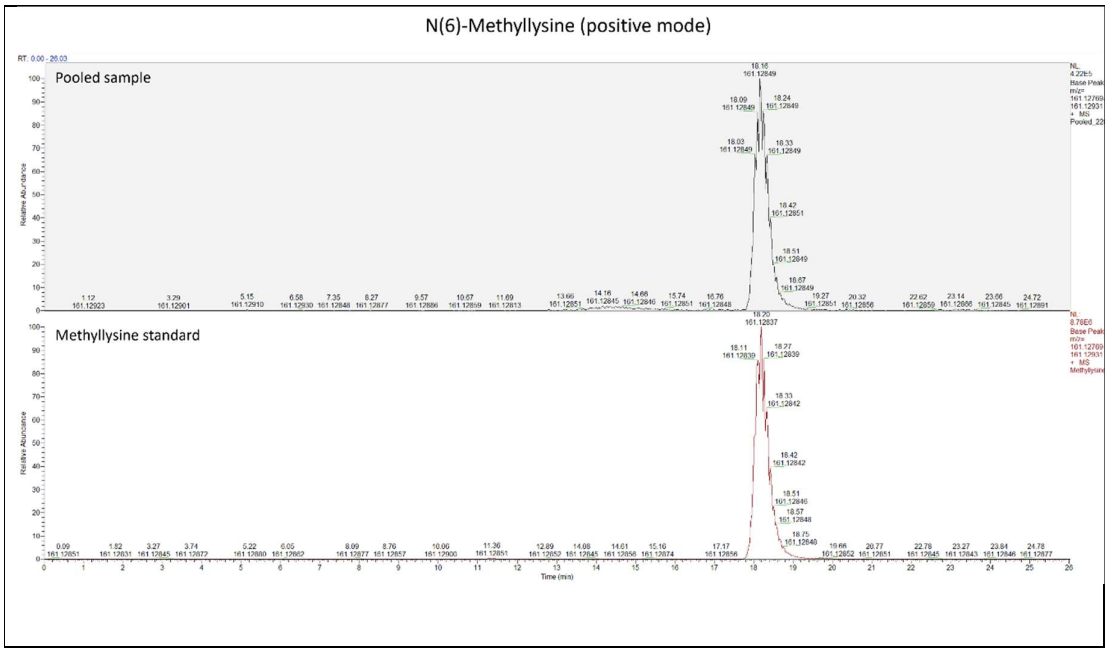

Figure 9-3: Chromatograms for N(6)-methyllysine in sample (top) and in standard (bottom).

Additional confirmation of metabolite identities was achieved with MS2 fragmentation data. *Figure 9-4* and *Figure 9-5* show the MS2 fragmentation spectra for betaine and N(6)-methyllysine respectively, both demonstrating a clear match.

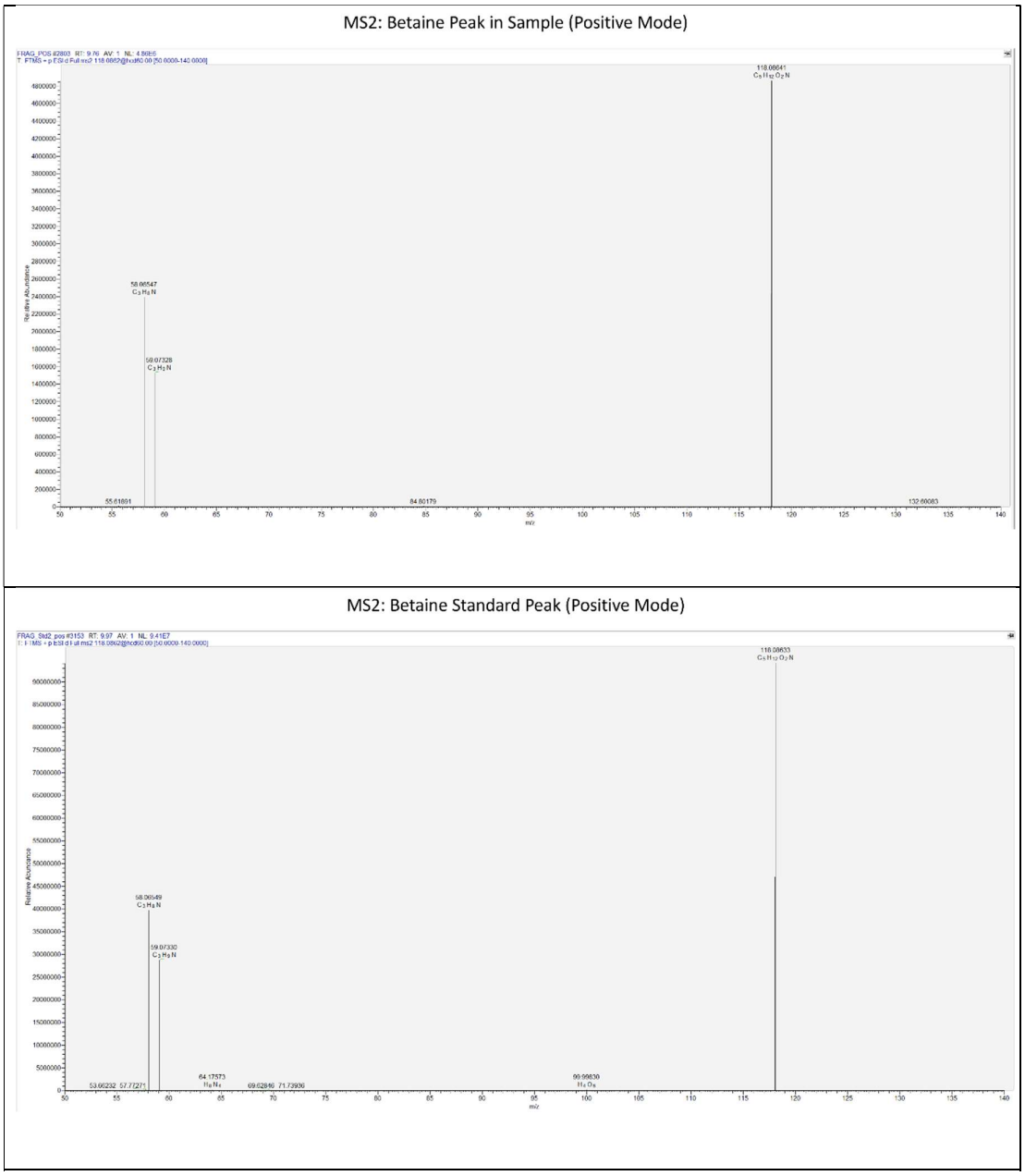

Figure 9-4: MS2 fragmentation spectra for betaine in sample (top) and in standard (bottom).

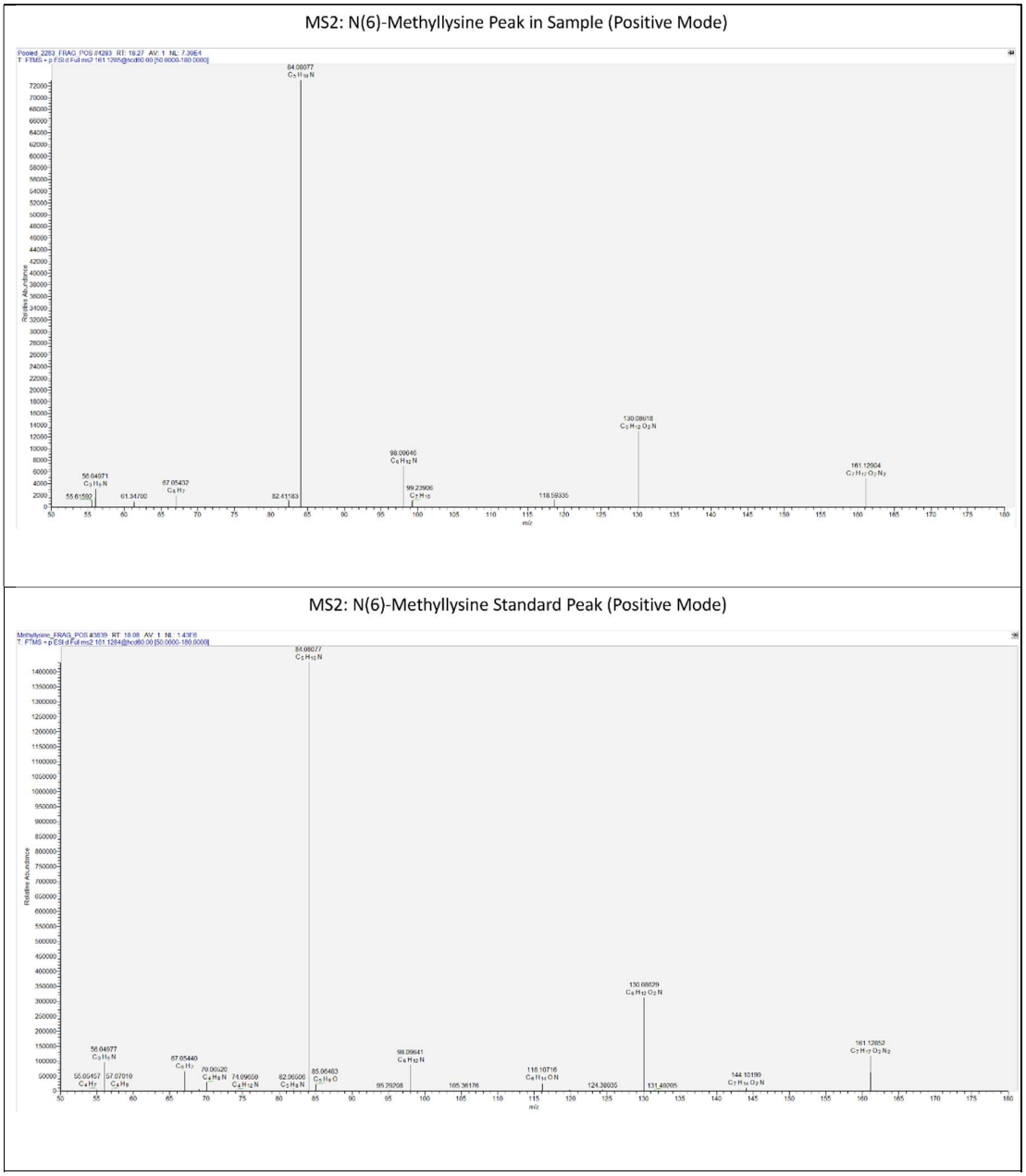

Figure 9-5: MS2 fragmentation spectra for N(6)-methyllysine in sample (top) and in standard (bottom).

9.3.2 Phosphatidylcholines

MS2 fragmentation spectra were also produced in positive ionisation mode for the four PC metabolites. (See *Figures 9-6*, *9-7*, *9-8* and *9-9*)

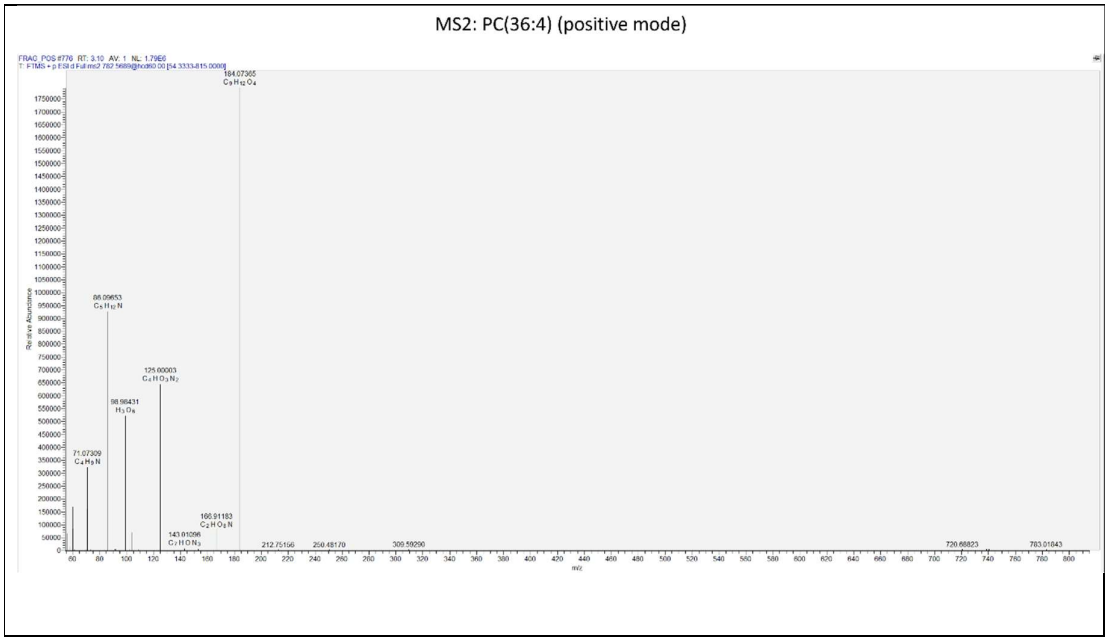

Figure 9-6: MS2 fragmentation spectrum for PC(36:4).

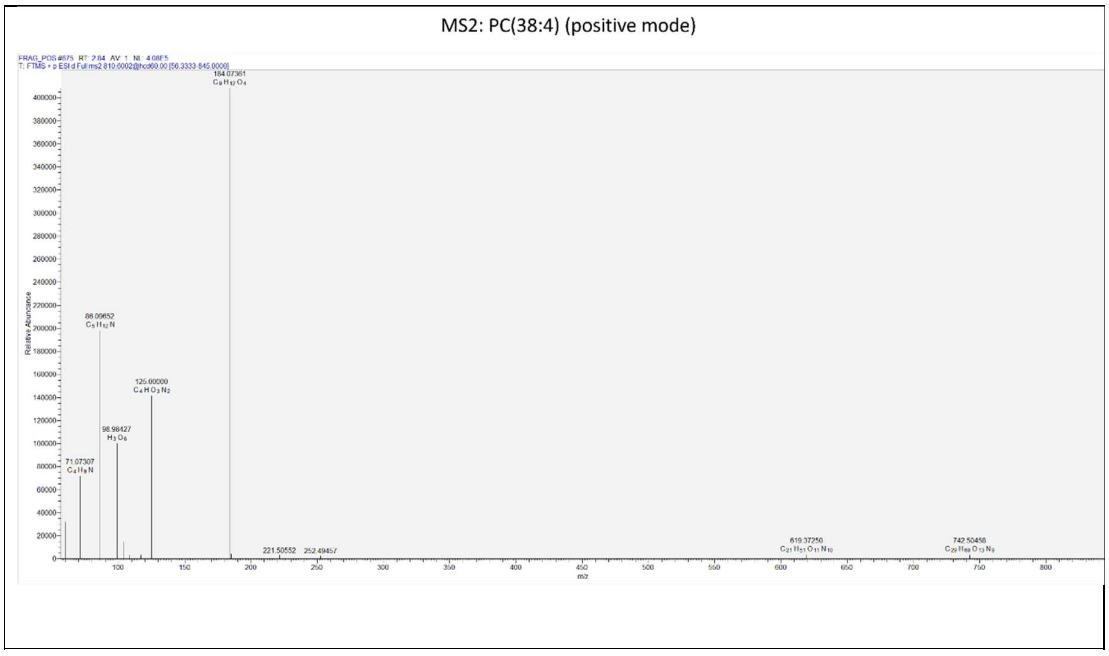

Figure 9-7: MS2 fragmentation spectrum for PC(38:4)

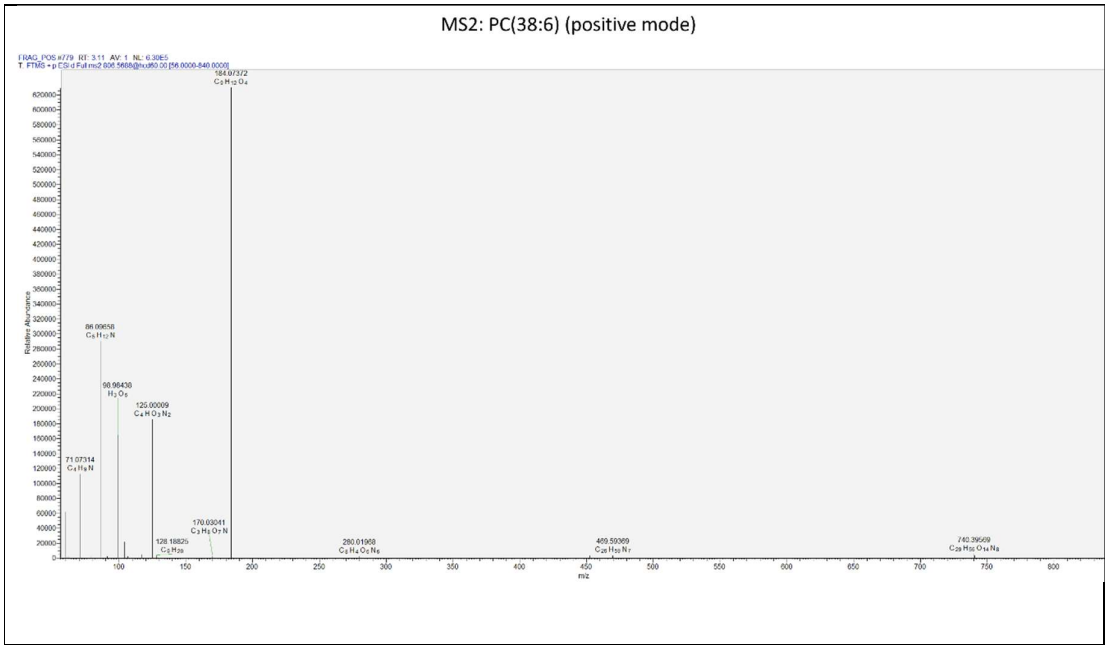

Figure 9-8: MS2 fragmentation spectrum for PC(38:6)

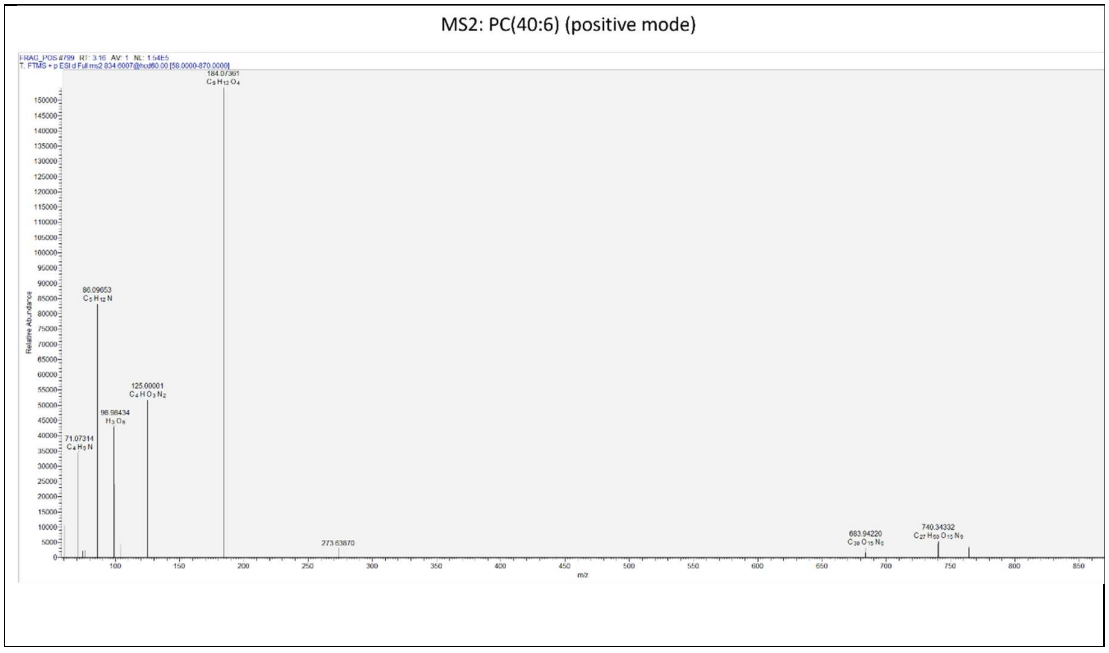

Figure 9-9: MS2 fragmentation spectrum for PC(40:6)

All four fragmentation spectra demonstrated characteristic fragments of a phosphocholine head, with *m/z* 60.08108, 71.0731, 86.0965, 98.9843, 125.0000 and 184.0736. Authentic standards were unavailable for the four PC species. Nevertheless, there was high confidence in the number of carbons and insaturations in each compound to confirm the identity of the four molecules using the fragmentation data.

9.4 Final Metabolite Identities

Six metabolites were used for the final predictive model for differentiating Gram positive and negative infections (See *Table 9-1* and *Figure 9-10*). Betaine was found to be significantly increased in Gram positive infections, while N(6)-methyllysine and the four PCs were increased in Gram negative infections.

Metabolite	Direct Parent	Molecular Formula	*m/z*	Retention Time (s)	Relative Intensity (mean ± SE)		Log2 Fold Change	q-value
					Gram Positive	Gram Negative		
Betaine	Alpha amino acids	$C_5H_{12}NO_2$	118.0863	588	26.499 ± 0.1077	26.244 ± 0.0739	0.014	0.043
N(6)-Methyllysine	L-alpha-amino acids	$C_7H_{16}N_2O_2$	159.1139	1049	19.109 ± 0.1133	19.544 ± 0.1023	-0.033	0.035
PC(36:4)	Phosphatidylcholines	$C_{44}H_{80}NO_8P$	782.5689	178	23.615 ± 0.0907	23.971 ± 0.0508	-0.022	<0.001
PC(38:4)	Phosphatidylcholines	$C_{46}H_{84}NO_8P$	810.6001	179	22.668 ± 0.0928	22.896 ± 0.0476	-0.014	0.01
PC(38:6)	Phosphatidylcholines	$C_{46}H_{80}NO_8P$	806.5688	177	22.563 ± 0.0627	22.821 ± 0.0496	-0.016	0.009
PC(40:6)	Phosphatidylcholines	$C_{48}H_{84}NO_8P$	834.6000	177	20.684 ± 0.0759	20.922 ± 0.0489	-0.017	0.024

Table 9-1: Metabolites significantly different between Gram positive and Gram negative infections. A q-value < 0.05 indicates statistical significance. SE: standard error.

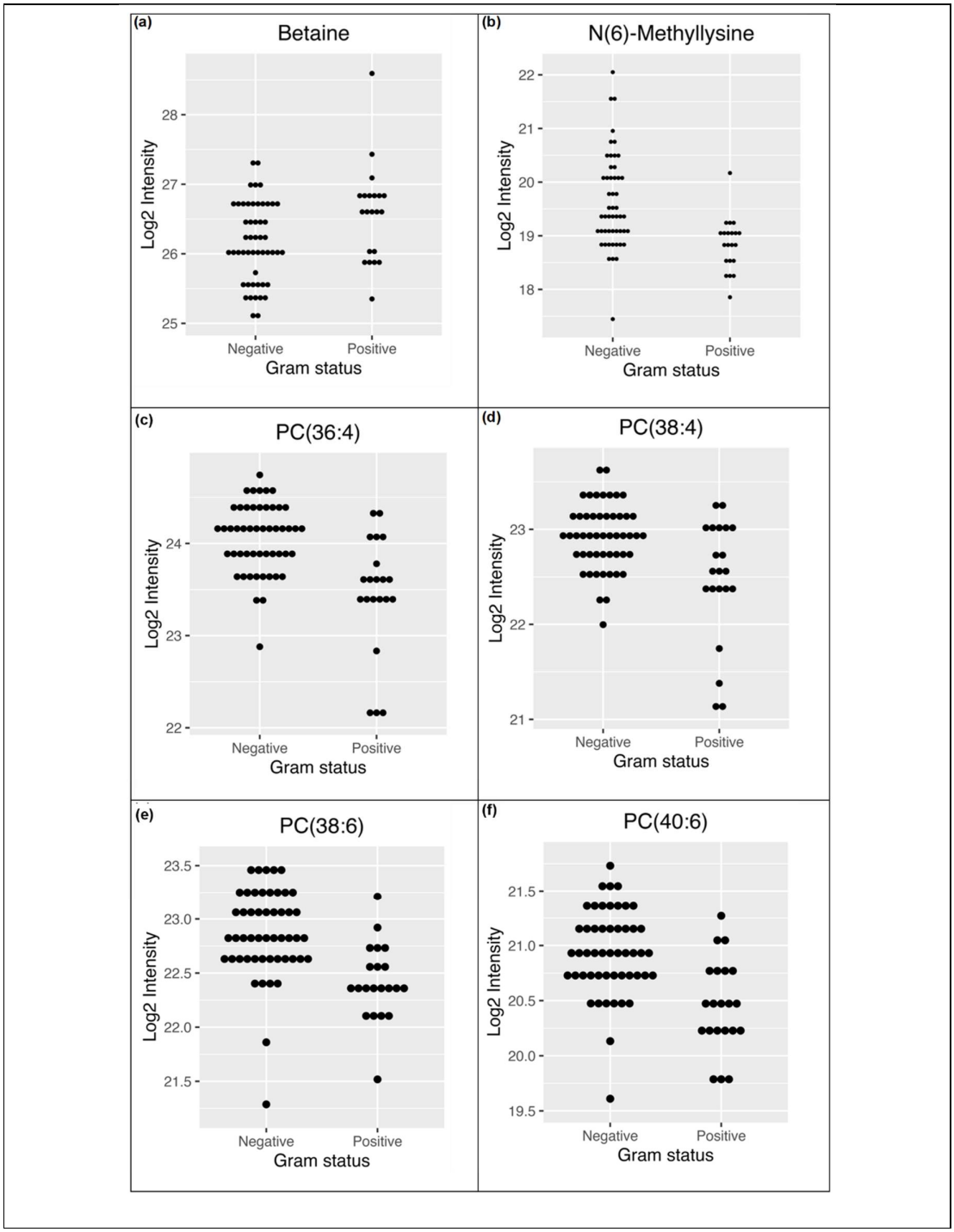

Figure 9-10: Dot plots depicting significantly different metabolites between Gram positive and Gram negative infections: (a) betaine, (b) N(6)-methyllysine, (c) PC(36:4), (d) PC(38:4), (e) PC(38:6), (f) PC(40:6)

9.5 Model Performance

Figure 9-11 depicts the ROC curve with sensitivity and specificity for determining Gram positive and negative infections, with an AUROC of 0.88 ± 0.074.

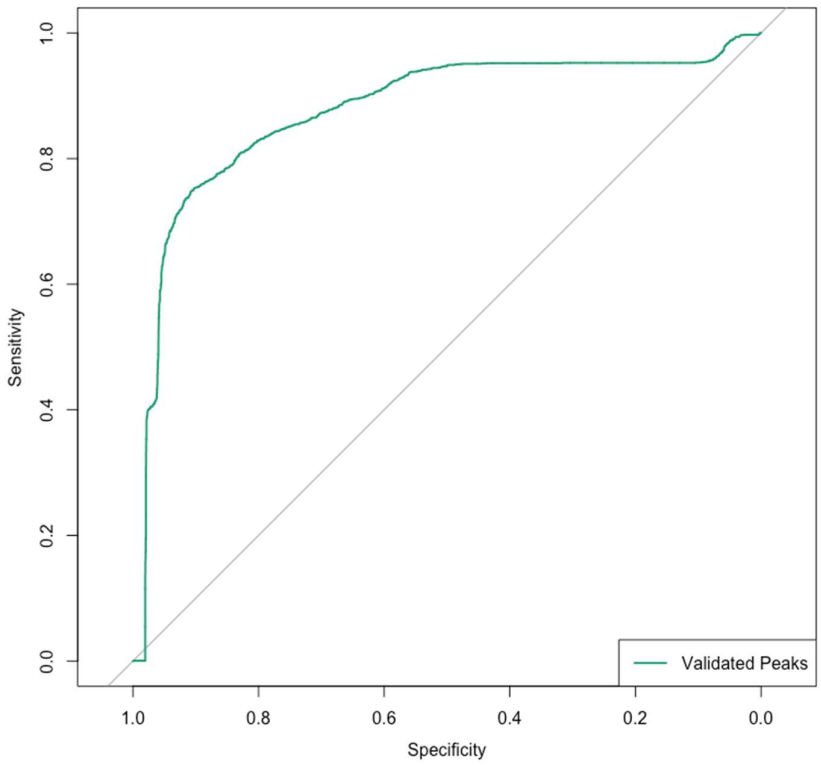

Figure 9-11: Receiver operating characteristic curve demonstrating test performance of the metabolomic panel of the six validated peaks for differentiating Gram positive and Gram negative infections.

9.6 Discussion

The machine learning algorithm produced a model which could confidently differentiate Gram positive and negative infections using a panel of six metabolites. Few prior metabolomics studies have sought to differentiate the nature of infections according to Gram staining. The study by Kelly *et al*. attempted to examine this, but unfortunately the sample size was too small and there were no clear metabolomic differences between gram positive or negative infections (434). The metabolomics study by Schmerler and colleagues found that a simple metabolite panel consisting of the acylcarnitine C10:1 and the phosphatidylcholine PCaaC32:0 was sufficient to differentiate sepsis patients from non-infectious SIRS. However, when they validated these findings, the model much better at identifying Gram positive infections than Gram negatives, suggesting these two metabolites may be more specific for Gram positive infections (448).

The study by Zheng *et al*. used a panel of ten metabolites which was capable of differentiating infections by Gram stain with an AUROC of 0.8. The model in the present study had an AUROC 0.88, suggesting that this metabolite panel is better at differentiating the Gram status of infections than the model produced by Zheng *et al*. (453).

9.6.1 Betaine

Betaines are naturally occurring compounds found in all plants, animals and microorganisms. Their structure is characterised by a quaternary ammonium group and a carboxylic group (680). Betaine glycine is also known as trimethylglycine due to its resemblance to a glycine amino acid with three additional methyl groups. Betaine glycine was the first betaine to be discovered, and so it is often simply referred to as betaine (681). Betaine was first identified in sugar beets which is where its name was derived from, however it is found in a variety of other foodstuffs including wheat, spinach and shellfish (682). Betaine may be acquired from the diet, but may also be synthesised endogenously from choline (681,683).

Betaine acts as a methyl donor during the cyclic process of hepatic and renal synthesis of methionine. Betaine passes its methyl group onto homocysteine, producing methionine (See *Figure 8-11*) (684). Methionine can then go on to produce SAM, which also acts as a methyl donor. Demethylation of SAM produces SAH, which goes on to reform homocysteine, which can continue the cycle by taking another methyl group from betaine (681). Of note, SAM passes its methyl group onto guanidinoacetate in the formation of creatine.

Other effects of betaine include osmoprotectant activity, particularly within the brain, kidneys and liver where it can accumulate and prevent water influx, reducing the risk of osmotic damage to cells (681). Betaine also exerts anti-inflammatory effects and reduces free radical formation (683). Betaine can suppress signalling via NF-κB, resulting in decreased pro-inflammatory cytokine release (685). Moreover, betaine can inhibit LPS signalling and suppress the formation of the NLRP3 inflammasome, further demonstrating anti-inflammatory effects (686). Betaine increases anti-oxidant defences, helping to mitigate ROS induced oxidative injury (687). Accordingly, betaine has been suggested to have beneficial and protective effects against several diseases including Alzheimer's dementia (688), diabetes mellitus (689), cardiovascular disease (690) and hepatic steatosis (691).

Metabolomics studies have conflicting results in terms of the response of betaine to sepsis. In a porcine model of sepsis, betaine was found to be depleted in septic animals compared to healthy controls (692). Conversely, a mouse model of sepsis by Muratsu and colleagues found that septic mice had increased betaine levels (693). The study by Liu found that in sepsis, increased betaine was associated with worse survival (489).

In terms of differentiating the nature of infections, Gram-positive and negative organisms appear to accumulate different types of betaines with different preferences. *S.aureus* appears to preferentially accumulate betaine glycine in the presence of other betaine molecules, whereas *E. coli* is less selective (694). Interestingly, *in vitro* studies have demonstrated that cultures of Gram positive organisms including *S. aureus* and *Enterococcus* species contained higher betaine concentrations than cultures of gram negative organisms such as *K. pneumoniae, E. coli*, *A. baumannii* and *P. aeruginosa* (695,696). This suggests that Gram positive organisms secrete betaine in higher concentrations than Gram negative species. As such, Gram positive infections may be associated with increased plasma betaine. Interestingly, the machine learning algorithm in the study by Zheng and colleagues also used betaine as part of their model, adding further evidence that this compound can help differentiate between Gram positive and negative infections (453).

9.6.2 N(6)-methyllysine

N(6)-methyllysine is a monomethylated derivative of the amino acid lysine. The ε-amine of a lysine molecule can bind with up to three additional methyl groups, and thus can be mono-methylated, di-methylated or tri-methylated (697). Lysine methylation plays a key role in epigenetics: the phenomenon of modifying gene expression without amending DNA base pairs (698). Nucleosomes are bundles of genetic information composed of a DNA strand wrapped around eight histone proteins (699). The lysine amino acids within the histones are targets for methylation, which reduces how tightly DNA is bound. This can alter the expression of genes within the DNA segment (700).

Gram negative infections may increase lysine methylation. In response to LPS stimulation, increased methylation of histone H3K4 occurs at promotor regions of IL-6 and TNF-α, suggesting that histone methylation is involved in upregulating pro-inflammatory signalling in response to Gram negative infections (701). Conversely, methylation at histone H3K9 is transiently decreased in response to LPS stimulation, further increasing additional pro-inflammatory gene expression. Remethylation of H3K9 occurs sub-acutely and appears to be associated with a reduction in pro-inflammatory signalling, promoting immunosuppression which could make patients more vulnerable to secondary infections (702,703).

Decreased lysine methylation at NF-κB binding sites has been observed in post septic mice. These mice went on to exhibit impaired wound healing and reduced production of pro-inflammatory cytokines in macrophages (704).

Non-histone proteins may also undergo lysine methylation as an important post-translational modification. Methylation of lysines affects protein activity and localisation, and impacts cellular growth and DNA repair (697,700). Methylated lysines can be liberated from proteins through enzymatic hydrolysis during protein catabolism (705). The increased protein turnover observed in sepsis may increase plasma N(6)-methyllysine. Moreover, there will be reduced renal excretion of methylated lysines in renal failure, thus sepsis induced acute kidney injury could lead to plasma accumulation of N(6)-methyllysine (705).

9.6.3 Phosphatidylcholines

As described in chapter 4, PCs are the most abundant phospholipid membrane constituents in eukaryotes. Prokaryotic lipid membranes most commonly contain PG and PE, however approximately 15% of all bacteria can produce PCs (706). The presence of PCs in bacterial membranes may be used for bacteria/host interactions, either for symbiosis of to increase pathogenesis (707). Organisms such as *Brucella* and *Legionella* appear to require the presence of PCs to exact full virulence (706). Gram positive human pathogens which contain PC in their membranes include C*lostridium* and S*treptococcus* species, whereas M*ycoplasma* species do not. Gram negative organisms including *Neisseria*, *Klebsiella*, *Legionella*, and *Pseudomonas* species can incorporate PCs into their membranes, while *Campylobacter*, *Escherichia* and *Haemophilus* species do not (707). *Klebsiella* and *Pseudomonas* were responsible for a number of respiratory tract infections in this study, which may account for the increase in PCs in Gram negative infections.

It is interesting to note that the metabolites differentiating Gram positive and negative infections demonstrated a greater statistical significance than those used in the model for identification of secondary infections. This raises the possibility that the biomarkers identified for differentiating Gram positive and negative infections may be well placed to identify secondary infections too. A future analysis combining betaine, N(6)-methyllysine and the four phosphatidylcholines along with creatine, S-methyl-L-cysteine and 2-hydroxyisovalerylcarnitine would be interesting to see if this boosts the performance of the model.

Alternatively, the greater significance for the Gram positive versus Gram negative biomarkers may reflect the inherent heterogeneity of secondary infections. There are likely to be different metabolic perturbations depending on the aetiology of the infection including pathogen type and infection site, as well as host immune responses and underlying comorbidities. Biomarker panels may be more specific to different types of infection such as Gram negative or positive infections, rather than attempting to identify secondary infections as a single heterogeneous condition. Future studies examining secondary infection subtypes may increase the specificity and reliability of metabolomics models, and produce more clinically useful biomarker panels.

9.7 Conclusion

This study has shown that HPLC/MS metabolomics is capable of producing profiles which can accurately differentiate Gram positive from Gram negative infections in critically ill patients with COVID-19. Betaine is increased in Gram positive infections, while N(6)methyllysine and the PCs PC(36:4), PC(38:4), PC(38:6) and PC(40:6) are increased during gram negative infection. These metabolites are involved in glycerophospholipid metabolism and lysine methylation pathways.

10 Final Discussion

10.1 Project Summary

Metabolomics is a modern discipline which can detect changes in the concentration of many thousands of metabolites. These metabolic perturbations provide a snapshot of the health of an individual at a particular point in time. Metabolomics studies can therefore elucidate biomarkers of disease states. Many metabolomics studies have sought to identify novel biomarkers in sepsis, and a wide range of metabolite candidates have been produced, particularly relating to energy metabolism, protein catabolism and lipid metabolism. However studies investigating biomarkers in secondary infections are lacking.

In severe COVID-19 the clinical signs and symptoms, as well as radiological and biochemical tests commonly used to identify the presence of an infection will often already be abnormal from the primary SARS-CoV-2 infection. This makes it highly challenging to detect secondary infections. Correctly identifying a secondary infection is crucial to ensure that appropriate antimicrobial therapy is commenced as early as possible, while avoiding unnecessary administration of antibiotics to patients who do not require them.

This study was able to produce a panel of three metabolites capable of identifying secondary infections with high accuracy. A separate panel of six metabolites was able to accurately differentiate between patients with Gram positive and Gram negative infections.

10.2 Limitations

There were several limitations to this study. The number of patients recruited was greater than many of the sepsis metabolomics studies discussed in Chapter 5. Nevertheless, only 40 of the recruited patients developed a secondary infection during the trial period. A greater number of participants would increase the confidence in detected metabolite differences. Moreover, having a greater number of patients would permit additional analyses, for instance examining if specific metabolomic profiles are produced for different infecting pathogens.

The inclusion criteria for this study required patients to have tested positive for SARS-CoV-2 in the previous 7 days, but did not take into consideration when they had first tested positive. Additionally, patients were eligible for recruitment if they were requiring level 2 care at the time of recruitment, but there was no limit on how long patients had spent in critical care prior to recruitment. This means that different patients will have been recruited to the study at different stages in the disease process. In a future study, it might be more appropriate to specify that patients would only be eligible for recruitment to the study in the first 24-48 hours of admission to critical care.

Given that it was not possible to predict when a patient would develop a secondary infection, this study employed a strategy of collecting three blood samples across a ten day period, with an additional fourth sample if the patient yielded positive microbiological cultures or had a clinical deterioration in keeping with the development of a secondary infection. It is likely that these blood sampling times will not have always happened on the exact same day that a secondary infection developed, and so it is possible that the peak changes in metabolite concentrations may have been missed. Variation in sampling time points also made it impractical to directly compare infection markers such as CRP and WCC between patients with and without secondary infections. In future, daily blood sampling would ensure that a blood sample would always have been taken on the day that a secondary infection develops. To provide more accurate comparisons between the two groups, a propensity score could be developed to identify non-infected controls with similar baseline characteristics to patients with secondary infections, and a comparison at the equivalent time point of development of the secondary infection could be made between cases and controls.

The data collection for this study was performed using Excel. This method of data collection is prone to error, as data cells are not locked and so data could inadvertently be changed or deleted. A repeat collection of 10% of data was performed which showed no major discrepancies (See *Appendix I*). Performing a 10% recheck of the data was chosen for time efficiency reasons, however a more rigorous but resource intensive method would be to perform a complete recheck of the entire data set using an independent third-party participant. During the 10% data check, the BMI of one patient was found to have been miscalculated, but this made no difference to the mean BMI when the correct value was provided. This gives greater confidence in the rigour of the data collection and suggests that no significant systematic data input errors occurred. However, the fact that an error was found highlights that Excel is an imperfect error-prone tool. In future a better method would be to use an online repository such as the Research Electronic Data Capture (REDCap) (708). This software permits collaborative access to secure electronic case report forms, and has built in mechanisms to minimise error such as dropdowns and number ranges. Once data is entered, the case report form is locked and cannot be edited inadvertently. This is therefore a much more secure and robust method of data collection.

This study focussed on using metabolomics as a diagnostic rather than prognostic tool for secondary infections. As such, mortality was not included in the statistical model. However, mortality is an important clinical outcome in COVID-19, and patients with secondary infections have a significantly higher mortality rate than those without. Therefore, it may have been worthwhile assessing mortality as an outcome in this study. As previously discussed, it is often challenging to differentiate genuine secondary infections from colonised or contaminated microbiological cultures. Biomarker panels which are associated with both secondary infections and mortality are more likely to represent genuine infection rather than colonisation, and so inclusion of mortality may help to differentiate between the two.

During analysis, the q-value significance was determined *post hoc*. This is acceptable for exploratory studies as it allows for flexibility and potentially captures metabolites of importance which would be missed with a stricter cut-off. However, a more lenient significance level increases the risk of false positives. In a future validation study, an *a priori* significance value should be determined to minimise the risk of bias and type I error. Conversely, additional pathway analysis of metabolites close to the significance level may have yielded more relevant biomarkers. Exploratory examination of metabolites with q-values just above the significance cut-off may have shown other candidate biomarkers which also sit on these pathways. This helps to reduce type II errors which may be missed by strictly adhering to a statistical significance cut-off.

Visual inspection was used to compare mass spectra in this study. This is a subjective measure which is therefore open to bias. A more rigorous method would be to utilise quantification software such as matchms (709). This is an open-source Python package which will compare mass spectra in a standardised manner, permitting reproducibility and reducing potential bias.

The machine learning algorithm utilised in this study uses a 10-fold cross-validation procedure and was repeated 100 times which demonstrated good internal validity within the model. However, the use of an external validation cohort of patients would have provided even higher confidence in the results and to ensure that the findings were not simply due to chance. In future, recruiting patients to a validation group alongside the test group would mean that there would be a separate cohort which the final model could be validated against. A repeat targeted study could then be performed with patients from other cohorts worldwide to ensure that the findings are generalisable to all populations globally and not just confined to our cohort.

10.3 Future Work

The number of patients who require admission critical care due to COVID-19 has massively decreased since the beginning of the pandemic. It is unlikely that a repeat study would be able to match the recruitment of this study. However, within the last three decades three separate CoV pandemics have occurred as well as a pandemic caused by the H1N1 influenza virus. Another cause of viral pneumonia could arise at any point in the future. Furthermore, there is an annual surge every year in respiratory diseases caused by viruses such as the respiratory syncytial virus and influenza viruses. There will always be a proportion of these patients who develop severe disease and will be at risk of a secondary infection. A future project could investigate metabolomic signatures for secondary infections in different causes of viral pneumonia. A repeat study could investigate whether the metabolite panel identified in this study is applicable to identifying secondary infections in critically ill patients with other causes of viral pneumonia.

There have been a number of advances in metabolomics technology in recent years, with increased throughput, range of detectable features and significantly reduced costs (710). Unfortunately, the time associated with sample preparation and metabolite extraction, and the complexity associated with the data analysis currently limits the clinical application of metabolomics within the ICU. Quick decision making in the ICU is essential to ensure appropriate antibiotic therapy can be given at the earliest opportunity, and as such the current time constraints associated with metabolomics make the technique prohibitively impractical and not appropriate for real-time identification of secondary infections. With further innovation in MS technology, for instance increased availability of portable MS platforms (711), then point-of-care metabolomic analysis may be possible in the future. However, once a metabolomic panel has been validated externally there would be no need to repeat full LC-MS metabolomic analysis. Therefore, production of a new point of care colourimetric assay using the validated panel would allow for a faster and more cost-effective method of secondary infection identification (418).

Secondary infections are inherently diverse and heterogeneous conditions. Differences in infection types, sites, and host responses will invariably lead to variations in metabolism. Therefore, attempting to identify a ‘one-size-fits-all’ biomarker panel for all secondary infections may not sufficiently encompass the full scale of heterogeneity associated with the condition. This may explain why studies investigation biomarkers and therapies for sepsis have had such disappointing results (351,712).

Precision medicine is a diagnostic and therapeutic strategy which attempts to considers individual patient characteristics rather than grouping all patients with the same condition together. Enrichment strategies aim to select the patients who are most likely to develop outcomes of interest are most likely to respond favourably to specific treatments (351). Within clinical metabolomics, this strategy is still within its infancy but is generating much interest regarding sepsis metabolomics studies. A recent study by Antcliffe *et al*. was able to determine that three separate patient sub-phenotypes exist within septic shock. One of these sub-phenotypes demonstrates a rise in lipid species, and persistence of this sup-phenotype is associated with better outcomes (713). This suggests that serial monitoring of these lipid metabolites may aid with prognostication in patients with septic shock.

Excitingly, precision medicine approaches may help to uncover undiscovered therapeutic pathways which could identify novel effective drug therapies. The GENOMICC trial determined that patients with a mutation in the *TYK2* gene were more likely to develop severe COVID-19. Increased *TYK2* gene activity increases JAK/STAT proinflammatory signalling (714). As a result, the RECOVERY trial investigators added the JAK/STAT inhibitor baricitinib as an intervention arm to their study. This drug subsequently demonstrated improved survival when given to patients with severe COVID-19 (149).

Correspondingly, metabolomics could help to produce biomarker panels for specific secondary infection endotypes and subphenotypes. This could permit characterisation of the likely pathogen to enable appropriate early antimicrobial therapy to be initiated, while also helping to classify patients into those who may benefit from immunomodulatory therapies (712). Pharmacometabolomics could play a central role in the ICU in future, as metabolomic analysis of critically ill patients could help to provide tailored treatments for patients, while serial monitoring could track patient responses to therapies (434).

The clinical challenge of how to correctly identify secondary infections is not unique to COVID-19. Any condition which presents with a SIRS response can make identification of an infection problematic. Conditions such as pancreatitis, polytrauma and severe burns can all cause SIRS, potentially masking the development of an infection. Patients with chest wall trauma may develop secondary infections through hypoventilation and inadequate cough. Identification of a metabolomic signature of infection in this group could help to reduce the unnecessary use of antibiotics in patients without chest infections.

Patients with haematological malignancies can also present to critical care with SIRS. These patients are at significant risk of infectious complications, and fungal infections in particular can have devastating consequences. Future studies investigating infections in other forms of critical illness would be interesting to study if metabolomic signatures of infection are the same between different aetiologies of critical illness.

A multi-omics approach to a future ICU study may provide complementary data regarding the underlying disease pathobiology observed during infections. Functional genomics aims to identify culprit genes involved in disease processes and their downstream effects. Exploring functional genomics in secondary infections would help to gain a clearer idea of the pathways involved in the cause of metabolite perturbations and the responsible genetic polymorphisms. By identifying genes associated with secondary infections and pairing this with downstream metabolic changes, a detailed map of the pathways involved in the observed immunometabolic dysfunction could be created, which would help to identify potential new drug targets (434). Moreover, a multi-level integrated genomics and metabolomics analysis would provide additional validity, as associations between candidate genes could be corroborated with corresponding metabolomic data, strengthening the biological plausibility of the results (458). The addition of proteomics into a multi-level analysis would provide further strength to a future study, and would act as an intermediate level between genomics and metabolomics (715). Inflammatory molecules such as cytokines and other acute phase proteins could be captured and included as part of a secondary infection biomarker panel, providing an additional synergistic dimension to the biological data (458).

10.4 Conclusion

This project has demonstrated an association between metabolomic biomarker panels and secondary infections in COVID-19. These panels appear to be capable of identifying secondary infections as well as differentiating between Gram positive and Gram negative infections within this patient group. This project has demonstrated the vast potential for using metabolomics as a tool for biomarker discovery in critical illness and infectious diseases. Future directions for this project include larger integrated multi-omics studies examining secondary infections in other critical illness aetiologies.

11 Appendices

11.1 *Appendix A*: Table of the Most Studied CoVs

Host	Virus	Abbreviation
Alphacoronaviruses		
Human	*Human CoV-229E*	*HCoV-229E*
	Human CoV-NL63	*HCoV-NL63*
Bat	*Bat coronavirus CDPHE15*	*BtCoV CDPHE15*
	Rousettus bat coronavirus HKU10	*BtCoV HKU10*
	Rhinolophus ferrumequinum alphacoronavirus HuB-2013	*BtRf-AlphaCoV*
	Rhinolophus bat coronavirus HKU2	*Rh-BatCoV-HKU2*
	Miniopterus bat coronavirus 1	*Mi-BatCoV-1A*
	Miniopterus bat coronavirus HKU8	*Mi-BatCoV-HKU8*
	Myotis ricketti alphacoronavirus Sax-2011	*BtMr-AlphaCoV*
	Nyctalus velutinus alphacoronavirus SC-2013	*BtNv-AlphaCoV*
	Pipistrellus kuhlii coronavirus 3398	*Pi-BatCoV-HKU5*
	Scotophilus bat coronavirus 512	*Sc-BatCoV-512*
Pig	*Porcine respiratory coronavirus*	*PRCV*
	Transmissible gastroenteritis virus	*TGEV*
	Porcine epidemic diarrhoea virus	*PEDV*
	Swine acute diarrhoea syndrome-coronavirus	*SADS-CoV*
Dog	*Canine coronavirus*	*CCoV*
Cat	*Feline CoV*	*FCoV*
Mink	*Mink coronavirus 1*	*MCoV*
Betacoronaviruses		
Lineage A (Embecoviruses)		
Human	*Human CoV-HKU1*	*HCoV-HKU1*
	Human CoV-OC43	*HCoV-OC43*
Cow	*Bovine CoV*	*BCoV*
Pig	*Porcine hemagglutinating encephalomyelitis virus*	*PHEV*
Horse	*Equine CoV*	*ECoV*
Camel	*Dromedary camel coronavirus HKU23*	*DcCoV-HKU23*
Rat	*China Rattus coronavirus HKU24*	*ChRCoV-HKU24*
	Myodes coronavirus 2JL14	*MrufCoV 2JL14*
Mouse	*Mouse hepatitis virus*	*MHV*
Lineage B (Sarbecoviruses)		
Human	*Severe acute respiratory syndrome coronavirus 1*	*SARS-CoV-1*
	Severe acute respiratory syndrome coronavirus 2	*SARS-CoV-2*
Bat	*Bat SARS-related coronavirus WIV1*	*Bat SARSr-CoV WIV1*
	Bat coronavirus RaTG13	*Bat-Cov-RaTG13*
Lineage C (Merbecoviruses)		
Human	*Middle East respiratory syndrome coronavirus*	*MERS-CoV*
Hedgehog	*Hedgehog coronavirus 1*	*EriCoV*
Bat	*Pipistrellus bat coronavirus HKU5*	*Pi-BatCoV-HKU5*
	Tylonycteris bat coronavirus HKU4	*Ty-BatCoV-HKU4*
Lineage D (Nobecoviruses)		
Bat	*Rousettus bat coronavirus HKU9*	*Ro-BatCoV-HKU9*
	Rousettus bat coronavirus GCCDC1	*Ro-BatCoV-GCCDC1*
	Eidolon bat coronavirus C704	*Ei-BatCoV* ***C704***
Deltacoronaviruses		
Pig	*Porcine coronavirus HKU15*	*PorCoV HKU15*
Bulbul	*Bulbul coronavirus HKU11*	*BuCoV HKU11*
Eurasian Tree Sparrow	*Sparrow coronavirus HKU17*	*SpCoV HKU17*
Common Moorhen	*Common moorhen coronavirus HKU21*	*CMCoV HKU21*
Munia	*Munia coronavirus HKU13*	*MunCoV HKU13*
Black-crowned night heron	*Night heron coronavirus HKU19*	*NHCoV HKU19*
Gray-backed thrush	*Thrush coronavirus HKU12*	*ThCoV HKU12*
Japanese white-eye	*White-eye coronavirus HKU16*	*WECoV HKU16*
Eurasian wigeon	*Wigeon coronavirus HKU20*	*WiCoV HKU20*
Gammacoronaviruses		
Whale	*Beluga whale coronavirus SW1*	*BWCoV-SW1*
Goose	*Canada goose coronavirus*	*CGCoV*
Chicken	*Infectious bronchitis virus*	*IBV*

Adapted from (8,11,14,81,716–722)

11.2 *Appendix B*: Literature Review Search Strategy

The following search strategy was used, based off the meta-analysis conducted by (480):

1 (((((Sepsis or septic or Severe) adj1 Sepsis) or Py?emia or Py?emias or Pyoh?emia or Pyoh?emias or Septic?emia or Septic?emias or Blood) adj1 Poisoning*) or Bacter?emia or Bacter?emias or Endotox?emia or Endotox?emias or Fung?emia or Fung?emias or Candid?emia or Candid?emias or Parasit?emia or Parasit?emias or Vir?emia or Vir?emias).tw.

2 sepsis/ or bacteremia/ or fungemia/ or septic shock/ or septicemia/ or urosepsis/

3 1 or 2

4 exp metabolic fingerprinting/

5 metabolome/ or metabolomics/ or metabonomics/ or nutrimetabolomics/ or pharmacometabolomics/

6 (Metabolomics or Metabolomic or Metabonomics or Metabonomic or Nuclear magnetic resonance spectroscopy or NMR or liquid chromatography-mass spectrometry or LC-MS or mass spectrometry or MS or profiles or profiling or fingerprint or fingerprints or fingerprinting).tw.

7 4 or 5 or 6

8 diagnosis/ or diagnostic accuracy/ or diagnostic test/ or diagnostic test accuracy study/ or differential diagnosis/ or early diagnosis/ or laboratory diagnosis/ or molecular diagnosis/ or quick diagnosis unit/

9 (diagnos* or detect* or identif*).tw.

10 8 or 9 13290082

11 3 and 7 and 10

This search strategy generated 6766 unique hits. The following studies were excluded:

- Review articles
- Case reports, case series, protocols, letters to the editor and meeting abstracts
- Animal studies and *in vitro* studies
- Paediatric, neonatal or obstetric studies
- Studies not in English
- Other "omics" studies (proteomics/transcriptomics/genomics in absence of metabolomics)

11.3 *Appendix C*: Healthy Volunteer Information Sheet

The use of Metabolomics for the Early Recognition of Sepsis

NHS
Greater Glasgow and Clyde

HEALTHY VOLUNTEER INFORMATION SHEET

1. Study Title
The use of Metabolomics for the Early Recognition of Sepsis

2. Introduction
You are invited to participate in a research study. Before you decide, it is important for you to fully understand why the research is being done and what it will involve. Please take your time to read the following information carefully. Ask your study doctor or nurse if there is anything that is not clear or if you would like more information.

3. What is the purpose of the Study?
The purpose of the study is to evaluate new methods for diagnosing infection. This is when the body's organ systems are severely affected by infection. This research will determine if these methods can both diagnosis of infection earlier than the methods currently used and define different types of infection.

4. Why have you been invited to take part?
You are being invited to take part because you have are a healthy volunteer.

5. Do you have to take part?
Your participation in the study is voluntary. It is up to you to decide whether to take part or not. If you decide to take part, you will be given this Healthy Volunteer Information Sheet to keep and be asked to sign a patient Informed Consent Form. You are free to change your mind or withdraw from the study at anytime and without giving reason. Your decision will not affect in any way the standard of care you receive. If you decide to withdraw from the study blood samples and data collected up until that point may still be analysed.

6. What will happen if you take part?
Your doctor or nurse will take 5 mls (1 teaspoons) of blood. We would also like to store any left-over sample for use in future ethically approved research. In addition, some information will be collected from your medical notes to allow any association with e.g. infection type, to be determined.

7. What information is being collected?
The results of tests on your blood will be collected as well as some general information about you, information relating to the treatment you received, and the outcomes of this.

8. How is the information going to be used?
All the information will be entered into a computer database to determine the changes in the markers of infection. The new (metabolomic method) we are testing will be compared to the existing methods of diagnosing infection using the biochemical markers such as C Reactive Protein and Procalcitonin.

9. How secure is the information?
If you decide to take part all the information which will be collected about you during the study will be kept strictly confidential. Your study doctor and nurse will have access to your case notes as well as authorised personnel from the Board for data monitoring and audit purposes. You will be assigned a study number which will be used to label data and samples and therefore, although data and samples will be sent to the University of Glasgow for analysis, no identifiable personal information will leave the hospital. Any published report of the data will not identify you.

10. What are the possible disadvantages or risks of taking part?
When possible, blood samples will be collected from an existing intravenous or arterial catheter. If this is not possible a venipuncture will be required. For most people, needle punctures for blood sampling do not cause any serious problems. However, they may cause dizziness or bleeding, bruising, discomfort, infections, and/or pain at needle site.

11. What are the possible benefits of taking part?
There would be no direct benefit to you from taking part in this study, however, information gained from the study would hopefully enhance knowledge of severe sepsis.

12. How long does the research intervention last?
A blood sample (5mls) will be collected once.

13. Who is organising and funding the research?
The study is sponsored by the NHS Greater Glasgow and Clyde and funded by a grant from the Chief Scientist Office, Scotland.

14. Who has reviewed this study?
The study has been reviewed by the NHS Scotland A Research Ethics Committee.

15. What if I have any concerns?

If you have a concern about any aspect of this study, please contact the study investigator Dr Malcolm Sim (Consultant in Anaesthesia and Intensive Care Medicine) on 0141 452 3033. For an independent view from a clinician not involved in the study you can contact Dr Sandy Binning (Consultant in Anaesthesia and Intensive Care medicine) on-0141 452 3033. They will do their best to answer your questions.

If you remain unhappy the usual NHS complaints mechanisms will still be available to you and your relative. This can be accessed thought the Citizens Advice bureau, or the Boards complaints office on 0141 201 4500 (Email:complaints@ggc.scot.nhs.uk)

11.4 *Appendix D*: Patient With Capacity Information Sheet

The use of Metabolomics for the Early Recognition of Sepsis

NHS
Greater Glasgow and Clyde

PATIENT INFORMATION SHEET

1. Study Title
The use of Metabolomics for the Early Recognition of Sepsis

2. Introduction
You are invited to participate in a research study. Before you decide, it is important for you to fully understand why the research is being done and what it will involve. Please take your time to read the following information carefully. Ask your study doctor or nurse if there is anything that is not clear or if you would like more information.

3. What is the purpose of the Study?
The purpose of the study is to evaluate new methods for diagnosing infection. This is when the body's organ systems are severely affected by infection. This research will determine if these methods can both diagnosis of infection earlier than the methods currently used and define different types of infection.

4. Why have you been invited to take part?
You are being invited to take part because you have developed COVID-19 infection and it is within this group of patients the research needs to be carried out.

5. Do you have to take part?
Your participation in the study is voluntary. It is up to you to decide whether to take part or not. If you decide to take part, you will be given this Patient Information Sheet to keep and be asked to sign a patient Informed Consent Form. You are free to change your mind or withdraw from the study at anytime and without giving reason. Your decision will not affect in any way the standard of care you receive. If you decide to withdraw from the study blood samples and data collected up until that point may still be analysed.

6. What will happen if you take part?
Your doctor or nurse will take 5 mls (1 teaspoons) of blood once a day for a total of 3 days (today in 3days and 10days from now) and further sampled may be taken if we suspect you have developed an infection other than covid 19 while you are a patient in intensive care to look at biochemical markers of infection. Where possible this will be taken at the same time as routine blood samples. We would also like to store any left-

over sample for use in future ethically approved research. In addition, some information will be collected from your medical notes to allow any association with e.g. infection type, to be determined.

7. What information is being collected?
The results of tests on your blood will be collected as well as some general information about you, information relating to the treatment you received, and the outcomes of this.

8. How is the information going to be used?
All the information will be entered into a computer database to determine the changes in the markers of sepsis. The new (metabolomic method) we are testing will be compared to the existing methods of diagnosing infection using the biochemical markers such as C Reactive Protein and Procalcitonin.

9. How secure is the information?
If you decide to take part all the information which will be collected about you during the study will be kept strictly confidential. Your study doctor and nurse will have access to your case notes as well as authorised personnel from the Board for data monitoring and audit purposes. You will be assigned a study number which will be used to label data and samples and therefore, although data and samples will be sent to the University of Glasgow for analysis, no identifiable personal information will leave the hospital. Any published report of the data will not identify you.

10. What are the possible disadvantages or risks of taking part?
When possible, blood samples will be collected from an existing intravenous or arterial catheter. If this is not possible a venipuncture will be required. For most people, needle punctures for blood sampling do not cause any serious problems. However, they may cause dizziness or bleeding, bruising, discomfort, infections, and/or pain at needle site.

11. What are the possible benefits of taking part?
There would be no direct benefit to you from taking part in this study, however, information gained from the study would hopefully enhance knowledge of severe sepsis.

12. How long does the research intervention last?
Samples will be collected daily once a day for a total of 3 days (today in 3days and 10days from now) and further samples may be taken if we suspect you have developed an infection other than covid 19 for the duration of your stay in Intensive Care.

13. Who is organising and funding the research?

The use of Metabolomics for the Early Recognition of Sepsis

The study is sponsored by the NHS Greater Glasgow and Clyde and funded by a grant from the Chief Scientist Office, Scotland

14. Who has reviewed this study?
The study has been reviewed by the NHS Scotland A Research Ethics Committee.

15. What if I have any concerns?
If you have a concern about any aspect of this study, please contact the study investigator Dr Malcolm Sim (Consultant in Anaesthesia and Intensive Care Medicine) on 0141 452 3033. For an independent view from a clinician not involved in the study you can contact Dr Sandy Binning (Consultant in Anaesthesia and Intensive Care medicine) on-0141 452 3033. They will do their best to answer your questions.

If you remain unhappy the usual NHS complaints mechanisms will still be available to you and your relative. This can be accessed thought the Citizens Advice bureau, or the Boards complaints office on 0141 201 4500 (Email:complaints@ggc.scot.nhs.uk)

11.5 *Appendix E*: Patient With Recovered Capacity Information Sheet

The use of Metabolomics for the Early Recognition of Sepsis

NHS Greater Glasgow and Clyde

PATIENT INFORMATION SHEET RECOVERED CAPACITY

1. Study Title
The use of Metabolomics for the Early Recognition of Sepsis

2. Introduction
During your recent admission to hospital you were unable to give consent for entry into a study, we therefore asked your nearest relative or welfare attorney or guardian who gave consent on your behalf to enter this study. Before you decide, it is important for you to fully understand why the research is being done and what it will involve. Please take your time to read the following information carefully. Ask your study doctor or nurse if there is anything that is not clear or if you would like more information.

3. What is the purpose of the Study?
The purpose of the study is to evaluate new methods for diagnosing infection. This is when the body's organ systems are severely affected by infection. This research will determine if we can use new biochemical tests to diagnosis ~~sepsis~~ infection earlier than the methods currently used and define different types of infection.

4. Why were you invited to take part?
You were admitted to hospital for treatment of COVID-19 and your nearest relative or welfare attorney or guardian agreed that you could join the study. However, you are now capable of making an informed decision about whether you wish to continue in the study or not

5. Do you have to continue to take part?
Your participation in the study is voluntary. It is up to you to decide whether to take part or not. If you decide to take part, you will be given this Patient Information Sheet to keep and be asked to sign a patient Informed Consent Form. You are free to change your mind or withdraw from the study at anytime and without giving reason. Your decision will not affect in any way the standard of care you receive. If you decide to withdraw from the study blood samples and data collected up until that point may still be analysed.

6. What has happened to you so far and what will happen if you continue to participate?
Your doctor or nurse has taken 5 mls (1 teaspoons) of blood daily for a total of 3 days (today in 3days and 10days from now) and a further sample may be taken if we suspect you have developed an infection other than covid 19 while have been a patient in intensive care to look at biochemical markers of infection. If you wish to

continue then further 5 ml blood samples will be taken ~~once a day~~ during your stay in intensive care. Where possible this will be taken at the same time as routine blood samples. We would also like to store any left-over sample for use in future ethically approved research. In addition, some information will be collected from your medical notes to allow any association with e.g. infection type, to be determined.

7. What information is being collected?
The results of tests on your blood will be collected as well as some general information about you, information relating to the treatment you received, and the outcomes of this.

8. How is the information going to be used?
All the information will be entered into a computer database to determine the changes in the markers of infection. These new biochemical markers will be compared to the existing methods of diagnosing sepsis using current biochemical markers.

9. How secure is the information?
If you decide to take part all the information which will be collected about you during the study will be kept strictly confidential. Your study doctor and nurse will have access to your case notes as well as authorised personnel from the Board for data monitoring and audit purposes. You will be assigned a study number which will be used to label data and samples and therefore, although data and samples will be sent to the University of Glasgow for analysis, no identifiable personal information will leave the hospital. Any published report of the data would not identify you.

10. What are the possible disadvantages or risks of taking part?
When possible, blood samples will be collected from an existing intravenous or arterial catheter. If this is not possible a venipuncture will be required. For most people, needle punctures for blood sampling do not cause any serious problems. However, they may cause dizziness or bleeding, bruising, discomfort, infections, and/or pain at needle site.

11. What are the possible benefits of taking part?
There would be no direct benefit to you from taking part in this study, however, information gained from the study would hopefully enhance knowledge of severe sepsis.

12. How long does the research intervention last?
Samples will be collected daily for a total of 3 days (today in 3days and 10days from now) and further samples may be taken if we suspect you have developed an infection other than COVID 19 for the duration of your stay in Intensive Care.

13. Who is organising and funding the research?
The study is sponsored by the NHS Greater Glasgow and Clyde and funded by a grant from the Chief Scientist Office, Scotland.

14. Who has reviewed this study?
The study has been reviewed by the NHS Scotland A Research Ethics Committee.

15. What if I have any concerns?

If you have a concern about any aspect of this study, please contact the study investigator Dr Malcolm Sim (Consultant in Anaesthesia and Intensive Care Medicine) on 0141 452 3033. For an independent view from a clinician not involved in the study you can contact Dr Sandy Binning (Consultant in Anaesthesia and Intensive Care medicine) on 0141 452 3033. They will do their best to answer your questions.

If you remain unhappy the usual NHS complaints mechanisms will still be available to you and your relative. This can be accessed thought the Citizens Advice bureau, or the Boards complaints office on 0141 201 4500 (Email:complaints@ggc.scot.nhs.uk)

11.6 *Appendix F*: Nearest Relative Information Sheet and Consent Form

NHS
Greater Glasgow and Clyde

PARTICIPANT INFORMATION SHEET AND CONSENT FORM

Nearest Relative/Guardian or Welfare Attorney

"The use of Metabolomics for the Early Recognition of Sepsis"

You are being invited to consider giving your permission for your relative to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Please ask me if there is anything that is not clear or if you would like more information. Thank you for reading this.

What is the purpose of the study?
The purpose of the study is to evaluate new methods for diagnosing infection. This is when the body's organ systems are severely affected by infection. This research will determine if we can use new biochemical tests to diagnosis sepsis earlier than the methods currently used and define different types of infection.

Why has the patient been chosen?
Your relative been asked to take part as they have been diagnosed with COVID-19.

However, they currently lack the capacity to make an informed decision about whether they can take place in a research study. We are therefore asking you as their nearest relative, welfare attorney or guardian if you will give consent on their behalf to join this study. This is permissible under the Adults with Incapacity (Scotland) Act 2000.

Do they have to take part?
No. It is up to you to decide whether they take part in the research or not. If you decide that should take part you are free to change your mind at any time and without giving a reason and this will not alter their care in any way, now or at any stage in the future.

What will happen to your relative if they take part in the research?
The doctor or nurse looking after your relative will take 5 mls (1 teaspoon) of blood every day for a total of 3 days (today in 3days and 10days from now) and further samples may be taken if we suspect you have developed an infection other than COVID19 while they are a patient in intensive care. Where possible this will be taken at the same time as routine blood samples. For an average patient we expect 4 samples to be taken in total. The sample will be analysed to look at biochemical

markers of infection. We would also like to store any leftover sample for use in future ethically approved research.

In addition some information will be collected from your relative's medical notes. This will allow us to look for any association with e.g. different infection types. Further, results of some blood tests will be collected as well as some general information about them, the treatment they are receiving and its effect.

All the information will be entered into a computer database to determine the changes in the markers of infection. These new biochemical markers will be compared to the existing methods of diagnosing sepsis using current biochemical markers.

What are the possible benefits of taking part?
There is no direct benefit to your relative of taking part in this study. However, infection and COVID-19 is a disease process which in some patients can carry a high mortality. Earlier diagnosis with new techniques will hopefully benefit other patients in the future.

What are the possible disadvantages and risks of taking part?
When possible blood samples will be collected from an existing intravenous or arterial catheter. This is by far the most likely scenario in Intensive Care. If this is not possible a venepuncture (taking blood with a needle and syringe) will be required. For most people, needle punctures for blood sampling do not cause any serious problems. However, they may cause dizziness or bleeding, bruising, discomfort, infections, and/or pain at needle site.

What if there is a problem?
If you remain unhappy the usual NHS complaints mechanisms will still be available to you and your relative. This can be accessed thought the Citizens Advice bureau, or the Boards complaints office on 0141 201 4500 (Email:complaints@ggc.scot.nhs.uk)

In the unlikely event that something goes wrong and your relative is harmed during the research and this is due to someone's negligence then you may have grounds for a legal action for compensation against NHS Greater Glasgow and Clyde but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you.

What happens when the study is finished?
The data and samples will be stored for two years following the data publication of the study.

Will taking part in the study be kept confidential?
All the information we collect during the course of the research will be kept confidential and there are strict laws which safeguard the privacy of the patient at every stage. Study researchers will need access to your relative's [medical records/data] to carry out this research. Your relative will be assigned a study number which will be used to label data and samples. Although data and samples will be sent to the University of Glasgow for analysis, no identifiable personal information will leave the hospital. Any published report of the data would not identify your relative.

With your consent we will inform your relative's GP that they are taking part. To ensure that the study is being run correctly, we will ask your consent for responsible representatives from the Sponsor (Dr. Maureen Travers, Research and Development) to access your relative's medical records and data collected during the study, where it is relevant to them taking part in this research. The Sponsor is responsible for overall management of the study and providing insurance and indemnity. As above no identifiable data will leave the hospital.

What will happen to the results of the study?
The study will be written up in a peer reviewed scientific journal and presented at a scientific conference. Your relative will not be identifiable in any published results.

If your relative is interested a summary of the results of the study can be obtained by contacting Dr. Malcolm Sim on 0141 452 3033 or E-mail malcolm.sim@ggc.scot.nhs.uk.

Who is organising the research and why?
The study is sponsored by the NHS Greater Glasgow and Clyde and funded by a grant from the Chief Scientist Office, Scotland.

Who has reviewed the study?
The study proposal has been reviewed by several senior Intensive Care clinicians. All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee. A favourable ethical opinion has been obtained from Scotland A REC. NHS management approval has also been obtained.

If you have any further questions about the study please contact:
Dr. Malcolm Sim on 0141 452 3033 or E-mail malcolm.sim@ggc.scot.nhs.uk
(Consultant in Anaesthesia and Intensive Care Medicine)

If you would like to discuss this study with someone independent of the study please contact:
Dr Sandy Binning (Consultant in Anaesthesia and Intensive Care medicine) on 0141 4523033. E-mail: Sandy.Binning@ggc.scot.nhs.uk

If you wish to make a complaint about the study please contact:
NHS Greater Glasgow and Clyde Complaints team on:
0141 201 4500. Email: complaints@ggc.scot.nhs.uk)

Thank you for taking the time to read this information sheet

Nearest Relative/Guardian or Welfare Attorney
Consent Form

"The use of Metabolomics for the Early Recognition of Sepsis"

Participant ID:

[Contact details of person taking consent]

Please initial box

1. I confirm that I have read and understand the information sheet (as specified in this document header) for the above study and have had the opportunity to consider the information and ask questions. ☐

2. I understand that my relative's participation is voluntary and that I am free to withdraw my relative at any time, without giving any reason and without my relative's medical care or legal rights being affected. ☐

3. I understand that relevant sections of my relative's medical notes and data collected during the study may be looked at by individuals from the Sponsor [Dr. Maureen Travers], from Research and Development, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I agree to my relative's data and blood being stored and used for future ethically approved studies and I agree to my relative's anonymised data and blood being stored and used in future studies ☐

5. I agree to my relative's General Practitioner being informed of their participation in this study ☐

6. Having considered my relatives likely wishes are regarding medical research I agree to my relative taking part in the above study ☐

I confirm that I am the nearest relative for _____
and that no other nearest relative or welfare attorney or guardian exists.

Relationship to patient _____

I confirm that I am the Welfare Attorney or Guardian for_____

Name of person giving consent	Date	Signature
_____	_____	

Name of person taking consent (if different from Researcher)	Date	Signature
_____	_____	

1x original – into Site File; 1x copy – to Participant; 1x copy – into medical notes

11.7 *Appendix G*: Healthy Volunteer Consent Form

NHS
Greater Glasgow and Clyde

HEALTHY VOLUNTEER CONSENT FORM

Study Title: The use of Metabolomics for the Early Recognition of Sepsis

R+D Reference GN13MI010
IRAS Reference 122091

Principal investigator at QEUH site

Dr Malcolm Sim

Principal investigator at GRI site

Dr Kathryn Puxty

Principal investigator at RAH site

Professor Kevin Rooney

Please initial each box

1. I confirm that I have read and understand the information sheet version 1.0 dated 19th May 2020, for the above study and have had the chance to ask questions. ☐
2. I understand that my taking part is voluntary and that I am free to stop at anytime, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the Sponsor [Dr. Maureen Travers], from Research and Development, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐
4. I agree to my data/tissue being used for future ethically approved studies ☐
5. I agree to my General Practitioner being informed of my participation in this agree to take part in the above study. I understand that I will get a copy of this signed and dated consent form. ☐
6. I agree to take part in the above study ☐

_____	_____	_____
Name of Patient	**Date**	**Signature**
_____	_____	_____
Investigator	**Date**	**Signature**

Version 1.0 19/05/20

11.8 *Appendix H*: Patient Consent Form

NHS
Greater Glasgow and Clyde

PATIENT CONSENT FORM

Study Title: The use of Metabolomics for the Early Recognition of Sepsis

R+D Reference GN13MI010
IRAS Reference 122091

Principal investigator at QEUH site

Dr Malcolm Sim

Principal investigator at GRI site

Dr Kathryn Puxty

Principal investigator at RAH site

Professor Kevin Rooney

Please initial each box

1. I confirm that I have read and understand the information sheet version 3.0 dated 19th May 2020, for the above study and have had the chance to ask questions. ☐
2. I understand that my taking part is voluntary and that I am free to stop at anytime, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the Sponsor [Dr. Maureen Travers], from Research and Development, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐
4. I agree to my data/tissue being used for future ethically approved studies ☐
5. I agree to my General Practitioner being informed of my participation in this agree to take part in the above study. I understand that I will get a copy of this signed and dated consent form. ☐
6. I agree to take part in the above study ☐

_____	_____	_____
Name of Patient	**Date**	**Signature**
_____	_____	_____
Investigator	**Date**	**Signature**

Version 2.0 19/05/20

11.9 *Appendix I*: Patient Data Check Using Random Number Generator

Patient ID	Comment
P021	No discrepancies
R004	No discrepancies
P037	No discrepancies
G001	No discrepancies
P015	No discrepancies
P019	BMI incorrectly recorded as 38. Actual value is 47.8
P007	No discrepancies
P077	No discrepancies
P082	No discrepancies
P036	No discrepancies
P017	No discrepancies

11.10 *Appendix J*: Uncertain Culture Results Discussed With Clinical Microbiologist

Patient	Positive Growth	Discussion	Decision
P027	*Moraxella catarrhalis* in sputum, *S. epidermidis* in one blood culture.	Severe COVID-19 with critical hypoxia requiring proning. Minimal secretions. No raised WCC. Apyrexial. CXR in keeping with severe COVID. Treated with Co-amoxiclav but stopped after 9 days as no improvement in clinical condition	Colonisation, not secondary infection.
P032	*E. coli*, *S. aureus* and *Candida* in sputum.	Patient profoundly hypoxic and prone dependent. No change in clinical condition when these organisms were cultured. CXR showed bilateral changes in keeping with severe COVID-19. Minimal inflammatory response. Copious secretions throughout admission. Apyrexial.	Colonisation, not secondary infection.
P035	*Klebsiella oxytoca*, *Enterobacter gergoviae* and *Candida* in sputum	Organisms cultured at presentation, but patient died before result was available. Presented with mildly productive cough. CXR appearances consistent with severe COVID-19. Apyrexial, no raised WCC and minimal inflammatory response.	Colonisation, not secondary infection.
P043	*Morganella morganii*, *Haemophilus influenzae* and *Candida dubliniensis* from tracheal aspirate	This patient was making good clinical progress when these organisms were cultured. CXR consistently showed bilateral opacifications in keeping with severe COVID-19. Raised WCC, some mucopurulent secretions. Apyrexial.	Colonisation, not secondary infection.
P053	*Candida*, *S. aureus*, *K. pneumoniae*, and *S. pneumoniae* in sputum.	Borderline raised WCC. Apyrexial. Bilateral opacities on CXR. Minimal secretions.	Colonisation, not secondary infection.
P064	*S. capitus* in one blood culture, *E. coli* and *H. influenzae* in sputum.	Apyrexial. Moderate mucopurulent secretions. Stable clinical condition, no associated deterioration. Raised WCC but other inflammatory markers low.	Colonisation, not secondary infection.
P068	*E. coli* in urine, *Klebsiella pneumoniae* in sputum	After culture developed cardiovascular instability and pyrexia. Small amounts of purulent secretions. CRP spiked but WCC remained within the normal range	Chest and urine secondary infection
P085	*Candida* in sputum and urine	Initially *Candida* in sputum. Apyrexial, no significant secretions. Normal WCC but raised CRP and PCT. Extensive and progressive CXR changes. Subsequent growth of *Candida* in urine associated with clinical deterioration and climbing WCC.	Secondary infection - Invasive candidiasis.

11.11 *Appendix K*: Full List of Positive Microbiological Cultures

Blood Cultures	n
Staphylococcus epidermidis	22
Staphylococcus hominis	12
Propionibacterium acnes	6
Staphylococcus aureus	6
Staphylococcus capitus	5
Staphylococcus haemolyticus	2
Candida	1
Corynebacterium striatum	1
Diphtheroid bacilli	1
Enterococcus faecalis	1
Escherichia coli	1
Granulicatella adiacens	1
Kocuria varians	1
Pediococcus acidilactici	1
Staphylococcus saccharolyticus	1

Respiratory Cultures	n
Candida	50
Staphylococcus aureus	20
Haemophilus influenzae	9
Escherichia coli	8
Klebsiella pneumoniae	6
Proteus mirabilis	5
Aspergillus fumigatus	4
Streptococcus pneumoniae	4
Klebsiella aerogenes	3
Pseudomonas aeruginosa	3
Corynebacterium striatum	2
Enterobacter cloacae	2
Klebsiella oxytoca	2
Morganella morganii	2
Raoultella ornithinolytica	2
Acinetobacter pittii	1
Enterobacter gergoviae	1
Streptococcus agalactiae	1
Moraxella catarrhalis	1
Raoultella planticola	1
Serratia liquefaciens	1
Serratia marcescens	1
Stenotrophomonas maltophilia	1

Urine Cultures	n
E. coli	12
Proteus mirabilis	3
Heavy mixed growth	2
Candida	1
Enterobacter Cloacae	1
Enterococcus	1

GI cultures	N
Campylobacter jejuni	1

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