DETERMINANTS OF SEVERITY IN PATIENTS WITH PANDEMIC INFLUENZA

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William Jake Dunning

Centre for Respiratory Infection

National Heart and Lung Institute

Imperial College London

Abstract

Novel strains of influenza are a major threat to security, health and wellbeing, but their timing and impact on global health cannot be predicted. An influenza pandemic was declared in June 2009, caused by the novel influenza A(H1N1)pdm09 virus. Although illness was generally mild, the pandemic is thought to have caused more than 250,000 deaths worldwide.

To learn more about the causes of severe influenza, the Mechanisms of Severe Acute Influenza Consortium (MOSAIC) was formed to conduct an integrated study of host, pathogen and co-pathogen factors that may affect disease severity. Two hundred and fifty-five patients with influenza-like illness (ILI), including 172 (67%) with confirmed influenza, were recruited during the second pandemic wave (winter 2009/10) and the third, post-pandemic wave (winter 2010/11). Sequential samples were obtained from the respiratory tract and blood throughout illness and recovery. Detailed clinical data were also collected to facilitate the interpretation of laboratory findings. Patients with ILI caused by other viral and bacterial pathogens were also recruited, along with asymptomatic healthy controls.

Translation of the Consortium's aims into an achievable clinical study was a significant undertaking in this body of work. The development of clinical protocols, the standardised sampling of patients and the collection of clinical and microbiological data were all essential tasks. Despite these challenges and a limited window of opportunity for recruitment, a large biobank and a database of detailed clinical data have been established.

To address the contribution of differences in gene expression in pathogenesis, whole blood transcriptomics analysis was performed. Two major transcriptional profiles were seen in influenza patients at the first time point: a marked up-regulation of interferon-associated genes in those with illness that tended to be milder and of shorter duration, and up-regulation of neutrophil-associated and bacterial-response genes in those with more established, often critical illness. To supplement and enrich the gene expression findings, we measured cytokines and chemokines in serum/plasma and respiratory tract secretions. Exuberant immune mediator responses were observed in many patients, consistent with cytokine storm, but subgroups appear to have different response patterns.

Findings are expected to help reveal the contribution of bacterial infections in severe influenza, in addition to demonstrating an association between severity of illness and changes in the immune response. The results have potential applications in the development of improved diagnostics and therapeutics, as well as broadening our understanding of influenza pathogenesis in humans.

Dedicated to the memory of my father, William Arthur Dunning

Declaration

This document consists of research work conducted at the Centre for Respiratory Infection, National Heart and Lung Institute, Imperial College London, between 2009 and 2014. I declare that the work presented is my own, except where specifically acknowledged in the text.

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Abbreviations and Notations

°C	Degrees centigrade
A(H1N1)pdm09	Influenza A (H1N1) pandemic 2009
A(H3N2)	Influenza A (H3N2) virus
A(H3N2)v	Variant influenza A (H3N2) virus
A(H5N1)	Influenza A(H5N1) virus
A(H7N9)	Influenza A(H7N9) virus
AEC	Airway epithelial cell
ALT	Alanine transaminase
AMU	Acute medical unit
APC	Antigen presenting cell
ARDS	Acute respiratory distress syndrome
ASC	Apoptosis-associated speck-like protein containing a caspase recruitment domain
B cell	B lymphocyte
BAL	Bronchoalveolar lavage
BIIR	Baylor Institute for Immunology Research
BMI	Body mass index
BPI	Bacterial permeability increasing protein
C3	Complement component 3
C5	Complement component 5
CAMP	Cathelicidin antimicrobial protein
cBRC	Comprehensive Biomedical Research Unit
CD	Cluster of differentiation
CD200	CD200 ligand
CD20OR	CD200 receptor
CD4+	Cluster of differentiation 4-positive cell
CD8+	Cluster of differentiation 8-positive cell
CDC	Centers for Disease Control and Prevention
CFR	Case Fatality Ratio
CLRN	Comprehensive Local Research Network
CMI	Cell-mediated immunity
COPD	Chronic obstructive pulmonary disease

CPAP	Continuous positive airway pressure
CPE	Cytopathic effect
CRF	Case Report Form
CRI	Centre for Respiratory Infection
CRP	C-reactive protein
CSF	Cerebrospinal fluid
Ct	Cycle threshold
CTL	Cytotoxic T lymphocyte
CV	Coefficient of variation
CXCR	CXC chemokine receptor
DAD	Diffuse alveolar damage
DC	Dendritic cell
DEG	Differentially expressed gene
DH	Department of Health
DNA	Deoxyribonucleic acid
DOB	Date of birth
dsRNA	Double-stranded RNA
ECMO	Extracoporeal membrane oxygenation
ED	Emergency department
EDTA	Ethylenediaminetetraacetic acid
eIF2a	Eukaryotic translation factor 2a
ELISA	Enzyme-linked immunosorbent assay
ENA-78	Epithelial-derived neutrophil-activating peptide 78 (C-X-C motif chemokine 5)
ER	Endoplasmic reticulum
ETA	Endotracheal aspirate
ETV7	Ets variant 7
FC	Fold-change
FCGR1A	High affinity immunoglobulin gamma Fc receptor I
FCGR1B	Fc fragment of IgG, high affinity Ib, receptor
FDR	False detection rate
FFP3	Filtering face piece 3
Flu-CIN	Influenza Clinical Information Network
Flu-neg	Influenza-negative
Flu-pos	Influenza-positive

FWER	Family-wise error rate
g	Gravitational acceleration
GADD45	Growth Arrest and DNA Damage
GBP1	Guanylate binding protein 1
GBS	Guillain-Barré syndrome
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GM-LLOD	Geometric mean of the lower limit of detection
GM-ULOD	Geometric mean of the upper limit of detection
GO	Gene Ontology
GP	General Practitioner
GTP	Guanosine-5'-triphosphate
H. influenzae	*Haemophilus influenzae*
HA	Haemagglutinin
HAI	Haemagglutination inhibition
HC	Healthy control
HDU	High dependency unit
HIV	Human immunodeficiency virus
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA
HPA	Health Protection Agency
HPAI	Highly pathogenic avian influenza
HRV	Human rhinovirus
HuMPV	Human metapneumovirus
iCOS	Inducible T-cell COStimulator
ICU	Intensive Care Unit
ID	Identification
IFI27	Interferon, alpha-inducible protein 27
IFI6	Interferon, alpha-inducible protein 6
IFIT	Interferon-induced proteins with tetratricopeptide repeats
IFITM	Interferon-induced transmembrane protein
IFN	Interferon
IFN-α2a	Interferon alpha-2a
IFN-β	Interferon beta
IFN-γ	Interferon gamma
IFN-λ (IL-29)	Interferon lambda (interleukin 29)

Ig	Immunoglobulin
IL-10	Interleukin 10
IL-12p70	Interleukin 12-p70
IL-13	Interleukin 13
IL-15	Interleukin 15
IL-17	Interleukin 17
IL-1β	Interleukin 1-beta
IL-2	Interleukin 2
IL-23	Interleukin 23
IL-27	Interleukin 27
IL-32	Interleukin 32
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-8 (CXCL-8)	Interleukin 8 (C-X-C motif chemokine 8)
ILI	Influenza-like illness
IP-10 (CXCL-10)	Interferon-gamma-inducible protein-10 (C-X-C motif chemokine 10)
IPA	Ingenuity Pathway Analysis
IPS-1	Interferon-beta promoter stimulator 1 (MAVS; VISA)
IRF	Interferon regulatory factor
ISARIC	International Severe Acute Respiratory and emerging Infection Consortium
ISG	Interferon-stimulated gene
I-TAC (CXCL11)	Interferon-inducible T cell alpha chemoattractant (C-X-C motif chemokine 10)
IU	International units
JAK	Janus kinase
K. pneumoniae	*Klebsiella pneumoniae*
Kg	Kilogram
KPa	Kilopascal
L	Litre
LAIV	Live attenuated influenza vaccine
LAP3	Leucine aminopeptidase 3
LLOD	Lower limit of detection
LPAI	Low pathogenicity avian influenza
M	Influenza virus matrix protein

M.pneumoniae	*Mycoplasma pneumoniae*
MAPK	Mitogen-activated protein kinase
MBL	Mannose-binding lectin
MCP	Monocyte chemoattractant protein
MCP-1 (CCL2)	Monocyte chemoattractant protein 1 (Chemokine (C-C motif) ligand 2)
MCP-4 (CCL13)	Monocyte chemoattractant protein 4 (Chemokine (C-C motif) ligand 13)
MDC (CCL22)	Macrophage-derived chemokine (Chemokine (C-C motif) ligand 22)
mg	Milligram
MHC	Major histocompatibility complex
MIG (CXCL9)	Monokine induced by gamma interferon (C-X-C motif chemokine 10)
Min	Minute
MIP	Macrophage inflammatory protein
MIP-1α (CCL3)	Macrophage inflammatory protein-1-alpha (Chemokine (C-C motif) ligand 3)
MIP-1β (CCL4)	Macrophage inflammatory protein (Chemokine (C-C motif) ligand 4)
mL	Millilitre
MMP	Matrix metalloproteinase
MN	Microneutralisation
MOSAIC	Mechanisms of Severe Acute Influenza Consortium
mRNA	Messenger RNA
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSD	Mesoscale Discovery
MUC5AC	Mucin 5AC glycoprotein
Mx1	Myxovirus (influenza virus) resistance 1 interferon-Inducible protein
MxA	Myxovirus (influenza virus) resistance A interferon-Inducible protein
N/A	Not applicable
NA	Neuraminidase
NAI	Neuraminidase inhibitor
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHS	National Health Service
NIHR	National Institute of Health Research
NIMR	National Institute for Medical Research
NIV	Non-invasive ventilation
NK	Natural killer
NLRP3	NOD-like receptor P3

nmol	Nanomole
NOD	Nucleotide-binding oligomerization domain
NP	Nucleocapsid protein
NPA	Nasopharyngeal aspirate
NPFS	National Pandemic Flu Service
NS	Not significant
NS2	Influenza virus non-structural protein 2
OAS	Oligoadenylate synthetase
PA	Influenza virus polymerase subunit PA
PB1	Influenza virus polymerase subunit PB1
PB2	Influenza virus polymerase subunit PB2
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
PCT	Procalcitonin
pDC	Plasmacytoid dendritic cell
PFU	Plaque-forming unit
pg	Picogram
PHC	Paediatric healthy control
PHE	Public Health England
PKR	Protein kinase R
PMC	Polymorphonuclear cell
PREPARE	Platform for European Preparedness Against (Re-)emerging Epidemics
PRR	Pattern recognition receptor
QC	Quality control
RANTES (CCL5)	Regulated on activation, normal T cell expressed and secreted (Chemokine (C-C motif) ligand 4)
RIG-I	Retinoic acid inducible gene I
RIN	RNA integrity number
RNA	Ribonucleic acid
RNase	Ribonulcease
RSAD2	Radical S-adenosyl methionine domain containing 2
RSV	Respiratory syncytial virus
RT-PCR	Reverse transcriptase polymerase chain reaction

S. aureus	*Staphylococcus aureus*
S. pneumoniae	*Streptococcus pneumoniae* (Pneumococcus)
SAM	Synthetic absorptive matrix
SARS	Severe acute respiratory syndrome
SEPT4	Septin 4
SIGLEC1	Sialic acid binding Ig-like lectin 1
SNP	Single nucleotide polymorphism
SOCS1	Suppressor of cytokine signalling 1
SOP	Standard operating procedure
ssRNA	Single-stranded RNA
STAT	Signal transducer and activator of transcription
sTNFR	Soluble TNF receptor
T cell	T lymphocyte
T0	Time-point 0
T1	Time-point 1
T2	Time-point 2
T3	Time-point 3
TARC (CCL17)	Thymus and activation regulated chemokine (Chemokine (C-C motif) ligand 17)
Th	T helper cell
Th1	T helper 1 cell
Th17	T helper 17 cell
Th2	T helper 2 cell
TIV	Trivalent inactivated vaccine
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNFAIP1	Tumour necrosis factor, alpha-induced protein 1
TNF-α2a	Tumour necrosis factor-alpha-2a
TRIM25	RIG-I-tripartite motif-containing protein 25
ULOD	Upper limit of detection
UTM	Universal transport medium
Vit D	Vitamin D
WHO	World Health Organization
WP	Work Package
µL	Microlitre

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Chapter One

1 Introduction

1.1 The Influenza virus

1.1.1 Historical aspects

J. Hugger first described the term influenza in the English language in his 1703 thesis *De Catarrho epidemio, vel Influenza, prout in India occidentali sese ostendit*. The precise etymology remains unclear, but influenza may be derived from the Medieval Latin *influentia*, meaning influence or visitation. This was thought to be with reference to an unfavourable "influence from the stars", but was later extended to include 'influence from the cold' (1). At the time of the most well known influenza pandemic, the 1918-1919 Spanish influenza pandemic, it was commonly believed that influenza illness was caused by a bacterium. Richard Pfeiffer had isolated bacteria (Pfeiffer's bacillus or *Bacillus influenzae*) from influenza patients during the earlier respiratory illness pandemic of 1892. The same bacillus was isolated from a large proportion of subjects studied during the 1918-19 pandemic, leading many to make the erroneous conclusion that this particular bacterial species was the cause of influenza illness, rather than being a potential cause of associated bacterial complications (2).

Swine influenza virus was successfully isolated from pigs by Richard Shope in 1931 (3). Subsequent animal inoculation experiments using secretions from humans during the 1932-33 influenza epidemic identified an invisible but filterable infectious agent that caused comparable disease in ferrets. The development of a serological assay using ferret-derived antibodies, with demonstration of neutralising antibodies in sick and recovered human patients, supported these findings. Although published 15 years after the outbreak of the 1918-19 pandemic, the work conducted by Wilson Smith, Christopher Andrews and Patrick Laidlaw at the National Institute for Medical Research, lead to a paradigm shift in our understanding of the infectious origin of classical influenza illness (4).

1.1.2 Classification of influenza viruses

Influenza viruses belong to the family Orthomyxoviridae and are divided into three main groups based on immunological and biological properties: influenza A, influenza B and influenza C viruses. Influenza A viruses are capable of infecting and causing disease in a wide range mammalian species; but avian species, especially aquatic birds, are the natural reservoirs (5). Although guinea pig models of influenza B virus transmission have been developed (6), naturally-occurring influenza B infection

has only been described in humans and seals (7) and influenza C viruses are known only to infect humans, pigs and dogs (8).

Influenza A viruses are characterised into sub-types based on two transmembrane proteins, haemagglutinin (HA) and neuraminidase (NA). To date, 18 individual HA antigens (H1-H18) and 11 different NA antigens (N1-11) have been identified. The combination of the HA number and NA number give rise to the influenza subtype e.g. H1N1, but influenza A viruses can be further broken down into different strains, based on other factors including year of isolation and strain number. This is important since viruses of the same subtype may emerge at different points in time and display different biological characteristics. For example, A/duck/Alberta/35/76 (H1N1), a virus isolated from ducks alone, has different implications for human health compared to A/California/07/2009 (H1N1), one of the first strains of virus to be detected in humans during the A(H1N1)pdm09 influenza pandemic. Similarly, although the early A(H1N1)pdm09 virus strains share antigenic similarities with 1918 pandemic A(H1N1) virus strains e.g. A/South Carolina/1/18 (H1N1), there are significant genetic differences between the two, hence the need to differentiate H1N1 viruses (9, 10).

Influenza B viruses are not divided into subtypes based on HA and NA, but are further classified by strains. Influenza B viruses are relatively homogenous, although two major lineages evolved in the 1970s, characterised by differences in major antigens: the Victoria lineage and the Yamagata lineage (11).

1.1.3 Influenza virus structure

A typical influenza virion has spheroidal form and possesses three major components: an envelope, a lipid bilayer with underlying matrix layer and a ribonucleoprotein core. All influenza viruses are segmented, negative-strand RNA viruses. Influenza A and B both contain 8 RNA segments, whereas influenza C contains 7 RNA segments. The trimeric HA and tetrameric NA are on the outside of the envelope, projecting as antigenic ‘spikes’, along with the tetrameric M2 proton-selective ion channel; the M1 matrix protein is found underneath the envelope membrane. HA and NA are linked to cholesterol-rich lipid rafts within the lipid bilayer. HA, the major envelope protein is important for host receptor cell binding and is also recognised by neutralising antibodies. By contrast, NA removes the major cell-surface receptor, sialic acid, facilitating penetration of host mucin produced by the respiratory epithelium, the release of viral progeny and also host-to-host transmission. NA also removes sialic acid links between HA and NA on virions, preventing aggregation of newly formed particles as they are released from an infected cell (12, 13).

The M2 ion channel is much less abundant than HA and NA, but is important in disassembly processes and virus budding. The M1 lipid-binding protein in the matrix layer is involved in many stages of virus replication. The core of the virus is composed of helical ribonucleoproteins (RNPs) containing viral genomic RNA segments, along with nucleoprotein, nuclear export proteins, and polymerase complex (PA, PB1 and PB2) (14).

1.1.4 Influenza virus life cycle

Most influenza viruses initially infect the respiratory mucosa, although virus may enter through other sites, including mucous membranes and conjunctiva, depending on the particular subtype and strain (15). The influenza virus attaches to host cells by binding of HA (ligand) to sialic acid (receptor). The type of sialic acid expressed varies according to cell type and location and the HA affinity for particular sialic acid receptors also varies according to virus strain, with multiple implications for infectivity, transmission and pathogenesis. Human influenza viruses typically bind preferentially to α2,6-sialic acid-bearing receptors that are abundant in the upper and lower respiratory tracts. By contrast, avian influenza viruses typically bind to α2,3-sialic acid-bearing receptors, which in humans are present in the lower respiratory tract and conjunctiva (16). Once bound, the virus enters the cell via receptor-mediated endocytosis. Following HA-mediated membrane fusion, disassembly occurs; RNP is released and is transported into the nucleus, with subsequent transcription and replication of RNP. Messenger RNAs are exported to the cytoplasm where they undergo translation. Early viral proteins, required for replication and transcription, are transported into the nucleus. Later in the cellular infection cycle, M1 and NS2 proteins in the cytoplasm facilitate the exportation of RNPs from the nucleus (17) (figure 1.1).

New virus particles are then formed through the process of budding (involving bud initiation, bud elongation and bud release) and are eventually released from the apical plasma membrane of polarized epithelial cells, a process similar to cellular vesiculation. Essentially, newly formed viral components including RNP are packaged within the cell and undergo the reverse process to cell entry, with new buds projecting from the infected cell membrane and eventually being released by fission, ready to infect other cells (14).

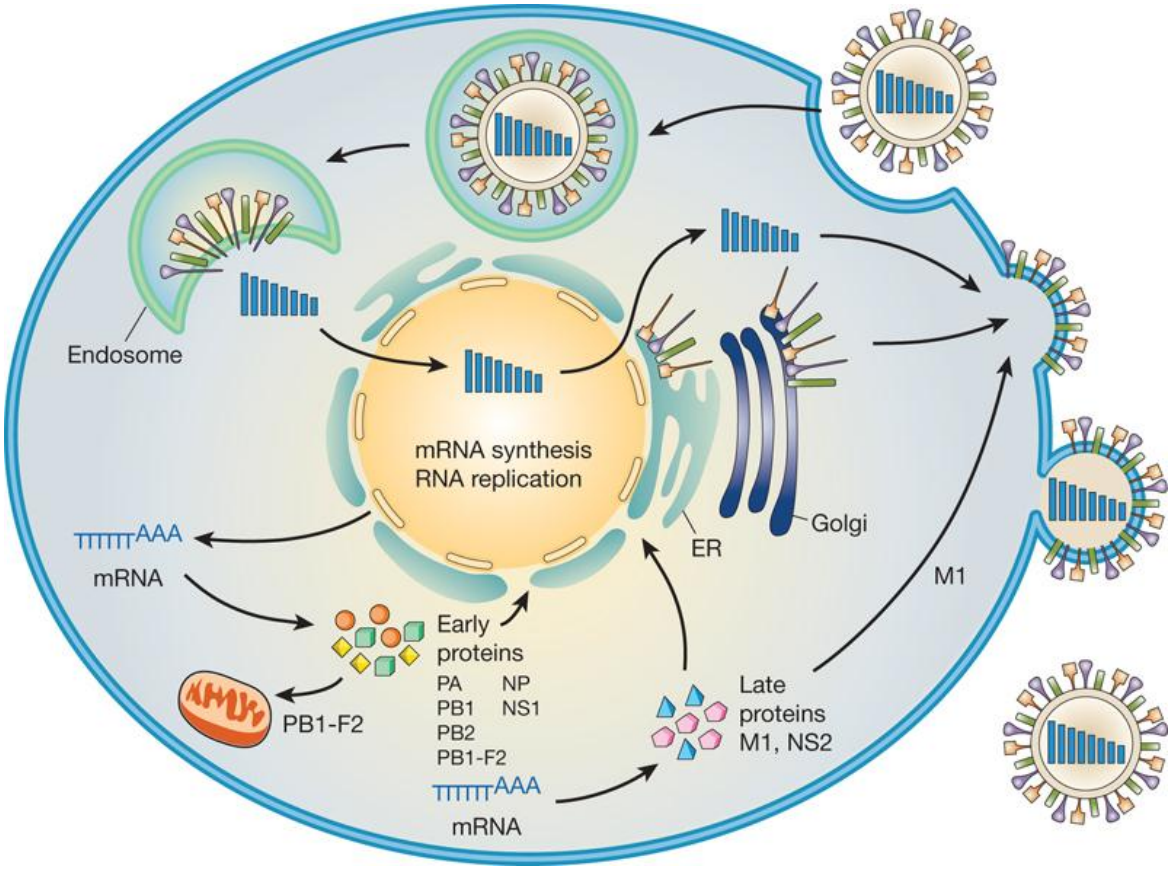


Figure 1.1 Schematic diagram of the influenza virus life cycle
After binding to sialic-acid bearing receptors, virus enters the host cell by receptor-mediated endocytosis (top right) and the virion undergoes disassembly in the cytoplasm. Viral ribonucloprotein complexes enter the cell nucleus, facilitating the synthesis of mRNA and RNA replication. Synthesised viral components are packaged and, following assembly and budding at the plasma membrane, fission from the host cell occurs and newly formed progeny virions are released. Reprinted by permission from Macmillan Publishers Ltd. G Neumann, T Noda, Y Kawaoka. Emergence and pandemic potential of swine-origin H1N1 influenza. Nature. 459 (7249): 931-939. Copyright 2009.

1.1.5 Influenza virulence factors and important viral proteins

The genome of influenza A virus contains three large RNA segments that encode polymerase proteins: PB1, PB2 and PA. The PB1 RNA segment also encodes PB1-F2 and PB1-N40 proteins and PA segment also encodes PA-X. Other RNA segments encode for HA, NA and nucleocapsid protein (NP). The two remaining segments encode M1 and M2, and NS1, NS2/NEP, respectively (18).

The influenza B genome also contains 8 segments, most of which encode the same proteins as the segments of influenza A. Unlike influenza A, the second polymerase segment of influenza B only encodes PB1 and the third segment only encodes PA, not PA and PA-X. The sixth segment encodes NB, a dispensable accessory protein, in addition to NA. Segment 7 encodes M1 and BM2; the latter protein is analogous to the M2 ion channel protein of influenza A (18).

1.1.5.1 Influenza non-structural protein 1 (NS1)

Several viral proteins are important in enabling the virus to counteract host immune responses. NS1 interacts with host cytoplasmic and nuclear factors to attenuate host interferon induction and mitigate the effects of interferon, thus modulating host antiviral responses and promoting viral replication. For example, inhibition of the host retinoic acid inducible gene I (RIG-I) pathway, an important cytoplasmic viral RNA sensing mechanism, leads to decreased interferon induction (19). This is achieved by NS1 inhibiting RIG-I-tripartite motif-containing protein 25 (TRIM25)-mediated activation of the interferon regulatory factor 3 (IRF3) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) transcription factors (20, 21). Additionally, by inhibiting the 3' end processing of interferon pre-mRNAs, host gene expression can be inhibited (22). NS1 also inhibits the synthesis and activity of the double-stranded RNA-dependent protein kinase R (PKR) (23) and 2'-5'-oligoadenylate synthetase (OAS) (24) and Mx proteins (25), three important IFN-inducible proteins involved in the host antiviral response.

Removal of the NS1 gene impedes viral replication (in the presence of a normal host IFN response)(26) and is associated with a marked pro-inflammatory immune mediator response and effective T-cell induction following infection of dendritic cells (27). Some interactions between host and virus NS1 appear to be strain-specific. For example, the NS1 of the 1918 H1N1 strain is a known virulence factor in non-human primate infection models, supported by associated changes in gene expression (down-regulation of antiviral response and lipid metabolism genes) (28). This contrasts with the NS1 from a contemporary seasonal H1N1 virus, which does not cause such marked down-regulation (29). NS1 has also been shown to have anti-apoptotic (30) as well as pro-apoptotic (31)

properties, which may occur at different, successive stages during infection. Reduced apoptosis occurring early in infection could promote viral replication, whereas enhanced apoptosis in later stages could promote release of viral progeny and clearance of defunct cells that lack the capability to support sustained replication.

1.1.5.2 Influenza polymerase complex

The influenza polymerase complex (PB1, PB2 and PA) assembles with NP and genomic RNA to form the RNP complex, essential for genome replication and gene expression. Specific nucleotide substitutions in genes encoding PB2, for example those arising from amino acid substitutions at position 627, facilitate adaptation of avian viruses in mammals. Such adaptation is often associated with enhanced polymerase activity and replication rates, leading to enhanced pathogenicity (32). Several functions of PB1-F2 have been proposed, although not all of them can be demonstrated to be relevant to pathogenesis in vivo (33). Perhaps the most important function, based on findings of in vivo models, is the ability of PB1-F2 from certain influenza strains to augment immune responses to influenza and/or co-infecting bacteria, resulting in demonstrable leukocyte recruitment and elevated levels of pro-inflammatory immune mediators. This may be mediated by an anti-interferon effect, but the precise underlying mechanism remains unclear (34, 35).

Similar to NS-1, deletion of PB1-F2 in vitro impacts on virus growth and virulence. Beneficial and detrimental nucleotide substitutions in viral polymerase genes may occur following cycles of transmission (36). The reassortant influenza A(H1N1)pdm09 virus contained a polymerase gene segment that was derived from a well-adapted seasonal H3N2 strain. This PB1-F2 was truncated and non-functional (37). This is in contrast to the highly virulent H5N1 avian influenza viruses which continue to express an active and inflammatory PB1-F2 protein (36). The 1918 H1N1 virus, which also expressed an active PB1-F2, was unusual in that the virus was thought to have “jumped” from birds to humans without established adaptation in mammals. It has been proposed that non-truncated PB1-F2 may contribute to human disease through its ability to enhance secondary bacterial infection, rather than modifying viral infection in the host (35). This may support the observation that the 1918-19 pandemic was associated with an excess of bacterial complications of influenza (38), although bacterial complications are infrequent in humans infected with highly virulent H5N1 influenza viruses which also express an active PB1-F2 (39).

1.1.6 Influenza virus evolution

Although aquatic birds are the natural reservoir of influenza A viruses, it is likely that influenza viruses have been infecting humans for many centuries. Transmission of influenza from aquatic birds to non-aquatic poultry occurs more commonly that transmission to mammals. Direct infection of

humans with highly pathogenic influenza A viruses from birds, such as infection with HPAI H5N1 influenza, is uncommon. A process of prior adaptation to mammalian species is more typical, often with genetic reassortment events occurring prior to emergence in humans (40).

Major genetic evolution may occur by reassortment of gene segments, or by the fixation of genomic errors that have occurred during transcription. With reassortment, genetic 'hybrids' are created by the mixing of genes from two or more influenza viruses, which may be unrelated subtypes of virus. If a host cell is infected by two different influenza A viruses at the same time, then there are 2^8 potential novel combinations of the eight RNA segments. Reassortment in avian species is thought to occur frequently and may result in novel viruses that are short-lived and of little significance to the infected host. However, with so many reassortment events taking place in birds and also in mammals e.g. pigs, the potential remains for a virulent, transmissible virus to emerge. Indeed, the most recent influenza pandemics arose from the introduction of reassortant viruses, perhaps with the exception of the 1918 H1N1 virus, where sequencing analysis suggests that it was an avian virus that had adapted directly to humans without reassortment (41, 42), but this has been contested by others (43, 44).

An extensive phylogenetic study, utilising a host-specific local molecular clock model, analysed >80,000 full-length viral genomes from humans, birds, pigs and horses revealed that all of the viruses studied shared a common ancestor dating back to the mid-to-late nineteenth century. This appears to have evolved into an equine H7N7 subtype and also a common avian strain that gave rise to almost all of the currently circulating avian, swine and human strains (45). Although reassortment is a naturally occurring phenomenon, changes at the human-animal interface, such as changes in land use, intensive farming practices and domestic bird-rearing techniques, have the potential to enhance or promote the natural process.

Fixation of genomic nucleotide substitutions may be influenced by multiple factors. The nature of the influenza polymerase results in a high rate of nucleotide substitutions for each infectious cycle, with segments encoding HA, NA, matrix (M) and NS being most affected. Selective pressures that may influence the fixation of a particular error/nucleotide substitution include the pre-existing host immune response or use of antiviral drugs. Furthermore, if a nucleotide substitution is not associated with a fitness cost to the virus, then it is more likely to become fixed (46).

Novel influenza A pandemics are associated with antigenic shift i.e. an abrupt and major change in influenza viruses. Antigenic shift is unique to influenza A viruses and it arises from changes in entire viral gene segments, with new HA and NA combinations that differ from those seen in established

circulating viruses in humans at the time a pandemic emerges. This gives rise to a new influenza subtype. Although a similar subtype may have circulated previously, the circulation would typically have occurred many decades before the pandemic, meaning that a large proportion of the exposed population will be naïve to the virus and thus lack pre-existing immunity (47). This was seen with the 2009-10 pandemic, when those born after 1957, the time when the 1918 H1N1 virus and its H1N1 descendants were replaced by the novel pandemic H2N2 virus, were particularly susceptible to infection with the novel virus and unlike many elderly persons, lacked cross-protective anti-H1N1 antibodies (48). Seasonal (non-pandemic) H1N1 viruses were circulating globally from 1976 onwards, following the American 1976 Fort Dix outbreak and the re-introduction of seasonal H1N1 virus in the Soviet Union in 1977, but A(H1N1)pdm09 virus is antigenically and genetically distinct from these viruses and the same observation of cross-protective immunity was not seen in those 30 to 50 years of age (49). Recipients of the 1976 H1N1 vaccine in the USA appeared to have higher titres of neutralising antibody against the novel A(H1N1)pdm09 virus, but only 25% of the United States population was vaccinated in 1976 (50). Interestingly, despite a wealth of known HAs and NAs, only viruses with combinations of H1-H3 and N1-N2 have been responsible for the four most recent influenza pandemics and some have suggested that there is HA restriction in this context, with cycles of virus evolution that are influenced by age-related herd immunity (51).

Antigenic drift occurs once a virus with a novel subtype has become established in the human population. Drift represents a more subtle variation in viruses than antigenic shift and is associated with changes in sites on the HA and NA proteins that are recognised by host antibodies generated by exposure to strains of the same subtype. In the absence of a completely cross-protective host immune response to influenza, gradual accumulation of amino acid changes in the HA and NA surface glycoproteins (at virus population-level rather than exposure within a single host) facilitates viral evasion of host immune responses. This accounts for recurrent influenza infections in influenza-experienced individuals and the need to review influenza vaccine strains of the same subtype on an annual basis (52).

Positive selection-pressure in a virus with a high replication rate and genetic turnover drives antigenic drift. The selection pressure exerts greatest effect on the HA1 domain of HA, since it contains the greatest number of epitopes targeted by neutralising antibodies. However, it has been proposed that factors other than antigenic drift may be more important in seasonal evolution of influenza virus e.g. migration and reassortment among multiple, co-circulating lineages of the same influenza subtype (53). Antigenic drift is as important as antigenic shift, since the biggest impact on public health is seen with seasonal influenza outbreaks, rather than pandemic influenza outbreaks,

and antigenic shift has been associated with particularly severe outbreaks of established seasonal strains (54).

The 2009-10 influenza pandemic provided an unprecedented opportunity to study virus evolution following the emergence of a novel influenza virus. Whole-genome sequencing and phylogenetic analysis of A(H1N1)pdm09 virus genomes from UK isolates obtained during the first and second pandemic waves revealed that many co-circulating lineages could be detected, some of which were geographically unique. Furthermore, persistence of virus lineage was seen between the two waves, with two UK-specific clusters being seen in both waves. Although the UK viruses and those analysed in other affected countries did not demonstrate significant antigenic divergence from the strains identified at the beginning of the pandemic e.g. influenza A/California/07/2009 virus, they were found to be genetically distinct (55).

The characteristics of the two waves in the UK were similar in terms of clinical severity and demographics of patients affected. However, the first post-pandemic influenza season (winter 2010/11) was particularly severe in the UK (56), providing an opportunity for MOSAIC and other studies to analyse virus evolution and whether it contributes to the change in severity observed in the immediate post-pandemic period.

1.1.7 Transmission of influenza virus

In susceptible populations, influenza virus can be highly transmissible and established strains typically cause seasonal outbreaks in temperate regions. In tropical regions, year-round transmission can occur, with peaks of activity that are related to rainy seasons in some but not all locales. Evidence suggests that influenza virus is transmitted between humans through airborne large-droplet transfer, droplet-nucleus inhalation and contact transmission. Viable viruses have been detected on selected environmental surfaces for up to seven days, facilitating acquisition of virus from a contaminated environment and subsequent self-inoculation through hand contact with mucous membranes (57, 58).

Multiple factors are likely to influence the effectiveness of transmission within a population. Virus-related factors may include differences in ability to bind to different sialic acid receptors (59), the infectious dose and antigenicity. Examples of potential host-related factors include immunological status, behavioural aspects and population density. Environmental factors may also play an influential role. Humidity affects transmission and also survival of the virus in the environment, and evidence suggests that decreases in absolute humidity are more influential than decreases in relative humidity in temperate regions (60). Some have proposed that transmission during cold and dry

periods in temperate regions occurs by aerosolisation, whereas transmission during humid and rainy periods in tropical regions occurs via fomites or through direct contact (61, 62).

1.1.8 Laboratory diagnosis of influenza

1.1.8.1 Virus culture

Influenza virus infection can be confirmed using a number of laboratory techniques. Viral cell culture is considered to be the gold standard technique. Respiratory tract specimens are inoculated into cell culture monolayers (primary or continuous cell lines) and cytopathic effect (CPE) is monitored by microscopy. In the presence of CPE, the virus is subsequently identified by immunofluorescent staining using specific antibodies. Molecular methods may also be used to confirm the presence of cultured influenza (see below). Although highly sensitive and the only method that confirms the presence of virus, isolation of virus may take 3-10 days (63) and may require additional biological safety measures to protect the operator from infection. Furthermore, growth of virus may be limited by host exposure to empirical antiviral therapy and culture is less successful if the specimen contains a relatively low amount of virus. This limits the usefulness of viral culture in the clinical setting and virus culture is now frequently reserved for confirmatory testing of influenza infection by reference laboratories. Rapid cell culture using shell vials and cell mixtures can reduce the test time to less than three days (64).

1.1.8.2 Rapid viral immunodiagnostic tests

Rapid viral immunodiagnostic tests have been used frequently to diagnose influenza infection over the last twenty years, since they can establish a diagnosis in just 10 to 15 minutes. The basis of these tests is the identification of virus protein antigens in respiratory tract samples using indirect or direct antibody staining. Tests may be packaged into immunochromatographic strips, making them easy to perform and interpret. There is significant variability in sensitivity and specificity of rapid tests, however, compared to viral culture or molecular techniques such as RT-PCR. Some tests are able to detect and differentiate between both influenza A and B, but others do not. Specimen type has been shown to influence diagnostic yield for certain tests. Accuracy also depends on influenza prevalence in the community, with false-positive results being more likely when prevalence is low and false-negative results being more likely when prevalence is high. Following reports of poor diagnostic performance of such rapid tests during the 2009-10 H1N1 influenza pandemic (65), PCR-based testing become the preferred, first-line diagnostic test in many laboratories, although molecular tests are more expensive than many rapid diagnostic tests.

1.1.8.3 Molecular diagnostic tests

Molecular diagnostic methods have transformed the detection of influenza. Reverse-transcriptase polymerase chain reaction (RT-PCR) is now the most commonly used diagnostic method in the UK, available in hospital laboratories in addition to reference laboratories. Same-day results are a major benefit and some cartridge-based systems offer an ease of operation and can produce results in less than one hour. With appropriate samples, sensitivity and specificity can approach 100%. Rapid distribution of approved primer/probe sets by public health laboratories helps ensure standardisation and improves the time taken to diagnose novel strains during outbreaks. Other benefits include the ability to detect different subtypes, antiviral resistance nucleotide substitutions and the influenza assay can be included in multiplex PCR assays to detect a panel of respiratory viruses. Quantitative or semi-quantitative PCR can also be performed to provide an estimate of viral load. Although neither qualitative nor quantitative PCR testing can indicate whether detected virus is viable (since it only detects RNA), serial PCR monitoring may be useful in monitoring the course of illness and help assess the response to treatment and emergence of antiviral resistance in an individual (66).

Regardless of the technique used, it became apparent during the 2009-10 H1N1 pandemic that the location of sampling may influence the result of the diagnostic test. In patients with influenza pneumonitis, especially critically ill patients with ARDS, influenza RNA may be detectable in lower respiratory tract secretions even though RNA may not be detected in upper respiratory tract secretions (67).

1.1.8.4 Detection of specific antibodies

Detection of influenza-specific antibodies in serum obtained from patients may also be used to diagnose influenza. However, because of the natural time course of antibody response following infection, serological methods are unlikely to provide a diagnosis in early infection. Serological methods have a more useful role in epidemiological and immunological studies, including the evaluation of vaccine immunogenicity. When used for diagnosis, demonstration of a significant increase in antibody titres (typically at least four-fold) in serum obtained in the acute-phase and convalescent phase is preferable. A single high antibody titre may be suggestive of, but cannot confirm recent influenza infection, irrespective of the patient's clinical status, vaccination history or prior influenza infection history. Strain-specific antibodies directed against HA typically arise between ten and fourteen days from onset of symptoms. Haemagglutination inhibition (HAI) and microneutralisation (MN) are the two commonly used assay techniques. MN is a highly sensitive and specific assay for the detection of a broad range of neutralising antibodies. HAI is less sensitive than

MN, it only measures antibodies directed against the viral HA. Both HAI and MN can measure subtype-specific and strain-specific antibody responses (68).

1.2 Seasonal influenza

1.2.1 Seasonal influenza viruses

Seasonal influenza A viruses are the established descendants of novel pandemic viruses. Because most influenza occurs outside of the context of a pandemic, seasonal viruses contribute the greatest, cumulative burden of disease and mortality. Similar to other temperate, northern hemisphere countries, England and Wales experience winter outbreaks of influenza every year. At the peak of an outbreak, there may be 200 healthcare consultations per 100,000 population per week, lasting for around six to eight weeks. An epidemic is declared when consultation rates exceed 200 per 100,000 population. Seasonal influenza outbreaks in the UK are estimated to cause 0.5-1 death per 1,000 cases of influenza, with severe disease and death mainly occurring in older persons. As many as 12,000 excess deaths are attributed to influenza in a single season in England and Wales, with even more excess deaths occurring during severe epidemic years (69). It should be noted, however, that these estimates of excess deaths are based on all-cause mortality and may not be directly attributable to influenza virus infection.

Globally, the World Health Organization estimates that one billion cases of influenza occur each year, including 3 to 5 million cases of severe illness and 300,000 to 500,000 deaths in industrialised countries (70). The burden of seasonal influenza in resource-limited countries remains unclear, but surveillance studies are now in progress in several regions. The economic impact of seasonal influenza outbreaks may also be considerable; the total financial burden in the USA has been estimated to be as high as 87 billion US dollars during an epidemic year (71). The currently circulating seasonal virus subtypes, which can be found globally, are influenza A(H3N2), A(H1N1)pdm09 and influenza B (Victoria and Yamagata lineages).

1.2.2 Seasonal (pre-2009) H1N1 influenza A viruses

Prior to the 2009/10 pandemic, a seasonal H1N1 virus was in circulation. In 1976, a novel, swine-origin H1N1 virus was detected in several hundred recruits at an army base in the USA. A vaccination programme was launched subsequently in the US, following concerns about the virus's pandemic potential. However, spread beyond the army base was not seen (72). In 1977, cases of illness caused by this virus were initially seen in Northern China and later in the Soviet Union. This virus was remarkably similar to the H1N1 viruses that circulated just before the 1957 H2N2 pandemic. The precise origins of this virus remain unclear, however. Sequence analysis implies that the virus may

have been circulating in 1976 and supports the notion that re-circulation in humans, for the first time in twenty years, may have occurred following release of a stock of the virus from an unidentified laboratory (73). Disease burden was concentrated in those born between 1957 and 1977. This virus continued to circulate until 2009, when the novel A(H1N1)pdm09 virus emerged. The displacement of the pre-pandemic H1N1 virus has not been extensively studied. It has been proposed that a sudden and large-scale increase in immunity against conserved regions of the H1 stalk and the N1 protein may have occurred during the pandemic and may have led to this displacement (47, 74). Irrespective of the mechanism, removal of the pre-pandemic H1N1 virus from circulation is welcome, since almost complete resistance to oseltamivir resistance in this virus emerged worldwide between 2007 and 2008 (75).

1.2.3 Seasonal H3N2 influenza A viruses

A novel H3N2 influenza A virus was the cause of the 1968-69 pandemic. The impact of this pandemic was limited in terms of mortality, perhaps because of pre-existing antibodies to the N2 neuraminidase of the previously circulating H2N2 virus. Like the H2N2 pandemic virus, the H3N2 pandemic virus was a reassortant, incorporating RNA segments from the 1918 pandemic H1N1 virus with other segments being introduced from avian viruses. As a seasonal influenza A virus, H3N2 has had greater impact than seasonal H1N1 viruses circulating before 2009. Severe epidemics of H3N2 have been seen and there is a greater risk of disease burden in years when there is vaccine-virus mismatch. The 1999-2000 influenza season, dominated by seasonal A(H3N2), is believed to have caused an estimated 21,497 excess deaths in England and Wales (76). In addition to seasonal H3N2, 13 human infections with the influenza A(H3N2) variant (H3N2v) virus were identified in the United States between 2011 and 2012. This reassortant virus contains the matrix (M) gene from the A(H1N1)pdm09 virus. Many of the individuals affected had histories of exposure to pigs, but two clusters of suspected human-to-human transmission were also identified. However, there is no evidence of sustained or widespread human-to-human transmission in the USA or elsewhere (77).

1.2.4 Influenza B viruses

Influenza B viruses are believed to have been in endemic in humans for centuries. In some years, they have contributed significantly to total influenza-associated morbidity and mortality, but generally not to the same extent as seasonal A(H3N2) viruses, possibly because there is less rapid antigenic drift in influenza B viruses. Furthermore, the lack of stable animal reservoirs makes the emergence of new variants unlikely. Although there were very few detections of influenza B in the UK during the 2009-10 H1N1 pandemic, influenza B virus accounted for approximately 25% surveillance programme detections during the winter of 2010/11 (56). In a study of influenza-related

mortality over five seasons in urban China, excess mortality attributed to influenza B infection during an influenza B-dominated season (2007-2008) was double that attributed to influenza A infection during seasons dominated by A(H3N2) and/or A(H1N1) viruses. Similar patterns have not been seen in other countries, even in countries with established influenza surveillance systems (78).

1.2.5 Illness caused by seasonal influenza viruses

1.2.5.1 Uncomplicated seasonal influenza illness

The typical incubation period for seasonal influenza is two days (range 1-4 days). Virus may be detected in upper respiratory tract secretions 24 hours prior to illness onset and levels of virus usually peak between 1-3 days. In previously healthy adults and older children with uncomplicated illness, virus replication typically continues for 3-5 days; longer replication (weeks to months) can be seen in certain populations e.g. infants and immunocompromised individuals, even if illness remains uncomplicated.

The classically described illness caused by seasonal influenza infection consists of sudden-onset fever accompanied by a combination of other symptoms including headache, myalgia, dry cough, lethargy and also variable coryzal symptoms and rhinorrhoea. Symptoms of uncomplicated seasonal influenza typically last for 3-5 days, although fatigue, cough and airway hypersensitivity may persist for several weeks. Peripheral blood lymphopaenia is a common finding, along with elevation of acute phase proteins such as c-reactive protein (CRP). Asymptomatic natural infection may also occur. Systematic review of data from virus challenge studies of seronegative healthy volunteers suggests that asymptomatic infection occurred in one third of successfully infected subjects, with no significant difference according to virus type/subtype or the infectious dose (79). Certain symptoms may be absent in specific populations. For example, fever and myalgia are less common in immunosuppressed transplant patients (80). Use of medications such as antipyretics, immunomodulators and analgesics may also influence the presenting symptoms.

1.2.5.2 Complicated seasonal influenza illness

Overall, the pattern of uncomplicated illness is similar for seasonal influenza A and influenza B viruses. However, virus type and subtype appear to influence rates of complications. Although children are at greatest risk of infection, the risk of complicated illness requiring hospitalisation is greatest at the extremes of age, especially in those who are over 65 years of age. The risk of complications and death increases in the presence of underlying medical conditions, including chronic respiratory conditions, pregnancy and immunosuppression (81). The reported intensive care admission rates for seasonal influenza range from 5-17% and mortality in hospitalised patients is in

the range 3-8% (higher rates are reported in studies of patients who were not treated with antivirals) (81).

The chief pulmonary complication of seasonal influenza infection is pneumonia, which may be caused by influenza virus infection alone, secondary bacterial infection (see below), or mixed viral-bacterial pneumonia. It is estimated that up to 1 in 5 community-acquired pneumonia cases requiring hospitalisation are caused by influenza virus infection (82, 83). Viral pneumonia typically presents with dry cough and dyspnoea, which may vary in severity, in addition to the classical features of influenza. Upper respiratory tract symptoms may have diminished by the time viral pneumonia is present. Viral pneumonia may cause type one respiratory failure with failure of oxygenation and progression to acute respiratory distress syndrome (ARDS) may occur. Acute bronchitis and exacerbations of existing lung diseases, especially asthma and chronic obstructive pulmonary disease (COPD) are also well recognised (84, 85).

Extra-pulmonary complications attributable to influenza virus infection have also been described, although it is not clear how virus type and sub-type influences all of these phenomena. Neurological complications include encephalopathy, encephalitis and Guillain-Barré syndrome (GBS). Encephalitis and encephalopathy appear to be more common in infants than in adults and older children, and there may also be a racial predilection for these complications (86). Influenza virus RNA is typically absent in cerebrospinal fluid (CSF) and tissue samples, suggesting that other factors, such as aberrant immunological or metabolic responses to infection, may contribute to the pathogenesis (87, 88). Although much attention is paid to influenza vaccine and association with GBS, recent influenza virus infection (influenza A and influenza B) has a much stronger association with GBS, with an estimated four to seven cases of GBS per 100,000 influenza cases, compared to one GBS case per 1,000,000 recipients of inactivated seasonal influenza vaccine. This association is supported by epidemiological observations and by specific sero-epidemiological studies (89).

Cardiac complications have also been described. Acute coronary syndromes appear to be more common in the immediate period following influenza infection (90, 91), which may be caused by destabilisation of atheromatous plaques by the inflammatory response to influenza infection (92). Myocarditis and pericarditis may also occur in seasonal influenza, although rarely as isolated clinical syndromes. It is unclear whether virus-induced cytolysis, immune-mediated inflammation, or both contribute to these complications (93). Interestingly, in an autopsy study of 45 fatal influenza B infections, cardiac injury was detected in tissues from 69% of cases examined. Most of these cardiac complications were in paediatric cases (94).

1.2.5.3 Bacterial co-infections in seasonal influenza

Rene Laënnec is recognised as being the first physician to have described secondary pneumonia following influenza illness, in his description of an epidemic of *la grippe* that occurred in Paris, France, in 1803 (95). Bacterial infection in patients with confirmed influenza may occur secondarily (occurring as the second phase of a biphasic illness, often after virus is no longer detectable) or concurrently (active viral and bacterial infection contributing to illness at the same time). In seasonal influenza, most identified bacterial complications are caused by *Streptococcus pneumoniae*, *Staphylococcus aureus, Haemophilus influenzae, and Klebsiella pneumoniae*. Invasive infections of *Neisseria meningitides* and Group A *Streptococcus* species are also recognised. Bacterial complications vary in severity, ranging from mild upper respiratory tract infections and otitis media, through to fulminant pneumonia, bacteraemia and meningitis (96). Exactly how much bacterial pneumonia contributes to severe influenza illness is difficult to estimate, because epidemiological surveillance is typically crude, often combining influenza and pneumonia as a single outcome measure (97). Furthermore, conventional methods for detecting bacteria lack sensitivity and specificity (98) (especially in the presence of antibiotic therapy) and there is no reliable test for determining whether bacteria detected outside of a 'sterile' biological compartment are actively pathogenic in any given individual.

The reasons why rates of bacterial complications differ by region, by season and across influenza-infected populations are unknown. It has been proposed that seasonal variation in carriage of bacteria may play a role, leading to variability in colonisation with specific strains of particular bacterial species during an influenza outbreak. These bacterial strains may possess different properties that influence outcome of infection, for example the ability to produce toxins (99). It has also been proposed that pairings of a specific influenza virus and a specific bacterial species/strain in an individual may lead to mixed pathological infection (100).

There have been a limited number of human studies of bacterial complications of influenza, with the most consistent data coming from studies conducted during influenza pandemics. Animal models - predominantly murine models using mouse-adapted influenza strains and strains of common respiratory tract bacteria - have been used to study co-infections. Infection of mice with influenza virus, followed by infection with *S. pneumoniae* seven days later, results in significantly greater mortality than is seen with infection by either agent alone (101). Furthermore, significant variation in mortality is seen with different strains of *S. pneumoniae* following influenza infection in animal models (102).

Virus characteristics may promote bacterial infection and such characteristics may vary between different influenza viruses (103). Examples include compromise of the respiratory epithelial barrier and cilliary clearance mechanisms by direct viral damage (104, 105), removal of sialic acid by viral neuraminidase which exposes oligosaccharide receptors and promotes bacterial adhesion (106), and modulation of neutrophil functions through the binding of virus to sialic acid receptors on neutrophils (107).

The host immune response to initial viral infection may also create a state of susceptibility to subsequent, pathological bacterial infection. Influenza-infected mice deficient for type I interferon-α/β receptor signalling demonstrate improved survival when subsequently infected with *S. pneumoniae*, compared to wild-type mice, which demonstrate impaired production of the chemokines CXCL-1 and CXCL-2. These chemokines are important in neutrophil-mediated clearance of bacteria from the lung (108). IFN-γ is also produced in abundance in response to influenza infection, but in mice has been shown to suppress phagocytosis of bacteria by alveolar macrophages (109). Although a robust interferon response may help clear virus, these animal studies suggest that it may also promote secondary bacterial infection. Murine studies also suggest that a state of relative immunosuppression may exist following influenza infection. Mice that have recovered from influenza infection appear particularly susceptible to lethal pneumococcal infection and have higher levels of the anti-inflammatory cytokine IL-10 compared to control mice, and administration of anti-IL-10 antibodies prior to pneumococcal challenge appears to be protective (110).

Overall, it is plausible that the development of secondary bacterial infection is multifactorial, involving specific host, pathogen (virus) and co-pathogen (bacteria) factors acting together in a single individual. It may be that bacterial infection occurs when an influenza virus expressing multiple virulence factors infects an individual whose respiratory tract has been successfully colonised by a strain of bacteria with invasive potential, and the individual has an immunological predisposition (genetic and/or environmental) that facilitates bacteria-virus synergy.

1.3 Avian influenza

1.3.1 Avian influenza virology

Birds are the primary reservoir for influenza A viruses. Avian influenza A viruses are divided into two groups, based on the severity of illness they cause in birds: low pathogenicity avian influenza (LPAI) and highly pathogenic avian influenza (HPAI). Highly pathogenic viruses – which are always H5 or H7 viruses - can kill entire stocks of poultry in one to two days, whereas LPAI viruses often cause asymptomatic infection in wild birds and poultry stocks. This classification does not reflect the ability

of avian viruses to cause significant illness in humans or other mammals, however. For example, infection currently circulating H5N1 HPAI viruses is associated with significant morbidity and mortality in both birds and humans (39), whereas the recently emerged A(H7N9) LPAI virus has caused complicated illness – and significant mortality - in the majority of humans known to have been infected yet it has had very little impact in infected poultry (111). LPAI H5 and H7 viruses can evolve into HPAI viruses following nucleotide substitution events in poultry, but it is unclear which factors drive such mutations (112).

In contrast to LPAI viruses, highly pathogenic avian viruses possess multiple basic amino acids at the cleavage site of the HA precursor protein, HA0, which makes the virus susceptible to the action of multiple proteases throughout the infected host. This promotes active HA and the replication of infectious virus in multiple tissues (113, 114). LPAI viruses are shed in the faeces of infected birds, facilitating widespread transmission in ducks and other aquatic birds (115).

Sporadic infections in humans have been described, particularly in countries where the viruses are endemic in birds, including HPAI A(H5N1) virus (116), LPAI A(H9N2) virus (117, 118), and most recently LPAI A(H10N8) virus (119) and LPAI A(H7N9) virus (120). However, many of the known human cases of avian influenza infection have reported contact with birds (especially poultry), poultry products or environments where birds are kept; substantive evidence of human-to-human transmission of non-adapted avian viruses, especially HPAI A(H5N1) virus, is limited (121) and multiple sero-epidemiological studies suggest that mild or asymptomatic HPAI A(H5N1) virus infection in humans (populations residing in endemic areas) is uncommon (1-2% seroprevalence) (122).

In contrast to established strains of seasonal influenza, the HA of avian viruses binds preferentially to sialic acid-bearing receptors with α2,3-linkages, rather than α2,6-linkages (123). The latter are more prevalent in the human respiratory tract, which may in part explain why bird-to-human transmission is limited. Reverse genetics-engineered viruses bearing avian glycoproteins demonstrate restricted replication and extent of infection when incubated with human ciliated airways epithelium maintained at the typical temperature of human proximal airways (32°C) but not when incubated at the typical temperature of human distal airways (37°C); the temperature of the avian enteric tract typically is 40°C. Since proximal airways facilitate infection in humans, this temperature restriction may also limit zoonotic transmission (124).

In addition to the highly cleavable HA, *in vitro* and animal studies of different H5N1 isolates have revealed other virulence factors including PB2 amino acid substitutions that facilitate mammalian

adaptation and enhance replication (including replication in the upper respiratory tract) (125), an NS1 substitution that appears to attenuate the antiviral effects of IFNs and TNF-α (126, 127) and marked induction of pro-inflammatory cytokines in human macrophage cell lines (128). The observation of familial clusters of HPAI A(H5N1) virus infection as well paucity of infection in some groups of virus-exposed poultry workers, suggests that host genetic factors may also influence human infection and illness, although this remains to be proven (129, 130). Following the first identified case of human HPAI A(H5N1) virus infection in 1997, on-going research has identified different clades and sub-clades of virus with differing geographical distributions and evolving virus genetics. Observed differences in mortality in patients infected with clade 1 and clade 2 viruses may reflect geographical differences in medical care, rather than different biological characteristics of the viruses.

Experimental modification of A(H5N1) viruses has been shown to produce viruses that are transmissible in non-avian animal models with relatively few nucleotide substitutions, although improved transmissibility may be associated with decreased virulence (131, 132). Human and avian isolates of A(H7N9) viruses have HA sequences that are consistent with preferential binding to α2-6 sialic acid receptors. Substitutions in PB2 that are associated with mammalian adaptation have also been identified (111, 133). However, the true pandemic potential of evolving HPAI A(H5N1), LPAI A(H7N9) viruses and potential avian-derived reassortant viruses remains unclear.

1.3.2 Illness caused by avian influenza virus infection in humans

1.3.2.1 Highly pathogenic avian influenza A(H5N1) virus infection

Since 2003, 649 human cases of HPAI A(H5N1) virus infection have been reported to WHO by fifteen countries. Of these cases, 384 (59%) have died. Indonesia, Vietnam and Egypt have reported the greatest number of cases (134). The reported median time from onset of symptom to presentation is 4 days, with a median time to death of 9.5 days from onset. The typical manifestation is severe bilateral pneumonia, which often progresses to ARDS. Upper respiratory tract infection without pneumonia is also recognised, especially in children. Diarrhoea has been reported to occur in as many as 50% of patients in some series, although the rates differ significantly according to the population studied. Virus RNA was detected in lower respiratory tract samples at one month in one patient and RNA and infectious virus has been detected in extrapulmonary tissues, secretions and excreta. Leukopaenia, lymphopaenia, thrombocytopaenia and transaminitis are common findings. A limited number of autopsy studies have demonstrated histopathological changes consistent with ARDS. Haemphagocytosis and disseminated intravascular coagulation has also been seen in some cases. Bacterial complications appear to be uncommon, however (39, 116).

Compared to seasonal influenza, HPAI A(H5N1) virus-infected patients with severe illness have higher levels of proinflammatory cytokines (e.g. IL-6 and IFN-γ), anti-inflammatory cytokine IL-10 and neutrophil-attracting chemokines (135). The findings of one study suggest that levels of these mediators in plasma correlate with upper respiratory tract viral load (39). This observation is supported by *in vitro* experiments using cell lines whereby A(H5N1) virus infection induced greater up-regulation of multiple cytokines than was seen with seasonal influenza virus infection (136, 137).

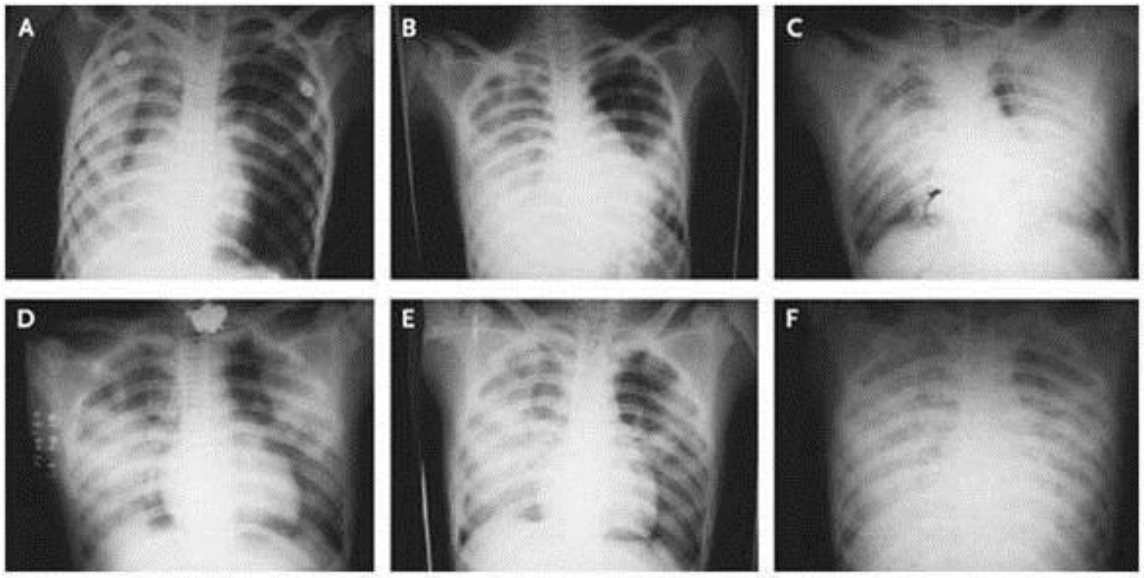


Figure 1.2 Chest radiographs of A(H5N1) virus-infected adult patients
Upper panels demonstrate widespread consolidation, collapse and interstitial shadowing in 3 individuals (A, B and C). Lower panels demonstrate radiographic progression in a single infected patient on days 5, 7 and 10 of illness (panels D, E and F, respectively). Reproduced with permission from *Avian Influenza A (H5N1) in 10 Patients in Vietnam*. Hien T. T. *et al*. N Eng J Med 2004; 350:1179-1188. Copyright Massachusetts Medical Society.

1.3.2.2 Low pathogenicity avian influenza A(H7N9) virus infection

In March 2013, human infection with a novel low pathogenicity avian influenza (LPAI) A(H7N9) virus was described for the first time in China (111). Unlike HPAI A(H5N1) virus, A(H7N9) virus does not cause significant illness in birds, making it difficult to monitor and control in farmed poultry. Small outbreaks of illness in humans caused by other H7 viruses have been reported previously by several countries. These were generally associated with poultry outbreaks and resulting in mild upper respiratory tract infections and/or conjunctivitis (138). In humans, severe illness caused by the novel A(H7N9) virus bears similarities to that caused by HPAI A(H5N1), with severe bilateral pneumonia, ARDS and multi-organ failure being common. Also similar to HPAI A(H5N1) virus infection, human-to-human transmission of A(H7N9) virus is rare, with indications that the current outbreak may also be zoonotic (139, 140).

Epidemiological data suggest many – but not all – of those infected had contact with birds or the environments where they are kept or sold, especially poultry (live or dead). Following the first wave of cases in early 2013, which lead to widespread closures of live poultry markets, a second wave appears to have commenced in December 2013. By mid-January 2014, 219 confirmed cases had been reported to World Health Organization, with an apparent increase in case reporting in the second wave. Fifty-five patients are known to have died (case-fatality ratio = 24%) (141). Although epidemiological analysis is on-going and cases of severe respiratory illness, there is a suggestion that less severe cases are also being seen and asymptomatic infections have been detected through active case finding and household screening programmes. Specific antibodies have not been detected in studies of those living in affected areas, suggesting that exposure (and immunity) in the general population is low. Over 6% of poultry workers from affected areas were found to have specific antibodies against A(H7N9) virus (measured by HAI) (142), although significant antibody titres (measured by HAI and MN) were not present in serum samples obtained in 2012 from poultry workers in affected areas (143).

Despite being a virus that can infect humans, A(H7N9) virus is thought to be a reassortant virus, containing RNA segments encoding HA from wild duck viruses and NA from wild-bird viruses, and six RNA segments derived from two separate A(H9N2) viruses (144). Although some virus isolates demonstrate genetic markers consistent with mammalian adaptation (145), it remains to be seen whether the A(H7N9) virus will evolve to cause sustained human-to-human transmission, with or without a potential fitness cost that may alter severity of illness.

1.4 Influenza pandemics of the twentieth century

1.4.1 The 1918 A(H1N1) influenza pandemic

Historical descriptions suggest that the first recognised pandemic attributable to influenza took place in 1510, although influenza is likely to have been infecting humans and causing major outbreaks of illness for many thousands of years (146). The 1918-19 pandemic is the earliest pandemic for which an influenza virus was identified as the causative pathogen. It is estimated that over two years, this pandemic caused at least 50 million deaths globally, with an estimated 12.5 million deaths occurring on the Indian subcontinent alone. Although the case fatality rate varied by location and time, it may have been as high as 2% in some populations. At the time of the pandemic, it was not known that the outbreak was caused by a virus; RNA from the 1918 A(H1N1) virus was subsequently recovered in the late 1990s, from preserved lung-tissue samples obtained from victims. Sequence analysis suggests that this virus is very similar to avian viruses and may have 'jumped' directly to humans, but because earlier viruses are not available for comparison, the precise origin of the virus is not known and some argue that it may have been a reassortant virus (147).

Like most influenza pandemics, the 1918-19 pandemic was associated with waves of activity (figure 1.3), and each wave caused excess morbidity and mortality. In the northern hemisphere spring of 1918, many countries experienced a wave of relatively mild influenza-like illness. However, this was followed by at least one wave of activity later in the same year that was associated with greater severity of illness in adults aged 20-39 years and in younger children, especially those under 1 year of age. This age-associated mortality distribution, often referred to as the "W curve" (figure 1.4) was unusual compared to the mortality distributions seen with seasonal influenza epidemics. Many countries also experienced a third wave of activity in 1919, with mortality rates reaching a peak in either the second or third wave. Pregnant women also appeared to be at high risk of severe illness. By contrast, those over 65 years of age appeared to be relatively protected from the most severe illness (148).

Although many patients were thought to have developed a rapidly progressive viral pneumonia with clinical features consistent with profound respiratory failure, evidence – including retrospective bacteriological analysis of preserved lung tissue - suggests that the majority of deaths were caused, at least in part, by secondary bacterial infection. Although the bacterial species identified varied between studies and populations, *S. pneumoniae*, *S. pyogenes* and *H. influenza*e were most commonly identified (38). The 1918-19 pandemic occurred at a unique time in history, following a major world war and prior to the advent of vaccines against common viral and bacterial respiratory

pathogens, and before antibacterial and antiviral agents had been developed. However, *in vitro* and animal experiments using 1918 A(H1N1) viruses reconstructed through reverse-genetics techniques, along with sequencing analysis of all eight RNA segments, has identified virulence factors (41, 149, 150). In addition to findings from gene substitution experiments which suggest that the 1918 A(H1N1) virus PB1-F2 modulates host immune response to influenza infection, expression of the 1918 A(H1N1) virus PB1-F2 enhances the development, severity and immunopathology of secondary bacterial pneumonia in mice (35).

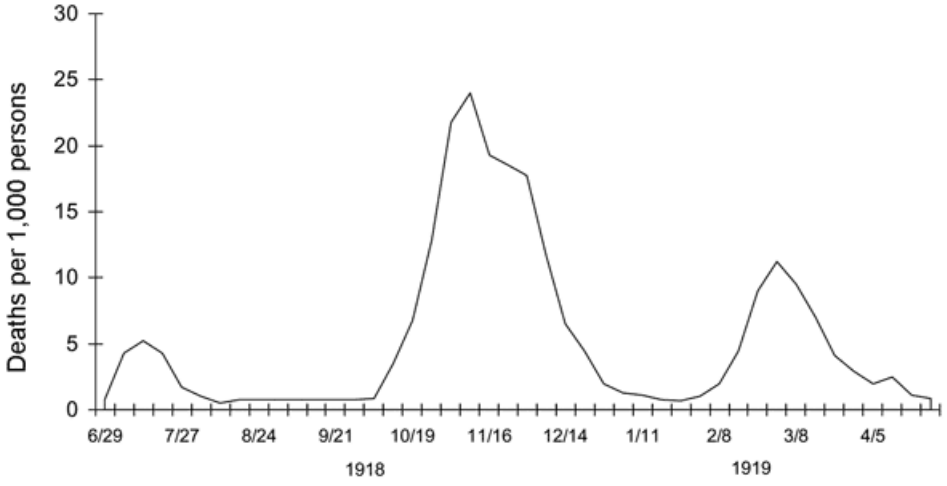


Figure 1.3 Three pandemic waves: weekly combined influenza and pneumonia mortality, United Kingdom, 1918–1919
Reproduced with permission from *1918 Influenza: the Mother of All Pandemics*. J Taubenberger and D Morens. Emerging Infectious Diseases (2006). 12(1):15-22.

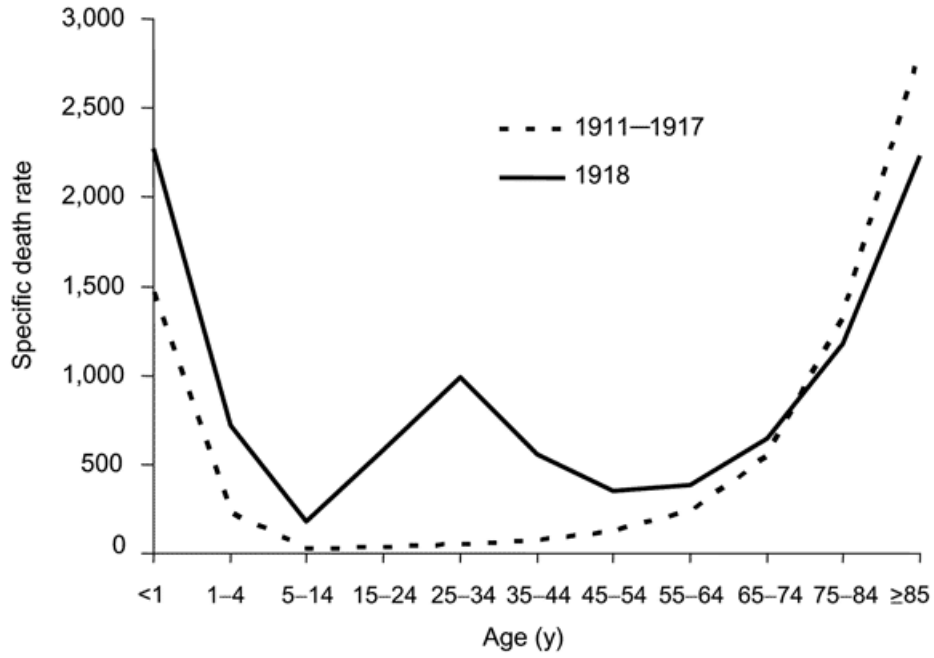


Figure 1.4 "U-" and "W-" shaped combined influenza and pneumonia mortality, by age at death, per 100,000 persons in each age group, United States, 1911–1918
Influenza- and pneumonia-specific death rates are plotted for the interpandemic years 1911–1917 (dashed line) and for the pandemic year 1918 (solid line). Reproduced with permission from *1918 Influenza: the Mother of All Pandemics*. J Taubenberger and D Morens. Emerging Infectious Diseases (2006). 12(1):15-22.

Outbreaks of influenza caused by descendants of the 1918 A(H1N1) virus occurred up to 1957, when a novel influenza virus emerged and caused a new pandemic. The 1957 A(H2N2) pandemic virus is believed to have spread from Southern China and caused an outbreak of influenza in Hong Kong in the spring of 1957, before going on to cause a global pandemic. The first pandemic wave in the UK occurred in the autumn of 1957 and many countries experienced a second wave in early 1958 (151). Subsequent analysis of the viral RNA segments revealed that this was a reassortant virus, whereby the descendants of the 1918 A(H1N1) pandemic virus had acquired segments encoding HA, NA and PB1 from a pool of avian viruses (152).

Although descriptions of clinical illness were similar for those described for the 1918 pandemic, the 1957-58 pandemic did not cause such striking excess mortality in younger adults. However, this pandemic still caused significant morbidity and mortality in those at the extremes of age and is considered to be the second most severe pandemic of the twentieth century. Bacterial complications (especially severe pneumonia) were also seen, but this time *Staphylococcus aureus* was the predominant species identified (38, 153). Although too late to be useful in mitigating a pandemic, this was the first time that a vaccine was produced in response to the emergence of a novel influenza strain, and by 1958 over 49 million doses of vaccine were available for use in the United States alone (154). As is typical for novel pandemic influenza viruses, waves of excess mortality attributed to the novel influenza virus were seen in the years following the 1957 pandemic (155), although the timing and magnitude of the post-pandemic waves often varies between countries (figure 1.5).

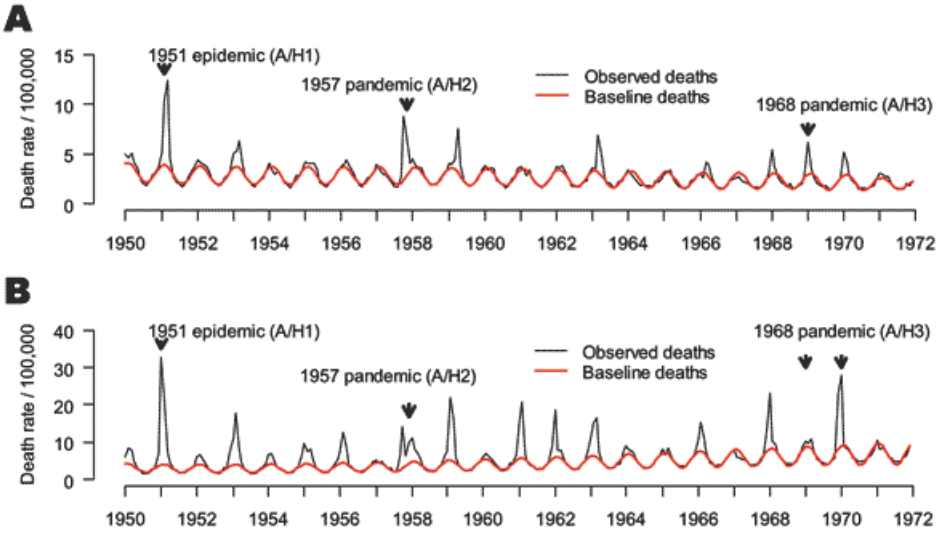


Figure 1.5 Time series of monthly mortality from pneumonia and influenza (P&I, represented as death rate/100,000) from 1950 to 1972 in A) Canada and B) England and Wales
Black line: observed deaths; red line: baseline deaths predicted by a seasonal regression model. Note the two arrows for the 1968 pandemic in England, representing the two waves of the smouldering A/H3N2 pandemic (1968–69 and 1969–70, respectively). Reproduced with permission from *1951 Influenza Epidemic, England and Wales, Canada, and the United States*. Viboud C *et al.* Emerging Infectious Diseases (2006). 12(4):661-668

1.4.2 The 1968 A(H3N2) influenza pandemic

Following the 1957 pandemic, just eleven years passed before the next pandemic occurred. Again, it was in China that the first outbreaks were reported and a novel influenza A virus, A(H3N2), was characterised following an outbreak in Hong Kong in July 1968. This virus was also reassortant, formed when avian influenza-derived HA and PB1 were acquired by the existing H2N2 virus (156). Subsequent waves of influenza activity caused by A(H3N2) virus were seen globally between 1968 and 1970. England experienced three pandemic waves, with the greatest excess mortality seen in the third and first waves.

Similar to the 1957 pandemic, most excess morbidity and mortality was seen in individuals who were at the extremes of age, especially those with underlying comorbidities. The overall impact of the 1968 A(H3N2) pandemic was relatively mild compared to the 1918 and 1957 pandemics, with an estimated 1-3 million fatalities and 30,000 deaths in the United Kingdom, most of which occurred during the second wave (157). It has been suggested that this lesser impact may have been due to the presence of pre-existing, cross-protective antibodies against N2 in those who had already been exposed to the 1957 A(H2N2) virus and its descendants (158). However, the 1968 A(H3N2) pandemic virus gave rise to A(H3N2) viruses that dominated seasonal outbreaks and severe influenza epidemics for over 40 years, until the A(H1N1) pandemic of 2009-10 (155). Despite the emergence of the novel A(H1N1)pdm09 virus in 2009, seasonal A(H3N2) viruses continue to circulate and were the dominant influenza A viruses during the 2011-12 influenza season in the Northern Hemisphere, although interim data suggest that the current 2013-14 season in the United States was dominated by A(H1N1)pdm09 virus (159).

1.5 The 2009 H1N1 influenza pandemic

1.5.1 The emergence of A(H1N1)pdm09 influenza virus

Influenza A(H1N1)pdm09 virus infection in humans was first reported in California, USA and in Mexico in April 2009 (160). Influenza A viruses that could not be subtyped were obtained from two American children with mild influenza-like illness. These viruses were analysed as part of the US national surveillance programme and rapid sequencing revealed the presence of viral RNA segments from triple-reassortant known to be circulating in pigs in North America, as well as other RNA segments that were closely related to those belonging to viruses circulating in pigs in Europe and Asia. In the same month, Mexican authorities also notified WHO of unexpectedly high numbers of ILI cases, following an outbreak of influenza-like illness that occurred in February 2009 in La Gloria, Mexico.

Following recognition of a novel influenza virus with pandemic potential, primers and probes for diagnostic assays were distributed rapidly. The virus was subsequently detected, retrospectively in samples obtained during the February outbreak in Mexico. After cases emerged across North America, the first cases in the UK were detected, initially in travellers returning from Mexico at the end of April 2009. By 21 May 2009, 41 countries had reported over 11,000 confirmed cases and on 11 June 2009, WHO declared that the criteria for declaring a pandemic (pandemic phase 6) had been met i.e. a novel influenza virus had caused sustained community level outbreaks with evidence of human-to-human transmission in multiple countries across multiple geographic regions (161).

1.5.2 The pandemic waves

Most temperate, Northern Hemisphere countries experienced two distinct waves of A(H1N1)pdm09 virus activity. In the UK, cases steadily increased from April 2009, reaching a peak in mid-to-late July 2009. Activity then decreased, probably because transmission was interrupted by the school summer holidays. A second wave was seen in the autumn, with a peak occurring in mid-October 2009. General practice consultations for influenza-like illness were more numerous during the first wave than during the second wave, possibly because of greater public concern in the early phase of the pandemic and also the introduction of the National Pandemic Flu Service (NPFS) during the second wave, which was designed to reduce demand for GP consultations and to provide rapid access to antiviral agents. By contrast, the number of hospitalisations and critically ill cases was greater during the second wave, but severity of illness was comparable between the two waves. Occasional cases were seen in the UK in the spring of 2010, but overall activity had markedly declined. After monitoring influenza activity during the Southern hemisphere's traditional influenza season, WHO was satisfied that the global levels and patterns of transmission of A(H1N1)pdm09 virus infection had changed and therefore declared the pandemic to be over on 10 August 2010. The declaration came with the caveat that post-pandemic activity should be expected, including further cases in young and previously healthy adults and children, adding that continued vigilance was required.

1.5.3 Epidemiological features of the 2009-10 pandemic

Unlike seasonal influenza, persons aged 65 years and over were relatively spared from infection during the 2009-10 pandemic. This is similar to the epidemiology of previous influenza pandemics. The majority of those hospitalised with illness caused by A(H1N1)pdm09 virus infection were adults aged 18-64 years. In the UK Flu-CIN Study of hospitalised patients, conducted during the first and second pandemic waves, only 5% of those admitted to hospital were over 65 years of age. Patients aged 5-54 years were overrepresented, compared with winter seasonal admission rates for acute

respiratory infection. School-age children accounted for fewer hospitalisations in the second wave compared to the first wave, but when they were hospitalised, they were more likely to have severe illness. Overall, a W-shaped curve for age distribution was seen, similar to the curve seen in the 1918-19 pandemic (162).

The total mortality for laboratory-confirmed cases and also estimated excess deaths was relatively low, compared to estimates of seasonal excess deaths due to influenza and pneumonia that are seen in most countries during seasonal influenza epidemics. This reflects differences in methodologies (laboratory-confirmed cases versus estimates of excess deaths) but also the differences in the ages of the most affected i.e. elderly persons versus younger adults and children. For laboratory confirmed adult cases in England, the median age at time of death was 43 years. However, the potential societal impact – in terms of years of life lost – was higher for the 2009-10 pandemic than for seasonal influenza outbreaks, because most deaths were in younger adults and children rather than in older persons. In the UK, the estimated overall symptomatic case fatality ratio (CFR) was 0.4 per 1,000 clinical cases. In those aged six months to 64 years and with a recognised risk factor for severe illness, the estimated CFR was 2 per 1,000 clinical cases (range 0.2 to 0.9), compared to 0.4 per 1,000 cases in the absence of a risk factor. Although elderly patients were less likely to be infected, those over 65 years with risk factors for severe illness had the highest CFR, 15 per 1,000 clinical cases (163). Statistical modelling suggests that the global pandemic respiratory mortality in the last nine months of 2009 was approximately ten-fold higher than WHO's laboratory confirmed mortality count (18,631 deaths), with up to 20-fold greater estimated mortality in North and South America (164). A further study estimated that there were 201,200 respiratory deaths (range 105,700 – 395,600) and an additional 83,300 cardiovascular deaths (range 46,000 – 179,900) associated with A(H1N1)pdm09 virus infection during the first twelve months of global virus circulation (165).

1.5.4 Risk factors for complicated A(H1N1)pdm09 virus infection

Identified risk factors for complicated A(H1N1)pdm09 virus infection have been relatively consistent across different international series (166-170). The UK Flu-CIN study described the clinical characteristics of 631 hospitalised cases admitted to 55 UK hospitals. Over half of the patients admitted to secondary care facilities did not have an underlying condition or identifiable risk factors for complicated influenza and of 29 patients who died, 59% were previously healthy. Comorbidity was a more frequent finding in adults (51%) compared to children (34%). Similar to reports from other countries, pregnant women were over-represented as a risk group, accounting for 4% of total admissions and 18% of women of child-bearing age (171). The greatest risk appears to be in the

second and third trimesters, although the risk also appears to extend to the early post-partum period (172, 173).

Across industrialised countries, asthma was identified as a common risk factor for complicated illness, accounting for approximately 30% of hospital admissions, although there was marked variation in the relative frequency of asthmatic patients across different countries. Mechanisms other than immunosuppression from prescribed corticosteroids appear to increase risk in asthmatics, since up to half of patients with asthma requiring hospitalisation did not receive long-term steroids prior to hospitalisation (66). In the UK Flu-CIN study, multivariate analysis suggested that dyspnoea, severe respiratory distress and a requirement for supplemental oxygen was more common in patients with asthma, but overall they were less likely to require critical care or have a fatal outcome, compared to patients without asthma. Analyses from the same study suggest that prior inhaled steroid use combined with prompt admission to hospital was associated with improved outcomes in patients with asthma (174).

Obesity also appears to be a risk factor for severe illness. An analysis of pooled data from ICU patients in four countries (n=859) identified obesity, defined as a body mass index (BMI) >30, and morbid obesity, defined as a BMI >40, in 30% and 13%, respectively. In the Flu-CIN study, obesity data were obtained in a limited number of patients, but obesity appeared to be associated with a severe outcome (likelihood ratio 6.96 (95% CI: 1.46 to 27.28; p=0.008) (171). Other co-morbidities identified across multiple international case series include immunosuppression, neurological disease (especially in children), chronic lung diseases other than asthma, cardiovascular disease, metabolic disease including diabetes mellitus, chronic renal disease and chronic liver disease (66, 175).

1.5.5 The post-pandemic period

In the twelve months that followed the WHO declaration marking the end of the 2009-10 pandemic, most countries experienced typical seasonal influenza activity, despite many Northern European countries experiencing a particularly harsh winter. In contrast to the pandemic waves, influenza B virus detections were seen in increasing numbers in the winter of 2010/11, although influenza A still dominated. Seasonal A(H3N2) viruses were also detected, but A(H1N1)pdm09 virus remained the dominant subtype in many countries. The UK was unusual in experiencing a third (post-pandemic) wave of A(H1N1)pdm09 virus activity during the winter of 2010/11. This was a more severe outbreak than the two pandemic waves, with a greater number of hospitalisations and critically-ill cases (56).

A sharp increase in hospitalisations was reported prior to an increase in community influenza-like illness (ILI) consultation rates in early December 2010, despite the existence of established

community surveillance systems (56). At the peak of activity in the third week of December 2010, 782 critical care beds in England were occupied with confirmed influenza cases (figure 1.6). This represents 20% of the UK critical care bed capacity being occupied at a single point in time by influenza patients (mainly adults aged 16-64 years and children) and contrasts with 172 critically-ill cases at the peak of the second pandemic wave (56, 176). Confirmed fatal influenza infections were also greater, mainly affecting middle-aged and younger adults. A slight, proportional increase in hospitalisation rates of those over 65 years was also observed (figure 1.7). Overall, complicated A(H1N1)pdm09 virus infection appeared to affected adults rather than children during the first post-pandemic season, something which has also been described for other pandemic viruses following the emergence of previous influenza pandemics (177, 178).

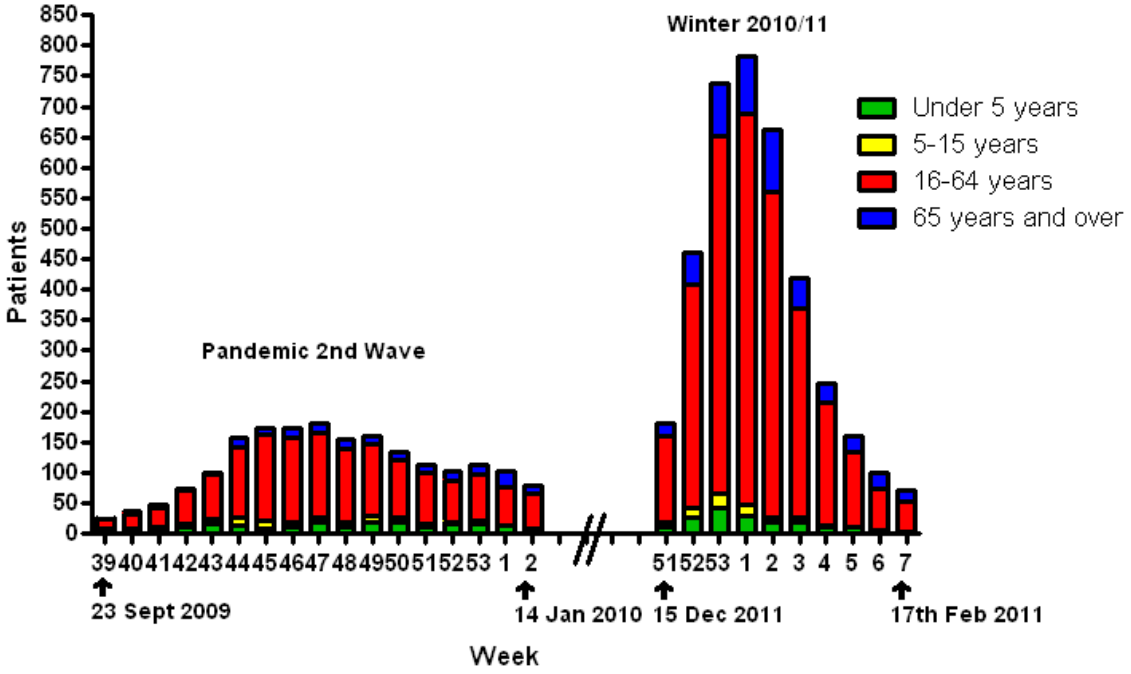


Figure 1.6 Patients with influenza occupying critical care beds in England by week Sept 2009 – Feb 2011

This graph was generated using data published in the Health Protection Agency (HPA) online weekly influenza bulletins (2009-10) and the UK Department of Health online *Winterwatch* bulletins (2010-11).

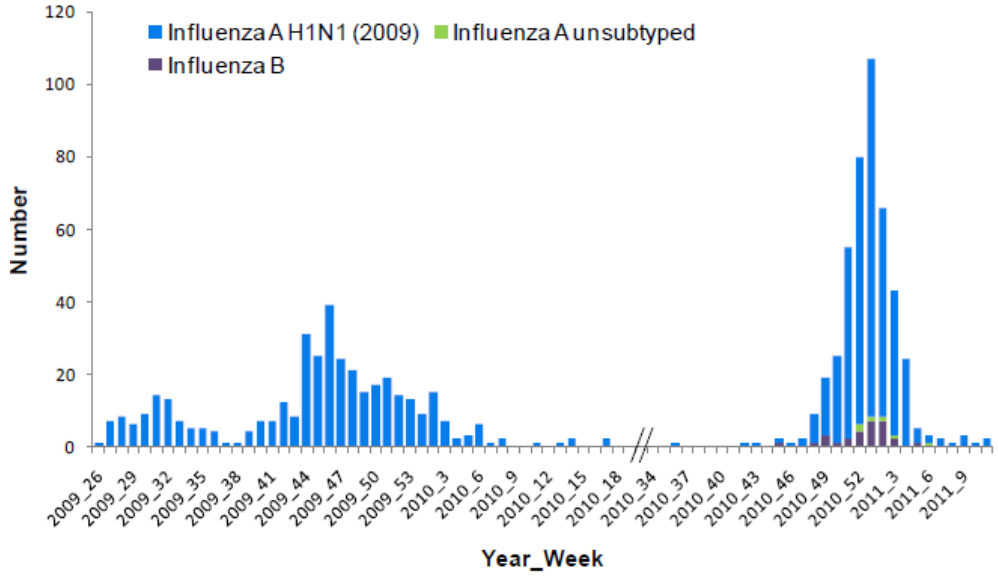


Figure 1.7 Number of fatal confirmed influenza cases by week of death and sub-type reported in England June 2009 – May 2011

Reproduced with permission from *Surveillance of influenza and other respiratory viruses in the UK, 2010-11 report.* Health Protection Agency, UK.

Why the UK experienced such a severe A(H1N1)pdm09 influenza season in 2010/11 remains unclear. However, post-pandemic waves of severe illness have been described in earlier influenza pandemics (155). Although retrospective studies conducted in Greece and Germany indicate that these countries also experienced an excess of severe cases in 2010/11 (179, 180), few countries reported similar findings to the UK and none appeared to experience such a large burden of critically-ill cases. Sero-epidemiological data from studies performed at the end of the UK's second wave lead some to suggest that a third wave of A(H1N1)pdm09 virus activity in 2010/11 was unlikely, given the high sero-prevalence in susceptible individuals (68).

Potential factors that may have contributed to an excess of severe cases have been proposed. An increased rate of bacterial co-infections was suggested by one study, but the surveillance data were limited and true association with influenza could not be clearly demonstrated (181). A more restrictive community antiviral prescribing policy was introduced following the 2009-10 pandemic, although modelling suggests that, in those who were hospitalised, potential differences in pre-hospital antiviral use did not fully explain the increase in severity seen in 2010/11 (177). Vaccine strain-match was appropriate in both the second and third waves and uptake was also at a higher level prior to the peak of the 2010/11 season compared to a comparable time point in the 2009/10 winter season (182). This suggests that "vaccine escape" (or even vaccine augmentation) did not contribute to third wave severity.

Although temperatures were lower in the UK in the winter of 2010/11 than during the winter of 2009/10, which may have promoted more effective influenza transmission (61), other countries in Europe experienced even lower temperatures and did not experience such high levels of influenza activity in 2010/11. Although the A(H1N1)pdm09 virus evolved as expected, 2010/11 isolates did not differ significantly, at least in terms of antigenic drift, from isolates obtained in 2009/10. Furthermore, there were no obvious major changes in the sequences of viruses obtained from those with mild and severe disease in 2010/11 (183). However, genome-wide changes in the 2010/11 isolates have been reported and more detailed analysis may reveal genetic changes that could affect virus behaviour *within host*. Although comorbidities that increase the risk of severe influenza are more common in those of advanced age, the age shift associated with infection seen during 2010/11 was from children to younger and middle-aged adults as the predominant group, rather than to older adults. Additionally, a significant proportion of hospitalised adults did not have underlying comorbidities (56).

In conclusion, there are no robust explanations for the severe post-pandemic wave of 2010/11, although it is possible that multiple contributing factors may have come together to create a

"perfect storm" scenario. Adaptation to a seasonal epidemiological pattern typically takes 3-10 years following a pandemic (184) and severe waves in other countries should be expected in the post-pandemic period.

1.5.6 Virology of influenza A(H1N1)pdm09

1.5.6.1 Origins and evolution of A(H1N1)pdm09 virus

Influenza A(H1N1)pdm09 virus is a reassortant virus, incorporating six RNA segments (PB1, PB2, PA, HA, NP, and NS) from the North American triple-reassortant swine lineage (containing H1N2 and H3N2 swine viruses) and two segments (NA and M) from the Eurasian avian-like swine lineage (containing H1N1 and H3N2 viruses) (17, 185). The North American triple-reassortant A(H3N2) and A(H1N2) viruses were first detected in pigs in the late 1990s and consisted of RNA segments from the human H3N2 A/Sydney/5/97-like strain, North American avian and classical A(H1N1) swine viruses (figure 1.8).

Influenza A(H1N1)pdm09 virus was shown to be antigenically distinct from existing human and swine H1N1 viruses (186). Although multiple groups of A(H1N1)pdm09 viruses were identified across different geographical regions (183, 187), they were antigenically similar and the subsequent vaccine strains were well matched with isolates obtained during the pandemic and in the immediate post-pandemic period (157). Although mixed infections with different virus subtypes are recognized (188), reassortment events between A(H1N1)pdm09 virus and seasonal A(H3N2) viruses or seasonal (pre-pandemic) A(H1N1) viruses have not been described. Mathematical models of opportunities for reassortment, along with *in vitro* and ferret studies using reassortant viruses, suggest that the potential for reassortment events exist in nature, but the effect on transmission and pathogenicity in humans remains unclear (189, 190). However, nine A(H1N1)pdm09/endemic swine influenza virus reassortants were described in the United States in 2011 (191).

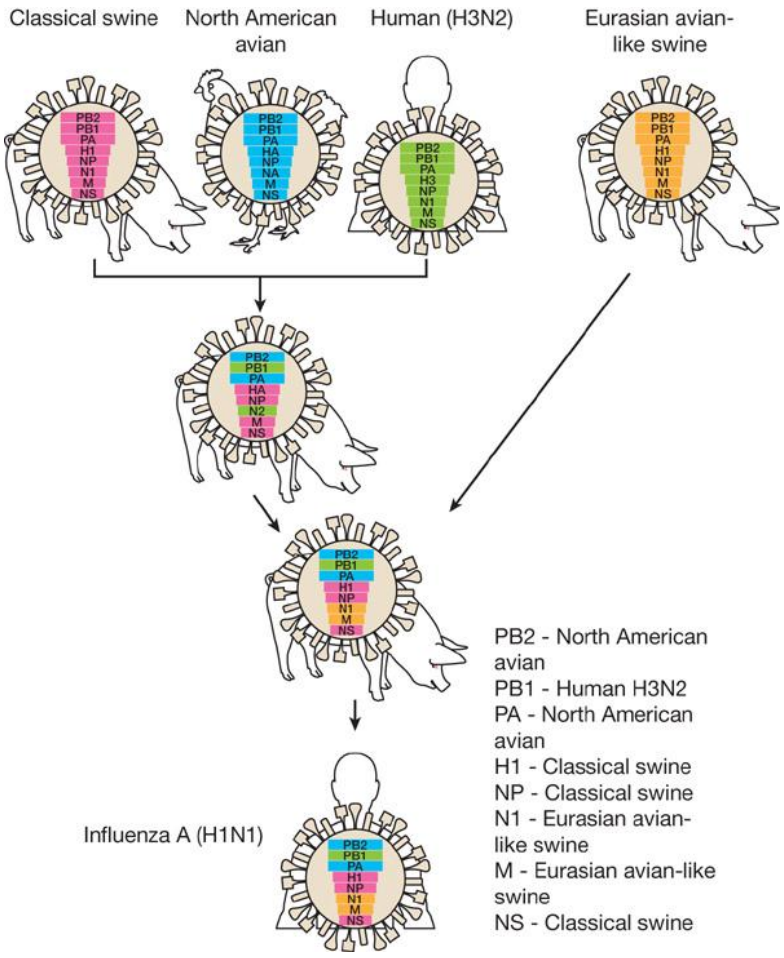


Figure 1.8 The origins of influenza A(H1N1)pdm09 virus
The A(H1N1)pdm09 virus (labelled A(H1N1)) is a reassortant virus consisting of six RNA segments from the North American triple-reassortant swine lineage and two RNA segments from the Eurasian avian-like swine lineage. Reproduced by permission from Macmillan Publishers Ltd. G Neumann, T Noda, Y Kawaoka. Emergence and pandemic potential of swine-origin H1N1 influenza. Nature 459 (7249): 931-939. Copyright 2009.

1.5.6.2 Tissue tropism and replication

In animal experiments, the level of A(H1N1)pdm09 virus replication is higher than the replication of seasonal H1N1 viruses (192, 193), but this difference was not seen with infection of *ex vivo* cultures of human tissues or *in vitro* cultures of human cells (194). Unlike seasonal A(H1N1) viruses, influenza A(H1N1)pdm09 virus is capable of replicating in human conjunctiva and whereas A(H1N1)pdm09 virus is capable of replicating in upper and lower respiratory tract tissue, swine A(H1N1) viruses appear to have a more limited tissue tropism and replication potential (15).

1.5.6.3 Virulence factors

Gene segment 2 of A(H1N1)pdm09 virus is derived from a human seasonal A(H3N2) virus; therefore, both viruses have a truncated and non-functional PB1-F2, associated with decreased virulence in animal models and decreased replication in cell-based models. This is in contrast to the PB1-F2 for all of the pandemic viruses of the last century and also HPAI A(H5N1) virus (36). Furthermore, deliberate mutation of A(H1N1)pdm09 virus to facilitate expression of full-length PB1-F2 was not associated with increased virulence in animal models, suggesting that this mutation on its own is not a significant determinant of severity in A(H1N1)pdm09 virus infection (37).

Amino acid substitutions in the PB2 segment of influenza A viruses, such as E627K, are proposed virulence factors, with the potential to influence transmission dynamics and pathogenicity. However, isolates of A(H1N1)pdm09 virus bearing this mutation have not been associated with enhanced transmission or greater severity of illness (195).

D222G (D225G in HA numbering system) is an HA substitution associated with increased binding to α2,3 sialic acid-bearing receptors and thus increased binding to cells of the human lower respiratory tract. This substitution has been seen in HPAI H5N1 and in some 1918 H1N1 isolates. Various studies have suggested that D222G is seen more commonly in isolates obtained from critically ill patients and fatal cases, compared to isolates obtained from patients with mild illness. Analysis of sequential samples and phylogenetic studies suggest that D222G-containing viruses arise within an infected patient, rather than being the infecting virus, and are often present as quasi-species populations (minor variants). Where paired upper- and lower-respiratory tract samples were obtained from critically ill patients, D222G-containing viruses were present in both samples in some, but not all patients. Since sequential lower respiratory tract samples tend not to be obtained from patients with mild illness, the significance of these findings is difficult to assess. It may be that D222G simply represents adaptation of virus over time, within host, that is more common in patients with established severe illness and pneumonitis (196, 197).

1.5.7 Pathological features of A(H1N1)pdm09 virus infection

Histopathological findings from human autopsy studies are generally consistent, revealing varying degrees of diffuse alveolar damage (DAD) with lymphocytic infiltrates, necrotising bronchiolitis and tracheitis. These findings are not specific to influenza caused by A(H1N1)pdm09 virus and can be seen in other fatal cases of influenza and ARDS due to other causes. Haemorrhagic pneumonitis and alveolar haemorrhage have also been described. In some autopsy series, pulmonary emboli were common and evidence of bacterial co-infection (typically with *Streptococcus pneumoniae* or *Staphylococcus aureus*) was found in up to 40% of cases. However, other series have reported lower rates of these complications; late presentations, differences in underlying comorbidities, specific therapeutic interventions e.g. extracorporeal membrane oxygenation (ECMO) and variable methodologies for detecting pathogens may have contributed to these discrepant findings. Extrapulmonary complications have also been described at autopsy, including myocarditis, haemophagocytosis, hepatic necrosis and acute tubular necrosis, but these findings appear to be uncommon. Influenza A(H1N1)pdm09 virus antigens have been detected in respiratory tract tissue (198-202).

1.5.8 Clinical characteristics of A(H1N1)pdm09 virus infection

The incubation period of A(H1N1)pdm09 virus infection is 1-3 days, similar to that of seasonal influenza. International case series suggest that the majority of those infected have mild to moderate disease and do not require hospitalisation. Early analyses suggested that at least one third of those infected may have mild illness without fever and some may remain asymptomatic (66, 203). A subsequent, prospective study of five successive cohorts of households across England, that included the 2009-10 pandemic and post-pandemic wave (six influenza periods in total during 2006-2011) found that approximately 20% of subjects had serological evidence of influenza and approximately 75% of all community influenza infections were asymptomatic or subclinical (204).

Mild illness due to A(H1N1)pdm09 virus infection typically manifests as influenza-like illness (ILI) i.e. fever with additional, variable symptoms including upper respiratory tract symptoms, headache, cough and malaise. Rhinorrhoea and myalgia are reportedly less common than in seasonal influenza, occurring in less than half of infected individuals, but nausea, vomiting and diarrhoea appear to be more common than in seasonal influenza infection (approximately one quarter of those with confirmed A(H1N1)pdm09 virus infection). Despite patients with A(H1N1)pdm09 virus infection being younger and having fewer comorbidities than patients with seasonal influenza, the risk of pneumonia appears to be higher in adults (4% vs. 1%) and the risk of extrapulmonary complications also appears to be greater with A(H1N1)pdm09 virus infection (23% vs. 16%) (205).

Shortness of breath is not a feature of uncomplicated influenza and suggests progression to more severe illness, particularly viral pneumonitis, the principal complication of A(H1N1)pdm09 virus infection in adults. Diffuse lung infiltrates, typically bilateral, are common radiographic findings in those with confirmed infection and pneumonitis and radiographs from critically ill patients are not dissimilar to radiographs obtained from hospitalised patients with HPAI H5N1 viral pneumonitis (figure 1.9). Although infiltrates affecting a single lobe have been attributed to primary viral pneumonitis, it is impossible to differentiate between bacterial and viral aetiologies of pneumonia on radiological findings alone, although some suggest that focal or dense infiltrates are suggestive of secondary or concomitant bacterial pneumonia. Resolution of lung infiltrates can be seen following successful treatment and recovery (206) (see chapter 3 for examples of chest radiographs from subjects recruited to this study).

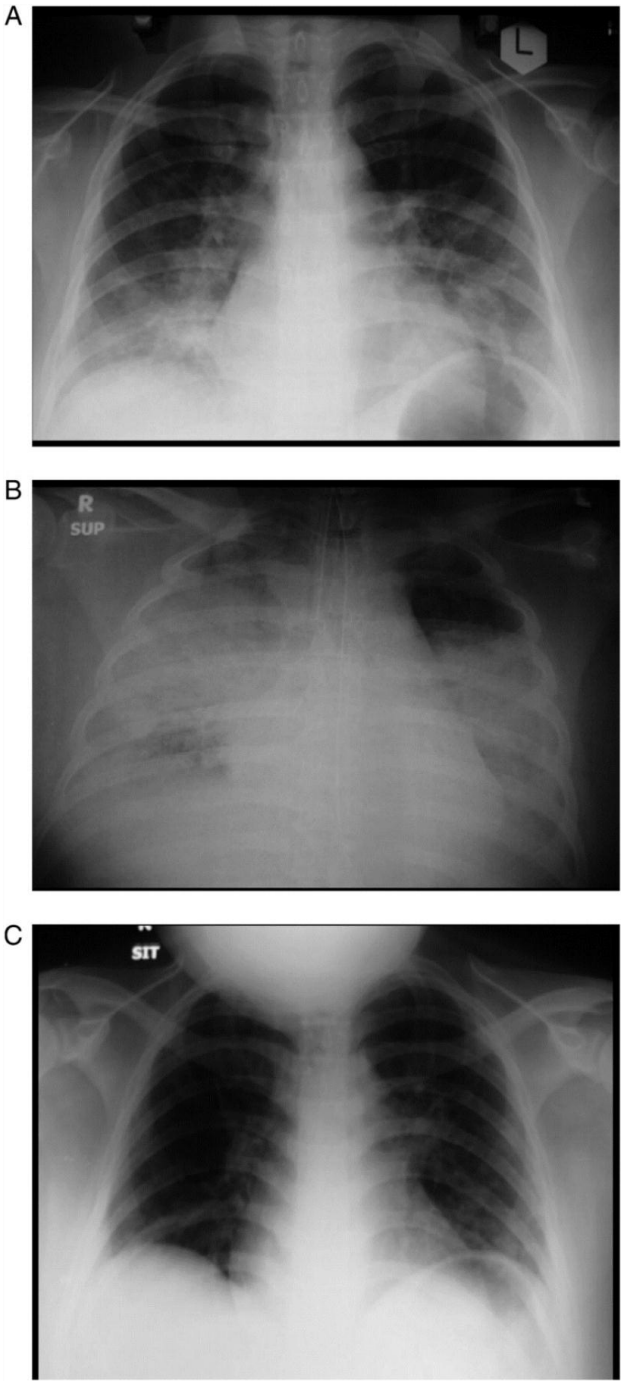


Figure 1.9 Sequential chest radiographs from a hospitalised patient with A(H1N1)pdm09 virus infection

Panel A shows a radiograph taken on admission to hospital (day 7 of illness) and reveals typical, bilateral patchy infiltrates, affecting the mid zones and lower zones. Panel B demonstrates progression of infiltrates at day 10, with more extensive infiltrates seen bilaterally. Panel C demonstrates resolution of chest infiltrates at day 21, following a period of invasive mechanical ventilation and treatment with oseltamivir. Reproduced with permission from *Clinical Management of Pandemic A(H1N1) Influenza*. D. S. Hui, N. Lee and P. K. S. Chan. Chest. 2010;137(4):916-925.

Clinical progression can be rapid in those with demonstrable lower respiratory tract involvement. Type I respiratory failure typically occurs around day 4-5 of illness and patients with the most severe illness, usually ARDS with variable multi-organ dysfunction (including septic shock and renal failure), tend to be admitted to critical care units within the first 24-48 hours of hospitalisation. Management of such patients can be challenging, and even advanced methods of invasive ventilation may fail to achieve adequate gas exchange, resulting in alternative attempts being made to oxygenate blood, including ECMO (207). Similar to other respiratory viral infections, exacerbations of underlying lung disease, such as COPD or asthma, can also be triggered by A(H1N1)pdm09 virus infection. Infants may experience bronchiolitis and young children may develop croup, sometimes requiring critical care (66, 206).

Atypical presentations may also be seen, including mild and afebrile illness and primary presentations of encephalitis and encephalopathy (especially in children), myositis, rhadbomyolysis and myopericarditis. Patients with compromised immune systems may not experience fever and other classical influenza symptoms, such as myalgia, may also occur less frequently in this group. At presentation to hospital, common laboratory findings include normal or low blood leukocyte counts, often with lymphopaenia, and also elevated levels of liver transaminases, CRP, creatine kinase and urea and creatinine. Although routine blood test results cannot adequately predict A(H1N1)pdm09 virus infection, metabolic acidosis and thrombocytopaenia appear to be associated with worse outcomes in those with severe illness (66). Additionally, a CRP level of $\geq$100 mg/L in the presence of radiologically confirmed pneumonia has been shown to predict the need for critical care and death (171).

1.5.9 Bacterial complications in pandemic influenza

Mice infected with non-lethal doses of A(H1N1)pdm09 virus, followed by infection with *S. pneumoniae* 48 hours later, demonstrated 100% mortality and significant weight loss and marked inflammatory responses and lung damage. By contrast, mice infected with A(H1N1)pdm09 virus alone or a seasonal H1N1 virus strain followed by *S. pneumoniae* infection did not die and demonstrated much less severe lung inflammation (208). Bacterial species associated with bacterial pneumonia have been identified in a significant minority of hospitalised patients with A(H1N1)pdm09 virus infection, but a clear correlation with severity has not been seen.

Although reported to be a common complication of 1918 A(H1N1) virus infection (38), rates of bacterial infection in patients hospitalised with A(H1N1)pdm09 influenza were approximately 20%-30% in most observational series (66, 209-212). In autopsy studies the frequencies have been much higher, however, with respiratory bacteria having been identified in up to 50% of cases (198). The

discrepant frequencies may reflect differences in how secondary bacterial infections are identified and defined, and also differences in the time to presentation and underlying comorbidities in those who died. However, the most frequently identified species tend to be the same: *S. pneumoniae*, *S. aureus* and *Haemophilus influenzae*. Invasive infections caused by group A *Streptococcus* and *Neisseria meningitides* were also reported during the 2010/11 A(H1N1)pdm09 outbreak in the UK, at a higher rate than had been seen during the 2009-10 pandemic waves (181). It has been proposed that inflammation-related, increased temperature of the respiratory tract seen in influenza infection may assist commensal bacterial species, such as *N. meningitides*, avoid host immune responses, leading to invasive infection (213).

The timing of onset of bacterial infection is not clear, but data suggest that the classically described biphasic illness may not apply in patients hospitalised with severe A(H1N1)pdm09 virus infection. In an observational study of 683 critically ill adults with confirmed A(H1N1)pdm09 virus infection in the USA, 207 (30%) were deemed to have bacterial pneumonia. The mean onset of bacterial co-infection was 6 days following onset of influenza infection (range 1.3-11.1 days), coinciding with a time period when viral replication is occurring. However, bacterial infection this study was defined by detection of bacteraemia or documented *presumed* bacterial pneumonia (214).

The true incidence of bacterial co-infection is difficult to define, due to the heterogeneity of study designs and the methods used to identify bacteria. Furthermore, with the exception of true sterile site infections e.g. detection of bacteraemia, a common problem for all studies is the difficulty in separating detection of harmless bacterial colonisation from pathogenic bacterial infection of the respiratory tract.

1.6 Influenza therapeutics

1.6.1 Vaccination

The primary approach for preventing influenza infection is through immunization. Currently available vaccines, whether based on live attenuated, inactivated or killed virus strains, provide immunity against specific strains of specific subtypes of influenza. The underlying principle is to induce host immunity to HA, which is conventionally assessed by the ability to produce neutralising, specific antibodies against an influenza strain, although other immune mechanisms may also contribute to protection. Because established influenza strains demonstrate antigenic shift, recommendations for the component vaccine strains are announced by WHO each year for both Hemispheres, ahead of the respective influenza seasons (215). Additionally, vaccines are produced in response to the emergence of novel influenza viruses with pandemic potential in humans, such as

A(H1N1)pdm09 virus. Ultimately, the effectiveness of seasonal influenza vaccines are most affected by the similarity of vaccine strains to circulating vaccines; since vaccine strains are selected ahead of time and are based on virus surveillance and predictions of virus evolution, effectiveness varies from season to season.

In addition to generation of neutralising antibodies against HA, antibodies against NA and other antigens may also contribute to protective immunity following vaccination with inactivated virus. Increases in cytotoxic T lymphocytes (CTLs) targeted against conserved internal proteins of influenza and increases in HA-specific $CD8^+$ T-cells can also be seen soon after vaccination (216, 217). Various factors may influence the production of neutralising antibodies, including the dose-schedule and timing of vaccination, the HA content in a vaccine, the use of immunogenic adjuvants e.g. squalene derivatives, and also host factors, such as the age of the recipient, prior experience of naturally-occurring influenza, and comorbidities or interventions impairing the immune response. In healthy adults aged 18-65 years with low antibody levels at baseline, peak neutralising antibodies (measured by HAI) are typically seen at 2-4 weeks following immunisation with a vaccine containing 15μg HA per strain. Antibody titres then decline with time, with a 50% reduction seen after six to twelve months. Although vaccine uptake tends to be greatest in those over 65 years of age, antibody titres following vaccination with 15μg HA tend to be lower in this group, possibly due to immune senescence and the effect of comorbidities that become more common with increasing age. Similarly, young infants have an altered response to standard vaccine and generally require two doses to mount an adequate antibody response following “first ever” vaccination. This is thought to be due to a combination of immunological immaturity and a lack of priming through influenza naivety (218).

Inactivated vaccines for use in humans were first produced in 1945. Current inactivated vaccines consist of chemically inactivated whole virus or split-virion products where the virus has been disrupted with detergents. Trivalent inactivated vaccines (TIVs) have been used since 1977, containing strains of H3N2, seasonal H1N1 and influenza B viruses. Since 2013, a quadrivalent vaccine has been used, to provide coverage against the two distinct lineages of influenza B virus that co-circulate, in an attempt to improve overall effectiveness. Both inactivated and live virus strains are cultures in embryonated chicken eggs, although egg-free manufacture is used to produce vaccines for use in those with egg allergy. Egg-based production is associated with a number of problems and alternative methods, such as mammalian and plant-based cell culture systems are being developed.

Although injection-site reactions and influenza-like illness are recognised side effects of inactivated influenza vaccines, mass surveillance of immunisation programmes confirms that these reactions tend to be mild, short-lived and infrequent (219, 220). Guillain-Barré syndrome (GBS) and narcolepsy are conditions for which an underlying cause often is not identified, although GBS may occur more frequently following influenza outbreaks. It has been suggested that GBS and narcolepsy may be associated with influenza vaccination programmes, although causation has not been clearly demonstrated. However, it is possible that a specific vaccine in a specific population may produce an abnormal reaction in a proportion of those vaccinated, albeit at a lower rate than may be seen following an outbreak of influenza (221, 222).

A meta-analysis of the studies assessing the effectiveness of inactivated vaccines in preventing laboratory-confirmed (PCR or virus isolation) A(H1N1)pdm09 virus infection suggests that vaccination provides at least moderate protection. The median vaccine effectiveness for monovalent vaccine was 69% (range 60-93), based on the results of five studies that met inclusion criteria (223). Subsequent meta-analysis of pooled results that included studies from the immediate post-pandemic period suggest that monovalent pandemic H1N1 vaccine effectiveness may be as high as 86% (range 60-95) (224). High rates of vaccine effectiveness appear to have persisted during the A(H1N1)pdm09 virus-dominated 2013/14 season in Canada, with a recent negative-test case-control study suggesting vaccine effectiveness of 74% (95% confidence interval: 58–83), based on interim analysis with medically-attended, laboratory-confirmed infection as the endpoint. This appears to be explained by a lack of significant drift in circulating A(H1N1)pdm09 virus, despite a clade switch in the preceding season (225).

Live attenuated influenza vaccines (LAIVs), delivered intranasally, have recently been licensed in Europe and the USA and are being used as part of trivalent vaccination campaigns for paediatric populations. Strain specific viruses are derived from a cold-adapted, attenuated master donor virus. The resulting reassortant viruses remain attenuated and only replicate effectively at temperatures lower than the replication temperature for wild type viruses. Although shedding of LAIV virus does occur, studies have demonstrated that transmission to other individuals is rare. The vaccine appears to be well tolerated in both children and adults, with mild upper respiratory tract symptoms being the most commonly reported adverse event (218). Although few studies have compared inactivated and live-attenuated influenza vaccines directly, data suggest that live attenuated vaccines are 50% more effective than inactivated vaccines in preventing infection in children aged 12 to 59 months (226, 227). The benefit appears to persist even when circulating viruses have drifted and vaccine-

match would be expected to be reduced. In adults, however, a limited number of studies have not shown greater effectiveness of LAIV over vaccination with inactivated virus (228, 229).

There is clearly room for improvement in vaccination strategies for preventing influenza. Antigenic drift and “best-guess” predictive vaccine strain selection limits the effectiveness of existing vaccines. The very young and the elderly are most at risk from complications of seasonal influenza, but only have modest responses to existing vaccines. To be a truly effective counter-measure, following the emergence of a novel strain with pandemic potential, the production of vaccine needs to be accelerated. To address these problems, research is now focussing on developing vaccines that target conserved epitopes e.g. M2 protein and the stalk domain of HA, and that induce heterosubtypic immunity. The overall aim is to produce a “universal vaccine” that is immunogenic, long lasting and can be rapidly manufactured. Clinical trials of candidate vaccines are in progress (230, 231).

1.6.2 Antiviral therapy

Neuraminidase inhibitors (NAIs) for influenza are the most commonly used agents for influenza, including pandemic and avian influenza. Although adamantanes, such as amantadine and rimantadine, and ribavirin have activity against influenza virus when administered as monotherapy, their use is limited by side effects, the emergence of antiviral resistance and limited efficacy data. Furthermore, influenza B viruses and A(H1N1)pdm09 virus are inherently resistant to the M2 ion-channel blockade effect of adamantanes (96). However, combined therapy using these agents with oseltamivir is being investigated in clinical trials (232, 233).

In the UK, two NAIs are licensed for the treatment and prevention of influenza A and influenza B infection: oseltamivir phosphate and zanamivir. NAIs target the NA surface protein and inhibit cleavage of viral HA from sialic acid residues expressed on the host cell-surface, preventing newly formed virions from detaching and infecting other cells. The net effect is restriction of virus replication to one cycle (234). Oseltamivir is administered orally (or via a nasogastric tube) and zanamivir is administered via a diskhaler. Aqueous solutions of both oseltamivir and zanamivir are investigational agents, but have been made available for compassionate use in selected patients since the 2009-10 pandemic. Alternative NAIs are available in other countries, including intravenous peramivir and the long-acting inhaled NAI, laninamivir (235).

Oseltamivir remains the most widely used antiviral agent for the treatment of influenza. Oseltamivir phosphate is absorbed across the gastrointestinal tract (bioavailability approximately 80%) and undergoes hepatic metabolism to form the active compound, oseltamivir carboxylate. The active

compound is detectable within 30 minutes of dosing and near-maximal concentrations in plasma are reached within 3-4 hours (234, 236). Because oseltamivir is renally excreted, dose-adjustments are required for those with renal impairment, but ECMO alone appears to have little impact on levels of the active compound in plasma (237). The licensed dosing schedule for the treatment of influenza in adults in 75mg twice daily for five days (238). Although UK prescribing guidelines recommend that oseltamivir should be commenced within 48 hours of symptom onset, observational data from the 2009-10 pandemic suggest that reductions in viral load can be seen even with delayed onset (239). However, multiple observational studies of patients hospitalised with A(H1N1)pdm09 virus infection demonstrate that the earlier treatment is started, the less likely a patient will progress to more severe illness (240-244). Therefore, national and international guidelines recommend empirical antiviral treatment (oseltamivir or zanamivir) when influenza is suspected, rather than waiting for laboratory confirmation of infection (245, 246).

Since 2009, WHO guidelines have recommended NAI treatment in patients who have uncomplicated influenza (suspected or confirmed) who also have risk factors for complicated illness, as well as treatment of patients with severe or progressive clinical presentations of suspected or confirmed influenza (246). Some guidelines also suggest increasing the dose to 150mg twice daily in critically ill patients (66, 247). Although plasma levels of the active compound appear to be adequate with standard dosing in such patients, increased dosing is recommended because of concerns about achieving adequate levels in damaged and inflamed lungs. Similarly, increasing the treatment duration to ten days is recommended in critically-ill or immunocompromised patients (247), since "viral rebound" (detection of previously suppressed viral RNA) has been seen following shorter courses of oseltamivir (248).

The optimal duration and dosing for these special populations remain to be determined. Resistance to oseltamivir, most commonly mediated by the H275Y substitution, is recognised, although it remains uncommon in circulating A(H1N1)pdm09 virus isolates (249). However, rapid and almost total resistance to oseltamivir emerged in isolates of seasonal H1N1 during 2008 (75). Resistant A(H1N1)pdm09 viruses can be transmitted, but most resistance occurs on-treatment, typically in patients with profound immunosuppression (250).

Inhaled zanamivir is used less frequently than oral oseltamivir. Because minimal absorption occurs from the respiratory tract, inhaled zanamivir (or nebulised aqueous zanamivir) is not recommended in patients where a systemically-acting agent is desirable, for example in those with critical illness. In such patients, the intravenous preparation of zanamivir should be used. Furthermore, the inhaled preparation should be used with caution in those with a history of asthma or bronchospasm (247).

Because zanamivir and oseltamivir differ in structure and have interact differently with the binding site of viral NA, zanamivir is not affected by the same substitution that confers resistance to oseltamivir (and peramivir), H275Y. Although resistance mutations for zanamivir do exist, they are rare (251, 252). In the UK, zanamivir is recommended as the first-line agent for patients with confirmed oseltamivir-resistance influenza and for patients who are at significant risk of acquiring on-treatment oseltamivir resistance e.g. transplant patients (245).

The effectiveness of oseltamivir and zanamivir in treating uncomplicated influenza remains a matter for debate. Pooled analyses of data from observational and clinical trials are limited by variations in study design, definitions of influenza and study endpoints, and also by differences in influenza viruses and treatment of specific patient populations. Meta-analyses suggest that, based on data from studies with variable endpoints that were conducted before the 2009-10 pandemic, the apparent benefit to individuals with mild seasonal influenza in the community is limited, in terms of reducing the duration of symptoms and preventing complications such as pneumonia. A Cochrane Collaboration review suggests that in adults with uncomplicated seasonal influenza, oseltamivir reduces time to alleviation of symptoms by 21 hours compared to placebo, with no evidence of an effect on subsequent hospitalisation rates (253). By contrast, a separate meta-analysis of oseltamivir for the treatment of pre-2009 pandemic seasonal influenza concluded that treatment was associated with an overall 28% reduction in lower respiratory tract complications requiring antibiotic treatment and a 37% reduction in 66% of all patients who had laboratory-confirmed influenza (254). These discrepancies may reflect the differing inclusion criteria for each meta-analysis.

While randomised controlled studies and head-to-head comparisons of NAIs were not conducted during the 2009-10 pandemic, many observational studies of hospitalised patients with confirmed A(H1N1)pdm09 virus infection have reported their findings. Although the effects of other interventions cannot always be excluded, attempts at propensity-score matching and multivariate analyses were undertaken by several studies. There appears to be a clear signal that, for hospitalised adults with A(H1N1)pdm09 virus infection, early treatment is associated with the best clinical outcomes, including lower mortality rates in critically-ill patients and a decreased likelihood of requiring increased levels of care overall. A lesser degree of clinical and virological benefit may also be seen even when treatment is commenced four days or greater after symptom onset. It has been proposed that these apparent benefits are due to a reduction in viral load, which may ameliorate the inflammatory immune response to influenza virus (96, 240, 255, 256). A meta-analysis of antiviral treatment outcomes in 29,234 hospitalised influenza patients (78 studies, 2009-2011) found that, compared with no antiviral treatment, NAI treatment in adults with influenza (predominantly

A(H1N1)pdm09 virus infection) was associated with a reduction in mortality risk (adjusted odds ratio [OR] 0·81; 95% CI 0·70–0·93; *p*=0·0024). Propensity scores were applied to control for biases associated with NAI receipt and a time-dependent Cox regression model was used to control for immortal time bias. The greatest benefit was seen in those who commenced treatment within two days of symptom onset, with an increase in mortality hazard rate for each day of delay up to day 5 of symptoms, but even delayed NAI treatment appears to be better than no treatment (especially in critically-ill patients). Interestingly, the reduction in mortality risk was less pronounced and statistically non-significant in children (257). The reasons for this discrepancy are not clear and may reflect differences in study design, although biological differences e.g. higher viral loads need to be explored.

Cumulative experience obtained during the pandemic supports the findings of clinical trials and post-marketing surveillance, in that the administration of neuraminidase inhibitors in patients with confirmed influenza infection is well tolerated. The most common side effects of the most commonly used agent, oseltamivir, appear to be gastrointestinal disturbance and headache, occurring in less than 10% of treated patients (258).

1.6.3 Treatment of complications

In addition to antivirals targeting the influenza virus, antibiotics may be required in patients with confirmed or suspected bacterial co-infections. Because it is difficult to exclude bacterial infection based on clinical findings, and since influenza infection is known to be associated with an increased risk of bacterial infection, hospitalised patients with influenza tend to receive empirical antibiotics in addition to antivirals. The antibiotics recommended by most guidelines are similar to those recommended for community acquired pneumonia and should provide cover against the most commonly reported species, S*. pneumoniae, S. aureus and H. Influenzae* (247, 259). Consideration should also be given to antibiotic-resistant bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA), with antibiotic prescribing reflecting local incidence of drug resistant organisms and the results of cultures and sensitivity testing (66, 81, 247).

In addition, supportive measures may be required according to the degree of organ dysfunction, for example renal replacement therapy for patients with acute kidney injury. Multiple non-pharmacological interventions have been used to treat patients with A(H1N1)pdm09 virus infection and ARDS, including advanced invasive ventilation techniques, nitric oxide, prone-positioning and extracorporeal membrane oxygenation (ECMO) (207). The benefits (and potential harms) of particular interventions remain unclear, but a UK cohort study of 80 adult patients with

A(H1N1)pdm09 virus infection referred to ECMO centres (with 86% patients receiving ECMO) suggested a lower hospital mortality compared with matched non-ECMO-referred patients (260).

1.6.4 Immunomodulators and novel therapeutics

Given the evidence suggesting that an exuberant, pro-inflammatory immune response contributes to the pathogenesis of severe influenza illness, it seems reasonable to consider modulating the immune response, in addition to targeting viral replication. A variety of agents have shown benefits *in vitro* and in animal models of influenza, including HMG-CoA reductase inhibitors (statins) (261), macrolides (262), peroxisome proliferator-activated receptor agonists and cyclo-oxygenase-2 inhibitors (263). However, clinical trials of proposed agents are lacking and studies have failed to consistently demonstrate mortality reductions in patients with influenza who were receiving statins or those treated with macrolides (264-266). Systemic and often high-dose corticosteroids frequently have been given to influenza patients with pneumonitis and/or ARDS. Although randomised trials of corticosteroids have not been conducted in influenza patients, data were collected during the 2009-2010 pandemic. Analysis of pooled observational data suggests that, at best, steroid use in hospitalised patients was not associated with increased mortality (267). Two large, retrospective observational studies that utilised propensity-score matching have demonstrated increased mortality with high-dose steroids, however (268, 269). In these studies, corticosteroids were also associated with an increased risk of nosocomial pneumonia and invasive fungal infection. Although the timing of initiation and dosages used have varied between studies, and the patient populations have not been homogeneous, available evidence suggests that administration of high-dose corticosteroids specifically for influenza pneumonitis should be avoided in routine practice (247).

1.7 Influenza-like illness

1.7.1 Definition of ILI

Influenza-like illness (ILI) often refers to the clinical syndrome caused by pathogens other than influenza, with symptoms similar to those seen in most patients with seasonal influenza virus infection. Definitions of ILI vary. WHO defines ILI as sudden-onset fever (>38°C) with cough or sore throat, in the absence of other diagnoses (270). US CDC adapted the WHO definition to include “in the absence of a known cause other than influenza”. The definition used by ECDC is more comprehensive: sudden-onset of symptoms with at least fever or feverishness, or malaise, or headache, or muscle pain, and at least one additional symptom (cough, sore throat or shortness of breath) (271). This latter definition is more in keeping with the UK clinical criteria used for assessing patients with suspected A(H1N1)pdm09 virus infection during the 2009-10 pandemic (176).

1.7.2 Non-influenza pathogens capable of causing influenza-like illness

Over 200 viruses are capable of causing symptoms similar to those seen with the 'common cold' and many viruses cause an illness that is difficult to distinguish from that caused by influenza viruses. Bacterial, fungal and parasitic infections can also mimic influenza illness, especially in the early stages of infection. Influenza-like symptoms can also be seen illness that does not have an infectious aetiology e.g. vasculitides and cancer. While the circulating non-influenza respiratory viruses differ according to location, patient population and often time of year, respiratory syncytial virus (RSV) and human rhinovirus (HRV) are the most well recognised causes of ILI. Other viruses often included in ILI sentinel surveillance programmes include enterovirus, adenovirus, coronavirus, parainfluenza and human metapneumovirus.

1.7.2.1 Respiratory syncytial virus

Human RSV is a paramyxovirus, originally identified in chimpanzees and their handlers and subsequently in infants with severe lower respiratory tract infections. Subtypes A and B tend to co-circulate or alternate. Similar to influenza virus, seasonality is seen in temperate regions, with circulation typically increasing in late autumn and peaking between December and February, whereas year-round circulation can be seen in tropical regions, sometimes with two peaks of activity during a 12-month period. Unlike influenza, humans are the only natural host for human RSV, although bovine RSV and other species-specific RSV strains also exist (272).

Most children experience RSV infection before the two years of age and although antibodies are produced in response to infection, they do not protect against future infection. Following an outbreak, the risk of re-infection in the following season appears to be proportional to the magnitude of the outbreak (273, 274). In young infants, bronchiolitis is the most common lower respiratory tract complication and can necessitate admission to critical care units, although pneumonia and croup also occur. Upper respiratory tract infections, middle-ear effusions and non-specific ILI are also described in children and adults (275). The severity of illness tends to decrease with successive infections; in adults and older children, mild upper respiratory tract infection or asymptomatic infection is the norm. However, certain populations, such as the elderly, those with underlying chronic lung disease and immunocompromised patients, may experience significant morbidity and mortality, primarily due to lower respiratory tract infection (276). Although deaths in otherwise healthy infants are uncommon in Europe and North America, excess mortality in infants is seen in infants in resource-limited countries (277).

Transmission of RSV occurs through direct contact of virus with nasopharyngeal or conjunctival membranes, or less commonly, by large droplet transmission (275). Following infection, RSV is highly

restricted to the respiratory epithelium (272). Observational and *in vitro* studies suggest that subtype A is more commonly associated with severe disease than subtype B, possibly because infection with subtype A induces a more marked pro-inflammatory immune response (278, 279). Elevated levels of IL-6, IL-8, TNF-α and IL-1β can be detected in airway secretions of infected children. MIP-1α (CCL3) in respiratory secretions appears to be associated with severe disease, but elevated levels of other chemokines can be detected in infected children, including MCP-1 (CCL2), CCL5 (RANTES) and CCL11 (eotaxin). It remains unclear as to whether the reported elevated levels of soluble immune mediators in severe disease represent an aberrant immune response or are proportionate when there is a higher RSV viral load (280-282).

Treatment of RSV is primarily supportive. Ribavirin is active against RSV *in vitro*, but limited evidence of clinical benefit limits its use in most patients. However, nebulised ribavirin may be used in combination with high anti-RSV titre immunoglobulin and/or corticosteroids in selected patient groups, such as immunocompromised patients with severe lower respiratory tract infection (283). Although an effective and safe vaccine does not currently exist, a humanised monoclonal antibody, palivizumab, has been developed. Palivizumab targets the highly conserved F glycoprotein of RSV and is used in immunoprophylaxis. Because of the high cost, the use of palivizumab is restricted to selected paediatric patients e.g. infants born pre-term, during months when RSV typically circulates.(284).

1.7.2.2 Human rhinovirus

Human rhinovirus (HRV) is a picornavirus, most frequently associated with the common cold. However, infection with HRV is also a well-recognised trigger for exacerbations of asthma in adults and children, as well as causing non-specific ILI symptoms. Over 100 serotypes of HRV have been identified and this, in combination with the high rate of virus evolution caused by an error-prone RNA polymerase, helps explain why adults typically experience one to two HRV infections each year, with an even greater annual infection rate being seen in children. As with influenza virus and RSV, children are the major reservoir for HRV. Fever is more commonly seen in children than in adults (285, 286).

Infection occurs through direct contact of virus-containing materials with nasal or conjunctival membranes, usually via fomites, although small- and large-droplet transmission is also described. The nasopharynx is the primary site of replication (285). Most human rhinoviruses bind to the intracellular adhesion molecule 1 (ICAM-1) glycoprotein immunoglobulin, which is usually expressed on non-ciliated epithelial cells but can also be found on endothelial cells and ciliated epithelial cells. ICAM-1 occurs in a soluble form, in addition to a membrane-bound form and may exert antiviral

properties. Following infection, the soluble form is down regulated, whereas the membrane bound form is up-regulated, promoting infection of the respiratory epithelium (287, 288).

Following binding, HRV enters the host cell. Elevated levels of IL-8 can be seen in respiratory secretions soon after infection and promote influenza of polymorphonuclear cells (PMCs) (289). Although there is little damage to the nasal epithelium (290), the increased levels of IL-8 is associated with neutrophil degranulation and chemotaxis of eosinophils, T cells and basophils and correlate with symptom scores in human challenge studies (291). Bradykinins are also produced, but only in symptomatic infection and application of exogenous bradykinin to the nasal mucosa of healthy volunteers produces symptoms of the common cold (292). It remains unclear as to whether asthma exacerbations are caused by direct infection of the lower respiratory tract (which is known to occur), or by indirect, immune-mediated mechanisms. Studies have demonstrated that early apoptosis of infected cells is reduced in patients with asthma, associated with deficient IFN-β regulation of apoptosis in primary bronchial epithelial cells. This ultimately promotes sustained virus replication and may increase the likelihood of more complicated illness (293).

In human challenge studies, HRV typically remains detectable (by isolation methods) in nasopharyngeal secretions for two to three weeks, which can result in shedding for one to two weeks after symptoms have abated (294). Treatment of HRV infection is supportive; preventative vaccines and specific antiviral agents for clinical use do not exist.

1.8 The innate immune response to influenza virus infection

1.8.1 Overview

The innate immune system is a fundamental component of the host response to infection with influenza virus and other respiratory viruses. Conserved molecules called pathogen-associated molecular patterns (PAMPs), derived from viruses replicating in respiratory tract epithelial cells, are recognised by pattern recognition receptors (PRRs) located within infected epithelial cells and also in circulating dendritic cells (DCs), natural killer (NK) cells and macrophages (295). When PRRs are triggered, a signalling cascade ensues and host antiviral defences are activated. Soluble immune mediators, including interferons, are produced and these stimulate other cells to up-regulate multiple genes that are involved in antiviral response, protecting them from infection (296). Other soluble mediators stimulate an inflammatory response and also facilitate activation of the subsequent, adaptive immune response. Although the inflammatory response serves to eliminate the invading virus, if it is not appropriately regulated then host tissues may suffer greater damage from the immune response to the virus, rather than from the virus itself (295). The magnitude and

duration of the innate immune response may be influenced by pathogen factors e.g. influenza virulence factors, or by host factors, such as an inherited genetic polymorphism that alters the normal innate immune response (297) or underlying medical conditions that alter the immune response.

1.8.2 Innate sensors

There are three important classes of PRR that recognise influenza virus: the Toll-like receptors (TLRs) TLR3, TLR7 and TLR8, RIG-I, and NOD-like receptor P3 (NLRP3) (298). Following infection of a cell, virus-derived single-stranded RNA (ssRNA) bearing 5'-triphospates binds to and is recognised by ubiquitously expressed RIG-I, located within the cytosol. This complex then interacts with interferon-beta promoter stimulator 1 (IPS-1) expressed on the surface of mitochondria, triggering activation of NF-κB, which induces transcription of cytokine genes, and IRF3, which induces transcription of interferon-related genes (299, 300).

In uninfected sentinel cells, such as plasmacytoid dendritic cells, viral ssRNA is recognised by TLR7 and TLR8, located within the endosome. The activated TLRs engage signalling via MyD88, which in turn activates IRF7 and NF-κB, leading to production of type I interferons and pro-inflammatory cytokines (including IL-6, TNF-α and IL-1β), respectively. TLR3 activation in the endosome also contributes to a pro-inflammatory immune response, but TLR3 recognises viral dsRNA rather than ssRNA and the response does not involve type I interferons (299).

NLRP3 forms part of the cytosolic inflammasome and is expressed by DCs, neutrophils, monocytes and macrophages (301). Once activated, NLRP3 recruits apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC). This leads to activation of caspase-1 and the processing of pro-IL-1β and pro-IL-18 to their active forms, which influence the development of adaptive immune responses and the priming of NK cells and cytotoxic T cells to produce IFN-γ, respectively (302, 303).

1.8.3 Role of interferons

Interferons (IFNs) are cytokines that are produced by most cell types. They form a fundamental component of the innate immune response to infections caused by influenza virus other respiratory viruses. Needless to say, viruses have evolved to develop mechanisms that counteract host interferon responses. For example, the NS1 of influenza A viruses which binds to RIG-I and suppresses RIG-I signalling (304, 305).

Type I IFNs, including the subtypes of IFN-α and IFN-β, are released early in the course of influenza infection and serve to ameliorate damage and on-going virus replication in infected cells and protect other cells from becoming infected. Plasmacytoid dendritic cells (pDCs) are a particularly rich source of type I IFNs in early viral infection (306). Type I IFNs are also produced in response to bacterial infection, but may not confer the same benefits to the host as are seen in viral infections, sometimes facilitating bacterial propagation and damage to host cells and tissues (307). In influenza virus infection, although type I IFNs may halt replication of susceptible virus, the production of chemokines such as CCL2 is also triggered, leading to recruitment of monocyte-derived DCs and macrophages, which contribute to respiratory tract pathology (308). The production of CXCR2 ligands is also inhibited by type I IFNs, reducing neutrophil recruitment and creating favourable conditions for secondary bacterial pneumonia (108).

Type II interferon, IFN-γ, is also produced in the early stages of influenza infection, but appears to be less crucial for antiviral defence than type I IFNs. Mice lacking IFN-γ can still mount an effective antiviral response (309). Type III interferon (IFN-λ, IL-28/29) is induced by the same RIG-I-associated mechanisms that induce type I IFN. Although type I and type III IFNs bind to different receptors, they share a common end-effect, activation of the JAK-STAT signalling pathway and induction of ISGs. The cellular expression of type III IFNs is much more restricted than that of type I IFNs, with most expression occurring at cells within mucosal surfaces where respiratory viruses such as influenza are first encountered (310, 311).

In humans, several hundred interferon-stimulated genes (ISGs) exist and their expression is up-regulated by type I IFNs. The cytoplasmic proteins MxA and MxB are GTPases that recognise viral RNP in humans. MxA targets viral nucleocapsid-like structures and prevents it from entering the nucleus of the infected host cell, helping to restrict viral replication. In mice, similar Mx proteins exist, Mx1 and Mx2, but their mechanisms of action differ from MxA and MxB (312-314). Standard laboratory mice lack the Mx1 gene, but genetically engineered homozygotes expressing Mx1 are protected from lethal infection with influenza strains including HPAI H5N1 and 1918 H1N1, whereas wild-type mice experience significant mortality following infection (315).

2'-5' Oligoadenylate synthetase (OAS) is another important innate antiviral defence protein, again found in the cytoplasm. When type I IFNs stimulate IFNαβ-receptor following infection, expression of OAS proteins is increased. OAS recognises dsRNA and converts ATP into 2'-5' oligoadenylate, which in turn activates ribonuclease RNaseL and causes cleavage of both viral and cellular ssRNAs, inhibiting protein synthesis (316, 317).

Protein kinase R (PKR) is the third, well-recognised ISG protein, with diverse antiviral functions. Mice lacking PKR are rendered susceptible to several viruses, including influenza virus. PKR is a serine/threonine kinase phosphorylates the a-subunit of eukaryotic translation factor 2a (eIF2a). Binding of viral dsRNA to PKR within the cytosol inhibits translation and inhibits protein synthesis (of both cellular and viral products). PKR has additional functions, including modulation of NF-κB signalling pathways, apoptosis, cell cycle and autophagy mechanisms. It also stabilises IFN-α and IFN-β mRNA (318-320).

1.8.4 Pre-existing soluble inhibitors

Influenza virus must contend with a number of antiviral defence mechanisms from the moment in enters the host airway. Soluble innate effectors found within the airways include mucins, gp-340 and pentraxins. Mucins, such as mucin 5AC glycoprotein (MUC5AC), are high molecular weight glycoproteins produced by airway epithelial cells. Secreted mucin contains a variety of anti-viral peptides and also sialic acid residues that act as receptor decoys to which influenza binds. In mice, infection with different influenza A strains induces MUC5AC expression at RNA and protein levels (321). Collagenous lectin molecules called collectins are also found in blood and respiratory secretions during infection and act as pattern recognition molecules. Examples include surfactant protein A (SP-A), surfactant protein D (SP-D) and mannose binding lectin (MBL). Collectins bind to and neutralise influenza virus in early infection, but also bind to and modulate the activity of a variety of cells, including neutrophils. Influenza A(H1N1)pdm09 virus has been demonstrated to be resistant to the direct antiviral actions of MBL *in vitro* (322) although interestingly, MBL knockout mice do not lose as much weight, nor do they have such marked proinflammatory cytokine and chemokine responses as wild-type mice, following infection (323). However, in an observational study of children with A(H1N1)pdm09 virus infection, MBL levels were lower in those with critical illness, compared to those who were not critically ill (324). MBL is a unique collectin in that it promotes complement activation. Although the role of complement as part of the human innate response to influenza is unclear, C3 and C5 are involved in T cell priming and $CD8^+$ T cell responses in mouse models of influenza virus infection (325).

1.8.5 Innate cellular responses

Patrolling alveolar macrophages also recognise and phagocytose influenza virus. Once influenza had bound to airway epithelial cells, a variety of innate cells are mobilised to the site of infection, following activation of the inflammasome and release of IL-1β. The cells include DCs, pDCs, monocytes (which can differentiate into DCs and macrophages), NK cells and neutrophils (326).

Monocyte-derived DCs stimulate cytotoxic T-cells into killing infected cells. NK cells assist with clearance of virus and macrophages and neutrophils phagocytose dead, virus-infected cells (327).

Dendritic cells exert their innate immune actions against influenza through activation of PRRs, but some DCs also mature and migrate (via up-regulation of CCR7 chemokine receptors) to draining lymph nodes. Here, they prime naïve T cells and B cells through antigen presentation, activate them through co-stimulation, and promote differentiation by secreting specific cytokines. Thus, DCs are unique in having roles in both innate and adaptive immune responses (328-330).

1.8.6 Innate responses and their roles in the pathogenesis of influenza illness

Innate immune responses to influenza virus are responsible for the commonly described clinical manifestations of influenza illness, such as fever, myalgia and the various respiratory tract symptoms. Infection of healthy volunteers with seasonal influenza strains has revealed relationships between symptoms, viral load and levels of soluble immune mediators in different biological compartments. Following infection, peak symptoms tend to coincide with peak viral load, which is also accompanied by a peak in pro-inflammatory IL-6 and IFN-α in nasal secretions. As the symptoms and virus load subsequently decrease, levels of IL-8 and TNF-α in nasal secretions peak. In these studies, blood levels of the same increased mediators are generally lower than those seen in nasal secretions, or in the case of certain mediators, increases are absent in blood (331, 332).

In naturally-occurring seasonal and A(H1N1)pdm09 virus infection, observational studies have demonstrated that blood levels of pro-inflammatory IL-6, IL-8 and CXCL-10 (IP-10) peak at the same time as peak viral load (measured in nasal secretions), typically on day 3-4 of symptoms. In some studies, increases in IL-6 have been shown to correlate with the severity and duration of illness (333).

In adults in the early stages of A(H1N1)pdm09 virus infection, elevated serum levels of pro-inflammatory innate mediators (IP-10 [CXCL-10], MCP-1 and MIP-1β) have been reported in mild and severe illness, consistent with findings in patients with seasonal influenza. Elevated levels of serum IFN-γ, IL-6, IL-8, IL-9, IL-17, TNF-α, IL-15 and IL-12-p70 were seen in hospitalised patients, but not in community patients with mild illness (334). Furthermore, in the same study, elevated IL-15, IL-12p70 and IL-6 appeared to be a "hallmark" of critical illness. Interestingly, an observational study comparing mediator profiles in patients hospitalised with seasonal influenza infection and those with A(H1N1)pdm09 virus infection demonstrated that Th1 and Th17 cytokines may be *suppressed* in patients with A(H1N1)pdm09 virus infection. The investigators proposed that this results in weakened adaptive immune responses in A(H1N1)pdm09 virus infection (335).

In models of infection of primary human cells *in vitro* (alveolar epithelial cells, bronchial epithelial cells, dendritic cells peripheral blood-derived macrophages), replication of A(H1N1)pdm09 virus is similar to that seen with seasonal influenza infection, as are the innate soluble immune mediator responses (336). By contrast, many pro-inflammatory mediator responses tend to be more marked in most, but not all, of the different cell type models of HPAI A(H5N1) virus infection (136, 137, 337). In ferret models, infection with HPAI A(H5N1) virus or 1918 A(H1N1) viruses is associated with more severe lung pathology than is seen with A(H1N1)pdm09 virus or seasonal influenza infection. With HPAI H5N1 virus infection, interferon signalling e.g. IP-10 (CXCL-10)-associated pathways, and other innate pathways are more markedly up-regulated than with A(H3N2) seasonal influenza virus infection, whereas T-cell and B-cell signalling pathways tend to be more markedly down-regulated in HPAI A(H5N1) virus infection (315, 338).

Similar to infection in ferrets, HPAI A(H5N1) virus and 1918 A(H1N1) virus infections in mice are associated with the greatest viral replication in lung tissue, as well as more marked pro-inflammatory cytokine induction and lung pathology, compared to A(H1N1)pdm09 virus infection. However, all three viruses still demonstrate increased pathology and cytokine induction overall, when compared to infection with seasonal strains. Marked macrophage and polymorph infiltration, along with markedly increased levels of IL-6, IFN-γ, MCP-1 and MIP-1α, is seen in the lungs of mice of infected with 1918 A(H1N1) or HPAI A(H5N1) viruses (339). Mice knockouts lacking functional IL-6 or MCP-1 do not differ from wild-type mice following infection with HPAI A(H5N1) virus, probably because of redundancy of innate immune signalling pathways i.e. influenza activates different innate pathways, but because the pathways lead to a common end-effect, interference with a single pathway does not affect the overall end-point e.g. survival (340). In macaque models, similar trends to other animal models are seen overall, with the severity of infection, lung pathology and pro-inflammatory cytokine response being greatest in HPAI A(H5N1) virus infection and 1918 A(H1N1) virus infection, followed by A(H1N1)pdm09 virus infection, followed by infection with seasonal influenza virus (28, 341).

Immune homeostasis and anti-inflammatory pathways have also been studied in mice infected with influenza virus. For example, the CD200R receptor of pulmonary alveolar macrophages (also present on dendritic cells) is activated by CD200 ligand expressed by lung epithelial cells, and constitutive expression of the CD200R is high. Receptor activation helps regulate activation and function of pulmonary alveolar macrophages, decreasing secretion of IL-6 and TNF-α. CD200 knockout mice have expanded populations of these macrophages. Following influenza virus infection, viral clearance is greater but pro-inflammatory cytokine production, cellular infiltration of lung tissue,

weight loss and mortality are all greater in the knockout mice compared to wild-type mice. Infection in wild-type mice up-regulates CD200R expression, especially in pulmonary alveolar macrophages. Furthermore, administration of CD200R agonist to wild-type mice decreases lung pathology and weight loss. Overall, this suggests that the CD200-CD200R mechanism helps to prevent 'bystander' inflammatory damage when the innate immune system attempts to eliminate influenza virus from infected cells (342). Effector T cells are also known to infiltrate lungs and secrete the anti-inflammatory cytokine IL-10, following influenza infection. Blockade of IL-10 receptor in mice infected with influenza virus results in decreased survival, but without an effect on clearance of virus (343).

1.9 The adaptive immune response to influenza virus infection

1.9.1 Antibody-mediated (humoral) immunity

The neutralising antibodies that are produced during influenza virus infection help to reduce replication of virus and infection of uninfected cells, in addition to facilitating clearance of the invading pathogen. Furthermore, through memory B cells and plasma cells, long-lasting immunity against the invading strain ensues (homosubtypic protection), at least in the normal host. At a population level, the same neutralising antibodies, primarily targeting viral HA, exert significant selective pressure on circulating viruses of the same subtype, leading to antigenic drift. Therefore, an influenza-experienced individual may be infected by a 'drifted' virus of the same subtype in the future. However, more broadly cross-reactive antibodies, directed against conserved regions of influenza virus, are also produced, which may perhaps prevent future severe illness, at least against drifted strains of the same virus subtype (52).

Following infection and the initiation of the innate immune response, B-cell responses are initiated in lymph nodes draining the primary sites of infection i.e. areas within the respiratory tract. Antibody-secreting plasma cells are formed quickly in these lymph nodes (during active viral replication), although in mice, antibody-secreting cells are detected later in the spleen and lung. In humans, plasmablasts are detected six to eight days after influenza infection or vaccination, but serum antibodies tend not to be detected for two to three weeks. Specific antibodies may remain detectable in serum for years (160), with secreting cells being produced by bone marrow and lung tissue (344-346). Memory B cells are also maintained in many tissues long-term and can respond rapidly and differentiate, leading to the production of antibody, following re-exposure to virus. Memory B cell responses are not routinely measured when assessing immunity against influenza, but plasmablasts that are thought to represent activated memory B cells can be seen transiently in the blood of vaccine recipients (347, 348).

In active influenza virus infection, virus-specific IgA is secreted by plasma cells within the respiratory tract mucosa, protecting epithelial cells from infection (349, 350). Although IgA-deficient mice do not have significantly worse outcomes compared to wild type mice during influenza virus infection (351), IgA is a component of antibody mediated immunity in influenza, albeit not an essential one. This is supported by the observation that IgA-deficient patients do not suffer an excess burden of influenza-related morbidity and mortality (352, 353).

In contrast to the upper respiratory tract, the lower respiratory tract lacks a lamina propria and therefore also lacks plasma cell population capable of producing IgA, so IgG contained in inflammatory transudates (derived from blood) provide protection (354). IgG can be found in abundance in lung secretions following infection in mice. Levels of specific IgG in blood correlate highly with vaccine-induced protection from influenza (355). Specific IgG-producing cells are also found in lymph nodes draining the respiratory tract during acute infection, and can be found later in spleen, lung and bone marrow (356). IgG antibodies help clear influenza virus through opsonisation (encouraging phagocytosis by alveolar macrophages) and by facilitating NK cell clearance. Both mouse and observational human studies suggest that the early appearance of IgM in serum correlates with a subsequent, robust and neutralising IgG response following class-switching. The precise roles of specific IgM in influenza infection in humans remains unclear, although mouse studies suggest a direct role in virus elimination, perhaps by complement-mediated cell lysis (354).

1.9.2 Cell-mediated immunity

In addition to the antibody-mediated arm of the adaptive immune response to influenza virus infection, cell-mediated immunity (CMI) also has an important role in limiting disseminated viral replication, enhancing clearance of virus from the host and ultimately, decreasing disease severity. The key effectors of this response are thymus-derived $CD4^+$ and $CD8^+$ T lymphocytes These cell types recognise peptide fragments – derived from intracellular proteins of infected cells – bound to class I and class II major histocompatibility complex (MHC), respectively (357).

Antigen-specific T cells recognise peptide-MHC complexes via clonally expressed T-cell receptor $\alpha\beta$ heterodimers. There is a large and diverse repertoire of TCRs, regulated in an individual by thymic selection to maintain balance between recognition of “foreign” versus “self”. When influenza infection occurs, naïve T cells are exposed to peptide-MHS complexes presented by migrated respiratory-tract DCs. This is facilitated by a cocktail of soluble immune mediators and takes place within lymph nodes draining the primary site of infection. The end result is activation of naïve and memory antigen-specific $CD4^+$ and $CD8^+$ T-cells (358, 359).

Once activated, these T cells undergo clonal expansion. In mice, an expanded pool of antigen-specific $CD8^+$ T cells can be seen – initially in lung and then in lymph nodes – as virus load begins to decrease, typically between days five and eight of infection. After peaking at around day ten, $CD8^+$ numbers decline, but a population of memory T cells persists, providing a rapid and specific response to future virus challenge (360, 361). During influenza virus infection, $CD8^+$ cytotoxic T lymphocytes (CTLs) are the principal population involved in clearing virus, but $CD4^+$ T cells may also assist with virus clearance, in addition to helping B cell responses and promoting cytotoxic T cell memory (362, 363). Following presentation of peptide-MHC1 complex to the TCR, an activated CTL releases cytolytic granzymes and perforin on to the infected cell, leading to its apoptosis (364, 365). Cytokines are also released by activated CTLs, including IFN-γ, TNF-α, which have antiviral and pro-inflammatory effects, and IL-2, which encourages the proliferation and survival of CTLs (366).

Both activated $CD4^+$ and $CD8^+$ cells produce cytokines at the sites where antigen is presented. The cytokines released by $CD4^+$ T cells influence the actions of DCs, trigger isotype switching of B cells, promote germinal centre formation (via IL-21) and B cell affinity maturation, as well as promoting the development of long-lasting B cell memory (367). Virus-specific $CD4^+$ cells acting in concert with IL-27-producing innate immune cells regulates production of IL-10 by lung-residing, virus-specific $CD8^+$ T cells, important in preventing excessive inflammation in response to infection (368).

1.9.3 A model of immune responses to influenza virus infection

The sequences of classical innate and adaptive immune responses to influenza virus infection have been described individually, but they should be viewed as part of a continuum of the events involved in influenza illness. Following established viral infection and replication, innate responses transition into adaptive responses, around the time that illness (and acute lung injury, if present) reaches a peak. As the immune system switches from a process of viral eradication and inflammation to a state of damage-repair, two main outcomes are proposed: resolution and recovery, or on-going inflammation and illness. The latter outcome could represent secondary bacterial infection, whereby the profound immune innate immune response to the initial pathogen (influenza) may have left the host vulnerable to attack by other pathogens (bacteria). This model is represented in figure 1.10.

The turning-point for the overall outcome of infection may come well before this time, however. Indeed, there may be a succession of events that culminate in a particular outcome. Furthermore, the published evidence suggests that there is more than one clinical phenotype for A(H1N1)pdm09 virus infection and this suggests that different mechanisms of pathogenesis and severity affect different patient populations. It seems increasingly unlikely that a single mechanism will explain severe illness, although a significant mechanism may be identified in a specific sub-population e.g.

patients with asthma. IFITM3 appears to be a non-redundant component of the innate immune response and the rs12252-C polymorphism of *IFITM3* has demonstrable, biological relevance, having been identified in a large proportion of Chinese patients with influenza and correlating with severity of illness in this group (369). However, in the small proportion of MOSAIC patients studied to date, the polymorphism was only present in a very small proportion of those with severe illness (297), suggesting that factors other than this polymorphism will be involved in pathogenesis of severe influenza in the majority of patients in the MOSAIC study.

In patients who lack underlying risk factors for complicated illness, and excluding the influence of extrinsic factors such as antiviral and antibiotic therapy, it may be that a “perfect storm” or a “lock and key” scenario is at play, operating at the level of the affected individual. For example, a host with a subtle variation in host immunity (perhaps inherited as a genetic trait, but one that does not predispose to recurrent severe infections) may be infected by an influenza virus with a discrete mutation or set of mutations that leads to a profound innate, interferon-dominated immune response. This response may effectively eradicate the virus, but paradoxically may lead to excessive inflammation and perhaps an opportunity for colonising bacteria to subsequently invade respiratory tract epithelium.

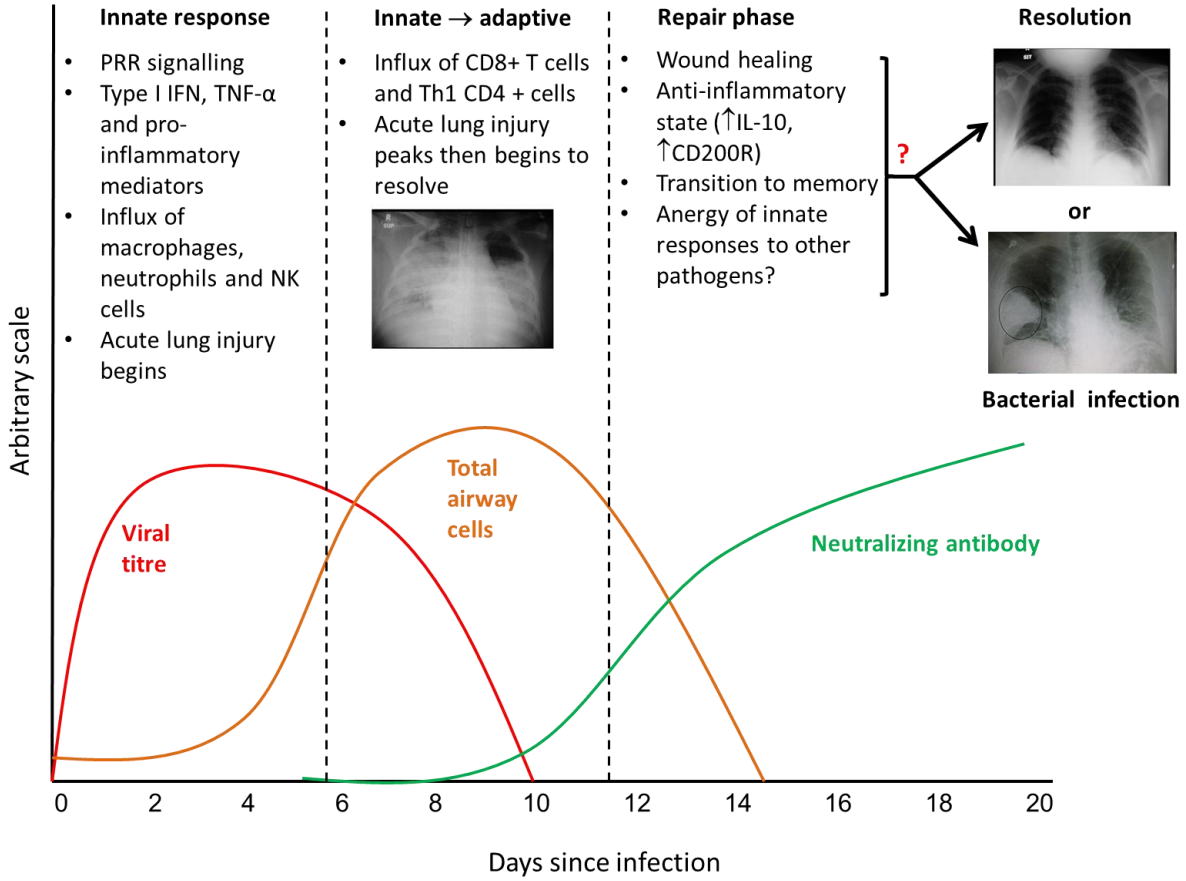


Figure 1.10 A model of the immune response to influenza virus infection and its role in pathogenesis

Following infection of respiratory epithelial cells, effective virus replication is established. Pattern recognition receptors (PRRs) are activated and the innate immune response attempts to control virus replication. Virus replication in the normal host peaks around day 3 of infection. The innate response is both antiviral and pro-inflammatory, and may lead to inflammation in lung and other tissues. Transition to an adaptive immune response follows the peak of the innate response, with an influx of CD4+ and CD8+ T lymphocytes into affected tissues. In an unvaccinated individual naïve to influenza, specific antibodies are not produced until day 8-10 of infection, after viral load has decreased. If lung injury has occurred, it peaks around this time. The overall response to infection now switches to repair, with anti-inflammatory responses including production of IL-10 and up-regulation of CD200R. Although the expected outcome is resolution of illness and lung injury (where present), on-going inflammation is seen in others, sometimes with invasive bacterial infections including pneumonia.

1.9.4 Background

The Mechanisms of Severe Acute Influenza Consortium was established in 2009, following the emergence of A(H1N1)pdm2009 virus and a need to improve understanding of the pathogenesis of severe influenza disease in humans. The Consortium was co-ordinated by the overall Lead Investigator, Peter Openshaw, and the Centre for Respiratory Infection at Imperial College London. MOSAIC established a network of 11 hospitals in London and Liverpool where hospitalised patients could be recruited, along with a network of nine specialist research centres (45 investigators) across England and Scotland that could analyse samples collected from patients with influenza-like illness. The Consortium was launched in time to capture the UK's second pandemic wave (winter 2009/10), and also the winter of 2010/11, the so called "third wave" of A(H1N1)pdm09 virus activity. A total of 255 adults and children were recruited to the MOSAIC prospective observational cohort study over these two periods. Healthy adult and paediatric controls were also recruited.

MOSAIC operated in three phases. Phase 1 involved study design, protocol development, establishment of networks and infrastructure, establishment of appropriate funding and acquisition of regulatory and ethical approvals. This phase took approximately seven months to complete, from May to November 2009. Phase 2 consisted of the active recruitment of patients, collection of samples and clinical data. This phase utilised existing clinical research networks, including support from National Institute of Health Research (NIHR) Comprehensive Local Research Networks (CLRNs) in London and Liverpool, maximising patient recruitment and sampling. Phase 3 involved sample analysis and clinical data analysis. This phase commenced at the same time as the first patients were recruited during phase 2 and continued after phase 2 had been completed.

Funding for the study was provided via a joint award (090382/Z/09/Z) from the Wellcome Trust and the Medical Research Council. Support was also provided by the Imperial College London Comprehensive Biomedical Research Centre (cBRC). Patient recruitment ceased in early 2011 and the study will close in July 2014. A biobank of residual samples was established and the clinical database is maintained, so additional analyses and studies may be performed in the future (with appropriate ethical approval in place).

1.9.5 MOSAIC's objectives

MOSAIC's goal is to allow the enormous variation in severity of influenza to be understood during a single, relatively monotypic influenza outbreak in a population with little prior immunity and so to gain insight into pathogenesis that will enable novel treatments to be found.

The original objectives of the Consortium were:

1. To develop a UK-wide research network through which a number of distinct but complementary and linked pathogenesis studies can be co-ordinated

2. To establish links with other national and international networks conducting pandemic influenza research

3. To coordinate the rapid collection, storage and sharing of data and samples from patients

4. To refine study design based on initial experience

5. To rapidly disseminate results of influenza pathogenesis studies, where possible, with those managing patients

6. To plan future trials and research priorities on the sample-archive and clinical database

1.9.6 An integrated approach to investigating influenza pathogenesis

It is assumed that multiple factors contribute to the pathogenesis of influenza, broadly falling into three categories: host, pathogen and co-pathogen factors. Rather than study a single category, MOSAIC studied all three categories to attempt to form an integrative model, linking contributing factors from three different components. To achieve this, different work packages were formed to investigate host (cellular immunology, soluble immune mediator responses, transcriptomics and genomics), pathogen (influenza virus genetic and antigenic characteristics, viral shedding and viral load) and co-pathogen (bacteria and non-influenza viruses) that may be associated with disease severity in humans. In addition to detecting population-level signals, MOSAIC's design allows full linkage of diverse information on a case-by-case basis, thereby enabling the impact of genetic variations on influenza illness phenotype to be determined. Moreover, intensive sequential sampling allows clinical and laboratory data to be linked in the same manner.

1.10 Aims and objectives of this study

The main objectives of this study were to characterise the whole blood transcriptional responses and the soluble immune mediator and biomarker responses in patients infected with influenza virus and other pathogens causing influenza like illness, within the context of a pandemic caused by a novel influenza virus.

Our original hypotheses were:

1. Whole blood transcriptomic profiles in patients with influenza virus infection are distinct from those seen in non-influenza ILI

2. In patients with influenza virus infection, whole blood transcriptomic profiles differ according to the severity of illness

3. Changes in whole blood transcriptomic profiles are associated with bacterial co-infection in patients with influenza virus infection

4. Soluble immune mediator/biomarker profiles in patients with influenza virus infection are distinct from those seen in patients with ILI

5. In patients with influenza virus infection, specific soluble immune mediator/biomarkers are associated with different severities of illness

To address these hypotheses, clinical research protocols and related documents were developed, patients were recruited to the MOSAIC study and sequential samples of their blood and respiratory tract secretions were collected. Samples were also obtained from appropriate healthy controls. High-throughput microarray analysis of RNA extracted from whole blood was performed and gene expression analysis was undertaken, following an analytical approached used by collaborators to successfully study gene expression profiles of other infections in humans, including seasonal influenza and tuberculosis. Soluble immune mediators in serum/plasma and respiratory tract secretions (nasopharyngeal aspirate, nasal synthetic absorptive matrix eluates and broncho-alveloar lavage fluid) were measured using an electrochemiluminesence multiplex assay and enzyme-linked immunoassay (ELISA). Additional biomarkers were measured in serum and plasma by collaborators using validated clinical assays. Results from these assays were interpreted using MOSAIC clinical data collected using an established clinical data collection tool for pandemic influenza (FluCIN). Data collection was refined by the development of a supplementary case report form, introduced during the second, larger period of patient recruitment.

Retaining the original focus of MOSAIC, severe influenza pathogenesis in humans following the emergence of a novel pandemic strain, the following aspects of the immune response to influenza virus infection in hospitalised adults have been examined:

1. The clinical characteristics of patients hospitalised with influenza and other causes of ILI

2. The whole blood transcriptomic response to influenza virus and other causes of ILI during acute illness and how gene expression profiles correlate with selected clinical variables

3. The soluble immune mediator and biomarker response to influenza virus and other causes of ILI during acute illness and how these correlate with selected clinical variables

This thesis has chapters covering each of these areas.

Chapter Two

2 Materials and methods

2.1 Participants

Patients with influenza-like illness (ILI) and adult healthy controls (HCs) were recruited after informed consent (appendix 1), declaration of opinion from a personal or nominated consultee (for adults who lacked capacity; appendix 2) or parental consent/assent (in those under 16 years of age). The study protocol, age-appropriate consent/assent forms, patient information leaflets, study promotional materials and all protocol amendments were approved for England-wide use by the Northwest London Research Ethics Committee. Local research and development approvals were also obtained for each participating NHS Trust. Clinical investigators and recruitment staff received comprehensive training, including training on sampling methods and clinical data collection, and followed International Conference on Harmonisation Good Clinical Practice guidelines (370). All data and records are maintained according to NHS and Imperial College London data protection and information governance guidelines.

2.1.1 Patients

Hospitalised patients were recruited between 01 December 2009 and 03 March 2011. Patients (or their representatives) were approached for recruitment across five Imperial College London-associated hospitals (St Mary's Hospital, Hammersmith Hospital, Charing Cross Hospital, Chelsea and Westminster Hospital, and the Royal Brompton Hospital) and across four Liverpool University-associated hospitals in Liverpool and the Wirral (Royal Liverpool Hospital, Liverpool Women's Hospital, Alder Hey Hospital, and Arrowe Park Hospital). Seven of these hospitals have emergency departments and three offer specialist paediatric services. Additional specialist services are provided by several sites, including HIV medicine, oncology, haematology and transplantation medicine. Critical care is available at most of the centres and three hospitals provide paediatric intensive care, three have maternity high-dependency units and one (the Royal Brompton Hospital) provides specialist lung-injury intensive care including extra-corporeal membrane oxygenation (ECMO). Two geographically distinct cities were chosen with the aim of avoiding bias that may arise from difference in access to or types of health care services provided, region-specific differences in circulating pathogens and co-pathogens and regional differences in ethnicity.

There was no upper or lower-age limit for recruitment. Patients were approached for recruitment to the study if they met one of the following criteria:

1. Clinician-suspected influenza infection at admission to hospital or during an existing hospital admission for another indication

2. Influenza infection confirmed by NHS polymerase chain reaction (PCR) testing at admission to hospital or during an existing hospital admission for another indication

The only exclusion criterion was confirmed absence of influenza infection based on NHS PCR testing of an appropriate sample at the time influenza illness was suspected *and* prior to being approached for recruitment. If these patients underwent subsequent testing and were found to be positive, they were not included. To optimise recruitment, we did not apply restrictions based on day-of-illness at the time of recruitment. Since underlying health conditions are relevant to the development of severe influenza illness, patients with comorbidities were not excluded. History of prior treatment with influenza antivirals, antibiotics or receipt of influenza vaccination did not result in exclusion, since none of these interventions guarantee prevention of influenza infection or influenza-like illness.

To optimise recruitment further, instructions to notify study investigators of a suspected case were included in local clinical guidelines for the management of influenza at each site. Additionally, a referral hot-line and active case-finding was employed at London sites. Furthermore, to encourage recruitment and facilitate rapid diagnosis, MOSAIC staff at London sites also obtained diagnostic swab samples on behalf of the NHS Trust and delivered them to the appropriate clinical laboratories for immediate testing, where possible, to encourage referral of patients to the study and ensure that diagnostic testing was performed.

During both recruitment periods, clinicians generally followed the Health Protection Agency (HPA) clinical diagnostic algorithm for A(H1N1)pdm09 virus infection i.e. to suspect infection if the patient had a body temperature >38°C and the presence of two or more relevant symptoms e.g. cough, sore throat, or headache. Patients did not have to meet these criteria to be recruited to the study, however; any case clinician-suspected influenza illness could be referred for recruitment. Furthermore, the HPA guidance suggested influenza testing for any severe of life-threatening illness suggestive of an infectious process. This reflected an awareness that not all patients with confirmed influenza fulfilled the clinical diagnostic criteria, especially for those in certain at-risk groups e.g. immunosuppressed patients. Recruitment staff also worked closely with local NHS virology laboratories, reviewing the results of daily influenza PCR assays and approaching patients who had positive results but had not yet been referred to the research team. Again, this maximised

recruitment opportunities and ensured that the majority of patients presenting with influenza symptoms were recruited as close to the point of admission as possible.

2.1.2 Controls

Thirty-six healthy adult controls were recruited at Imperial College London and Chelsea and Westminster Hospital, between June and September 2011. Following consent, healthy controls were screened by direct questioning and were excluded if they had any underlying health condition including atopic conditions such as allergic rhinitis, were taking regular medications other than the oral contraceptive pill, smoked tobacco or consumed recreational drugs or an excessive amount of alcohol, or reported a history of influenza-like or cold-like illness in the preceding 4 weeks. Recent influenza vaccination did not result in exclusion, but vaccination histories were recorded, along with demographic data.

For transcriptomic analyses, it was necessary to recruit a larger number of healthy controls, matched to patients by age, sex and ethnicity. It was not possible to match each individual patient with a healthy control of the exactly the same age, sex and ethnicity. Therefore, we used selective recruitment of controls to ensure that the relative proportions of patients of a particular age range (using ten year intervals for adults), sex, and ethnic group were similar in the control group. The demographic summaries are shown later (see Table 4.1). Over 100 adult healthy controls were recruited by collaborating clinical staff from the MRC National Institute for Medical Research between 2010 and 2012 and their whole blood transcriptomics samples were shared with MOSAIC, in accordance with the terms of the NIMR study's ethics approval.

A small number of paediatric healthy controls were also recruited by researchers from the Department of Paediatrics at Imperial College London, St Mary's Campus, between 2011 and 2012. These children were recruited and screened as part of an existing paediatric study (IRIS), with ethics permission to collect samples for, or share samples with MOSAIC collaborators.

2.2 Clinical Data

2.2.1 Data collection tools

Clinical data from patients were collected using two main collection tools. Additionally, demographics and the outcome of clinical assessment at T3 were recorded in the subject's research folder. Age, sex and ethnicity were cross-checked between the documents and any discrepancies were resolved by deferring to those recorded in hospital databases. Screening details and demographics for 36 adult healthy controls were recorded on a dedicated case report form.

2.2.1.1 Influenza Clinical Information Network (Flu-CIN) form

All patients had a Flu-CIN form completed (appendix 3). This form was developed by a Department of Health committee of influenza investigators as an audit tool for the 2009/10 pandemic. The MOSAIC Lead Investigator, Prof Peter Openshaw, was a member of this committee. The form captured data including demographic characteristics, past medical history, pre-hospital medication, clinical presentation, inpatient care timelines, clinical findings on admission to hospital including baseline chest imaging, investigations and care escalation from levels 0 to 3, discharge and death. The forms were completed by trained staff following the Flu-CIN Investigators' completion instructions, populating data fields through retrospective case-note analysis and NHS electronic records where appropriate. Admission-discharge summaries and medical certificates of cause of death, as appropriate, were obtained to complete the outcome section. Daily assessment data were collected for the first 14 days of admission to a MOSAIC site or for the first 14 days from when influenza was suspected in the case of existing inpatients admitted for another reason. Where appropriate, transfer documents from referring hospitals provided details about the history of the presenting complaint and findings on initial admission to hospital.

2.2.1.2 MOSAIC 2010/11 Case Report Form (CRF)

Following recruitment during the 2009/10, it became apparent that many clinical data cannot be captured through retrospective case note analysis, because the data are not usually recorded by NHS staff. Therefore, an additional 2-page case report form was introduced during the 2010/11 to supplement the Flu-CIN forms (appendix 4). This form was completed at the time of recruitment and involved direct questioning and assessment of the patient, including data concerning pre-hospital antibiotic, antiviral and antipyretic use, antimicrobials received in the emergency department, smoking history, and influenza, pneumococcal and *Haemophilus influenzae B* vaccination history. Recruiters endeavoured to obtain height and body-weight measurements, but when this was not possible a visual estimate of obesity was made, using previously published pictorial assessment tools.(371, 372) The CRF also included a section that captured key clinical data relating to day of illness, severity of illness and underlying comorbidities. These data were not intended to be used as part of final analysis, but served as a real-time, rapid assessment of patient profiles to provide feedback to investigators during recruitment.

2.2.1.3 Obtaining missing influenza vaccination data

Vaccination histories were infrequently recorded in the hospital case notes and specific questioning about vaccination history was only introduced during 2010/11 (via the supplementary CRF). An attempt was made to retrospectively obtain vaccination histories from patients with missing data.

With permission from the ethics committee, 53 surviving patients who had been admitted to Imperial College Healthcare NHS Trust hospitals (or their guardian/representative) were contacted by mail, with a request to provide influenza vaccination histories (requesting the same information that was collected in the CRF). Permission was sought to contact the patient's general practitioner if the patient was unsure of their vaccination history. Thirty-seven patients did not reply. Ten patients replied that they were unsure about their vaccination histories and so their respective general practitioners were contacted by mail, but replies were not received. Six patients stated that they had never received vaccine and these findings were added to the vaccination section of the MOSAIC database.

2.2.1.4 Determination of health status at T3

For those who underwent T3 sampling, a clinical assessment form was completed, recording the location of the patient (ward inpatient, critical care inpatient or discharged) and their general health status (on-going influenza/ILI illness, new complications or recovered).

2.2.2 Clinical data management

2.2.2.1 MOSAIC Database

The MOSAIC Data Clearing House developed a bespoke MOSAIC database, using Microsoft Access to collate all clinical data from all sources. Over 3,000 variables were defined and each was assigned an identifier, which was documented in a codebook. Twelve trained research staff following a standardised protocol, with access to a trained supervisor to address specific queries, entered data.

2.2.2.2 Consolidation of data from different sources

For 2010/11 patients, Flu-CIN and the MOSAIC CRF recorded some of the same clinical variables. A meticulous search for duplicate and conflicting entries was performed. Where discrepancies arose, these were investigated individually. Obvious errors e.g. a calculated 'adult' age for a paediatric subject in Flu-CIN were investigated by referring back to the original case notes. Otherwise, Flu-CIN data took precedence over CRF data for a single variable at all times e.g. data of onset of illness. Where a variable had been captured by the CRF and not by the Flu-CIN tool, the CRF data were used for that particular variable e.g. influenza vaccination history.

2.2.2.3 Clinical data validation and quality control

Records of who entered data into the database were maintained throughout. Validation of entered data was performed by a separate member of staff. Validation of accuracy of data entry from

information provided on the form was completed on the entire dataset following a strict protocol and with ample supervision. Any change, query or issue identified at validation was recorded. All queries were discussed with the team lead and head supervisor and necessary changes were made. While data entry validation ensured accuracy of replicating the data form, it did not necessarily check accuracy of the data gathered. Given the volume of data collected on the FLUCIN form, validation of transcription of the full form from the notes was not possible; however, key information was checked for the following data: oxygenation; onset of illness; age; gender; ethnicity. These variables were entered into their own databases and merged with the final clinical dataset in the statistical package (Stata 12MP, StataCorp, Texas, USA).

2.2.2.4 MOSAIC Severity Score

Existing scoring systems for pneumonia, sepsis and multi-organ dysfunction were assessed for suitability for the scoring severity of illness in MOSAIC subjects. Two meetings were convened to review available literature and identify existing scoring systems were tested against Flu-CIN data from 160 MOSAIC patients (adults and children). For non-ICU patients, Community Assessment Tools (CATs) (373, 374) and Pandemic Medical Early Warning Score (PMEWS)(375) For ICU patients, the Acute Physiology and Chronic Health Evaluation II (APACHE II)(376) scoring system was evaluated. The outcome measure of the analysis was ability to predict intervention/clinical outcome. Only CATs could be assessed in both adults and children. Scores were initially tested using data from the time of presentation to hospital and the point of maximum severity of illness.

Data required for calculation of scores were frequently missing. The results of the analysis revealed that Flu-CIN data available for the subset were insufficient to calculate CAT, MEWS and APACHE II scores. In addition, the review group agreed that although these established scores are of value in predicting interventions and outcomes, a more pragmatic approach to specifically determine severity of illness in a given patient was required. Since published series demonstrate that severity of illness in A(H1N1)pdm09-infected patients is driven primarily by respiratory failure, MOSAIC developed its own severity scoring system specifically for use in this study. This four-point scale, whereby the severity grade is determined by the presence or absence of respiratory compromise and the interventions required to correct any respiratory compromise, is described in detail in chapter 3.

2.2.2.5 Clarification of comorbidities

Occasionally, comorbidities documented in Flu-CIN were not clear in terms of the actual condition or which World Health Organisation risk group category the illness belonged to. A panel of senior

clinical investigators reviewed these cases, with referral back to the original case notes where necessary.

2.2.3 Statistical analysis

Statistical analysis of MOSAIC clinical data was performed using Microsoft Excel (Microsoft Corporation, Seattle, USA), Stata 12MP (StataCorp LP, Texas, USA) and GraphPad Prism version 5.00 (GraphPad Software, San Diego California USA). Analyses including chi-square analysis and Fisher's exact test for comparison of proportions and t-tests, Mann-Whitney rank sum, and sign rank tests for comparisons of central tendency. The individual tests used and reasons for the chosen test are provided in the relevant results chapters.

2.3 Sampling

2.3.1 Development of sampling schedules, protocols and sampling kits

New sampling protocols were developed to acquire standardised, serial samples. Sampling was designed to be easy to perform and require minimal training. Separate clinical protocols and sampling schedules were designed for adults and children, and also to patients who were clinically fragile e.g. patients requiring intensive care. Additional considerations were made to minimise the risk of spreading infection to other patients and staff.

To facilitate this process, pictorial standard operating procedures (SOPs) were developed for research staff, and kits containing all sampling equipment and consumables required for each time point were prepared in advance, in London and Liverpool. Advice was received from fellow investigators with experience in conducting clinical studies and adaptations were made to the protocols and sampling schedules where appropriate. Adaptations were also made following the first season of recruitment (2009/10). For each subject, detailed records were completed at the time of sampling, including samples taken and time of sampling, time of delivery to the specimen reception and any deviations from the protocol. On-site training was provided in London and Liverpool to encourage standardisation. When non-research clinical staff were required to obtain samples (e.g. sputum, urine, stool), specific instructions were provided, along with guidance on how to temporarily store samples and notify the research team that samples had been collected.

All patients underwent sampling within 24h of recruitment to the study, the first time point (T1). This time point was intended to be as close to the time of admission to hospital as possible for the majority of patients recruited. Additionally, all patients underwent clinical diagnostic sampling for influenza, referred to as T0 sampling. The T0 sample may have been taken by NHS clinicians, or by

researchers on behalf of the clinical team. T0 may have been taken prior to T1 or at the same time as T1 (see additional note on diagnostic sampling in children, below). Occasionally, T0 may have been taken shortly after T1. Upper respiratory tract samples e.g. flocked nasal/nasopharyngeal swabs in universal transport medium or nasopharyngeal aspirates (NPAs) were the typical T0 diagnostic sample obtained by clinicians and/or MOSAIC research staff. Where appropriate and at the discretion of the attending clinician, lower respiratory tract diagnostic samples e.g. BAL fluid or endotracheal aspirates, may have been taken alone or in addition to upper respiratory tract samples, to establish the diagnosis.

Subsequent sampling differed between the two recruitment seasons. During the 2009/10 recruitment period, subjects only proceeded to the second acute time point, T2 (48-72h from recruitment), if influenza virus RNA was detected by PCR in the T0 sample; all influenza-negative ILI cases only provided T1 samples. During the 2010/11 recruitment period, all subjects were able to provide samples through to the third and final time point, T3, irrespective of T0 influenza infection status. T3 was designed to be a convalescent sampling time point, ideally taken at 4-6 weeks from T1. However, a decision was taken during recruitment to obtain T3 samples from patients who remained in hospital, predominantly those who remained critically ill and were not expected to recover. Furthermore, due to limited resources and for logistical reasons, some T3 sampling was allowed to take place beyond 6 weeks. The health status at T3 was always recorded, along with the date of sampling.

Additionally, during the 2010/11 season, the requirement for flocked viral throat swab sampling of every patient was removed for logistical reasons. The option to obtain an endotracheal aspirate (ETA) at each time point from invasively ventilated patients, in addition to upper respiratory tract samples, was also introduced during the 2010/11 recruitment period. Extra volumes of sodium heparin blood were also obtained at T3 during 2010/11 recruitment, for collection of peripheral blood mononuclear cells (PBMCs).

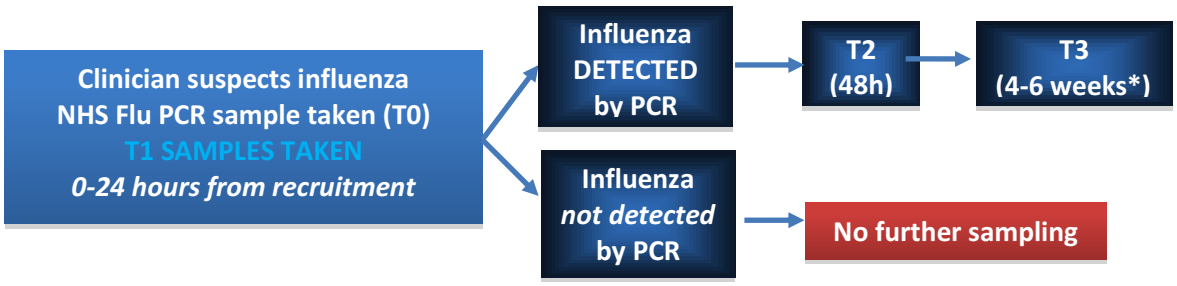
Certain samples were obtained only if they were produced by the patient (stool, urine and sputum) or were obtained for clinical reasons and an additional sample volume could be spared for research e.g. bronchoalveolar lavage (BAL) fluid. These samples were not linked to a specific time point, but date and time of collection was recorded. Bronchoscopy and BAL were performed according to the clinicians' own protocols. The solution used for BAL, the volume instilled and the volume returned was recorded in the subject's research folder where possible, along with the date and time of the procedure.

Results of research microbiological and virological analyses, along with cellular immunology profiles, may have clinical relevance that could impact on the care of the patient, even when not performed in real-time i.e. during the patient's hospital episode relating to their influenza/ILI. Therefore, a reporting mechanism was established to notify clinicians of clinically relevant results, following review of results by a designated senior investigator. All such events were recorded in the subject's research folder, as appropriate.

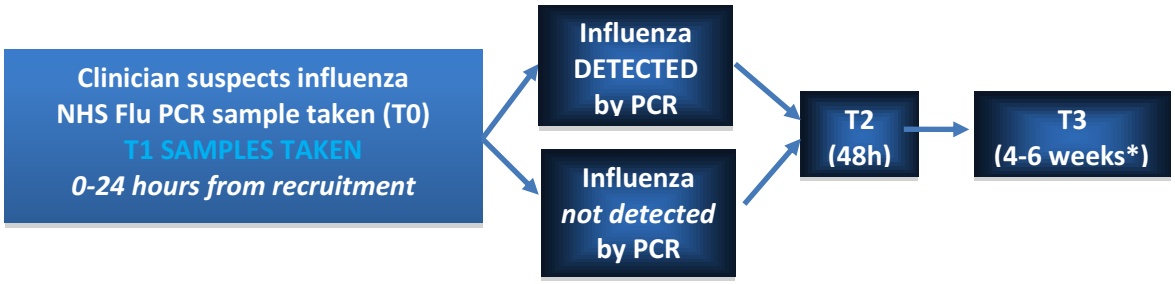
2.3.2 Sampling of adults

An overview of the primary clinical samples obtained from adults is outlined in table 2.1. Researchers aimed to obtain full sample sets, but this may not have been possible in all cases due to clinical circumstances or for reasons of patient preference. Primary samples or aliquots were distributed amongst work package laboratories, as appropriate. Full details of sampling equipment, blood volumes and adult sampling standard operating procedures (SOPs) can be found in appendix 5. Figure 2.1 provides a summary of the sampling schedule for adult patients and the sample types obtained.

A



B



C

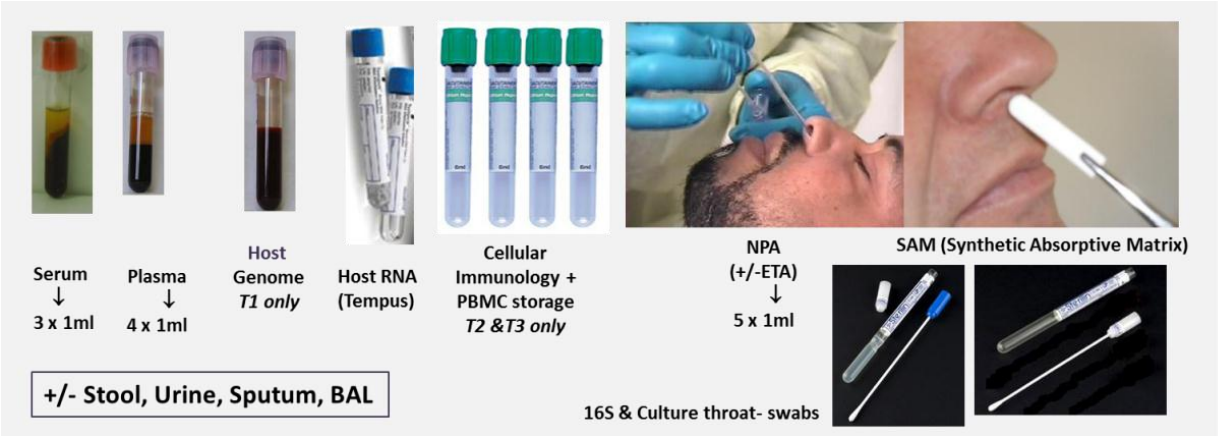


Figure 2.1 Sampling schedule for adult patients. Panel A shows the sampling schedule for patients recruited during the 2009/10 season and panel B shows the schedule for patients recruited during the 2010/11 season. Sampling occurred at two acute time points (T1 and T2) and at a late time point (T3). The target time for T3 was 4-6 weeks from T1, but may have been performed earlier or later in some patients (see chapter 3 for actual sampling times). Panel C shows the sample types specified in the sampling schedule for adults. Stool, urine and sputum samples were obtained where possible. BAL fluid was obtained when BAL was performed for clinical reasons and sample volume could be spared for research purposes.

Sample	T1 (0-24h from recruitment)	T2 (48-72h from recruitment)	T3 (≥4 weeks from recruitment)	Non time-point samples (if available)
Whole clotted blood	✓	✓	✓	
K2-EDTA blood	✓			
Sodium heparin blood		✓	✓	
Tempus blood	✓	✓	✓	
NPA	✓	✓	✓	
Nasal SAM	✓	✓	✓	
Flocked throat swab in UTM (2009/10 subjects only, unless NPA could not be obtained in a 2010/11 subject)	✓	✓	✓	
Throat swab in Amies medium	✓	✓	✓	
Plain rayon throat swab	✓	✓	✓	
ETA (intubated patients only, 2010/11 subjects only)	✓	✓	✓	
Sputum				✓
BAL fluid				✓
Urine				✓
Stool				✓

Table 2.1 Sample sets for adult patients recruited to MOSAIC
Sample sets differed according to the sampling time point. Sputum, BAL fluid, urine and stool were obtained when available. Adult healthy controls provided the same samples as outlined for T3 patient sampling.

2.3.3 Sampling of children

The sampling schedule in children was essentially the same as that for adults, but specific changes were made according to age and body-weight, which determined the blood samples and volumes of blood that could be obtained. A sampling hierarchy was developed, based on blood volume limitations recommended in the Manchester Clinical Research Network (MCRN) guidelines (REF). The hierarchy is outlined in appendix 6. Additionally, to minimise discomfort caused to paediatric subjects, researchers could omit the NPA sample at T1 if an NPA was also obtained for diagnostic purposes (the T0 sample); one single NPA sample would be divided for clinical diagnostic testing and for research requirements.

2.3.4 Sampling of healthy controls

Following informed consent, each of the 36 adult healthy controls recruited in London provided a single set of samples at recruitment. This sample set was identical to the T3 sample set obtained from adult patients. All procedures, including sample transport, storage and processing, were performed in an identical manner to the T3 sampling conducted in hospitalised patients. Additional Tempus blood samples were collected and processed from a separate group of healthy adult controls recruited by NIMR research staff following their own SOP and healthy-control screening, but obtained samples in the same way as samples collected for MOSAIC.

2.4 MOSAIC Sample Analysis

2.4.1 MOSAIC Laboratory Network

The analyses performed by partner laboratories are outlined in table 2.2, below. Selected laboratory outputs were used for the analyses presented in this thesis.

MOSAIC Laboratory	Analyses	Sample types analysed*
West of Scotland Specialist Virology Centre, Glasgow	Multiplex detection and PCR quantification of viruses and bacteria	Frozen NPA, flocked swabs in UTM, ETA, BAL fluid, EDTA-plasma, and stool
Simmonds Laboratory, Roslin Institute, Edinburgh	Molecular detection of rhinovirus, enterovirus and bocavirus	Frozen NPA, flocked swabs in UTM and BAL fluid
Virus Reference Division, Public Health England (formerly Health Protection Agency), Colindale, London	Influenza virus isolation and propagation; pyrosequencing for NAI resistance mutations; serum influenza antibody titration by haemaglutination inhibition and microneutralisation	Frozen NPA, flocked throat swabs in UTM, BAL fluid, ETA, and serum
Barclay Influenza Laboratory, St Mary's Campus, Imperial College London	Assessment of phenotypic consequences of genotypic changes in influenza viruses *in vitro* and in animal models	Frozen virus isolates
Wellcome Sanger Institute, Cambridge	Influenza virus genome sequencing	Frozen virus isolates and original clinical samples (NPA, flocked swabs in UTM, BAL fluid, ETA & selected T0 samples)
Baylor Institute for Immunology Research (BIIR), Dallas, USA	Whole blood RNA extraction & purification; microarrays	Frozen blood in Tempus tubes
Weatherall Institute of Molecular Medicine, Oxford	Cellular Immunology	Fresh blood in sodium heparin tubes (T2 and T3 only)
National Heart and Lung Institute (NHLI), Saint Mary's Campus, Imperial College London	Soluble immune mediator quantification; PBMC separation and storage	Frozen serum, plasma, NPA, SAM eluate, BAL fluid; fresh blood in sodium heparin tubes (T3 and HCs only)
Roslin Institute, University of Edinburgh, and Wellcome Sanger Institute	Human genomics (GWAS, SNP analysis & exome sequencing)	Frozen EDTA whole blood
Moffatt/Cookson laboratory, NHLI, Royal Brompton Campus, Imperial College London	16S ultra-deep sequencing for bacteria	Frozen plain rayon throat swabs; NPA; sputum

*frozen aliquots of original samples were provided where appropriate

Table 2.2 Summary of work undertaken and samples analysed by participating laboratories within the MOSAIC Network

2.4.2 General sample collection, handling and storage

Following collection, samples labelled with identifiers that were unique to the subject, time point and sample type were transferred from the clinical environment to local specimen receptions in cooled, sealed containers containing a single freezer gel pack that was not in direct contact with the samples. The target time to delivery to the specimen reception was between 30 and 60 minutes from the time of sampling, but on occasion may have been longer for logistical reasons. The date and time of sample collection was always recorded, as was the date and time of delivery, initial processing and, where applicable, freezing of the sample or aliquots. The clinical samples were initially processed and stored at three designated specimen receptions, one in London (Imperial College Respiratory Research Unit) and two in Liverpool (Institute of Child Health, Alder Hey Children's Hospital and Liverpool School of Tropical Medicine). All laboratories employed trained staff that followed a single SOP. Frozen samples were stored at -80°C and freeze-thaw cycles were avoided and documented. Freezers were monitored with remote alarms and had CO_2 back-up units fitted. Specialist couriers performed transfer of frozen samples between sites, with samples placed in temperature-controlled units containing dry ice.

MOSAIC analyses were performed by a number of collaborating laboratories in England, Scotland and the United States. A laboratory information management system (LIMS) was used to co-ordinate the storage and distribution of samples (LabCollector, AgileBio) within the laboratory network. Sample collection, handling and storage followed health and safety guidance published by Public Health England (formerly Health Protection Agency). Clinical sample processing in specimen reception laboratories was performed at Containment Level 2.

2.4.3 Laboratory analyses undertaken by the candidate

I performed initial specimen reception processing for the majority of clinical samples obtained from over 100 patients and healthy controls personally recruited by me. I performed the majority of 2009/10 soluble immune mediator assays (electrochemiluminescence and ELISA) and a proportion of the 2010/11 assays; a MOSAIC technician who was trained and supervised by me performed the remaining 2010/11 assays. I also performed urinary pneumococcal antigen testing on MOSAIC-obtained urine samples. Although I did not perform RNA extraction/purification and microarrays for the transcriptomics analyses, I have ensured that I am familiar with the processes involved and have observed the same processes being performed at NIMR. These processes were identical to those performed at the Baylor Institute for Immunology Research (working in collaboration with NIMR), which were used to generate the analyses of raw microarray data presented in this thesis. MOSAIC laboratories or local NHS laboratories performed all other laboratory analyses.

2.5 Whole blood transcriptomics

2.5.1 Sample collection and storage

Tempus blood RNA tube (Applied Biosystems/Ambion) was used to collect blood for transcriptomics analyses. The tubes were pre-filled with a solution containing guanidine hydrochloride, halide salt and proprietary agents that lyse whole blood cells and stabilize RNA upon mixing. The Tempus system was chosen over other proprietary collection tubes, such as PAXgene blood RNA tube, for several reasons, including investigator familiarity and published studies demonstrating superior RNA stability over time, superior RNA yields, low variability in expression measures and more consistent and predictable differential gene expression results *in vitro,* in validation studies of stimulated whole blood (377).

For adults, 3 ml of whole arterial or venous blood were collected into Tempus tubes (Applied Biosystems/Ambion) by standard phlebotomy (appendix 5). Despite using the Vacutainer collection system, care was taken to avoid over- or under-filling, as either can have a negative impact on cell lysis and RNA stability. Once the tubes were filled, blood was immediately and vigorously mixed with the reagent by inverting the tube ten times. Following rapid transfer of temperature-controlled containers to specimen reception laboratories, the uniquely-labelled samples were frozen at −80°C before RNA extraction. A frozen state was maintained throughout transfer to the collaborators' laboratory. Specimens were brought to room temperature only on the day of RNA extraction.

Proprietary collection tubes that can accommodate a smaller volume of blood and contain a smaller volume of Tempus reagent are not readily available. Therefore, modified collection tubes were developed in-house for sampling children for whom <3ml blood was available for transcriptomics sampling. Briefly, under aseptic conditions, 2 ml of Tempus reagent was aspirated from an adult tube and pipetted into a 15 ml polypropylene BD Falcon tube. A fill-line was marked on each tube and tubes were sealed and kept upright until use. Following phlebotomy, to maintain the manufacturer's recommended 2:1 ratio of reagent:blood, 1 ml of non-clotted venous or arterial blood added to the modified tube and mixing, transfer and storage of samples occurred as per the adult protocol outlined above.

2.5.2 RNA extraction and purification

RNA extraction and purification was performed at BIIR by following an established BIIR-NIMR protocol. RNA was isolated using 1.5ml mixed blood and the MagMAX-96 Blood RNA Isolation Kit

(Applied Biosystems/Ambion), according to the manufacturer's instructions. 250 µg of isolated total RNA was globin reduced using the GLOBINclear 96-well format kit (Applied Biosystems/Ambion) according to the manufacturer's instructions. Total and globin-reduced RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies), with algorithm analysis of the electropherogram to determine the RNA integrity number (RIN) for each sample. RNA yield was assessed using a NanoDrop8000 spectrophotometer (NanoDrop Products, Thermo Fisher Scientific).

2.5.3 Hybridisation and microarray

Biotinylated, amplified antisense complementary RNA (cRNA) targets were then prepared from 200–250ng of the globin-reduced RNA using the Illumina CustomPrep RNA amplification kit (Applied Biosystems/Ambion). Seven hundred and fifty nanograms of labelled cRNA was hybridized overnight to Illumina Human HT-12 V4 BeadChip arrays (Illumina), which contained more than 47,000 probes. The arrays were washed, blocked, stained and scanned on an Illumina iScan, as per manufacturer's instructions. GenomeStudio (Illumina) was then used to perform quality control and generate signal intensity values. All procedures were performed by the same small group of experienced operators, for samples from both periods of recruitment. Samples were randomised before assignment to the arrays, to avoid experimental bias. 2009/10 season samples were analysed *en masse* with their matched control samples in 2011, and 2010/11 season samples were analysed *en masse* with their matched control samples in 2012.

2.5.4 Selection of Patient Groups for Analysis

The number of paediatric cases recruited to the study was relatively small. 2009/10 paediatric samples lacked matched paediatric healthy controls. Although samples from age-matched controls were obtained for the 2010/11 patients, age-matching was more difficult than in adults, especially for babies and young infants. Furthermore, RIN values tended to be lower for paediatric samples than adult samples. For this reason, only adult patients and adult healthy control microarray data from both waves have been analysed to date and will be presented in this thesis.

2.5.5 Data analysis of microarray results

2.5.5.1 General approach

While alternative approaches and several analytical programmes are available to analyse complex microarray data, the computer-based analysis of microarray results in this study followed an

approach that has been used successfully by NIMR and BIIR collaborators in numerous studies of other infectious diseases in humans, including studies of differential gene expression and biomarker discovery (378-381). GeneSpring 12.6 (Agilent Technologies) was used to perform visualisation and analyses of microarray data, unless otherwise stated.

2.5.5.2 Experiment set-up in GeneSpring GX 12.6

Raw, non-transformed, non-normalised microarray data were received from BIIR as flat-text data files. Each subject's sample had a unique sample ID assigned at BIIR prior to processing and microarray. For each season, a separate Microsoft Excel spread-sheet contained the BIIR sample IDs corresponding to the subject ID and sampling time point was created. The raw microarray data from each wave were imported into two separate projects in GeneSpring and a new master experiment was set up for each. Using finalised, validated clinical data-sets provided by the MOSAIC Data Clearing House, sample attributes were entered into a spread-sheet (Microsoft Excel), containing responses denoting infection status (influenza-positive, influenza-negative, healthy control) and time point (T1, T2, T3 or HC). Individual experiments were set-up in each project by searching for samples in the master experiment containing the appropriate variables e.g. healthy controls and influenza-positives at T1. Once created, the samples included in each experiment were cross-checked against the BIIR sample ID file and additionally infection status was cross-checked against the validated MOSAIC clinical data-set.

Once entered into a new experiment, raw data were pre-processed to minimise systemic non-biological differences (to reveal true biological differences). Data underwent $\log_2$ transformation (threshold raw signal was set to 1.0) and percentile shift normalisation was performed, whereby the 75^{th} percentile was calculated for each array and this value was subtracted from every value on the array. Transcript intensities were then rescaled to the same relative abundance level, centring around zero. This baseline transformation step was to the median of all samples (patients and healthy controls combined) and facilitates identification of similar expression patterns but doesn't affect any subsequent statistical analyses.

2.5.5.3 Quality control by Principal Component Analysis

To eliminate poor-quality samples and reduce low-reliability genes, quality control (QC) was performed at sample level and at entity (gene RNA transcript or "entity") level. Sample-level QC was performed using principal component analysis (PCA). After assigning influenza infection status using the experiment grouping function, each sample was plotted according to its values for the first three

principal components. Principal components are vectors that capture the most variable in the data and PCA assumes that samples within an experimental condition e.g. HCs should be more similar to each other than to those from different conditions e.g. influenza-positives. Variable reduction is performed by an eigenvalue-eigenvector decomposition performed on the covariance matrix of the gene expression values around zero. The eigenvector corresponding to the largest eigenvalue is called the first principal component. Successive principal components are eigenvectors corresponding to each similar eigenvalue. In the three-axis PCA plot, clustering according to condition could be visualised and any outliers close to the first component (X axis) could be considered for removal. To avoid potential software-associated errors with normalisation following removal of samples at the PCA step, if outliers were identified for removal, then the experiment was set up from scratch with removal of the identified samples at the outset.

2.5.5.4 Quality control by filtering probe-sets by flags

For QC at entity level, filtering of probe-sets (a collection of probes designed to interrogate a given sequence) was performed. First, filtering on flag calls was performed. Flags are attributes that denote the quality of the entities and are generally specific to the technology or the array type used. Following the BIIR-NIMR method, after background subtraction each probe was attributed a flag to denote its signal intensity detection p-value. Flags were used to filter out probe sets that did not result in a "present" call in at least 10% of the samples, where the "present" lower cut off equalled 0.99 i.e. where signal precision was less than 0.01.

2.5.5.5 Filtering probe-sets by expression

Following filtering by flags, the remaining transcripts were filtered to select the most variable probes: those that had a minimum of x-fold expression change compared with the median intensity across all samples, in greater than 10% of all samples, where x is the desired positive integer. This was achieved in GeneSpring by filtering and retaining entities that were greater than x-fold above the median intensity and greater than x-fold below the median intensity, and then combining the generated entity lists using Venn analysis, to create a new entity list.

For the analyses presented in this thesis, expression filtering was performed separately in each experiment using two-fold ($\log_2(2)$) and three-fold expression change ($\log_2(3)$). Because expression filtering at the level of two-fold expression change was found to generate excessively large and generally unworkable entity lists (following subsequent statistical analysis), three-fold expression change was used to generate the results presented. Three-fold expression change was considered to be an appropriate level to generate lists of entities of interest, given the number of samples and heterogeneity of patient phenotypes in each experiment.

2.5.5.6 Statistical filtering

Following expression filtering, entity lists underwent statistical filtering in GeneSpring. All statistical tests were non-parametric, since it has been shown that their validity and power are minimally affected by outlying values and since normality assumption justifying parametric testing is often untenable in microarray studies using samples from clinical studies (382). Equally, power is expected to be maintained because of the large number of samples in each experiment.

For comparison of healthy controls and either the influenza-positive or the influenza-negative group at a single time point, the Mann-Whitney unpaired test was used. For comparison of healthy controls against both groups, the Kruskal-Wallis test was used.

Since statistical tests perform one test per entity, if 10,000 entities were analysed with $p = 0.05$, five hundred entities are likely to appear significant by chance and be inappropriately included in the filtered entity list. Although performing the aforementioned QC steps prior to statistic filtering decreases the number of false positives, performing multiple testing correction further decreases the number of false positives. Two tests were employed in the analyses: Benjamini and Hochberg False Discovery Rate (FDR) and Bonferroni Family-wise Error Rate (FWER). The former is known to generate more false positives, whereas the latter is known to generate more false negative results. Following analysis of MOSAIC data-sets using both methods and after reviewing the size and biological plausibility of the lists generated, Bonferroni FWER has been used to generate the results presented here, unless stated otherwise.

Following the BIIR-NIMR approach, a corrected *p* value cut off of 0.01 was used throughout, using the asymptotic method. Based on collaborator experience, it was felt that this cut-off achieved an acceptable balance between the likelihood of type I errors e.g. genes are labelled as differentially expressed when they are not, a false positive, and the likelihood of type II errors e.g. genes are not labelled as differentially expressed when actually they are, a false negative. Following the null hypothesis that 'entity A' has equal mean expression level in the healthy control population sample and the disease sample e.g. influenza-positive patients, if the *p* value was below 0.01 for that entity, it would be removed automatically from the subsequent entity list. For selected experiments, lower *p* value cut-offs were tried (0.005, 0.001) but they did not appear to influence the results to a notable degree, so a $p < 0.01$ was accepted as a cut-off.

2.5.5.7 Cluster analysis

Clustering is a method for identifying patterns within datasets. Clustering algorithms partitions inputs, such as entities, into groups based on similar expression patterns. Clustering may be

performed at entity level, which infers that entities that are grouped within a cluster may be co-regulated and thus functionally related. This analysis is part-supervised, since expression profiles for all samples belonging to a specific condition (e.g. all influenza-positive) will be grouped together. This is useful for initial exploration of trends associated with a particular group and may inform the operator how to proceed with subsequent experiments.

Hierarchical clustering was used for the majority of clustering analyses. Following QC, expression filtering and statistical filtering, entity lists underwent hierarchical clustering in GeneSpring, using Pearson's uncentred (cosine) similarity metric. The similarity metric takes two expression patterns and produces a correlation value (-1 to 1) based on the degree of similarity. Clinical variables supplied by the Data Clearing House were entered into a Microsoft Excel spread-sheet containing corresponding MOSAIC subject IDs and microarray sample IDs. The selected clinical variables are described later (see chapter 4). Clinical data files were created for each of the 2009/10 and 2010/11 cohorts. For an experiment, the relevant data-file was imported into GeneSpring using the "experiment grouping" function; "interpretations" were then created to determine the conditions used to perform hierarchical clustering and investigate the clinical variable of interest, for example severity of illness. With the exception of age, sex and ethnicity, healthy control samples were always assigned the same "HC" identifier, rather than individual values or categorical definitions.

Hierarchical clustering at the combined entity and condition level identifies entities that are differentially expressed under different conditions e.g. whether the sample belongs to a healthy control or an influenza-positive patient. This is unsupervised, whereby the algorithm arranges similar vertical expression profiles together in groups in an unbiased fashion, without *a priori* knowledge of the underlying group of interest.

The clustering output for entities and conditions is represented in a two-dimensional dendrogram, whereby the most similar expression profiles are joined together into a group and groups are further joined in a tree structure, in a step-wise fashion, until all data form a single group (so called agglomerative clustering). The distances between branches of the tree relate to the similarity of the expression patterns, and the distance between clusters is determined by the average of the distance between all points in each cluster. This 'average linkage' rule may be more accurately defined as when the distance between two sub-trees is the average of the pair-wise distance between members of the two clusters.

For each entity displayed, signal intensities relative to the baseline are expressed in the form of a heat-map, where baseline expression e.g. the median signal intensity for all samples, is yellow, the

greatest up-regulation (positive signal intensity) is bright red and the greatest down-regulation (negative signal intensity) is bright blue; variable shades of red or blue illustrate varying degrees of up- or down-regulation, respectively.

In addition, K-means clustering was used to investigate differential expression for selected clinical variables, for example, differences in expression between healthy controls and influenza-positive patients of varying severities of illness. K refers to the number of clusters selected into which selected entities (or conditions, if selected) are partitioned. The algorithm attempts to minimize *intra-cluster* variability and maximise *inter-cluster* variability. Entities are randomly divided in *K* clusters and each cluster's centroid, *n*-dimensional average expression is calculated. Each entity is then reassigned to its closest centroid. Each centroid is then recalculated based on new entities assigned to it. This process is repeated until entities have converged into immutable groups. A K-value of 10 was used for the analyses presented in this body of work, with the number of iterations set to 200 i.e. the maximum number of times that each centroid was recalculated after entities were reassigned to their closest centroid was set to be the maximum computational value that GeneSpring permits.

The output of K-means clustering on entities is visualised using expression profile plots, according to the chosen clinical interpretation. Heat-maps were then generated and pathway analysis performed on entity lists for clusters where profile plots revealed differences between the chosen clinical categories e.g. healthy controls and differing severity of illness grades for influenza-positive cases.

2.5.5.8 Biological interpretation of transcriptional signatures

To investigate the biological functions, processes and locations of identified entities, two different methods were used. GO Ontology (Gene Ontology Consortium) is a controlled vocabulary that describes gene products in terms of their associated cellular components, molecular functions and biological process in a species-independent manner (383). GO terms and their relationships are provided within GeneSpring and are updated periodically. The *p* value calculated for each GO term indicates the likelihood that the selected genes of interest fall into that category, just by chance. To compute *p* value, GeneSpring uses standard hypergeometric distribution, the number of entities in the entity list with the particular GO terms and its children, the number of entities with the GO term on the entire array, the total number of entities in the entity list and the total number of entities on the array. Multiple probes corresponding to the same Entrez ID (gene identifier) are collapsed before enrichment scoring is performed, in order to produce an unbiased calculation. GeneSpring applies the Benjamini and Yekutielli correction to account for testing of multiple null hypotheses.

Ingenuity Pathway Analysis (IPA, Ingenuity Systems Inc.) is widely used proprietary software for exploring biological functions of entities identified through microarray analysis. This sophisticated analysis tool is supported by the Ingenuity Knowledge Base, a repository of regularly updated, curated biological interactions and functional annotations, based on published literature and subject to quality control. IPA generates networks where differentially expressed entities can be related, according to previously known associations between genes or their products.

IPA was used to determine most significant canonical pathways associated with the differentially expressed genes of interest for a particular cluster or patient group. Canonical pathways are presented in a bar chart, with identified pathways displayed along the x-axis; negative-log (p-value) is shown on the x-axis. The taller an individual bar the greater the significance. A threshold line for p=0.05 is also shown as an overlay. P-value scoring is calculated using Fisher's exact test with Benjamini and Hochberg FDR. The ratio is also shown as an overlay line. Ratio is calculated as the number of molecules in a given pathway from the dataset that meet cut-off criteria, divided by the total number of molecules that are represented in that canonical pathway. The ratio provides an estimate of the strength of association and the percentage of genes in a pathway that were also found in the dataset. When directional fold-change data are also exported from GeneSpring, a stacked bar-chart can be generated, indicating the proportion of entities from the data set that are up-regulated (red) or down-regulated (green) or unchanged (grey).

2.5.5.9 Transcriptional Modular Analysis

Transcriptional modular analysis was performed using methodology and data analysis tools developed by BIIR (384). This approach allows microarray data to be compared against a set of transcriptional modules that categorise different components of the host immune response. The framework includes genes with coordinate expression within or across whole-blood sets derived from studies of nine human diseases, which were selected in multiple rounds of clique or paraclique clustering, as described by Guiducci *et al.* (385). Following GeneSpring analysis, the raw expression levels of all transcripts significantly detected by comparison with background hybridisation were compared between each sample and all the controls present in that dataset. The percentage of significantly expressed genes in each module are represented by the colour intensity (Student t-test, p<0.05), with red indicating over-expression and blue indicating under-expression.

2.6 Soluble Immune Mediator Profiling

2.6.1 Serum and plasma

Serum and plasma samples were collected from patients at each time point and from 36 adult healthy controls (see previous comment about sampling hierarchy used for children). Serum samples were used for soluble immune mediator assays presented here, although a smaller number of plasma samples have also been analysed (predominantly from children, or adults from whom serum was not provided or volumes were inadequate). Since mediator levels in plasma and serum may differ, it was decided that only results for serum samples would be compared and presented in this body of work.

2.6.1.1 Serum and plasma sample collection

For patients and healthy controls, blood was collected using the phlebotomy technique described in the sampling SOP (appendix 5). Venous blood was preferred and obtained from the majority of subjects and all healthy controls, although arterial blood may have been collected from patients on intensive care, at the request of the local Site Investigator or attending clinician. Blood source was documented in the subject's research folder. Where an indwelling vascular access device was used, the first 5 ml of blood drawn was discarded before obtaining the sample. For adults, 6ml blood was collected into a uniquely-labelled, plain BD Vacutainer plastic serum tube. This was the first blood sample to be drawn at T1, T2 or T3 in patients, or in healthy controls. The sample was kept cool and delivered to the local specimen reception as quickly as possible, as previously described.

2.6.1.2 Initial processing and storage of serum and plasma

On arrival at specimen reception, samples were centrifuged at 1000 x g for ten minutes in a cooled centrifuge. A minimum period of thirty minutes from time of phlebotomy had to have passed prior to centrifugation, to ensure clot formation. Several 0.5 – 1.0 ml aliquots of serum supernatant were pipetted into individual, labelled Cryovials and frozen immediately at -80°C. Samples were not allowed to thaw until the day of analysis.

2.6.1.3 Preparation of serum and plasma for assays

Serum aliquots were defrosted and then placed on ice. For a single experiment involving multiple assay plates and multi-channel pipetting, the required volume of serum for each sample was pipetted into corresponding wells of a sterile plastic 96 well plate, which was also placed on ice. A plate-plan was used, with care taken to ensure that samples were correctly pipetted into the appropriate wells.

2.6.2 Nasopharyngeal aspirate (NPA)

An illustrated description of NPA sampling can be found in the adult sampling SOP (appendix 5).

2.6.2.1 NPA sample collection

NPA was obtained using a 10F sterile Argyle suction catheter connected to a uniquely-labelled, sterile Universal sputum trap with a two-way connector, which was in turn attached to fixed-wall suction or a portable mechanical suction device. The catheter was gently inserted to reach the posterior nasopharyngeal wall and then moderate suction was applied and the catheter was slowly withdrawn, for a total period of 5 seconds. The tip of the catheter was then placed in a vial of 5 ml sterile normal saline (3ml for children) and suction was applied to aspirate the entire contents of the vial, to flush into the trap any secretions that remained in the catheter. The sealed sample was kept cool and transferred to the local specimen reception as quickly as possible, as described previously.

2.6.2.2 Initial processing and storage of NPA

On delivery to the specimen reception, the NPA sample was agitated in a vortex and 1ml aliquots were pipetted into individual, uniquely labelled CryoVials; these were frozen immediately at -80°C and not allowed to thaw prior to analysis.

2.6.2.3 Preparation of NPA for assays

Aliquots were thawed and then agitated in a vortex before being placed on ice. Required volumes of NPA were pipetted into a 96 well plate, as described in the method for serum.

2.6.3 Nasal Synthetic Absorptive Matrix (SAM)

SAM strips have been shown to bind soluble immune mediators from respiratory tract mucosal surfaces, including the nasal mucosa. Unlike Whatman's filter paper, the proprietary fibrous matrix (LeukoSorb, Pall) that is precision-cut by laser into nasal SAM strips has the advantage of binding mediators but not to an extent that mediators cannot be separated following elution-centrifugation. Additionally, SAM sampling is not subject to the significant dilution effect seen with nasal washing. Nasal SAM sampling was performed according to a method developed by collaborators at Imperial College London and described previously (386).

2.6.3.1 SAM sample collection

For each subject, the sampler identified the inferior nasal turbinate and inserted the 35 mm x 7 mm SAM strip via the nares, so that it came into direct contact with the mucosa of the lateral wall of the nasal cavity. If NPA had already been performed, the opposite nasal cavity was used. The patient or operator then occluded the relevant nares by applying pressure to the outside of the nose, to ensure

that contact was achieved. Light pressure was maintained for 2 minutes before the SAM strip was removed with sterile plastic forceps. The SAM strip was then placed in a uniquely-labelled CryoVial and transferred to the specimen reception in a cooled container as quickly as possible, as described previously.

2.6.3.2 Initial processing and storage of SAM

On arrival at the specimen reception, the labelled Cryovial containing the SAM strip was frozen immediately at -80°C and was not defrosted until the day of analysis.

2.6.3.3 Preparation of SAM samples for assays

On the day of analysis, each SAM strip was allowed to thaw in its original container. The SAM strip was removed before adding 500 µl of Milliplex Assay Buffer (Millipore). The buffer facilitated elution and contains phosphate-buffered saline (PBS), 1% bovine serum albumin (BSA), 0.05% sodium azide and 0.05% Tween-20. Five hundred microlitres of buffer were added to provide enough eluate for all of the planned assays. The soak strip was then placed in a Costar Spin-X centrifuge tube filter (pore size 0.22 µm) held within an Eppendorf tube. Sealed tubes were centrifuged at 4°C at 16,000 x g for 5 minutes. The eluate was retained in the Eppendorf tube and kept cool on ice until transfer to assay plates. The SAM strip and filter were discarded.

Total sample weight was calculated by subtracting the mean average weight of fifty unused SAM strips and the weight of the Eppendorf spin-filter + assay buffer from the weight of the Eppendorf spin filter + assay buffer + mean unused SAM weight + sample.

2.6.4 Bronchoscopic Alveolar Lavage (BAL) Fluid

BAL was performed at the discretion of the attending clinician and was only performed for clinical indications. A member of the research team was notified of a planned BAL and was in attendance at the time of the procedure.

2.6.4.1 BAL fluid Sample collection

BAL was performed by an experienced operator, according to the local clinical protocol. Sterile normal saline was instilled in all cases. The instilled volume and volume returned was documented in the subject's research folder. Five millilitres of BAL fluid in a sterile universal container was obtained for research purposes and transferred in a cooled container to the local specimen reception as quickly as possible, as described previously.

2.6.4.2 Initial processing and storage of BAL fluid

On arrival at specimen reception, the BAL fluid was agitated in a vortex and individual 1ml aliquots were pipetted into CryoVials. The samples were frozen immediately at -80°C and not defrosted until the day of analysis.

2.6.4.3 Preparation of BAL fluid for assays

BAL fluid was prepared as per the method used for preparing NPA samples (see 2.5.2.3)

2.6.4.4 BAL Healthy Control Values

A separate study undertaken by a collaborator (Dr David Jackson, Imperial College London) measured a variety of soluble immune mediators in BAL fluid from 11 adult healthy controls. The same MSD platform (MesoScale Discovery) was used to measure the mediators as was used for this study, although the chosen mediator panels were not identical. HC values were available for comparison for most, but not all mediators. Lower limits of detection were also provided.

2.6.5 Quantification of soluble immune mediators by MSD Electrochemiluminescence Multiplex Assays

2.6.5.1 MSD assay principles

The MSD electro-chemiluminescence quantitative patterned array detection method (Meso Scale Discovery, Gaithersburg, USA) has been used to investigate the role of bacterial and viral infection in acute exacerbations of COPD and asthma (387, 388). This method has the advantage of using very small sample volumes (25µL) to assay panels of up to 10 mediators with a dynamic range of 0.1-10,000pg/ml so that repeated measurements of multiple mediators at different dilutions is unnecessary.

In an MSD assay, specific capture antibodies for the analytes are pre-coated in arrays in each well of a 96-well carbon electrode plate surface (maximum of 10 different array spots per well). Mediators are detected in a sandwich immunoassay format. The sample and a solution of labelled detection antibodies are added to each well. The detection system uses a proprietary electrochemiluminescent compound, MSD SULFO-TAG. Mediators in the sample bind to capture antibodies immobilized on the working electrode surface and recruitment of the labelled detection antibodies by bound analytes completes the 'sandwich'. After addition of read-buffer and placement on the MSD SECTOR instrument, a voltage is applied to the plate electrodes, causing emission of light from bound labels. The instrument measures the emitted light intensity; quantitation is

achieved by comparing light emissions from samples against those from known standard dilutions of calibrators for each mediator included on the plate.

Background signals are minimal because the stimulation mechanism (electricity) is decoupled from the signal (light). Labels emit light at ~620 nm, eliminating problems with colour-quenching (the loss of photons along their paths to the photocathodes of the photomultiplier). According to the manufacturer's product information, few compounds interfere with electrochemiluminescent labels. Multiple excitation cycles of each label amplify the signal to enhance light levels and improve sensitivity.

2.6.5.2 MSD Mediator panels

The following panels of mediators were measured using the MSD platform:

- 7-plex: IFN-γ, IL-13, IL-15, IL-17, ITAC, MIG, MIP-1α
- 10-plex: GM-CSF, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, TNF-α
- 9-plex: Eotaxin-1, Eotaxin-3, MIP-1β, TARC, IP-10, IL-8, MCP-1, MDC, MCP-4
- 2-plex: IFN-α2a, IFN-λ
- Single-plex: IFN-β

Note that IL-8 is included in both the 10-plex and 9-plex assay plates, as this is how the non-customised plates were designed by the manufacturer. However, this served as a useful control for inter-plate variability.

2.6.5.3 MSD general assay method

All reagents and 96-well MSD assay plates were stored at an appropriate temperature prior to use, according to the manufacturer's instructions. Stock calibrator and control solutions were supplied in concentrated form, which varied according to the particular assay. The highest calibrator point (S1) was prepared by x-fold dilution in MSD Diluent 2, where x is the same as the concentration factor for the stock. Serial 4-fold dilutions, starting with S1, were then made to generate a total of 7 standards of decreasing concentration; the eighth standard (S8) was Diluent 2 alone (a zero calibrator). Initial experiments comparing dilution with Diluent 2 alone and Diluent 2 with 1% BSA added confirmed that Diluent 2 alone was appropriate for assays of all sample types, including serum-free samples. With the exception of the 2-plex assay, stock detection antibody solution (50x) was diluted to 1 x strength by diluting 60 µl stock solution in 2.94 ml of MSD Diluent 3. For the 2-plex assay, a 1 in 50

dilution was made to which a 1 in 500 dilution of streptavidin was added (60 µl of diluted detection antibody solution plus 6 µl of streptavidin in 2880 µl of Diluent 2). MSD Read Buffer T was diluted 2-fold in deionised water to create a 2x concentration solution (10ml of 4x Read Buffer T added to 10 ml deionised water for each plate). Plate washing solution was prepared by adding 250 µl Tween-20 (Sigma Aldrich) to 500 ml Dulbecco's phosphate buffered saline (Sigma).

For all assays except the custom 2-plex plate, 25 µl of Diluent 2 was added to each well and the plate was placed on a mechanical shaker (600 rpm) for 30 minutes at room temperature. Twenty-five microlitres of sample or calibrator were then added to each well of each plate, as appropriate. Standards and samples were run in duplicate (two wells per sample or standard), except for SAM eluate samples, which were run in singlet, according to a pre-defined plate plan. After two hours of mechanical shaking (600 rpm) at room temperature, the plates were washed three times with wash solution. Twenty-five microlitres of plate-specific detection antibody solution were then added to each well and the plate was shaken again under the same conditions. After two hours, the plate was washed three times, ensuring that no wash solution remained in the wells after the final wash. 150 µl of Read Buffer T were then added to each well, avoiding bubbles that may interfere with plate-reading, and each plate was analysed within 30 minutes on a manufacturer-calibrated MSD SECTOR 2400 Imager.

For the custom 2-plex plate, the plates were initially washed three times with the wash solution and then 25 µl of capture antibody solution (diluted to 1x in MSD Diluent 100) was added to each well. The plates were shaken (600 rpm) at room temperature for one hour and were then washed another three times. All subsequent steps were as described for the other plates, commencing with the 30 minute Diluent 2 incubation.

2.6.5.4 Quantification analysis and limits of detection for MSD assays

For each mediator, the calibrator standard curve was plotted by inputting the concentration of each standard in pg/ml into the MSD Discovery Workbench software. The curve was modelled using least squares fitting algorithms, so that signals from samples could be converted into concentrations. The software programmed utilizes a 4-parameter logistic model and includes a $1/Y^2$ weighting function to provide a better fit of data over a wide dynamic range.

The upper limit of detection (ULOD) is fixed for each mediator, defined by the concentration of the highest standard for the particular mediator. Since actual intensity signals vary between experiments, each plate's standard curve will differ, even when the same mediator is analysed. This effect is more marked for certain mediator that others e.g. eotaxin. Therefore, the lower limit of

detection (LLOD) calculated by the software programme was not accepted. Instead, a more conservative approach was adopted. Following inspection of the curve, the standard with the lowest detectable calculated concentrated mean value and a percentage coefficient variation cut-off of 10% was used to set the LLOD each mediator on each plate.

2.6.6 Enzyme linked Immunosorbent Assays (ELISAs)

2.6.6.1 ELISA assay principles

A monocolonal antibody specific for the mediator of interest is pre-coated onto a microplate. Standards and samples are pipetted into the wells of the plate and any mediator present is bound by the immobilized antibody. After removal of unbound substances by washing, an enzyme-linked polyclonal antibody specific for the mediator of interest is added. Following further washing to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and colour develops in proportion to the amount of mediator bound in the initial step. The colour development is then inhibited and the colour intensity is measured using a plate-reader with appropriate software.

2.6.6.2 Measurement of Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES)

RANTES was measured in serum (obtained as described previously) using the Quantikine Human CCL5/RANTES Immunoassay (R&D Systems Europe Ltd, Abingdon, UK). Single-thawed serum samples were diluted 100-fold (10 µL sample with 990 µL Calibrator Diluent RD6-11 (5X)). RANTES standard was reconstituted with 5 mL of Calibrator Diluent RD6-11 (5X), providing a stock solution of 200 pg/mL. 500 µL of the same diluent was pipetted into each of 7 polypropylene tubes. 500 µl stock solution was added to the first tube and then serial dilutions were made to produce 6 standards (31.2 pg/mL – 1000 pg/mL), with vortex mixing prior to transfer. The seventh (zero) standard was diluent alone.

One hundred microlitres of Assay Diluent RD1W was then added to each well, to which 100 µL of standard or sample was added, as appropriate. Plates were incubated at room temperature for two hours. Wells were then aspirated and washed three times with 400 µL Wash Buffer, prepared according to the manufacturer's instructions. Care was taken to remove any residual Wash Buffer following the final wash. Two hundred microlitres of RANTES conjugate was then added to each well and the plate incubated for one hour. The washing process was then repeated. Two hundred microlitres Substrate Solution (prepared according to manufacturer's instructions) was then added to each well. The plate was then incubated at room temperature, with protection from light, for

twenty minutes. Fifty microlitres of Stop Solution was then added to each well, ensuring thorough mixing to facilitate uniform colour change. The optical density was determined within 30 minutes using a microplate reader set to 450 nm.

To calculate results in pg/mL, the average zero standard optical density was subtracted from averaged duplicate readings for each standard and sample. Standard curves were generated using software capable of generating a four parametric logistic 94-PL) curve-fit. Sample concentration read from the standard curve was multiplied by the dilution factor to correct for sample dilution.

The manufacturer's quoted intra-assay and inter-assay precision, expressed as CV (%) was 2.5 and 6.4, respectively. The manufacturer states the mean minimal detectable dose to be 2.0 pg/mL.

2.6.6.3 Measurement of Neopterin

Neopterin was measured in serum using Neopterin ELISA (IBL International GMBH, Hamburg, Germany). Serum was collected and stored as described previously. As per the manufacturer's instructions, thawed serum was not diluted. Ten microliters of each manufacturer-prepared standard or sample were pipetted into the respective wells of a microtiter plate. One hundred microlitres of freshly-prepared Enzyme Conjugate (1:201) was then added to each well, followed by 50 µL of Neopterin Antiserum. The plate was sealed, protected from light and incubated for 90 minutes at room temperature on an orbital shaker (500 rpm). Incubation solution was discarded and the plate was washed four times with 300 µL Wash Buffer, diluted according to the manufacturer's instructions. Excess solution was removed after the washing process. 150 microlitres of TMB Substrate Solution was added to each well and the plate was incubated for ten minutes at room temperature. The substrate reaction was stopped by adding 150 µL of TMB Stop Solution to each well. Plates were shaken gently to ensure colour change. Optical density was measured within 15 minutes using a plate reader set to 450 nm. Results, expressed in nmol/mL, were measured using software in the same manner as described for all other mediators measured by ELISA. The manufacturer's quoted intra-assay and inter-assay precision, expressed as CV (%) is 3.6-6.8 and 7.6-10.3, respectively. The quoted limit of detection is 0.7 nmol/L. Results in nmol/L were multiplied by 0.253 to convert into ng/mL.

2.6.6.4 Measurement of Soluble Tumour Necrosis Factor Receptors 1 and 2 (STNFR1 & STNFR2)

STNFR1 and STNFR2 were measured in serum using the Quantikine Human sTNF RI/TNFRSF1A Immunoassay and the Quantikine Human sTNF RII/TNFRSF1B Immunoassay, respectively (R&D Systems Europe Ltd, Abingdon, UK). Single-thawed serum samples were diluted ten-fold in Calibrator

Diluent RD6O. Respective standards were reconstituted with 5 mL of Calibrator Diluent RD6O, providing stock solutions of 500 pg/mL. 500 microlitres of the same diluent was pipetted into each of 7 polypropylene tubes. Five hundred microlitres of stock solution was added to the first tube and then serial dilutions were made to produce six standards (7.8 pg/mL – 250 pg/mL) with vortex mixing prior to transfer. The seventh, zero standard was diluent alone.

Fifty microliters of Assay Diluent RD1-6 was then added to each well, to which 200 µL of standard or sample was added, as appropriate. Plates were incubated at room temperature for two hours. Wells were then aspirated and washed three times with 400 µL Wash Buffer, prepared according to the manufacturer's instructions. Care was taken to remove any residual Wash Buffer following the final wash. 200 µL of the respective conjugate was then added to each well and the plate incubated for 2 hours at room temperature. The washing process was then repeated. Two hundred microlitres Substrate Solution (prepared according to manufacturer's instructions) was then added to each well. The plate was then incubated at room temperature, with protection from light, for twenty minutes. Fifty microlitres of Stop Solution was then added to each well, ensuring thorough mixing to facilitate uniform colour change. The optical density was determined within thirty minutes using a microplate reader set to 450 nm. Results were calculated as described for the RANTES assay.

For STNFR1, the manufacturer's quoted intra-assay and inter-assay precision values, expressed as CV (%), were 3.7 and 3.6, respectively. The manufacturer states that the mean minimal detectable dose is 0.77 pg/mL. For STNFR2, the manufacturer's quoted intra-assay and inter-assay precision, expressed as CV (%) was 2.6 and 3.5, respectively. The manufacturer states that the mean minimal detectable dose is 0.6 pg/mL.

2.6.7 Soluble Immune Mediator Data Management

2.6.7.1 Quality control for collated soluble immune mediator data

All MSD and ELISA results were-cross checked against original plate-plans. Data were exported to Microsoft Excel and collated in a single spread-sheet, where each row represents an individual's patient's sample, with attributes added to denote subject ID, sample type, influenza infection status, time-point and per-time point severity scores. Attributes were imported from final verified clinical data-sets provided by the MOSAIC Data Clearing House.

A search was performed for duplicate values, to spot any data importation mistakes. Where duplicate values existed, the original signal intensity values were re-checked. Due to the large number of results, ten percent of results were randomly selected and cross-checked against the original values reported by the MSD or ELISA software.

2.6.7.2 Approach to left- and right-shifted mediator values

For the purposes of grouped analysis of all assays for a particular sample type, the geometric mean of the lower limit of detection (GM-LLOD) for a particular mediator measured by MSD was calculated using Microsoft Excel. Results below the GM-LLOD were re-assigned half of the value of the GM-LLOD for that particular mediator. The number of results falling below the GM-LLOD and the number of 'zero' results were recorded prior to re-assignment and original values were maintained in a separate spread-sheet. Results above the ULOD were re-assigned the ULOD value for that particular mediator.

2.6.8 Statistical analysis of soluble immune mediator data

Histogram analysis and D'Agostino-Pearson omnibus testing of mediator data-sets revealed non-normal distributions throughout. Therefore, non-parametric statistical tests were used. For statistical comparisons of two unpaired groups, the Mann-Whitney unpaired test was used. For comparison of more than two unpaired groups, Kruskal-Wallis test was used (α=0.05), with Dunn's post-test to compare constituent pairs. If the Kruskal-Wallis *p* value was >0.01 (non-significant), Dunn's test was not performed; this *p* value was set as a significance cut-off *in lieu* of formal multiple testing correction. For comparisons of the three paired time-points, analyses were performed using Friedman's Test with Dunn's post-test. Statistical testing was performed in GraphPad Prism 5.04 (GraphPad Software Inc.).

2.6.9 Biomarker and other assays performed by Clinical Chemistry

2.6.9.1 Sample preparation for clinical chemistry samples

Procalcitonin (PCT), c-reactive protein (CRP) and 25-hydroxy vitamin D were measured in previously unthawed aliquots of plasma from both periods of recruitment, and also in plasma samples from the 36 adult healthy controls described previously. Plasma was K_2-EDTA-derived. Cooled EDTA blood samples from each time point were centrifuged at 1000 x g for 10 minutes on arrival at the MOSAIC specimen reception laboratories and 0.5 ml aliquots of plasma supernatant were frozen immediately at -80°C. Following temperature-controlled transfer of samples and immediately prior to analysis, aliquots were defrosted and allowed to reach room temperature in the Imperial College Healthcare NHS Clinical Chemistry laboratory. Samples were split into batches; thawed batches were analysed in a single day (other batches were kept frozen).

2.6.9.2 Procalcitonin assay

Plasma procalcitonin (PCT) was quantified using the Elecsys BRAHMS PCT assay on a calibrated Cobas e602 platform, following the manufacturer's instructions. Briefly, the assay follows a sandwich principle, with a biotinylated monoclonal PCT-specific antibody and a monoclonal PCT-specific antibody labelled with a ruthenium complex. Following addition of streptavidin-coated microparticles and binding to the solid phase, microparticles were captured magnetically onto the electrode and unbound substances were removed. Electrochemiluminescent emission was measured by a photomultiplier and results were determined via an instrument-specific 2-point calibration curve and a master curve. The upper limit of detection (ULOD) for the assay is 100 ng/ml; for a minority of samples where the ULOD was reached, dilutions and repeat assays were not performed due to limited availability of reagents. The manufacturer states that the use of frozen samples can result in a lower recovery of up to 8%.

2.6.9.3 C-reactive protein assay

C-reactive protein (CRP) was measured using the Vario assay from Abbott, with analysis performed on the Architect platform (Main Automated Chemistry Analyser, Ci16200/C8000). Briefly, this was a latex immunoassay developed to measure CRP levels in serum and plasma. When an antigen-antibody reaction occurs between CRP in a sample and anti-CRP antibody, which has been adsorbed to latex particles, agglutination results. This agglutination is detected as an absorbance change (572 nm), with a rate of change being proportional to the quantity of CRP in the sample. The LLOD was 0.2 mg/L. Mean CV%, as reported by the manufacturer, 1.15% across the analytical range.

2.6.9.4 Vitamin D assay

Vitamin D was measured using LC-MS/MS (Walters Method). Measurement of 25-hydroxyvitamin D2 (25-OH-D2) and 25-hydroxyvitamin D3 (25-OH-D3) was performed in the laboratory by liquid-chromatography tandem mass spectrometry (LC-MS-MS, Waters® UPLC® with the ACQUITY® TQD, Waters Corporation, Milford, MA, USA). This method uses deuterated (3H) 25-hydroxyvitamin D3 (26,26,26,27,27,27-hexadeutero-25-hydroxyvitamin D3) as an internal standard.

A fixed volume of calibrator/control/patient sample was diluted with internal standard, which also acts as a protein precipitating agent. Samples were vortex mixed and vitamin D (including 25-hydroxyvitamin D and other hydroxy-metabolites) was extracted using n-hexane. Following vortex mixing and centrifugation, a proportion of the supernatant was removed into LC-MS glass vials and the solvent evaporated using a heated concentrator at 50°C. The residue was reconstituted in HPLC buffer and vortex mixed to dissolve the solute.

The extracted 25-OH D2 and 25-OH D3 were measured on an automated LC-MS-MS system controlled by MassLynx software. Calibrators, controls and patient extracts were sequentially injected onto a phenyl reversed phase column. Simultaneous measurement of 25-OH D2 and 25-OH D3 is achieved using the Turbulon Spray ionization source set to the positive ion mode followed by detection on the triple/quadruple tandem mass spectrometer by monitoring the molecular ions and specific fragmentation patterns caused by the bombardment of molecules with an argon collision gas. The LLOD was 10nmol/L (total vitamin D). Interassay precision was <11% CV for 25-OH D2 and <10% for 25-OH D3. Clinical reference ranges define deficiency as <40nmol/L, 40-70nmol/L as insufficient and 70-150 nmol/L as replete.

2.7 Detection of viruses

2.7.1 Virology sample collection, processing and storage

MOSAIC collected, processed and stored NPA samples for virus detection, following the method described for soluble immune mediator NPA (section 2.5.2), from patients at T1 and T2 and from 36 healthy adult controls. Flocked nylon throat swabs in universal transport medium (Copan, UK) were used to swab the palatopharyngeal arches of all patients during the 2009/10 season; during the 2010/11 season, flocked swabs were only obtained when NPA sampling was not possible. The tip of the swab was placed in a uniquely-labelled vial of universal transport medium (UTM, Copan), agitated and then transported in a cooled container as quickly as possible to the local specimen reception. Upon arrival at the specimen reception, the sample was frozen immediately at -80°C and was not defrosted until the day of analysis. BAL fluid and T1/T2 ETA from selected patients was also obtained; collection, storage and initial processing was the same as that described for BAL fluid soluble immune mediator analysis (section 2.5.4).

2.7.2 Virus detection by multiplex PCR

PCR detection of influenza and other viruses was performed by Investigators at the West of Scotland Specialist Virology Centre, Glasgow, with the exception of human rhinovirus typing, enterovirus and bocavirus detection, which was performed by Investigators at the Roslin Institute, University of Edinburgh.

For virus detection by multiplex PCR, total nucleic acid was extracted from specimens using the QI Aamp Viral RNA kit (Qiagen) according to the manufacturer's instructions. Real time RT-PCR was carried out in order to detect influenza A (a generic influenza A assay and a A(H1N1)pdm09-specific assay), Influenza B, respiratory syncytial virus (RSV), human rhinovirus, parainfluenza 1-4, human metapneumovirus, coronaviruses (229E, NL63, HKU1, and OC43) and adenovirus. Influenza C virus

detection was also performed, but as part of the bacterial multiplex PCR assay described below. The oligonucleotide primers and probes used have been described previously (389). These assays were developed at the West of Scotland Specialist Virology Centre and used as the frontline test for respiratory samples since 2005. All assays have been shown to be sensitive and specific by in-house development procedures and via participation in numerous external quality assessment schemes (EQA), including those provided by WHO, PHE (formerly HPA) and Quality Controls for Molecular Diagnostics.

All assays were carried out as described previously (389). Briefly, primers and probes were used at concentrations of 0.4 µM and 0.2 µM respectively in a 15 µl reaction volume; one-step rtRT-PCR was performed on 6 µl of RNA extract using the Platinum One-step qRT-PCR kit (Life Technologies) on an ABI Prism 7500 SDS real-time platform (Applied Biosystems). The following thermal profile was used: one cycle of reverse transcription for 15 minutes at 50°C; two minutes at 95°C for reverse transcriptase inactivation and DNA polymerase activation; 40 amplification cycles of 15 seconds at 95°C and 34 seconds at 60°C per cycle. Data acquisition occurred at the annealing step of each cycle, and the cycle threshold (Ct) for each sample was calculated by determining the point at which the fluorescence exceeded the threshold limit.

For all patients in the study, if A(H1N1)pdm09 RNA was detected by the viral multiplex in the T1 respiratory sample, all remaining samples available for that patient (such as EDTA-derived plasma, stool, other respiratory samples) and obtained at T1, T2 or T3 underwent A(H1N1)pdm09 quantification. Additionally, any patient for whom a T1 respiratory sample was not obtained had all remaining samples available tested.

Quantification of A(H1N1)pdm09 was carried out as described above, but with the inclusion in the dilution series of a commercially-available plasmid (Fast-Track Diagnostics). By comparing Ct values obtained from this dilution series to a virus sample of known quantity (donated by the Health Protection Agency), it was possible to assign a quantity of virus to each dilution of the plasmid. Using this information and comparing Ct values of plasmid dilutions to those from extracted samples, the ABI Prism 7500 calculated the quantity of virus present in each sample.

2.7.3 Interrogation of NHS clinical virology results

To optimise the detection of viruses in recruited patients, results servers for laboratories serving different clinical sites were also interrogated. If the local clinical laboratory screened for a similar panel of viruses using a clinically-validated respiratory virus multiplex PCR, then the results were included. Only results pertaining to the episode of influenza/influenza-like illness were included. For

per-time point analyses, the tested sample had to have been obtained 48h either side of the time-point. Valid samples included NPA, throat and nasal/nasopharyngeal swabs and BAL fluid.

Because MOSAIC-obtained samples were only collected at two acute time points, NHS results were also interrogated for evidence of serial sampling and PCR testing for influenza, to provide additional information of duration of influenza PCR positivity in a sub-group of patients (London sites only). All of the NHS clinical laboratories used HPA-approved PCR assays for the detection of A(H1N1)pdm09.

2.7.4 Determination of influenza infection status

Influenza infection status was assigned by the MOSAIC Virology Work Package (WP3), following a one-day meeting where all available WP3 and NHS virology results were scrutinised. Influenza-positive patients were defined as those with influenza viral RNA detected by PCR testing of an appropriately-obtained T0 (NHS diagnostic) or T1 (MOSAIC research) sample; influenza-negative status was assigned if the reverse was true. For one subject, PCR testing hadn't been performed but a rise in serum influenza-specific antibody titre between acute and convalescent periods was detected, with a confirmed absence of preceding influenza vaccination, so this patient was also classified as influenza-positive. Patients without PCR or serological evidence of influenza infection (or where paired serum samples were not available for antibody quantification) were classified as "influenza negative".

2.7.5 Data collation and quality control

All virology results were entered into a single database, with 100% validation by an independent operator who cross-checked entered data against the original data source.

2.8 Detection of bacteria

2.8.1 Bacteriology sample collection, initial processing and storage

T1, T2 and T3 NPA, ETA and (where available) BAL fluid samples were collected and handled in the same way as NPA samples for soluble mediator sampling (sections 2.5.2 and 2.5.4). Plain rayon throat swabs for 16S rRNA ultra-deep sequencing and culture throat swabs in Amies medium were collected from patients at T1, T2 and T3 and from 36 healthy adult controls. Taking care not to touch the tip of either swab, the palatopharyngeal arch was swabbed by making 5 circular motions and ensuring that the swab tip was in contact with the mucosa but avoiding contact with the tongue.

2.8.2 NPA, flocked throat-swab and BAL analysis by multiplex PCR

NPA aliquots, BAL fluid samples from patients and NPA from 36 adult healthy controls underwent bacterial multiplex PCR analysis. Extraction of RNA was carried out as described for the respiratory

virus multiplex (section 2.6.2). Real time RT-PCR was performed to detect *Staphylococcus aureus*, *Chlamydia pneumoniae*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Pneumocystis pneumoniae*, *Legionella* species, *Klebsiella pneumoniae*, *Salmonella* species, *Moraxella catarrhalis*, and *Bordetella pertussis*, using primers and probes from the FTD Respiratory Pathogens 33 kit (Fast-Track Diagnostics). Assays were carried out according to the manufacturer's instructions and using the AgPath-ID One-Step RT-PCR kit (Life Technologies). Data acquisition occurred at the annealing step of each cycle, and the threshold cycle for each sample was calculated as described above. Mycoplasma pneumoniae detection was also performed, but as part of the virus multiplex PCR assay described in section 2.6.2.

2.8.3 Culture of NPA, ETA throat swabs, BAL fluid and sputum

NPA, ETA and sputum samples, along with throat swabs in Amies medium were sent fresh (same day as collection) for bacterial microscopy, culture and sensitivity testing at local collaborating NHS microbiology laboratories (Imperial College Healthcare NHS Trust and Royal Liverpool and Broadgreen Hospitals NHS Trust). These laboratories follow recognised bench protocols and are subject to external quality assessment and clinical accreditation. Throat swabs were cultured on blood agar, *Fusobacterium*-selective agar and Sabouraud agar. Additionally, NPA samples were cultured on *Bordetella*-selective agar. Analysis and reporting of microscopy, culture and sensitivity results was performed by the respective clinical laboratories. All results were verified by senior microbiologists prior to release.

2.8.4 Urinary pneumococcal antigen detection

Urine samples were collected in sterile universal containers, transported to the local specimen reception as described previously and frozen immediately at -80°C. BinaxNOW *Streptococcus pneumoniae* Antigen Card (Alere Ltd) was used to detect pneumococcal antigen in the thawed sample at room temperature. Briefly, this immunochromatographic membrane assay detects pneumococcal soluble antigen in human urine and CSF. Rabbit anti-*S. pneumoniae* antibody, the 'sample line', is adsorbed onto nitrocellulose membrane. Control antibody is adsorbed onto the same membrane as a second stripe, the control line. Both rabbit anti-*S. pneumoniae* and anti-species antibodies are conjugated to visualizing particles that are dried onto an inert fibrous support. Testing was performed according to the manufacturer's instructions, with the inclusion of negative and positive controls supplied by the manufacturer. Results were read 15 minutes after application of the sample. The presence of a clear sample line and a clear control line indicated a positive test. If only the control line was visible, then the test was said to be negative. All samples tested produced a control line, indicating that the assays had been performed satisfactorily.

2.8.5 Interrogation of NHS clinical microbiology results

To enhance bacteriological investigations within MOSAIC, patients' NHS microbiology results were obtained from each participating clinical site. Only results pertaining to a patient's acute influenza/ILI episode were analysed. Blood culture and cerebrospinal fluid (CSF) microscopy, culture and sensitivity results have been obtained and collated; collation and analysis of respiratory tract sample results is on-going, therefore NHS respiratory tract culture results have not been included in this work.

Where a patient had been transferred from a non-MOSAIC hospital and a significant culture result (or urine antigen testing result) was documented in the transfer documents, the researcher contacted the original hospital's laboratory for confirmation and documented the result and data and time of sampling in the patient's subject folder.

2.8.5.1 Blood culture and cerebrospinal spinal fluid culture

Blood stream infections reported by NHS laboratories were assessed for significance according to the Matching Michigan criteria (390). All blood culture and CSF culture results were interpreted by an independent microbiologist from Imperial College Healthcare NHS Trust. For time-point analyses, only blood cultures obtained 48h either side of the relevant time-point were included.

2.8.5.2 NHS Urinary pneumococcal and Legionella antigen detection

Where testing had been performed, the results of NHS urinary pneumococcal and legionella antigen testing were obtained. For time-point analyses, only antigen results obtained 48h either side of the relevant time-point were included.

2.8.6 Bacteriology data collation and quality control

A standard operating procedure for data validation was developed by the Data Clearing House. For clinically-relevant respiratory tract organisms, data were validated in a single database by two individuals. Because data entry occurred on a *per-organism* basis and validation occurred *per patient*, the two individuals may have revalidated their own data. Therefore 25% randomly selected results underwent further validation by two other individuals. A validation tracking log was maintained to monitor these activities. Every respiratory organism identified was entered into the database, with the exception of 'upper respiratory tract flora',' oropharyngeal flora' and 'oral flora'. Results stating 'not detected' were also entered, along with identification of subjects who did not have samples submitted for testing. Entered data had to match absolutely with the reports and the agreed time period for that sample type. NHS blood stream infection, CSF and urine antigen data were entered by a single individual and validated by a different individual.

Chapter Three

3 Clinical and Microbiological Characterisation of Patients

3.1 Introduction

3.1.1 Pandemic influenza demonstrates unique clinical characteristics

Observational studies conducted during the 2009-10 pandemic identified a number of risk factors associated with acquiring influenza infection and also risk factors associated with severe influenza illness resulting in hospitalisation (66, 171). One of the most important differences between outbreaks of seasonal and pandemic influenza is the age distribution of those infected; in the 2009-10 pandemic, younger adults and children were particularly at risk of severe influenza, whereas complicated seasonal influenza is primarily an illness of older adults (391, 392). Although older adults were at lower risk of acquisition of A(H1N1)pdm09 virus infection, possibly due to pre-existing H1N1-specific antibodies, they were found to be at higher risk of complicated illness and death once they became infected (66, 393). In the UK Flu-CIN study, only 5% of those admitted to hospital with confirmed A(H1N1)pdm09 infection were over 65 years of age (171). Furthermore, complications of influenza are more likely at the extremes of age, irrespective of the underlying influenza sub-type. Although seasonal and pandemic influenza appear to carry a similar degree of risk of complications in hospitalised adults, after controlling for age and underlying medical conditions, the risk lower respiratory tract complications and critical illness was shown to be greater with A(H1N1)pdm09 influenza than with seasonal influenza infection. This is most apparent in younger adults with A(H1N1)pdm09, who were shown to have 2-4 times the risk of severe outcomes compared to age-matched adults with seasonal influenza (392).

3.1.2 Risk factors for complicated pandemic influenza

Multiple studies from around the world have highlighted common risk factors for complicated A(H1N1)pdm09 illness. For example, asthma, chronic lung disease, chronic cardiovascular disease, obesity, immunosuppression, diabetes mellitus and pregnancy were all identified as risks in studies from Europe, China and North America. In industrialised countries, asthma was found in up to 30% of patients requiring admission for A(H1N1)pdm09 virus infection (175). Notably, the UK Flu-CIN study reported that approximately 50% hospitalised adults with A(H1N1)pdm09 virus infection had no underlying risk factors for developing severe influenza (171, 174).

3.1.3 MOSAIC's approach to clinical data collection

MOSAIC's integrated and comprehensive approach to investigating the biology of influenza pathogenesis – primarily the pathogenesis of illness caused by A(H1N1)pdm09 virus infection – required detailed, standardised clinical data in order to interpret the findings of laboratory studies. MOSAIC recruitment commenced following the peak of the UK's second pandemic wave (referred to hereafter as the 2009/10 season) and was completed following the end of the UK's first post-pandemic wave, which is often called the 'third pandemic wave' (referred to hereafter as the 2010/11 season). Given the novel circumstances of an influenza pandemic and the narrow window of opportunity in which to recruit patients, MOSAIC utilised an existing Department of Health data collection tool, the Flu-CIN document. This was specifically designed to capture daily clinical data from patients hospitalised with A(H1N1)pdm09 virus infection and described by the Department of Health as a clinical audit tool.

Retrospective analysis of MOSAIC patients' case-notes using the Flu-CIN data collection tool has produced an extraordinarily detailed clinical data-set for hospitalised adults and children with influenza and influenza-like illness. Supplemented by a case-report form (CRF) that was introduced during the 2010/11 season, designed to overcome problems relating to missing data that were infrequently recorded in hospital case-notes, the data-set for the 2010/11 season is particularly rich and contains data relating to the majority of the confirmed influenza cases recruited to MOSAIC. Although only selected key clinical variables have been used for the initial analyses of transcriptomics and soluble immune mediator results presented in this thesis, the clinical data-set is a valuable resource to help answer other hypotheses posed by MOSAIC investigators in the future, and may also be used to address other research questions as they arise. Furthermore, at the time of writing, analyses of certain data-sets, for example NHS microbiology results and NHS haematology and biochemistry results, are still on-going.

3.1.4 Aims of this chapter

Only a selection of the final, validated clinical data from MOSAIC will be presented here. The aims of this chapter are:

1. To describe the main clinical characteristics of the MOSAIC cohort

2. To assess whether the cohort is broadly representative of hospitalised A(H1N1)pdm09-infected patients described elsewhere

3. To highlight some novel clinical findings from MOSAIC

The primary objective of this chapter is to provide appropriate clinical detail for the interpretation of transcriptomics and soluble immune mediator analyses that are presented in subsequent chapters. Although we have only presented transcriptomic and soluble immune mediator analyses for adult subjects, both adult and paediatric clinical characteristics are presented for completeness.

3.2 Data collection, analysis and validation

3.2.1 Data collection tools

The Flu-CIN tool collected data for up to 3,635 variables and contained 29 tables. Daily clinical assessment data were collected for up to 14 days from the time of recruitment, in addition to the collection of data on final outcome of illness. The supplemental MOSAIC Case Report Form (CRF) collected data for 443 variables and contained 3 tables, but was only used for the 2010/11 season following its introduction after 2009/10 recruitment. The inclusion of a supplemental CRF was prompted by an interim analysis of Flu-CIN results for patients recruited during the 2009/10 season, which identified variables that generally were not recorded in hospital notes. These include influenza vaccination history, pre-hospital antiviral and antibiotic use, and height and body-weight data. The majority of variables in the CRF were also included in Flu-CIN, but the relevant fields required real-time completion, rather than completion through retrospective data analysis. Therefore, the CRF was completed at the bed-side at the time of recruitment. Many clinical laboratory variables were also included in Flu-CIN, but these sections often were not completed adequately. Therefore, NHS laboratory databases also require interrogation to capture the required data; at the time of writing, this process is on-going.

3.2.2 Amalgamation of Flu-CIN and MOSAIC Case Report Form data

Because MOSAIC utilised two clinical data sources, the Flu-CIN form and the MOSAIC CRF, data collation rules were created to promote consistency and avoid bias. Flu-CIN was always used as the primary data source but when discrepancies arose for certain variables, individual cases were investigated by returning to the original clinical notes. Where Flu-CIN did not record an answer but the answer was available in the CRF, the CRF data were used. All data conflicts and the steps taken to achieve resolution have been recorded by the MOSAIC Data Clearing House.

3.2.3 Resolving missing clinical data and data anomalies

Of 255 Flu-CIN booklets completed, 163 were identified as missing key clinical data. Hospital case notes were recalled for these patients and the missing data were inserted into the forms and then transferred to the database, where possible. Problems relating to determination of severity of illness were resolved by a panel of clinical investigators, following review of original cases notes alongside

information recorded in Flu-CIN forms and in the CRFs. This process followed a set of pre-defined rules and was managed by the MOSAIC Data Clearing House.

Because respiratory severity was determined in part by interventions administered to correct respiratory failure e.g. invasive mechanical ventilation, it was apparent that certain patients were at risk of having an underestimation of their severity of illness. For example, a terminally-ill oncology patient may have met the commonly accepted criteria for mechanical ventilation, but may not have received the intervention, since the responsible clinicians considered it to be inappropriate. Therefore, the MOSAIC clinical review panel looked at the indication for respiratory support and not just whether it was given or not. This review included analysis of death certificates, where appropriate, looking for evidence of fulminant respiratory failure secondary to acute infection.

Day-of-illness at presentation to hospital and at sampling time points received particular attention in the data validation steps. For the 2010/11 season cohort, discrepancies were identified between the date of onset recorded in Flu-CIN and that recorded in the CRF. This resulted in an additional validation step for all Flu-CIN forms, whereby the day-of-illness at presentation was calculated and compared against that reported in Flu-CIN publications (171, 174, 211). If the recorded day-of-illness was within the interquartile range (IQR) for day-of-illness reported by Flu-CIN publications (1-4 days), the value was accepted. If it did not fall within the IQR, the case-notes and discharge summaries were re-examined. Care was taken to differentiate onset of acute influenza/influenza-like illness from the onset of exacerbations of other, more chronic illness. Again, this required review of case notes by the panel of clinical investigators for specific cases where it was the date of onset was not immediately apparent or appeared not to be in keeping with the expected duration of illness for influenza. The validation process resulted in the correction of date-of-onset in 49 Flu-CIN forms (36%).

3.3 Patient recruitment and sampling

3.3.1 General recruitment

Patients were recruited as close to the point of hospital admission as possible. Recruitment sites offered both specialist and non-specialist services, optimising recruitment of cases from the general patient population and also from specific patient populations e.g. immunosuppressed patients. However, quotas for specific categories of patient were not set and specific units were not deliberately targeted. This was done with the intention of the cohort being as representative of the general patient population as possible.

3.3.2 Recruitment criteria

Patients with laboratory-confirmed influenza or clinically-suspected influenza were recruited, to facilitate rapid biological sampling as early in the course of illness as possible and ideally prior to specific interventions e.g. antiviral and antibiotic therapy, having been administered. Clinical suspicion of influenza was guided by local and national clinical diagnostic algorithms for influenza. Guidelines were circulated during the pandemic period and were still in use in the winter of 2010/11. Briefly, clinicians were advised to consider A(H1N1)pdm09 infection if the patient presented with temperature >38°C and two or more symptoms consistent with ILI (headache, rhinorrhoea, sore throat, cough, shortness of breath, loss of appetite, myalgia, diarrhoea, vomiting, lethargy). However, clinicians were free to refer any patient to the study if they thought that influenza infection was still possible despite not meeting the clinical diagnostic criteria, as long as the intention was to test and/or treat the patient for influenza infection. This was particularly important for certain groups of patients who were more likely to have to have atypical presentations of influenza e.g. immunosuppressed patients, as they otherwise might have been missed. Influenza-negative ILI patients were defined as those who had symptoms compatible with influenza but for whom laboratory testing of appropriate clinical diagnostic or MOSAIC-obtained samples did not identify influenza infection. An alternative cause of ILI was sought in all cases but was not required for inclusion in the study, at either the point of recruitment or in final analysis.

3.3.3 Recruitment of specialist intensive care patients

Royal Brompton adult intensive care patients were recruited in a different manner to other patients. They accounted for just over half of the 40 adults who required invasive mechanical ventilation. Royal Brompton patients were initially admitted to local hospitals and then transferred for specialist care for the treatment of ARDS, including ECMO therapy. Therefore, these patients had already received a period of inpatient care prior to recruitment at a MOSAIC centre. Compared to other critically-ill adults admitted directly to a MOSAIC centre, Royal Brompton patients may have been at a later day of illness at the time of recruitment. These patients were included deliberately because they represented some of the most severe influenza cases in the UK during the recruitment periods. Although the Royal Brompton ICU patients had a longer overall duration of inpatient care prior to recruitment, the average 'delay' in recruitment because of transfer was not considered to be excessive (table 3.1).

	All recruitment sites	Sites other than Royal Brompton Hospital ICU	Royal Brompton Hospital ICU
Time from admission to MOSAIC site to recruitment (days)			
Mean	2.3	2.5	0.8
Median	1	2	1
Range	0-28	0-28	0-4
Time from admission at original hospital to recruitment (days)			
Mean	NA	NA	5.25
Median	NA	NA	5
Range	NA	NA	2-9

Table 3.1 Average time spent from point of hospital admission to recruitment
Data are shown for all MOSAIC patients from both seasons. Only adult patients were admitted to Royal Brompton Hospital ICU.

3.3.4 Identification of potential subjects

For ward-based patients and those being admitted from emergency departments, rapid recruitment following admission was achieved by the combined methods described in Materials and Methods (Chapter 2). Briefly, this involved clinician referral and active case-finding through daily interrogation of influenza PCR results lists. Five patients in London were referred by clinicians but were not approached for recruitment, because influenza had already been excluded by laboratory testing of an appropriately-sourced sample at the time of referral. Two critically-ill patients were referred by clinicians but because of their rapid decline (leading to their eventual demise), personal/nominated consultees were not approached to discuss recruitment, for ethical reasons. Although data were not formally recorded by all sites as part of the study protocol, local recruitment data for adult patients in London revealed that only seven adults declined the invitation to participate in the study and only one personal consultee stated that they did not believe that the patient would wish to participate. This suggests that sampling schedule was deemed acceptable by most patients following informed consent.

3.3.5 Season-specific differences in recruitment and sampling

During the 2009/10 season, patients who were recruited to the study and subsequently had a negative influenza diagnostic (T0) result underwent T1 sampling, but not T2 or T3 sampling. In the 2010/11 season all patients were eligible for sampling through to T3. Although the majority of patients found to have influenza-negative ILI were recruited during the 2009/10 season, these differences in follow-up sampling were necessary because of more limited resources during the first season. Two patients from 2009/10 were initially thought to be influenza-negative, but subsequent

testing of their MOSAIC-obtained research samples revealed that they did indeed influenza. For this reason, they did not have the opportunity to undergo T2 or T3 sampling.

3.3.6 Demographics

A total of 256 recruitment episodes took place: 85 during the 2009/10 season and 171 during the 2010/11 season. One individual was inadvertently recruited twice across both periods of recruitment; the patient's second episode (2010/11 season) was excluded from final analysis. Of the remaining 255 patients, 212 (83.1%) were adults and 43 (17%) were children (0-16 years of age). The demographic characteristics for adults are presented in table 3.2 and those for children are presented in table 3.3.

	Total (n=212) n (%)	**Influenza-negative (n=78) n (%)**	**Influenza-positive (n=134) n (%)**	***p* value**
Mean age in years (median)	43.1 (43.0)	45.5 (46.2)	41.8 (42.2)	0.09
Age range in years (IQR)	17.6 - 84.8 (29.7 - 52.2)	17.6 – 84.8 (30.7 - 54.6)	17.6 - 74.1 (29.1 - 51.6)	-
Male	101 (48.1)	33 (42.3)	69 (51.1)	0.20
Ethnicity				0.44
White	139 (65.6)	50 (64.1)	89 (67.7)	
Asian	19 (9.0)	10 (12.8)	9 (6.8)	
Black	36 (17.5)	13 (16.7)	23 (16.5)	
Other	18 (8.5)	5 (6.4)	13 (9.0)	
Recruited in 2010/11	141 (66.5)	30 (38.5)	111 (82.8)	<0.001
Recruited in London	173 (81.6)	63 (80.8)	110 (82.1)	0.81

Table 3.2 Demographic and recruitment characteristics for adult subjects recruited to the MOSAIC study

For *p* value calculations in comparisons of influenza-negative and influenza-positive cases, Wilcoxon rank sum tests of central tendency were performed to compare means and medians, as appropriate. Chi-squared test analysis was performed for contingency tables (contrast of proportions) unless n <5, in which case Fisher's exact test was performed.

	Total (n=43) n (%)	**Influenza-negative (n=5) n (%)**	**Influenza-positive (n=38) n (%)**	***p* value**
Mean age in years (median)	5.4 (3.9)	1.4 (0.8)	6.0 (4.0)	0.064
Age range in years (IQR)	0.08 - 15.7 (0.8 - 8.8)	0.2 - 4.7 (0.3 - 0.8)	0.08 - 15.7 (1.0 - 9.3)	-
Male	20 (46.5)	4 (80)	16 (43.2)	0.174
Ethnicity				-
White	32 (74.4)	5 (100)	27 (71.1)	
Asian	4 (9.3)	0	4 (10.5)	
Black	2 (4.7)	0	2 (5.3)	
Other	5 (11.6)	0	5 (13.1)	
Recruited in 2010/11	29 (67.4)	20 (1)	28 (73.7)	-
Recruited in London	12 (27.9)	0	12 (31.6)	-

Table 3.3 Demographic and recruitment characteristics for paediatric subjects recruited to the MOSAIC Study
For comparison of influenza-negative and influenza-positive cohorts, Wilcoxon rank sum tests of central tendency were performed to compare means and medians, as appropriate. Chi-squared test analysis was performed for contingency tables (contrast of proportions) unless n<5, in which case Fisher's exact test was performed.

In summary, MOSAIC successfully recruited 255 patients across two seasons: the winter of 2009/10 (the UK's second pandemic wave) and the winter of 2010/11 (the UK's first post-pandemic wave). The majority of influenza-positive cases were recruited during 2010/11. Most patients were middle-aged adults, although a reasonable number of influenza-positive children were also recruited. Influenza-positive adults tended to be middle-aged and children tended to be of infant school age. Multiple ethnic groups are represented, although white-European/white-British patients dominate the cohort.

3.4 Influenza virus detection

3.4.1 Influenza detections by season

In the 2009/10 season, 33 of 85 patients (38.8%) had confirmed influenza infection. 14 children (<16 years) were recruited, of whom 10 (71%) had confirmed influenza. Of 71 adults, 23 (32%) had confirmed influenza. In the 2010/11 season, 139 of 170 (82%) patients had confirmed influenza. Of 29 children, 28 (97%) had confirmed influenza and 111 of 141 (78.7%) recruited adults had confirmed influenza infection.

3.4.2 Influenza subtypes

Of 172 patients with confirmed influenza, 149 (86.6%) had A(H1N1)pdm09 detected, 5 (2.9%) had influenza A that could not be subtyped, 3 (1.7%) had H3N2 detected and 15 (9%) had influenza B detected. Of the 33 influenza-positive cases from the 2009/10 season, one patient had H3N2 infection; all other patients had A(H1N1)pdm09 infection. All influenza B detections were in patients from the 2010/11 cohort. One patient from 2010/11 had A(H1N1)pdm09 detected in their diagnostic sample and then influenza B was detected in a subsequent sample. Review of all results available for this patient, both those for NHS-tested samples and MOSAIC-obtained samples, confirmed that they had sequential infection with two different influenza viruses.

3.4.3 Source of influenza virus detection

Of the 172 influenza-positive cases, 170 had influenza RNA detected by PCR in their NHS diagnostic sample (T0). One patient from the 2009/10 season had influenza RNA detected by PCR in a MOSAIC T1 sample but not in their T0 sample. An additional patient did not have influenza RNA detected in either sample, but they did have a significant rise in A(H1N1)pdm09-specific antibodies following testing of acute (T1) and convalescent (T3) serum samples. Following confirmation that the patient had not received influenza vaccine, this case was subsequently classified as influenza-positive.

3.4.4 Antiviral resistance

Oseltamivir resistance testing was performed on all influenza-positive T1 samples, as part of the initial PCR screening of all T1 samples by the MOSAIC virology laboratory. Influenza A(H1N1)pdm09 virus with the H275Y substitution, which is associated with high-level resistance to oseltamivir, was detected in a T1 sample from an adult with leukaemia and a T1 sample from an infant with chronic lung disease. Both patients were recruited in Liverpool during the 2010/11 season and the cases were not related. Further details regarding their antiviral treatment histories are awaited. The adult patient also had clinical samples and a MOSAIC isolate submitted to HPA laboratories for pyrosequencing, which confirmed the finding (90% majority variant). All other patients who had

clinical samples submitted for resistance screening, at the request of the attending clinician, did not have evidence of mutations associated with oseltamivir or zanamivir resistance. Of 60 viruses isolated from MOSAIC-obtained samples from 49 patients, one further patient had H275Y virus detected by pyrosequencing in a single isolate, but it was a minority variant (10.7%).

Overall, despite intensive screening of MOSAIC-obtained samples by specific PCR of T1 samples and pyrosequencing of a smaller number of virus isolates, the incidence of H275Y-mediated oseltamivir resistance was low. Nationally and internationally, resistance was reported to be low (1-3% of isolated tested) during the recruitment period, so these findings are to be expected. Although details concerning antiviral treatment are awaited, both patients had underlying comorbidities and one had a condition that is often associated with immunosuppression, a recognised risk factor for the development of neuraminidase resistance (249).

3.4.5 Comparison of influenza viral load at T1 and T2

The MOSAIC study was not designed to assess the duration of influenza viral RNA detectability ('viral shedding'). However, for subsets of patients from both waves, quantitative PCR of A(H1N1)pdm09 influenza RNA was performed by the MOSAIC Virology laboratories. The results were converted and expressed as plaque-forming units (PFU)/mL. Of the twelve patients from the 2009/10 season with paired samples containing detectable viral RNA, six (50%) demonstrated a decrease in viral load at T2 compared to T1. Two (17%) patients had similar levels of viral RNA at T1 and T2 and four (33.3%) patients had higher viral loads at T2.

Of the 28 patients from the 2010/11 season with paired samples containing detectable RNA, 18/28 (64%) demonstrated a decrease in viral load at T2 compared to T1. Five patients (18%) had an increase in their viral load at T2 and five patients (18%) had similar viral loads at T1 and T2 (figure 3.1).

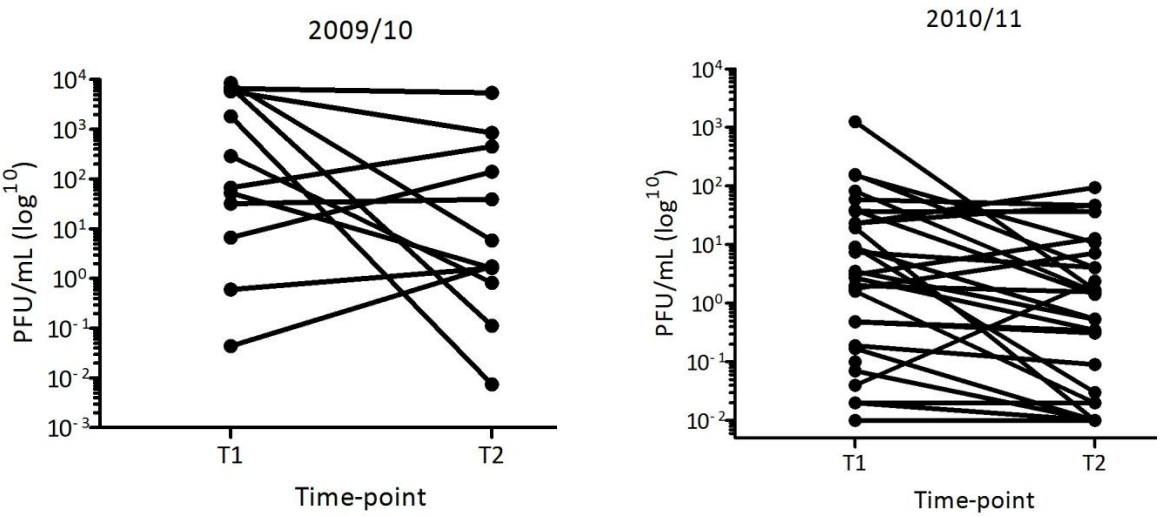


Figure 3.1 Influenza viral load at T1 and T2
Influenza viral load in NPA samples obtained at T1 and T2 obtained from patients recruited during 2009/10 (left-hand panel) and 2010/11 (right-hand panel) who had samples available for analysis. Viral load is expressed as PFU/mL(log_{10}).

For both seasons, over half of the patients (24/40) demonstrated a decrease in NPA viral load from T1 to T2 (an intervening period of 48-72 hours). It is expected that most patients would have received several doses of antivirals by the time the T2 sample was obtained and, based on the reported natural history of A(H1N1)pdm09 illness, that the majority patients with non-critical illness would be showing signs of clinical improvement by the time T2 sampling occurred (see also section 3.8). This will require confirmation through correlation with influenza antiviral treatment histories and daily clinical assessment data (analysis currently in progress). Correlation with comorbidity data and severity is also warranted.

3.4.6 Duration of influenza RNA detectability (shedding)

MOSAIC obtained samples at just two acute time points, so NHS virology databases were interrogated to identify patients in whom serial sampling had been performed (London sites only). These results were then combined with MOSAIC influenza detection results, in an attempt to enrich available data on the duration of viral RNA detectability. Patients were divided according to peak severity of illness (grades 1-4) and the numbers of days since onset of symptoms was applied to each result, along with the number of days since antiviral therapy had been commenced. It should be noted that subsequent non-detection of viral RNA was not established for the majority of patients and it is not known why serial sampling ceased in these patients. Furthermore, pending further data collation and analysis, it is not currently known whether patients were still receiving antiviral therapy at the time that final samples were obtained.

Only three patients with severity 1 illness had more than one sample obtained for influenza testing. All had two serial samples taken, 2, 4 and 8 days apart, respectively. All samples were obtained from the upper respiratory tract. The durations of illness when influenza was detected in the final samples obtained were 5, 6 and 9 days from onset of symptoms. The number of days that had passed from when antiviral therapy had been commenced was 3, 3 and 9 days, respectively. All three patients had significant comorbidities that were associated with immunosuppression.

Eight patients with grade 2 or 3 maximum severity of illness underwent serial sampling at the discretion of the attending clinicians. The mean number of samples taken was 3 (range 2-9 samples). The mean day of illness at the time the final positive serial sample was obtained was 18.5 days (range 4-85 days, median 8.5 days). All patients had sampling of the upper respiratory tract only. All patients had comorbidities and five had illnesses that were associated with immunosuppression. The patient with detectable virus at day 85 of illness had chronic myelogenous leukaemia. Influenza A(H1N1)pdm09 viral RNA was intermittently detected in nasopharyngeal secretions from this patient at day 85 and a subsequent sample taken at day 133 since onset of illness was negative.

Twenty-five patients with grade 4 maximum illness severity were identified. The mean day of illness at the time the final positive sample was obtained was 13.6 days (median 12 days, range 4-41 days). Serial sampling for clinical reasons was evident in 17 of these patients, including 3 children. The mean number of samples obtained per patient was 3.5 (median=3, range 2-9). The mean day of illness when the final positive sample was obtained was 14.5 days (median=12 days, range 4-41 days). Three patients had sampling of the upper respiratory tract only; BAL or ETA samples were obtained on at least one occasion in the remainder. Five patients (29%) did not have an underlying comorbidity or known risk factor for severe influenza illness. Only one patient was known to be immunosuppressed. Three patients had influenza B infection and the day of illness at the time the final positive sample was obtained was 7, 9 and 28 days, respectively. All other patients had A(H1N1)pdm09 virus infection. The patient with the longest known duration of A(H1N1)pdm09 viral RNA positivity (41 days from onset of symptoms) had grade I obesity but no other known risk factors. This patient had commenced antivirals 30 days prior to the sample being obtained.

Of note, influenza A(H1N1)pdm09 viral RNA was detected in lung swabs obtained at an autopsy performed 4 days after death, from a patient who had received an autograft transplant as part of treatment for multiple myeloma. The patient's A(H1N1)pdm09 virus infection was thought to have been acquired in hospital and they received 5 days of high-dose oral oseltamivir, followed by 10 days of intravenous zanamivir. At the time of death, they had not received antiviral therapy for over two weeks (for further details, see description for Case C in section 3.8).

In summary, clinicians chose to perform serial sampling for influenza detection in a subset of patients, most of whom had the most severe level of illness and required invasive mechanical ventilation. While comparison of patients' results is difficult due to the lack of a standardised sampling schedules and an absence of subsequent negative results in most patients, it is notable that the median day of illness at the time the final positive result was received was greatest in those who had the most severe illness. The longest known duration of detectability of A(H1N1)pdm09 viral RNA was seen in a patient with peak severity level of grade 2 and this patient had underlying immunosuppression. However, A(H1N1)pdm09 viral RNA was still detectable at day 41 in a critically-ill patient who had did not have a formal diagnosis of immunosuppression (the only documented comorbidity was grade I obesity). Serial samples from both patients were screened for mutations associated with antiviral resistance, but resistance mutations were not detected. Prolonged periods of RNA detectability were not unique to A(H1N1)pdm09 virus infection, with late detections also being seen in patients infected with influenza B. Detailed antiviral data, once available, will permit further investigation of these cases.

3.4.7 Summary of influenza virus detection findings

To summarise, just over two thirds of the MOSAIC cohort had confirmed influenza infection, predominantly A(H1N1)pdm09 virus infection. This is in keeping with the original aims of the study, to recruit patients affected by a pandemic strain. Recruitment during the 2009/10 season was dominated by influenza-negative ILI cases, probably because the peak of the pandemic second wave had already passed and other respiratory viruses were known to be circulating at this time (176). However, because the UK experienced a significant post-pandemic wave, influenza case numbers were boosted significantly in 2010/11, an outbreak that also was dominated by A(H1N1)pdm09 virus (56). The majority of cases were diagnosed on the basis of detection of influenza RNA in their NHS diagnostic sample, with or without subsequent detection of influenza RNA in their MOSAIC research samples. Only a small number of children did not have influenza, but over one third of adults with ILI did not have evidence of influenza infection. Antiviral resistance was rare, consistent with national surveillance reports (249). Most, but not all, patients with samples available demonstrated a decrease in viral load in NPA between T1 and T2. The duration of influenza virus RNA detectability was not specifically addressed by MOSAIC sampling, but analysis of serial NHS-obtained results suggests that viral shedding may be prolonged, especially in critically-ill patients. This is consistent with findings reported by others (394).

3.5 Comorbidities

The MOSAIC clinical panel reviewed comorbidity data and classified comorbidities according to World Health Organization criteria (395). Detailed reviews were conducted for specific subjects where comorbidity classification was not immediately apparent. Reported diseases and their comorbidity classifications are listed in table 3.4.

Asthma Physician-diagnosed asthma	**Diabetes mellitus** Type I diabetes mellitus Type II diabetes mellitus	**Chronic Renal Disease** Chronic renal impairment Renal transplant
Chronic Lung Disease COPD Cystic fibrosis Chronic lung disease (unspecified) Kyphoscoliosis Bronchiectasis Pulmonary sarcoidosis Tracheal stenosis	**Chronic Cardiovascular Disease** Heart disease (unspecified) Atrial fibrillation Hypertension Congenital heart disease Ischaemic heart disease Angina pectoris Heart failure Pulmonary hypertension	**Chronic liver disease (unspecified)** Cirrhosis Primary sclerosing cholangitis Chronic hepatitis C infection Liver failure Hepatocellular carcinoma
Cancer Active solid tumour or haematological malignancy or cancer requiring active treatment	**Haematological Disorder** Sickle cell disease Thalassaemia major Factor VII deficiency Myeloproliferative disorder	**Immunodeficiency** Immunodeficiency (unspecified) HIV infection (any CD4 count) Graft versus host disease Primary immunodeficiency Vasculitis requiring immunomodulatory therapy
	Chronic Neurological and Neuromuscular Disease Chronic neurological disease (unspecified) Rett's syndrome Stroke Cerebral palsy Lissencephaly Leigh's syndrome Microcephaly Developmental delay with any of: cerebral dysgenesis; absent corpus callosum; spinal muscular atrophy	

Table 3.4 Diseases reported in MOSAIC patients
Diseases for adults and children are grouped according to risk factors identified by the World Health Organization's recommendations for influenza surveillance programmes.

3.5.1 Asthma

Asthma was the most frequently observed comorbidity in adults, reported in approximately one third of all patients, one third of influenza-positive patients and one third of influenza-negative patients. In adults with confirmed influenza, those with known asthma (n=43) had a significantly shorter mean length of stay than those who did not have asthma (n=90) (mean 8.6 days versus 15.3 days; *p* 0.002, Kruskal-Wallis equality of populations rank test). A greater number of influenza-positive asthmatic patients required oxygen at the point of maximum severity of illness than influenza-positive patients without asthma (48.8% versus 33.3%, respectively; *p* 0.001), but they were less likely to require invasive mechanical ventilation (16.3% versus 37.8%, respectively; *p* 0.001). Thirty of forty-three (69.8%) adults with asthma and influenza had other risk factors for severe influenza (comorbidities and/or pregnancy).

Although objective measurements of acute airways obstruction, such as changes in peak-expiratory flow-rate, were not recorded, clinical records were examined for documentation of wheeze and clinician-defined exacerbation of asthma. Influenza-infection status did not appear to influence the incidence of wheeze or acute exacerbation in patients with asthma (influenza-positive 46%, influenza negative 43%).

3.5.2 Other common comorbidities

The second most common comorbidity was chronic cardiovascular disease, reported in 24.1% of all patients (28.2% influenza-negative cases and 21.6% influenza-positive cases). The only comorbidity category with statistically significant differences in reported frequencies between influenza-positive and influenza-negative cases was immunodeficiency (17.9% and 3.9%, respectively, p 0.002). When HIV patients were analysed separately, the majority were also influenza-positive (p 0.03). Overall, one in five adults did not have any comorbidity and was not pregnant (20.1% influenza positive and 19.2% influenza-negative adult patients) (table 3.5).

3.5.3 Obesity in adults

Twenty-three percent of all adult patients were known to be obese (20.2% influenza-positive cases and 28.2% of influenza-negative cases). Obesity was determined by analysing multiple variables from both the Flu-CIN forms and MOSAIC CRF. The latter required bed-side recording of height and weight and also completion of the visual estimate of obesity scale (appendix 4). For adults, BMI was calculated where height and weight data were available, defining obese as BMI ≥30. Patients were also classified as obese if BMI could not be calculated but 'obese' was documented in FluCIN or the CRF. Comparison of visual obesity scale scores with BMI data (in patients with BMI data and completed visual scores) revealed that a visual score of 'H' or above was consistent with BMI-

defined obesity. Therefore, any patient with a visual score of H or above but no BMI data was also classified as obese.

3.5.4 Comorbidities in children

In contrast to adults, neurological and neuromuscular disease was the commonest comorbidity reported in children (38.1% overall and 40.5% influenza-positive cases), followed by chronic lung disease (21.4% overall and 21.6% influenza-positive cases). In contrast to adults, having below-normal bodyweight was particularly common in children, rather than being obese; low body-weight (defined according to the World Health Organization classification of a z-score of at least -2) was reported in one third of all cases and 30% of influenza-positive cases. 42.9% all children and 40.5% influenza-positive children did not have an identified comorbidity (table 3.5).

	Total, n=212 n (%)	**Influenza-negative, n=78 n (%)**	**Influenza-positive, n=134 n (%)**	***p* value**
Asthma	69 (32.6)	25 (32.1)	44 (32.8)	0.91
Chronic Lung Disease	22 (10.4)	8 (10.3)	14 (10.5)	0.97
Cardiovascular Disease	51 (24.1)	22 (28.2)	29 (21.6)	0.28
Chronic Liver Disease	6 (2.8)	1 (1.3)	5 (3.7)	0.75
Chronic Renal Disease	11 (5.2)	4 (5.1)	8 (6.0)	0.75
Neurological/Neuromuscular Disease	10 (4.7)	2 (2.6)	8 (6.0)	0.33
Diabetes mellitus	22 (10.4)	8 (10.3)	14 (10.6)	0.97
Haematological Disorder	9 (4.3)	4 (5.1)	5 (3.7)	0.73
Immunodeficiency (all causes)	27 (12.7)	3 (3.9)	24 (17.9)	0.002
HIV	17 (8.0)	2 (2.6)	15 (11.3)	0.03
Obesity	49 (23.1)	22 (28.2)	27 (20.2)	0.18
Current or recent cancer	22 (15.6)	10 (12.8)	23 (17.2)	0.40
No known comorbidity and not pregnant	42 (19.8)	15 (19.2)	27 (20.1)	1.0

Table 3.5 Co-morbidities in adult patients recruited to the MOSAIC Study
Data from both recruitment seasons have been combined. For comparison of influenza-negative and influenza-positive patients, *p* values were calculated using Fisher's exact test (two-tailed).

	Total (n=42) **n (%)**	**Influenza-negative (n=5)** **n (%)**	**Influenza-positive (n=37)** **n (%)**
Asthma	3 (71.0)	0	3 (8.1)
Chronic Lung Disease	9 (21.4)	1 (20.0)	8 (21.6)
Cardiovascular Disease	4 (9.5)	1 (20.0)	3 (8.1)
Chronic Liver Disease	0		
Chronic Renal Disease	0		
Neurological/Neuromuscular Disease	16 (38.1)	1 (20.0)	15 (40.5)
Haematological Disorders	1 (2.4)	0	1 (2.7)
Immunodeficiency (all causes)	5 (11.9)	0	5 (13.5)
HIV only	0		
Under-weight	14 (33.3)	3 (60.0)	11 (29.7)
Current cancer	1 (2.4)	0	1 (2.7)
No known comorbidity	18 (42.9)	3 (60.0)	15 (40.5)

Table 3.6 Co-morbidities in paediatric patients recruited to the MOSAIC Study
Data from both recruitment seasons have been combined. For comparison of influenza-negative and influenza-positive patients, *p* values were not calculated due to the small number of influenza-negative children.

3.6 Pregnancy

Of 112 female adult patients, 21 (19%) were pregnant (accounting for 20% influenza-positive women and 20% influenza-negative women). 46 influenza-positive women were within the commonly accepted range for child-bearing age, 15-44 years, and of these, 13 (28%) were pregnant. This figure is in excess of the reported expected 6% prevalence of pregnant women of child-bearing in age in the general population in England and Wales.(162) Influenza B was detected in one pregnant woman and the remainder had A(H1N1)pdm09 virus infection. Seven (53.8%) of the women had comorbidities.

Maximum severity of illness scores were calculated and five pregnant women (38.5%) had a score of 1, three (23%) had a score of 2 and five (38.5%) had score of 3. Of the five women with the most severe illness, four were in the third trimester and one was in the second trimester. However, two of five women with the least severe illness were also in the third trimester (the other three were in the first trimester). Additionally, eight women of child-bearing age were pregnant but influenza virus RNA was not detected in their samples. Six (75%) of these women had a maximum severity score of 1, two (25%) had a score of 2, but none had a score of 3.

The gestation period in weeks at onset of illness was known for 20/21 women recruited. Four (20%), five (25%) and eleven (55%) of the women were in the first, second and third trimesters, respectively. Of the influenza-positive pregnant women, four (30.8%), two (15.4%) and seven (53.8%) were in the first, second and third trimesters, respectively. Five of thirteen (38.5%) influenza-positive pregnant women did not have any comorbidities. Five of the thirteen (38.5%) women required invasive mechanical ventilation (grade 4 maximum severity of illness) but an equal number had mild illness (grade 1 severity). Two of those requiring intensive care did not have underlying comorbidities and one woman, who also had asthma, died in hospital.

Consistent with findings from other studies (175, 242), pregnant women with influenza appear to be over-represented in the MOSAIC cohort, as compared to prevalence in the source population. Pregnant women with influenza were generally at an advanced stage of pregnancy. A significant proportion required intensive care, but an equal proportion had mild illness without respiratory compromise.

3.7 Duration of illness at presentation and at recruitment

Analysis of data from all patients recruited to MOSAIC revealed that the mean day of illness at first presentation to any hospital was 4.5 days (median 3 days, range 0-31 days). The mean day of illness at presentation did not differ significantly between influenza-positive and influenza-negative

patients (mean 4.6 and 4.3 days, respectively). Both groups had a median day of illness at presentation of 3 days.

The mean day of illness at the time of T1 sampling for all patients was 7.2 days (median 6 days, range 0-35 days). Influenza-negative patients had a lower mean day of illness at T1 than influenza-positive patients (mean 5.8 versus 7.9 days and median 4 versus 7 days, respectively; *p* 0.002). The distribution of day of illness at T1 for influenza-positive and influenza-negative adults is shown in figure 3.2.

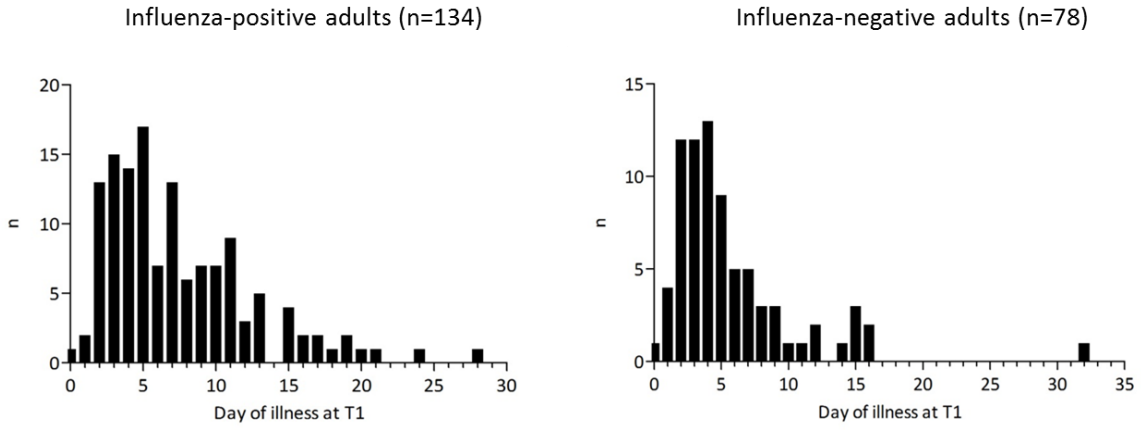


Figure 3.2 Day of illness at T1 for influenza-positive and influenza-negative adults
Data from both seasons have been combined (n = number of patients). Day of illness is the number of days passed from onset of compatible symptoms to the time T1 sampling occurred.

The majority of adult patients presented to hospital and subsequently underwent T1 sampling after experiencing a short duration of symptoms in the community, regardless of their influenza status. For influenza-positive adults, the longer tail of the distribution of day of illness at T1 may be influenced by recruitment of patients that had been transferred for tertiary intensive care. For day of illness at presentation and at T1, the highest values were greater than expected for patients with ILI. These extreme values, seen in patients with influenza-negative ILI, may represent a lack of sensitivity of the data collection tools and associated difficulties in differentiating chronic symptoms from acute symptoms in patients with underlying comorbidities e.g. COPD. Alternatively, they may represent true on-going infection, or possibly complications of a preceding infection, such as secondary bacterial infection following a respiratory virus infection.

3.8 Assessment of severity of illness

3.8.1 Original MOSAIC Severity Score

Because of the intensive nature of the study and limited resources available for patient recruitment, MOSAIC did not recruit non-hospitalised patients with mild influenza or influenza-like illness. However, a range of illness was observed in our hospitalised cohort. This varied from patients without respiratory compromise or evidence of other complications and who required a short hospital admission e.g. 48 hours duration, through to patients who developed ARDS and multi-organ failure and who required a long period of intensive care and/or who died in hospital.

International observational series have reported that respiratory failure was the dominant feature in patients with severe A(H1N1)pdm09 virus infection (66). Respiratory failure is also well recognised in hospitalised patients with influenza B or H3N2 infection (94, 396) and in ILI caused by other respiratory tract pathogens (397, 398). Furthermore, respiratory failure was reported to be variable in patients infected with A(H1N1)pdm09 virus infection, ranging from no respiratory compromise through to respiratory failure requiring intensive care, which is consistent with our own observations. Therefore, severity of illness in MOSAIC patients was graded according to the degree of respiratory compromise and the interventions that were required to correct the abnormality (table 3.7).

Severity grade	Definition
1	no respiratory compromise (i.e. O_2 saturation >93% on room air)
2	respiratory compromise (i.e. supplemental oxygen required for hypoxaemia or O_2 saturation ≤93% on room air) but mechanical ventilation not indicated
3	respiratory compromise justifying *non-invasive* mechanical ventilation
4	respiratory compromise justifying *invasive* mechanical ventilation

Table 3.7 The original four-point MOSAIC severity of illness grading system

Respiratory compromise was defined as oxygen saturation ≤93% (measured without supplemental oxygen) or the need for supplemental oxygen. Patients without respiratory compromise were classed as grade 1 severity. Interventions for respiratory compromise were then stratified as simple (oxygen delivered by mask or nasal cannulae only, severity grade 2), non-invasive (non-invasive mechanical ventilation, severity grade 3), or invasive (invasive mechanical ventilation with or without other organ support, severity grade 4). Justification for intervention was included in the definition, rather than receipt of an intervention alone, because in some cases it was deemed inappropriate to provide an intervention, for example when an underlying terminal illness made admission to an intensive care unit inappropriate. This approach aimed to avoid under-estimation of severity in such patients. It required detailed analysis of case-notes and in some cases, review of death certificates.

3.8.2 Example cases

Three examples of hospitalised adult subjects with mild (grade 1), moderate (grade 2), and severe (grade 3) illness are described in the case descriptions below.

Case A: Grade 1 severity of illness

A 28 year-old woman presented with a two-day history of fever, myalgia, sore throat, headache and diarrhoea. She had polycystic ovarian syndrome, but no other past medical history. The patient had not been immunised against influenza or *S. pneumoniae* and she had not taken any anti-infective agents prior to admission. Apart from pyrexia and tachycardia, routine physical examination on admission was unremarkable. Peripheral blood oxygen saturations were 99% on room air and the chest radiograph appeared normal. Blood tests revealed a normal white cell count but lymphopaenia ($0.5 \times 0.4 \times 10^9$/L). CRP was elevated (52 mg/L). A nasopharyngeal swab was obtained at admission and submitted for respiratory virus multiplex PCR testing. Blood cultures were negative. Empirical anti-infective agents were not commenced. The patient remained febrile throughout the second day of admission. On day 2, the patient was commenced on oral oseltamivir 75 mg twice-daily when the multiplex PCR result was received, confirming A(H1N1)pdm09 virus infection. The patient was afebrile on day 3 and her symptoms were improving. She was discharged with a five day course of oseltamivir.

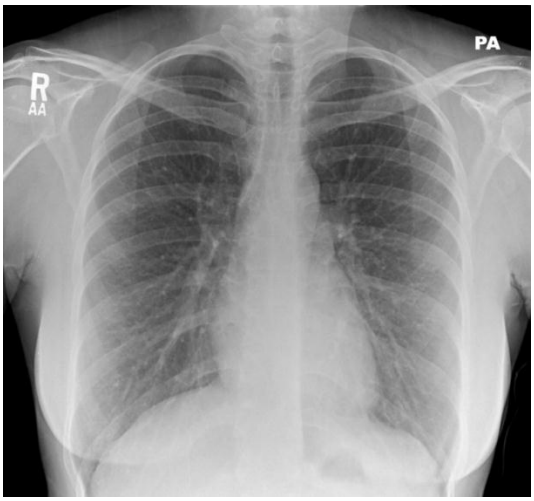


Figure 3.3 Admission chest radiograph for Case A
Posterior-anterior projection. Lung fields have a normal appearance and no other abnormality can be seen.

Case B: Grade 2 severity of illness

A 59 year-old woman presented with a four-day history of malaise, sore throat, fever, myalgia and vomiting. Past medical history was unremarkable and she had not received antimicrobials prior to admission, nor had she been immunised against influenza or pneumococcus. Observations at the time of admission revealed fever, tachycardia and peripheral blood oxygen saturations of 93% measured on room air. Chest auscultation revealed crepitations in both lower zones and bronchial breath sounds in the right upper zone, but no tachypnoea. A chest radiograph showed consolidation in the right upper lobe. Blood tests revealed a white cell count of 14.3 x 10^9/L, a neutrophil count of 14.3 x 10^9/L and a lymphocyte count of 0.4 x 10^9/L. Serum CRP was 346 mg/L. Additionally serum ALT was elevated at 125 IU/L and serum albumin was decreased at 27g/L. A combined nose and throat swab was submitted for respiratory multiplex PCR and blood and sputum samples were submitted for bacterial culture. Pneumococcal and *Legionella pneumophila* urinary antigens were not detected. Empirical oral oseltamivir 75 mg twice-daily, co-amoxiclav and clarithromycin were commenced. The patient received supplemental oxygen via a face mask. Infection with influenza A(H1N1)pdm09 virus was confirmed by PCR on day 2 of admission. The patient required supplemental oxygen for the first four days of hospital admission. Defervescence occurred on day 5. At the time of discharge on day 7, oxygen saturations were 97% on room air. The peripheral blood white cell count had normalised and the lymphocyte count had increased to 1.0 x 10^9/L. Serum CRP remained above normal range, but had decreased to 28 mg/L. A repeat chest radiograph showed partial resolution of the right upper lobe consolidation that had been seen previously.

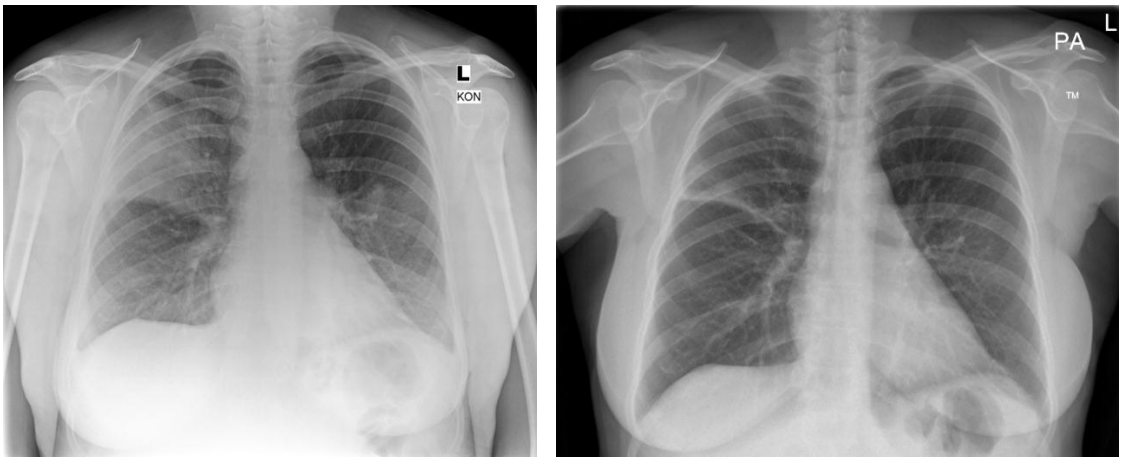


Figure 3.4 Chest radiographs for Case B
The left-hand radiograph was obtained at the time of admission and demonstrates right upper lobe consolidation. Partial resolution of right upper lobe consolidation can be seen in the radiograph obtained at discharge (right hand panel).

Case C: Grade 4 severity of illness

A 68 year-old man was admitted electively for a bone marrow transplant for treatment of multiple myeloma. His other past medical history included type II diabetes mellitus and hypertension. At the time of admission, he was not receiving immunosuppressants, but he was receiving prophylactic antimicrobials (ciprofloxacin, fluconazole and aciclovir). He had not received influenza or pneumococcal immunisation. Autologous bone marrow transplantation was performed on day 6 of admission and he was nursed in protective isolation due to his induced immunosuppressed state.

On day 7 of hospital admission, he developed fever and a cough productive of sputum. On day 9 of admission, he became tachypnoeic and tachycardic, but oxygen saturations on room air were 97%. Chest auscultation revealed crackles at the base of the left lung. Peripheral blood white cell count was 0.1 x 10^9/L (neutrophils 0.1 x 10^9/L, lymphocytes 0.1 x 10^9/L). The serum CRP level was 71.2 mg/L. Blood and sputum cultures were requested. A combined nose and throat swab was submitted for PCR testing for influenza. The chest radiograph revealed airspace shadowing in the left lower lobe. Ciprofloxacin was stopped and empirical antibiotics were commenced (piperacillin and tazobactam, teicoplanin and amikacin).

On day 10 of admission (day 3 of illness), detection of influenza A(H1N1)pdm09 virus RNA was reported and oral oseltamivir 75 mg twice daily was commenced. Blood tests showed increased serum creatinine and urea, consistent with acute kidney injury, and total neutropaenia and lymphopaenia. CRP had increased to 262 mg/L. His chest was reported to be clear on auscultation and peripheral blood oxygen saturations remained at 96% without supplemental oxygen. He developed new onset diarrhoea, without evidence of *Clostridium difficile* infection. Piperacillin/tazobactam was discontinued and meropenem was commenced. Pathogenic bacterial infections were not identified following culture of blood, stool, urine and respiratory secretions.

By day 12 of admission (day 5 of illness), the patient demonstrated desaturation on movement (oxygen saturations 93%; type I respiratory failure on arterial blood gas analysis). Chest auscultation revealed bi-basal crepitation and bi-basal consolidation was seen on the chest radiograph. Clarithromycin was commenced and oseltamivir was reduced to 75 mg once daily in view of renal impairment. Non-invasive mechanical ventilation (CPAP) was commenced on day 17 of admission (day 10 of illness), in view of worsening type I respiratory failure. On day 19 of admission (day 12 of illness), the patient required invasive mechanical ventilation for ARDS and haemofiltration for worsening acute kidney injury and fluid overload. Additionally, he required inotropic and pressor support for circulatory failure.

Oseltamivir was discontinued after 10 days of treatment and intravenous zanamivir was administered. Pyrosequencing of A(H1N1)pdm09 virus-positive respiratory samples did not identify substitutions associated with oseltamivir or zanamivir resistance. Zanamivir treatment was discontinued after 10 days of administration, following receipt of a negative influenza PCR result (upper respiratory tract sample). The patient continued to receive broad-spectrum antibiotics and empirical antifungal treatment was commented (liposomal amphotericin). Bacterial and fungal infections were not identified.

The patient continued to deteriorate and died on day 34 of admission (day 27 of illness). An autopsy was performed five days after death. Histological examination of tissues revealed myocarditis and fibrinous pericarditis, lung tissue changes consistent with ARDS and moderate chronic inflammation with foci of acute inflammation. Examination of bone marrow revealed features suggestive but not diagnostic of haemophagocytosis. Influenza A(H1N1)pdm09 virus RNA was detected in samples from the nasopharynx and the lower lobes of the lungs.



Figure 3.5 Serial chest radiographs for Case C
The left-hand radiograph was obtained at the onset of symptoms and demonstrates patchy infiltrates in the left lower zone. The central radiograph was obtained on day 5 of illness and reveals extensive bilateral, patchy airspace infiltrates. The right-hand radiograph was obtained on day 25 of illness and persistence of the bilateral infiltrates can be seen, along with changes consistent with evolving lung fibrosis.

3.8.3 Changes in severity of illness over time

Severity scores were assigned for severity at the time of admission to a recruiting site (initial respiratory severity) and at T1 and at T2. The maximum respiratory severity score for the acute episode of influenza illness/ILI was also assigned for each subject. The relative proportion of severity 1 patients was the same at presentation, at T1 and at T2 (approximately 45%). However, when maximum severity was measured, grade 2 severity was the most commonly observed severity of illness, accounting for almost 40% of patients. Severity 3 patients were relatively uncommon, irrespective of when severity was measured.

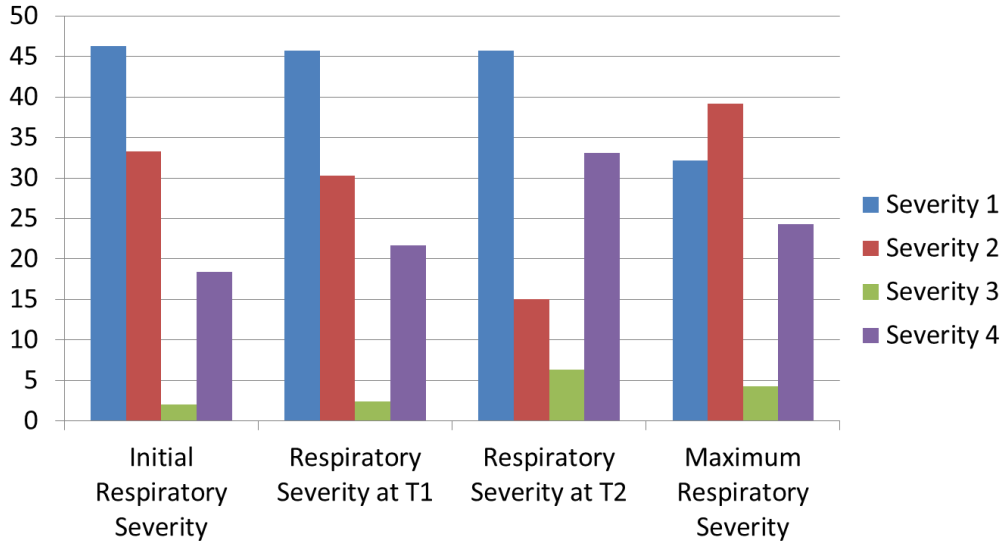


Figure 3.6 MOSAIC severity score at different times during hospitalisation
The severity score was assigned at presentation to hospital, at T1, and at T2. Peak severity of illness for the entire influenza/ILI episode was also measured. Data are for all patients from both seasons. Scores were calculated for 255 patients on presentation (initial respiratory severity), 254 patients at T1 and 127 patients at T2. Maximum severity score for the hospital episode was calculated for 255 patients. The percentage of patients is shown on the Y axis.

3.8.4 Severity by season

The majority of patients with grade 4 severity of illness were recruited during 2010/11; seven children and four adults with grade 4 severity at T1 were recruited in 2009/10, compared to twelve children and thirty-two adults with grade 4 severity at T1 recruited during 2010/11. Recruitment procedures did not differ between the two seasons, however. For patients who were transferred from a local hospital to a MOSAIC recruiting centre for specialist intensive care interventions, analysis of transfer documents revealed that 92% of patients were admitted to ICU at the time of admission or within 48h of admission.

3.8.5 Association between severity score at admission and maximum severity score

Comparison of severity scores on admission to a MOSAIC centre with the maximum severity score revealed that initial severity was predictive of maximum severity in the majority of patients. This was most noticeable for patients with grade 4 severity. Of 64 patients with grade 4 maximum severity of illness, 47 (76%) already had grade 4 severity at presentation to hospital (table 3.14).

		Maximum Severity			
		Severity 1	**Severity 2**	**Severity 3**	**Severity 4**
Initial Severity	**Severity 1**	69.5 (82)	25.4 (30)	2.5 (3)	2.5 (3)
	Severity 2		82.3 (70)	5.9 (5)	11.8 (10)
	Severity 3			60.0 (3)	40.0 (2)
	Severity 4				100 (47)

Table 3.8 Initial severity score and its relationship to the maximum severity score
Maximum severity score is the peak severity observed during a patient's episode. Initial severity is severity at time of presentation to hospital. Data are for all MOSAIC patients from both seasons.

Fifty-three patients (21%) experienced an increase in severity of illness following presentation, but the increase in severity occurred following T2 in the majority of patients. Of 255 patients, 127 patients (49.8%) underwent T2 sampling. 113/127 (89%) were influenza-positive patients and 25/127 (19.7%) were children. Only three patients demonstrated an increase in severity at T2. However, of 39 patients with severity 2 illness at T1, 21 (53.9%) had illness at T2 that had decreased to grade 1 severity. Of 47 patients with severity 4 illness at T1, 5 (10.6%) patients had also decreased in severity at T2 (table 3.15).

	Severity unchanged at T2 % (n)	**Severity increased at T2 % (n)**	**Severity decreased at T2 % (n)**
Severity 1 at T1 n=37	97.3 (36)	2.7 (1)	NA
Severity 2 at T1 n=39	41.0 (16)	5.1 (2)	53.9 (21)
Severity 3 at T1 n=4	100 (4)	0	0
Severity 4 at T1 n=47	89.4 (42)	N/A	10.6 (5)

Table 3.9 Changes in severity of illness between T1 and T2
Data are from 127 patients who underwent sampling at T1 and T2. Data shown are for influenza-positive and influenza-negative adults and children from both recruitment seasons.

3.8.6 Revised MOSAIC Severity Score used in transcriptomics and soluble mediator analyses

Due to the small number of patients with severity 3 illness and to facilitate analysis of transcriptomic and soluble immune mediator data, the original four-point severity score was revised to form a three-point score. Existing grade 3 severity patients (respiratory compromise and non-invasive mechanical ventilation justified) were combined with grade 2 severity patients (respiratory compromise but no need for mechanical ventilation), to create the revised, three-point score (table 3.16).

Revised severity grade	Definition
1	no respiratory compromise (i.e. O_2 saturation > 93% on room air)
2	respiratory compromise (i.e. supplemental oxygen required for hypoxaemia or O_2 saturation ≤93% on room air) but *invasive* mechanical ventilation was not justified or administered
3	respiratory compromise justifying *invasive* mechanical ventilation

Table 3.10 Revised three-point severity score used for transcriptomics and soluble immune mediator analysis

3.8.7 Comparison of severity for influenza-positive and influenza-negative adults

Comparison of the revised, three-point severity scores for influenza-positive and influenza-negative adult patients revealed that a significantly greater proportion of grade 3 severity patients had confirmed influenza (30.6% versus 11.5%, respectively; *p* 0.005). The proportions of adult patients with grade 1 severity and grade 2 severity were similar between the two groups (31% influenza-positive with grade 1 severity versus 46% influenza-negative with grade 1 severity; 38% influenza positive with grade 2 severity versus 42% influenza-negative with grade 2 severity). The mean duration of illness was significantly greater in the influenza-positive adults compared to influenza-negative adults (18.3 versus 12.9 days, respectively, p 0.04).

3.8.8 Fatalities

Of 134 influenza-positive adults for whom appropriate data were available, 18 patients had a fatal outcome (13.4%). This is in contrast to the influenza-negative adult cohort, where 3 of 78 patients (3.9%) died. Although this difference reached statistical significance (*p* 0.04), it should be remembered that given the high prevalence of patients with significant comorbidities in the MOSAIC cohort, influenza or other acute infections causing ILI may not have been the direct cause of death, although they may have contributed to death.

Of 18 fatal influenza cases, all had A(H1N1)pdm09 virus infection and 15 (83%) had comorbidities. Of 12 adults with influenza B virus infection, only one patient had severity 3 illness and none died. Six adults with influenza B virus infection had severity 2 illness and five adults had severity 1 illness. Of the three adults with H3N2 infection, two had severity 2 illness and one had severity 1 illness; none died.

3.8.9 Summary of severity and mortality findings

In summary, MOSAIC recruited patients with varying severities of illness, ranging from those without respiratory compromise through to patients requiring long periods of specialist intensive care, including ECMO therapy. Patients requiring non-invasive mechanical ventilation were uncommon and this necessitated revision of the original severity score into a new 3-point severity score that did not differentiate patients requiring non-invasive mechanical ventilation from patients requiring mask/nasal oxygen. For combined analysis of patients from both seasons, the relative proportions of patients in each severity group are similar, although there were relatively few influenza-negative patients with the most severe illness (severity grade 3). Although an increase in severity was uncommon at T2, approximately half of severity grade 2 patients had objective evidence of improvement at T2. Additionally, 10.6% of severity grade 4 patients had improved severity scores at T2.

3.9 Sampling and assessment of recovery status at T3

The third time point, T3, was intended to be a convalescent sampling time point performed at 4-6 weeks following recruitment. However, it was apparent that some patients were unlikely to survive beyond T3. Therefore, a decision was made to obtain T3 samples irrespective of convalescent status at T3. Furthermore, for logistical reasons, it was not possible to sample all patients within 4-6 weeks. For the purposes of correlation with biological data, it was important to classify patients' health status at T3. This was achieved by means of a simple clinical assessment performed by researchers when obtaining T3 samples. Patients were deemed to have recovered if they had been discharged from hospital and were free from symptoms compatible with on-going influenza/ILI and free of symptoms suggestive of a complication of influenza/ILI. For patients who remained in hospital at T3, a distinction was made between those who required on-going intensive care and those who were receiving ward-level care.

Of 255 patients recruited to the study, 90 underwent T3 sampling (35.2%). Nine patients (10%) were originally influenza-negative and 17 (18.9%) were children. The mean time from onset of illness to T3 was 47.7 days (median 44 days, range 26-97 days). At T3, 72 patients (80%) had recovered, 9 (10%) remained on intensive care and 9 (10%) were in-patients receiving ward-level care.

3.10 Non-influenza virus detections

3.10.1 Detections of respiratory viruses in non-influenza patients

Seventy-eight patients without evidence of influenza virus infection had respiratory tract samples submitted for multiplex respiratory virus PCR. Respiratory tract samples included MOSAIC T1 NPA

and any NHS samples obtained and tested between recruitment and T2. Thirty-five patients (45%) had non-influenza viruses detected. The most commonly detected viruses were rhinovirus (12 detections) and RSV (11 detections). Other viruses detected were: coronavirus (229E, NL63, OC43 and HKU1), human metapneumovirus, enterovirus, bocavirus, adenovirus and parainfluenza 4 virus (parainfluenza 1-3 were not detected in any patient). For some samples, more than one virus was detected.

3.10.2 Detections of respiratory viruses in patients with confirmed influenza

Of 134 influenza-positive patients, non-influenza viruses were detected in respiratory tract samples from 35 patients (26%). Samples were obtained and tested in the same manner as described for influenza-negative patients. Again, rhinovirus and RSV were the most frequently detected viruses (17 and 7 detections, respectively). Parainfluenza virus was not detected in any sample, but the other viruses detected were the same as those in influenza-negative patients and again, more than one non-influenza virus was detected in some samples.

3.10.3 Summary of non-influenza virus detections

Although causation of ILI cannot be assumed and respiratory virus detections will need to be considered alongside clinical information and other microbiology results, almost half of the influenza-negative patients had non-influenza viruses detected in respiratory tract samples (predominantly upper respiratory tract samples). Furthermore, one quarter of influenza-positive patients had other influenza viruses detected in samples obtained between recruitment and T2. This is a higher incidence of secondary viral infection/co-infection than was expected.

3.11 Detection of bacteria

3.11.1 Bacterial detection data collection

Collation and analysis of bacterial detection data is on-going. However final data sets are available for selected tests performed on MOSAIC samples (T1 NPA PCR and pneumococcal urine antigen testing) and for NHS-obtained pneumococcal urine antigen tests and blood cultures. Additionally, procalcitonin was measured in MOSAIC-obtained serum and plasma.

3.11.2 Detection of bacteria in upper respiratory tract samples from patients with influenza

Appropriate NPA samples were available for testing from 143 influenza-positive patients (27 patients from 2009/10 and 116 patients from 2010/11). Overall, fifty-four patients (37.8%) had bacteria detected. The rate of detection in 2010/11 was almost double that seen in 2009/10 (41% versus 22%, respectively), but this did not reach statistical significance. The most commonly detected

bacterial species were *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Haemophilus influenzae*. More than one bacterial species was detected in some NPA samples (data not shown) (table 3.8).

3.11.3 Blood culture results

Blood cultures were obtained at the discretion of attending clinicians. Therefore, blood cultures were not obtained from every patient. An analysis of results of blood cultures obtained 48h either side of T1 sampling from influenza-positive adults with available data was performed. In 2009/10, of 22 influenza-positive adults, 16 (72.7%) had blood cultures obtained. Only one patient had a positive bacterial culture and this was growth of a coagulase-negative *Staphylococcus*, thought to be related to an indwelling vascular catheter. In 2010/11, of 108 adult patients, 52 (48%) had blood cultures obtained. Six patients (11.5%) had bacteria detected in blood. *S. pneumoniae* was cultured from 3 patients, one patient had *S. aureus*, one had Group A *Streptococcus* and one had Group B *Streptococcus.*

3.11.4 Urine pneumococcal antigen results

Pneumococcal urinary antigen testing was performed when a MOSAIC urine sample was available. The samples were not linked to a specific time point, but the samples were obtained between T1 and T2. Urine samples were also obtained and tested by NHS laboratories, at the discretion of the attending clinician. For both seasons, 118 MOSAIC samples were tested. 4/18 (22.2%) children and 12/100 (12%) adults had urine antigen detected. Three of the detections were in influenza-negative adults; the remaining 13 detections were in influenza-positive patients.

3.11.5 Known invasive pneumococcal infection

By combining detections of *Streptococcus pneumoniae* by blood culture and/or detection of pneumococcal urine antigen in influenza-positive patients, 1/33 (3%) patients from 2009/10 and 10/139 (7.2%) patients from 2010/11 were shown to have evidence of invasive bacterial infection. Because appropriate and standardised testing for invasive infection was not performed in all subjects, these figures only represent cases that were known to have invasive pneumococcal infection (table 3.8).

3.11.6 Other infections in influenza patients

Two patients with A(H1N1)pdm09 had invasive bacterial infections detected by other means, based on analysis of NHS microbiology results. One child recruited in 2010/11 had meningococcal meningitis diagnosed by culture of cerebrospinal fluid at the same time that influenza infection was diagnosed. One critically-ill adult, also recruited in 2010/11, had invasive aspergillosis diagnosed, on

the basis of compatible radiological findings, growth of *Aspergillus fumigatus* from BAL fluid and detection of an elevated galactomannan level in serum. This infection occurred following completion of treatment of influenza and influenza RNA was not detected in upper or lower respiratory tract samples at the time. The patient had one risk factor for severe influenza (grade I obesity) but had received corticosteroids as part of treatment of ARDS. The patient died following massive pulmonary haemorrhage that was attributed to invasive aspergillosis.

3.11.7 Serum procalcitonin measurement

Procalcitonin (PCT) measured in T1 serum was also used as a surrogate marker of invasive bacterial infection or bacterial pneumonia. This is discussed in more detail in chapter five. Studies have shown that a low serum PCT (< 0.05 ng/ml) has a high negative predictive value i.e. a low value is a sensitive marker of *absence* of invasive bacterial infection or bacterial pneumonia (104, 399, 400). Low levels of PCT were detected in serum from approximately half of influenza-positive patients, regardless of the recruitment season (table 3.8).

3.11.8 Bacterial detections in influenza-negative patients

Although microbiological/virological diagnoses are still being finalised for the influenza-negative patients, patients from both seasons also underwent the same screens for bacterial infection at T1 that have been described for influenza-positive patients. Preliminary analysis of T1 NPA respiratory multiplex PCR results for reveals that 14/45 (31%) adult patients from 2009/10 and 12/31 (38.7%) adult patients from 2010/11 had bacteria detected.

In those with samples available for analysis	**2009/10 n (%)**	**2010/11 n (%)**	***p* value**
Patients with bacteria detected in T1 NPA (%)	6/27 (22)	48/116 (41)	0.08
Bacterial species detected in T1 NPA:			-
S. pneumoniae	3	17	
S. aureus	1	21	
H. influenza	1	16	
Other	3	16	
Patients with invasive *S. pneumoniae* infections	1	10	-
T1 serum/plasma PCT <0.5 ng/ml	14/29 (48)	63/124 (51)	0.34

Table 3.11 Detection of bacteria in influenza-positive adult patients by recruitment period
Bacterial detections in T1 NPA were identified by respiratory multiplex PCR (only detections with Ct value <35 were included). The number of detections of *Streptococcus pneumoniae* (Pneumococcus), *Staphylococcus aureus,* and *Haemophilus influenzae* are shown. Other bacteria detected were: *Mycoplasma pneumoniae*, *Moraxella catarrhalis,* and Klebsiella pneumoniae. More than one bacterial species was detected in some of the samples; therefore the total number of detections exceeds the number of patients with samples in which bacteria were detected. Invasive *S. pneumoniae* infection was defined as growth of the organism by blood culture and/or detection of pneumococcal urine antigen. Blood cultures and urine antigen testing was not performed for every patient. Serum procalcitonin <0.5 ng/ml is used a surrogate marker of absence of bacterial sepsis or pneumonia. For comparison of bacterial detection rates and for 2009/10 and 2010/11 seasons, *p* values were calculated using Fisher's exact test.

3.12 Antiviral, antibiotic and influenza vaccination data

3.12.1 Pre-hospital antibiotic and antiviral use in influenza patients

Pre-hospital influenza antiviral use was uncommon for influenza-positive patients from either season; only two patients reported receiving neuraminidase inhibitors prior to admission to hospital. This is contrast to pre-hospital antibiotic use. Of patients with appropriate data available for analysis, 43/170 (25.3%) influenza-positive patients had received newly prescribed antibiotics immediately before hospitalisation. Pre-hospital antibiotic prescribing rates in influenza-positive patients were similar for both seasons (27% for 2009/10 and 25% for 2010/11).

3.12.2 In-hospital antiviral use in influenza patients

Most patients with confirmed influenza received neuraminidase inhibitors in hospital. However, 1/33 (3%) influenza-positive patients from 2009/10 and 7/137 (5%) influenza-positive patients from 2010/11 did not receive antiviral therapy in hospital or at discharge. The reasons for not prescribing antivirals in these patients are not known. Of those who did receive neuraminidase inhibitors, approximately 85% of patients received oral oseltamivir, regardless of season of recruitment. Nine percent of influenza-positive patients received intravenous zanamivir (with or without preceding oseltamivir), again with no difference seen between the recruitment seasons. The mean time from onset of symptoms to commencing antivirals was 5.2 days (median 3 days) for 2009/10 patients and 7.4 days (median 5 days) for 2010/11 patients. Analysis of in-hospital antibiotic data is on-going.

3.12.3 Influenza vaccination data for influenza patients

Influenza vaccination data were available for 130/137 (95%) influenza-positive patients from 2010/11, but data were only available for 13/33 (39%) of influenza-positive patients from the 2009/10 season. Of the 2010/11 patients with data available, 21/130 (16%) reported having received influenza vaccine appropriate to strains circulating in that season at an appropriate time prior to onset of illness (at least two weeks prior to onset). Of those who did undergo appropriate vaccination but went on to develop influenza, all had underlying comorbidities. Asthma was the most commonly reported co-morbidity (12/21, 57%) and immunodeficiency was reported in 8/21 (38%), of which five were patients with HIV infection.

3.12.4 Summary of antiviral, antibiotic and vaccination data

Overall, pre-hospital antibiotic use was much more common than pre-hospital antiviral use. Most patients with confirmed influenza did receive antivirals, although they were generally commenced after the first 48h of symptomatic illness. Some patients developed influenza despite appearing to have been appropriately vaccinated. However, these patients tended to have underlying conditions,

or received treatments for underlying conditions, that may decrease the effectiveness of influenza vaccines.

3.13 Adult healthy controls

In addition to healthy controls recruited to provide whole blood transcriptomic samples, 36 healthy adult volunteers were recruited between 30 August and 01 December 2011. These adults provided a full set of MOSAIC samples, equivalent to the sample sets obtained from adult patients at T3 during the 2010/11 recruitment period. Questionnaire screening was used to exclude those underlying healthy problems, those with active or recent acute respiratory illnesses and those with a history of atopy. All patients were non-obese and were non-smokers. Twenty (55.5%) volunteers were male and thirty (83.3%) were white. The mean age was 31.8 years (median 30.5 years, range 22-57 years). Fourteen (38.9%) volunteers had never received influenza vaccination.

Eight (22.2%) healthy controls had *S. aureus* in their NPA samples, detected by respiratory multiplex PCR testing. One healthy control had *S. pneumoniae* detected. One patient had *M. catarrhalis* detected and this sample also contained rhinovirus RNA. Review of the screening questionnaire for this volunteer, along with a subsequently conducted interview, confirmed that the volunteer was free of symptoms at the time of sampling and that they did not develop ILI or any other illness following sampling. No other bacteria or viruses were detected in samples from the remaining volunteers. Serum procalcitonin was low in all healthy control subjects (mean 0.014 ng/mL; range 0.01-0.046 ng/mL).

3.14 Discussion

3.14.1 Summary of clinical aspects of MOSAIC

MOSAIC successfully recruited 255 hospitalised patients with influenza and other causes of influenza-like illness. Two thirds of recruited patients had confirmed influenza infection, predominantly A(H1N1)pdm09 virus infection; those who presented with ILI but did not have evidence of active influenza infection provide a useful comparison group. The cases were recruited during the UK's second pandemic wave (winter 2009/10) and the first post-pandemic winter (2010/11), although most were recruited during the latter period. MOSAIC achieved its aim of recruiting the majority of patients within 24 hours of admission to hospital, although most had already experience several days of symptoms in the community. Biological samples were obtained at two time points during acute illness and also at a third, delayed time point when patients were either convalescent or remained critically ill. The proportions of patients who underwent T2 and T3

sampling were acceptable for a clinical observational study of hospitalised patients with acute respiratory illness, although most were influenza-positive.

In terms of patient demographics and risk factors for severe influenza, the MOSAIC cohort is similar to other populations of hospitalised patients infected with A(H1N1)pdm09 virus (171, 175, 401, 402) and is, therefore, representative of the wider population. Although mild community cases were not recruited, there is a clear spectrum of severity of illness within the cohort, ranging from those without respiratory compromise through to those with significant lung injury attributable to acute infection. The spectrum of illness has been described using a severity score, which was based on the degree of respiratory compromise and the interventions required to correct it.

Influenza viral load appears to decrease at T2 in most patients, although showed an increase in viral load and others had evidence of prolonged virus shedding. Although analysis of bacterial and viral co-infection data is on-going, preliminary results suggest that detection of non-influenza viruses occurred in a greater number of patients than expected. By contrast, detection rates for bacterial co-infection appear to be similar to those described by others, despite the extensive investigations and data interrogation steps that took place. Influenza antiviral use was common once in hospital, but rates of pre-hospital antiviral use and influenza vaccination were low prior to hospitalisation, even in those who were at risk of developing severe influenza.

3.14.2 MOSAIC demonstrated that human pathogenesis studies are possible in a pandemic

MOSAIC was one of only a small number of studies to prospectively recruit patients during the 2009-2010 influenza pandemic (403). The Consortium was unique in its aim of studying all the factors (host, pathogen and co-pathogen) contributing to severity of illness, in an integrated, prospective observational study conducted during a short-lived outbreak. Conceived in April 2009, the study was only able to launch and recruit patients after both the peak of the second pandemic wave had passed. Despite intensive recruitment efforts, 85 cases were recruited during the actual pandemic period and only 33 of these had laboratory confirmed influenza. The larger number of non-influenza viruses detected in the first period of recruitment reflects the respiratory viruses that were circulating nationally during this time (176). However, the study remained open to recruitment and total case numbers and the number of confirmed influenza cases increased significantly during the winter of 2010/11 (total 255 cases, 172 with influenza). This third wave of A(H1N1)pdm09 virus activity was accompanied by increased numbers of hospitalisations and critically-ill cases across the UK, but the main groups affected – younger adults and children – remained unchanged when compared with the pandemic waves. Furthermore, although subtle changes in circulating

A(H1N1)pdm09 viruses have been described, the major antigenic determinants were reported not to have changed (183). Consistent with circulating viruses described in national surveillance data (404), a smaller number of patients infected with influenza B and H3N2 viruses were also recruited during 2010/11.

3.14.3 The ability to recruit patients during the severe first post-pandemic winter was important

Given the greater burden of hospitalised and critically cases seen nationally during the winter of 2010/11, even if MOSAIC had been able to recruit patients before the peak of the second wave, it appears likely, in retrospect, that the majority of confirmed A(H1N1)pdm09 virus infections would have been recruited during the 2010/11 season. It remains unclear why the UK experienced such a severe post-pandemic wave in 2010/11. Although other countries have reported a marked burden of severe illness during the same season, none reported a 'third wave' with such a large number of severe cases over a relatively short period (180, 405).

Serosurveillance studies suggested that following the second wave, the majority of children and adults across the UK had anti-A(H1N1)pdm09 virus antibodies and so a third wave was not expected in the absence of antigenic drift (68). However a longitudinal community cohort study of healthy adults in the UK demonstrated a significantly higher incidence of A(H1N1)pdm09 virus infection in 2010/11 than in 2009/10, with a higher risk for infection during 2010–11 among persons with lower pre-infection antibody titres (406). While this may explain a residual, susceptible population, it does not explain the apparent excess of severe cases seen in 2010/11. MOSAIC has measured anti-A(H1N1)pdm09 virus antibodies in patients' serum by haemagglutination inhibition and microneutralisation and the results are currently being analysed. MOSAIC is also currently comparing A(H1N1)pdm09 virus whole genome sequences for 2009/10 and 2010/11 virus isolates, to investigate whether changes not relating to major antigens may have contributed to an increase in severe cases in 2010/11.

It has also been suggested that the change in community antiviral prescribing guidance post-pandemic may have resulted in fewer patients receiving early antivirals, although data do not exist to support this theory (407, 408). Pre-hospital antiviral use and influenza vaccination rates were very low in the MOSAIC cohort overall, although the study lacks the power to compare differences between the two periods of recruitment.

In addition to A(H1N1)pdm09 infections, a smaller number of influenza B virus and influenza A(H3N2) virus infections were recruited, mainly during the 2010/11 season. This is consistent with the reports of circulating viruses described by national surveillance programmes during the

respective seasons and some of these patients also experienced complicated illness, similar to that seen in patients with A(H1N1)pdm09 virus infection. However, the respective numbers in each sub-group are too small to draw meaningful comparisons between patients infected with different influenza sub-types.

The success of the MOSAIC study reflects our decision to continue recruitment beyond the end of the pandemic. MOSAIC was able to refine its sampling protocols at the end of the first period of recruitment, without compromising data analysis. Furthermore, we were able to conduct an interim analysis of clinical data collection and identify clinical variables (e.g. vaccination data) that were not routinely recorded in case notes, leading to the introduction of the supplementary CRF which was completed at the time of sampling. Because most influenza-positive cases were recruited during the 2010/11 season, this means that we were able to achieve a more complete clinical data set during the main period of recruitment.

3.14.4 The MOSAIC cohort is representative of hospitalised A(H1N1)pdm09 patient populations

The ability to recruit at multiple hospitals in two cities, including those with specialist services, meant that MOSAIC recruited patients who were representative of the expected population. The average age of adults with influenza and that of children were similar to those reported by observational studies from the UK and elsewhere (176, 391, 404, 407). In the UK Flu-CIN study, which observed hospitalised patients during the first and second pandemic waves, the proportion of children aged 0-4 years (17%) and the proportion of adults aged 16-34 years (32%) were higher than expected, compared to the general population. The risk of severe outcome was generally greatest in children <1 year of age and adults >45 years of age, producing a W-shaped curve, which is similar to that seen during the 1918 influenza pandemic (162). National surveillance programmes suggested that the burden of influenza continued to affect adults of working age rather than the elderly, but that children were less prominently affected than during the pandemic (408). One study compared hospitalised influenza cases at the same NHS trusts during the pandemic and in the winter of 2010/11 and reported that the median age had increased from 20 years to 35 years (p<0.0001) (409).

Despite intensive efforts made to recruit both adults and children with influenza and ILI, MOSAIC mainly recruited younger adults during 2010/11. Even if one makes allowances for the accepted difficulties of recruiting large numbers of children to observational studies, the age distribution of the MOSAIC cohort does appear to be representative of the national (and international) experience of A(H1N1)pdm09 virus infections. Severe illness caused by infection with established seasonal

influenza viruses is typically something that affects adults over 65 years of age. The decreased incidence of A(H1N1)pdm09 virus infection in adults over 65 years of age has been attributed to pre-existing, cross-protective immunity (predominantly antibody-mediated) in those who were likely to have been exposed to 1918 H1N1 virus and its descendants between 1918 and 1957 (49, 410).

One fifth of recruited adults did not have underlying health problems, confirming that A(H1N1)pdm09 virus behaves like other pandemic strains in causing severe illness in a relatively large number of previously healthy adults and children, even in the immediate post-pandemic period. The proportion of previously healthy individuals with influenza in MOSAIC (24.6%) was less than that reported by Flu-CIN (55.1%). MOSAIC proactively measured body mass index during the second period of recruitment, which may be one explanation as to why there are fewer previously healthy adults compared to Flu-CIN. Other potential explanations may include the fact that MOSAIC recruited patients with non-influenza ILI whereas FluCIN only studied patients with confirmed A(H1N1)pdm09 virus infection and also that the majority of MOSAIC influenza cases were recruited after the pandemic, whereas Flu-CIN only analysed patients from the pandemic. Other studies in the UK and elsewhere have not reported such a high proportion of previously-healthy individuals that was seen in Flu-CIN, instead reporting similar proportions to those seen in MOSAIC (66, 175).

The five most common comorbidities reported in adults with influenza were similar to those reported by other observational studies of individuals hospitalised with A(H1N1)pdm09 virus infection (171, 175, 402) and also seasonal influenza (170, 205, 401): asthma and other chronic lung disease, cardiovascular disease, obesity, cancer and immunodeficiency. Many patients in MOSAIC had two or more comorbidities, making it difficult to assess the contribution of a particular condition to severe illness.

3.14.5 Adult patients with asthma are an important population in the MOSAIC cohort

In MOSAIC, the presence of asthma in patients with influenza was associated with a shorter mean length of stay. Although the need for supplemental oxygen was more common in patients with influenza and asthma compared to those with influenza but no asthma, these patients were less likely to require invasive mechanical ventilation. It is not clear why those with asthma have a propensity to be hospitalised with A(H1N1)pdm09 virus infection yet don't require escalation of care to the same extent as those with other comorbidities. A similar trend was seen in the Flu-CIN study, with the additional finding that patients with asthma tended to be admitted at an earlier stage of illness and an association between pre-hospital corticosteroid use and less severe outcomes (174). Data on corticosteroid usage in the MOSAIC cohort are currently being collected and analysed.

While there has been significant research on the relationship between asthma exacerbations and other respiratory viruses, little is known about the biological relationship between asthma and influenza. Deficient IFN responses – especially IFN-β and IFN-λ – have been described in human challenge models where patients with asthma were infected with rhinovirus. Abundance of IFN-λ was negatively correlated with airway symptom scores, changes in lung function, virus load, and markers of inflammation in one study (411, 412). Clinical features suggesting acute exacerbation of asthma did not appear to be present in the majority of asthma patients with influenza. This contrasts with the findings from the human rhinovirus challenge models. Behavioural aspects may also need to be taken into consideration in the over-representation of asthmatic patients in hospitalised patients with A(H1N1)pdm09 virus infection. Greater awareness of influenza in this population may have resulted in a lower threshold to present to secondary care and also to be admitted to hospital.

Patients with asthma were a prominent target group for influenza vaccination during the 2009-10 pandemic and the immediate post-pandemic period. While it is not surprising that patients with profound immunosuppression may not be protected by immunisation, it is interesting that asthma was reported in 57% of the influenza-positive patients in MOSAIC who had been appropriately vaccinated. A Cochrane Collaboration systematic review of the effectiveness of influenza vaccination in preventing influenza-induced exacerbations of asthma suggested that there is uncertainty over a protective effect (413). Antibody responses to seasonal influenza vaccination have been shown to be sufficient in patients with asthma, even those receiving long-term inhaled corticosteroids, so other reasons need to be considered in subsequent analyses, including the presence of other underlying conditions in these patients and the possible role of non-B-cell mechanisms in immunisation (414).

3.14.6 Obesity is a common comorbidity in adults with influenza

Obesity was also a common co-morbidity in adults with influenza but, similar to asthma, it frequently occurred alongside other co-morbidities. Apart from potential physiological complications of obesity, including those that may make mechanical ventilation more difficult, there may be underlying differences in immune responses to influenza. There is evidence that altered immune responses occur in obesity, but animal studies suggest that obesity may be associated with attenuated antiviral interferon response to influenza and other respiratory viruses (415, 416).

3.14.7 Pregnancy is an important risk factor for complicated influenza

Similar to the findings of observational studies of patients with A(H1N1)pdm09 virus infection (417), pregnant women with A(H1N1)pdm2009 virus infection were over-represented in the MOSAIC cohort, when compared to the background prevalence of pregnancy in women of child-bearing age (171). Consistent with the published findings, we found that the majority of pregnant women with

influenza were at later stages of pregnancy, primarily the third trimester. It is notable that only pregnant women with confirmed A(H1N1)pdm2009 virus infection were critically ill. Although pregnant women with influenza were shown to be at increased risk of complicated illness during previous influenza pandemics and during annual outbreaks of seasonal influenza (418, 419), it is interesting that mild illness (grade 1 severity) was seen in nearly 40% of influenza-positive women. This demonstrates that influenza in pregnancy does not necessarily result in critical illness and other factors may contribute to severe illness, such as the presence of comorbidities. Approximately half of the cases had documented comorbidities. Furthermore, three quarters of the influenza-negative women had the lowest severity of illness.

Taken together, these data may suggest that some pregnant women had mild influenza or ILI reported because of greater concern about the risks of influenza of in pregnancy, associated with a lower threshold to admit to hospital. This will require further exploration of the data to determine whether women were already in in hospital for pregnancy-related issues or were admitted because of influenza/ILI symptoms. However, it is believed that pregnancy is associated with an increased risk of influenza infection and also an increased risk of complications of influenza, which may be influenced by the physiological changes which occur during pregnancy and also by immunological changes facilitating immune tolerance of the foetus – including changes in maternal cell-mediated immunity and Th1/Th2 balance – which may lead to a state of relative immunosuppression in the mother (172, 420).

3.14.8 Children recruited to MOSAIC had a different risk profile for complicated influenza

Children infected with influenza demonstrated a different at-risk profile to adults. Similar to the findings of hospitalised case series (421, 422), neurological conditions, especially neurodevelopmental conditions, were particularly common in influenza-positive children recruited to MOSAIC. The explanation for this phenomenon is not clear, but compromised physiology, such as the ability to clear respiratory secretions or the presence of chronic respiratory conditions, might play a role. Furthermore, there may be difficulties in recognising infection in these children.

3.14.9 The MOSAIC severity score identified a spectrum of illness in hospitalised patients with influenza

MOSAIC examined existing scoring systems for pneumonia and sepsis and determined that none of them were appropriate for categorising influenza severity. Therefore, a new scoring system was developed, based on the presence of respiratory compromise and the interventions warranted to correct it. Since there were few patients with respiratory compromise requiring NIV, these patients

were subsequently combined with those with respiratory compromise and oxygen supplementation, rather than grouping them with invasively-ventilated patients who frequently had multi-organ dysfunction. This revised, three-point score resulted in distinct but comparable groups of patients across a spectrum of illness severity that has been described in published case series of patients with A(H1N1)pdm09 virus infection (66). The inclusion of tertiary centre ICU patients meant that some of the most severely ill patients in the UK were recruited, including those requiring ECMO. Although these patients were recruited later in their course of admission than other patients, ICU patients at other centres were recruited quickly following admission to hospital. The trend observed in MOSAIC was that critically-ill patients were admitted to ICU within 24-48h of hospital admission and, which meant that there was limited scope to monitor evolution of illness from mild/moderate through to critically ill. However, this is similar to the experiences of critically-ill patients reported by others (66, 247).

3.14.10 Bacterial respiratory pathogens were commonly detected in influenza patients

Collection and analysis of co-infection data remains on-going because MOSAIC has performed a thorough and detailed search of NHS microbiology and virology results, in addition to analysis of samples obtained for research purposes. However, initial analysis of MOSAIC respiratory multiplex PCR results has revealed that almost 40% of influenza-positive patients had bacteria in their T1 upper respiratory tract samples. This is higher than “bacterial co-infection” rates reported by the majority of observational series of patients with A(H1N1)pdm09 virus infection (66, 423, 424). The discrepancy may be explained by different methods used to detect bacteria and how bacterial co-infection is defined in different studies.

While PCR testing has higher sensitivity and may be less influenced by prior antibiotic exposure compared to culture methods, detection of bacteria by PCR does not necessarily reflect pathogenic infection. This notion is supported by the detection of *S. aureus* by PCR in one fifth of asymptomatic healthy adult control samples. Although *S. aureus* was detected by PCR in samples from influenza-positive patients, *H. influenzae* and *S. pneumoniae* were detected in similar proportions of patient samples, especially during 2010/11. These bacterial species were also the most commonly reported species in observational studies of patients with A(H1N1)pdm09 virus infection (66), irrespective of the detection methods used. Analysis of blood culture and urine antigen results suggest that at least ten influenza-positive adult patients had significant *S. pneumoniae* infections, although not all influenza-positive patients provided samples for urine antigen testing or blood culture.

During the 2010/11 season in the UK, there was concern that bacterial co-infections, especially with *S. pneumoniae*, *Group A Streptococcus* and *N. meningitidis*, may have been more common than during the 2009-10 pandemic (181). Although the MOSAIC NPA bacterial detection in 2010/11 rate was almost double that of 2009/10, the difference was not statistically significant. Invasive *N. meningitidis* and Group A *Streptococcus* detections were seen in MOSAIC influenza-positive patients recruited during 2010/11, but not in those recruited in 2009/10. It is not possible to draw conclusions about these findings, however, due to the small numbers of detections and the relatively low number of influenza-positive patients recruited during 2009/10.

3.14.11 Measurement of procalcitonin suggests that many influenza patients do not have significant bacterial co-infection

In a further attempt to determine the presence of clinically significant bacterial infection in influenza-positive cases, we measured procalcitonin (PCT) in serum and plasma. In patients with influenza virus infection, PCT has been demonstrated to have a good negative likelihood value but poor positive likelihood value, suggesting that its main purpose is as a rule-out test for concomitant invasive bacterial infection or bacterial pneumonia (400). Using a commonly accepted cut-off value of 0.5 ng/ml, in both recruitment seasons approximately half of influenza-positive adults did not appear to have significant bacterial infection at T1. Further stratification by severity of illness is required (see Chapter 5) but these findings suggest that bacterial co-infections, such as community-acquired bacterial respiratory tract infections, do not contribute to illness in a significant proportion of hospitalised patients, at least early on in the course of hospital admission.

3.14.12 Other respiratory viruses were commonly detected in influenza patients

Non-influenza virus detections, predominantly rhinovirus and RSV, were also more common than expected in MOSAIC influenza-positive patients overall, compared to the low rates of detection reports in published series of A(H1N1)pdm09 virus-infected patients (66, 209, 425). Again, this discrepancy may be explained by differences in detection methods employed in different studies. Similar to bacterial detections, it is not possible to state whether detection of viral RNA in nasopharyngeal secretions is representative of a pathogenic infection and what the non-influenza virus is contributing to the illness. Asymptomatic respiratory virus infections and proposed 'bystander' viruses in mixed infections have been well-described in the literature (426, 427). One MOSAIC healthy control had rhinovirus RNA detected (in addition to *Moraxella catarrhalis*), without evidence of preceding, active or subsequent symptoms. Further stratification of patients by age and by severity of illness may be helpful in exploring the relatively high rate of non-influenza virus detection in the MOSAIC cohort.

3.14.13 Conclusions

MOSAIC has collected what is probably the most detailed clinical data-set of any observational study of hospitalised patients with influenza and ILI and it appears to be representative of the generality of hospitalised patients with A(H1N1)pdm09 virus infection. The number of clinical variables collected meant that data entry and analysis was a significant and lengthy task, but this vast investment in meticulous data collection means that key variables are available for subsequent interpretation of soluble immune mediator and transcriptomics results. We have performed a thorough and detailed investigation of bacterial and viral co-infections and initial analyses suggest that bacteria and non-influenza viruses may be more common than we expected. However, our results suggest that pathogenic bacterial co-infection does not explain all cases of complicated influenza requiring hospitalisation.

The numbers of influenza-positive cases recruited are adequate for performing primary analyses, but analyses requiring comparison of specific sub-groups e.g. those with particular comorbidities, may be limited by small numbers of patients within each sub-group. Furthermore, it is difficult to assess the contribution of a specific underlying condition when many 'comorbid' patients having two or more underlying conditions.

The Flu-CIN data collection tool was used because it was immediately available at the time MOSAIC was launched. In retrospect, a more focussed clinical data collection tool that does not rely on retrospective data-collection (ideally a generic case report form (CRF) prepared in advance of an outbreak, with point-of-care electronic data capture) would have enabled clinical data to have been collected and analysed in a shorter period of time. The introduction of the supplemental CRF allowed us to collect important 'real-time' data on pre-hospital antimicrobial use and influenza vaccination. We have shown that while pre-hospital antibiotic prescribing appeared to be common, antivirals were rarely prescribed prior to admission, despite their widespread availability during and following the 2009-10 pandemic.

The final co-infection data-sets will be detailed and high-quality. The inclusion of NHS microbiology and virology results may not have provided significant enrichment of the existing research sample data-set, but evidence of invasive infections (especially from NHS blood cultures) is an important inclusion. While the influenza-negative group presented with the same symptoms leading to diagnostic testing for influenza, data from this group will be of greater use for comparison with influenza cases if the cause of their ILI can be established; this process of characterisation is on-going.

In conclusion, the MOSAIC influenza is reasonably representative of the patient population at the time of the 2009-10 pandemic and the 2010-11 influenza season. A broad range of illness severity has been identified in hospitalised patients and important patient populations are identified. A thorough search for co-infections has revealed a significant number of detections of bacteria and non-influenza viruses in patients with influenza. The MOSAIC sampling protocol was effective and detailed high-quality clinical data have been collected to facilitate the interpretation of laboratory analyses. Although data collection and analysis is on-going, final data-sets for key clinical variables have been provided. Further refinement of existing protocols and data collection tools will an important legacy of this study, helping to improve research efforts for subsequent outbreaks of influenza and novel pathogens.

Chapter Four

4 Whole Blood Transcriptomic Profiling

4.1 Introduction

4.1.1 Whole blood transcriptomic profiling in infectious diseases

Similar to other infections, the severity illness due to A(H1N1)pdm09 virus infection is only in part determined solely by the makeup of the infecting pathogen; host factors, particularly those affecting and relating to the immune response to infection are crucial. In addition to measuring downstream components of the immune responses, such as soluble immune mediators in blood and respiratory secretions, analysis of upstream responses at the level of transcription/gene-expression can be achieved through micro-array-based expression profiling of a vast number of transcripts. While inherited genetic polymorphisms, such as the minor *IFITM3* allele (SNP rs12252-C) that was identified in influenza patients by MOSAIC and its collaborators (297) may explain an altered course of influenza infection in some individuals, changes in mRNA expression may help to explain mechanisms of severity in others. In contrast to inherited polymorphisms, changes at the transcriptional level are dynamic, being influenced by acquired patients characteristics and extrinsic factors. Furthermore, patterns of gene expression – both up-regulation and down-regulation of gene activity – can provide insights to the overall patterns of immune responses seen in patients with influenza e.g. innate responses targeted against viral infection. The relationship between gene expression and soluble immune mediator production is not linear, however; the 'downstream' proteins that are produced following changes in expression may be part of pathways that themselves influence 'upstream' expression and both RNA and product are the balance of generation and destruction.

Whole blood transcriptomic analysis has been used to determine the immunological response to infection for a range of infectious diseases (428). Because different pathogens interact with different receptors and elicit different signalling profiles, the component immune responses will also differ, which is reflected in changes in the relative abundance of transcripts in tissues, lymphoid organs and blood, irrespective of whether the infection causes localised or systemic illness. In addition to providing mechanistic insights in studies of pathogenesis, expression profiling may have diagnostic capabilities, for both primary and secondary infections, and may assist in characterising different patterns of severity of illness.

There have been few studies of human transcriptomic changes in patients with influenza infection. The have so far been limited to seasonal influenza or have been performed and studied have been limited to studying children, or have used a small sample size, or have lacked detailed clinical characterisation and sometimes lacked appropriately matched healthy controls. Since high throughput microarray and advanced computational data analysis are relatively new, the 2009-10 pandemic presented the first opportunity to study a novel influenza virus outbreak using such techniques.

4.1.2 Hypotheses and aims

MOSAIC collected a large number of transcriptomic samples from adult and paediatric patients hospitalised with influenza and other causes of ILI. These samples were collected at two acute time points, obtained early in the course of hospitalisation in the majority of patients, and also at a third, delayed time point (representing recovery or on-going critical illness). For comparison, samples were also obtained from asymptomatic healthy controls matched by age, sex and ethnicity. Following an analytical approach that has been used successfully to study transcriptional changes in other infections (378), whole blood transcriptomic analysis of MOSAIC adult T1 samples has been performed.

This initial analysis addresses the following hypotheses:

1. Whole blood transcriptomic profiles in patients with influenza virus infection are distinct from those seen in non-influenza ILI

2. In patients with influenza virus infection, whole blood transcriptomic profiles differ according to the severity of illness

3. Changes in whole blood transcriptomic profiles are associated with bacterial co-infection in patients with influenza virus infection

The aim of this initial analysis was to compare the T1 transcriptomic profiles of influenza-positive and influenza-negative adults and to apply clinical characteristics, such as severity scores and evidence of co-infection, to explain changes in transcript abundance in influenza patients. Other, more complete analyses will follow at a later point.

4.2 Samples obtained for microarray

4.2.1 MOSAIC 2009/10 Cohort

Patients with influenza-negative ILI and confirmed influenza were recruited as described in chapter 2. Blood was collected into Tempus tubes at T1, T2 and T3 time-points, also described in chapter 2. For the 2009/10 cohort, 100 samples were collected across all time points and were available for RNA extraction/purification and microarray (all time points). Seven samples were obtained from children and 93 samples were obtained from adult patients. Samples were also obtained from 25 matched adult healthy controls. Matched healthy control samples were not available for children recruited during the 2009/10 period. RNA integrity number (RIN) scores were >5 for all purified extracts and RNA from all submitted samples was successfully hybridised to microarrays.

4.2.2 MOSAIC 2010/11 Cohort

For the 2010/11 recruitment period, 284 Tempus samples were obtained from adults and 32 samples were obtained from children (all time points). One hundred and thirty samples were also obtained from matched healthy adult controls and 12 paediatric healthy controls. RNA could not be isolated from four samples; three of these were from children. RIN scores were <5 for 10 samples (range 3.4-4.9). Four of these samples were from children, of which two had a degraded profile and therefore were not hybridised. Of the remaining six adult samples, four were obtained from two subjects sampled at different time points. Adult samples with RIN scores <5 were not excluded from subsequent analysis in GeneSpring, but their sample ID numbers were noted and outlier status was checked at PCA (see QC, below) and also in hierarchical clustering analysis.

4.2.3 Cases included in final analyses

Due to the relatively low numbers of recruited paediatric subjects and paediatric healthy controls, analysis of paediatric results has been deferred; only results from adult subjects are presented in this chapter.

Outlying samples were detected using principal component analysis (PCA) quality control, performed on all transcripts for each period of recruitment. Two samples from the 2009/10 cohort, 4030_A (LON0002 T1 sample) and 4017_A (HC 10150/1), were identified as outliers and were in close proximity to the X-axis. Rather than removing the samples at PCA, the respective GeneSpring experiments were set up once again, with removal of these samples from the experiments at the outset. QC steps in GeneSpring did not identify any 2010/11 patient samples that required removal. PCA plots for all cases at T1 for each season of recruitment are shown in figure 4.1.

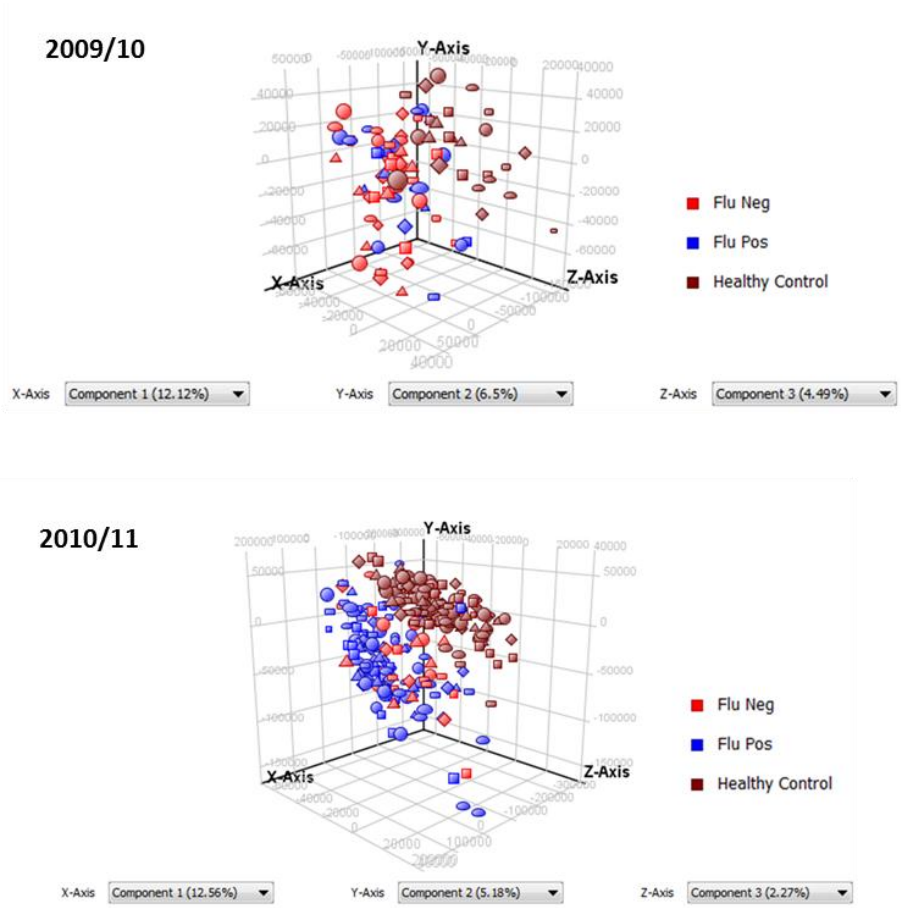


Figure 4.1 Principal component analysis of all transcripts significantly detected from background
Principal component analysis (PCA) was performed for all adult T1 samples and healthy control samples from the 2009/10 cohort (upper panel; 15,330 transcripts) and from the 2010/11 cohort (lower panel; 18,944 transcripts). Two outlying samples from the 2009/10 cohort were identified on earlier PCA of all samples (not shown) and removed at experiment set-up. The PCA analysis for the 2009/10 plot shown here was performed on remaining 2009/10 samples.

Adult samples available for transcriptional analysis are indicated in table 4.1. Microarray results from T1 have been analysed in this chapter, but the number of patients with complete sets of serially-obtained samples is also indicated, to facilitate subsequent discussion of future work.

	2009/10 subjects with T1 samples	**2009/10 subjects with T1 and T2 samples**	**2009/10 subjects with T1, T2 and T3 samples**	**2010/11 subjects with T1 samples**	**2010/11 subjects with T1 and T2 samples**	**2010/11 subjects with T1, T2 and T3 samples**
Adult influenza-positive	22	18	8	109	14	32
Adult influenza-negative	44	Not applicable	Not applicable	31	71	4
Adult healthy control	25	25	25	130	130	130
Total (including healthy control samples)	91	43	33	270	215	166

Table 4.1 Adult whole blood samples collected for transcriptomics analysis
For each recruitment period, the number of patients with T1 samples available for analysis is shown. The number of patients who provided samples at both acute time-points (T1 and T2) and at all three time points (T1, T2 and T3) is also shown. Matched adult healthy controls provided a single sample, but their samples can be included in all analyses. Note that in the 2009/10 recruitment period, influenza-negative subjects provided T1 samples only (marked “not applicable”).

4.2.4 Adult case-matching with healthy controls

For patients from each recruitment period, cases were best-matched according to age, sex and ethnicity, using demographic data associated with patients whose samples were submitted for RNA extraction and processed for microarray (see table 4.2).

	2009/10 patients	2009/10 healthy controls	2010/11 patients	2010/11 healthy controls
Total Number	66	25	140	130
Age in years				
Mean (range)	45 (18-83)	37 (21-54)	42 (18-85)	35 (20-68)
Median (IQR)	43 (30-60)	34 (29-48)	43 (29-52)	32 (26-41)
Female sex (%)	35 (53)	14 (56)	71 (51)	75 (58)
Ethnicity (%)				
White	34 (51)	14 (56)	101 (72)	90 (69)
Black	15 (23)	5 (20)	22 (16)	23 (18)
Asian	8 (12)	6 (24)	11 (8)	15 (11.5)
Other	9 (14)	0 (0)	6 (4)	2 (1.5)

Table 4.2 Demographic data for 2009/10 and 2010/11 adult patients and matched adult healthy controls
For ethnicity, patients categorised as "other" included patients of Arab ethnicity, as well as patients who were of mixed ethnicity.

4.3 All adult patients and healthy controls at T1

4.3.1 Association of transcriptomic response with Influenza infection status

For each of the two periods of recruitment, T1 samples from influenza-positive and influenza-negative, and those from healthy controls, were compared. For results from each period of recruitment, expression and statistical filtering was performed, as described in chapter 2. Briefly, transcripts were normalised to the median of all transcripts, then the resulting transcripts were filtered by flags, retaining transcripts if expression was significantly detected in at least 10% of samples. Expression filtering was performed on remaining transcripts, retaining only those with >3 fold-change from the median in at least 10% samples. Following non-parametric statistical filtering and multiple-testing correction, differentially expressed transcript lists were produced, containing 704 transcripts and 2426 transcripts for the 2009/10 and 2010/11 cohorts, respectively (figure 4.2)

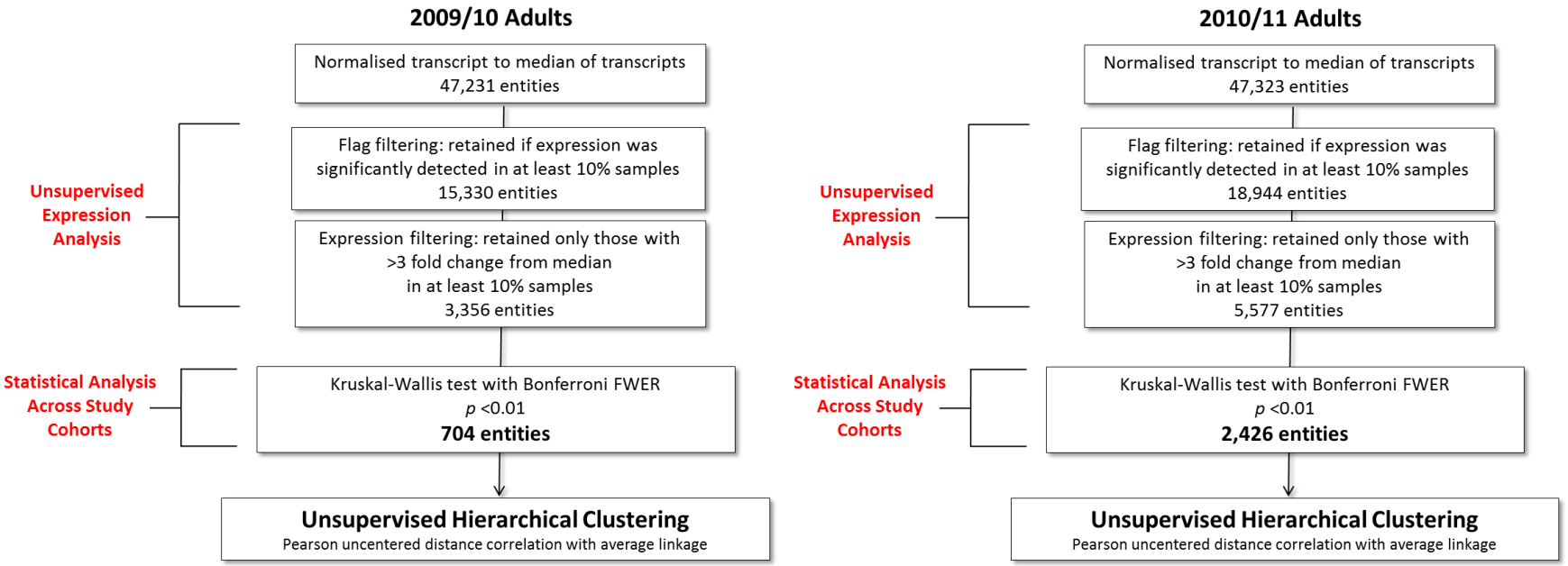
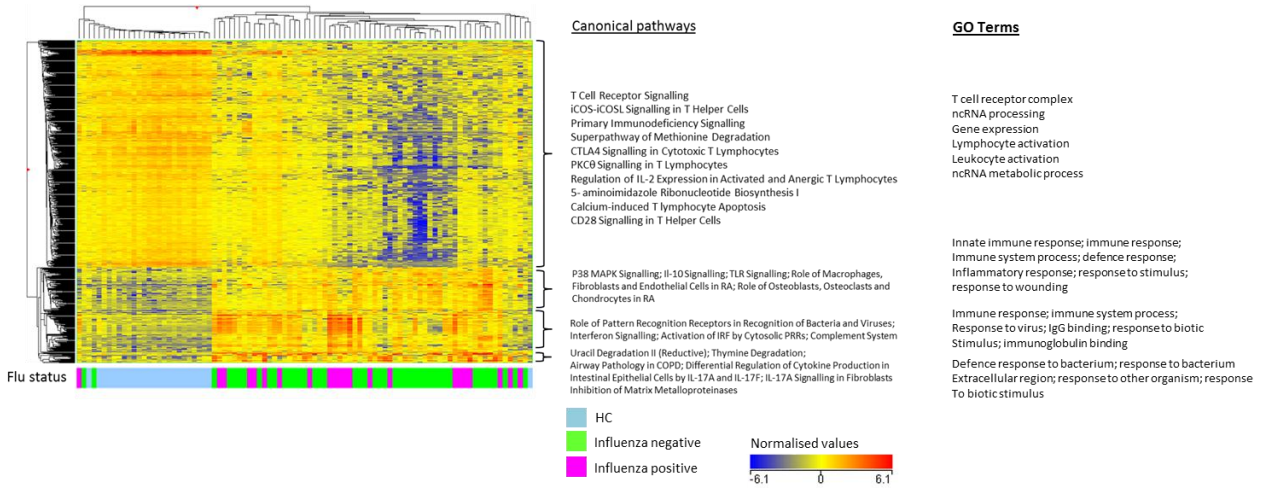


Figure 4.2 Analytical approach used for analysing microarray data in GeneSpring for influenza-positive adults at T1, influenza negative adults at T1 and healthy controls

Adult patients from 2009/10 and 2010/11 were analysed separately. Each recruitment cohort had its own set of matched healthy controls. Original normalised transcript lists (> 47,000 transcripts) underwent background detection (flag) filtering, followed by expression filtering with retention of transcripts that demonstrated > 3 fold change from the medain normalised intensity value in at least 10% samples. Statistical testing with mutliple testing corrections were then applied (Kruskal Wallis test with Bonferroni FWER). Hierarchical clustering was performed on the final entity lists (shown in bold).

The final transcript lists underwent hierarchical clustering using influenza infection status as the interpretation and heat-maps were generated for each recruitment period cohort. The heat-map for the 2009/10 cohort is shown above the heat-map for the 2010/11 cohort. Each column represents a patient or healthy control sample and each row represents an RNA transcript (entity). The infection status (influenza-positive, influenza-negative, healthy control) is indicated in the colour-bar below each heat-map. Transcript intensity values were normalised to the median of all transcripts; red transcripts are relatively over-abundant, whereas blue transcripts are relatively under-abundant (figure 4.3).

2009/10 (704 transcripts)



2010/11 (2,426 transcripts)

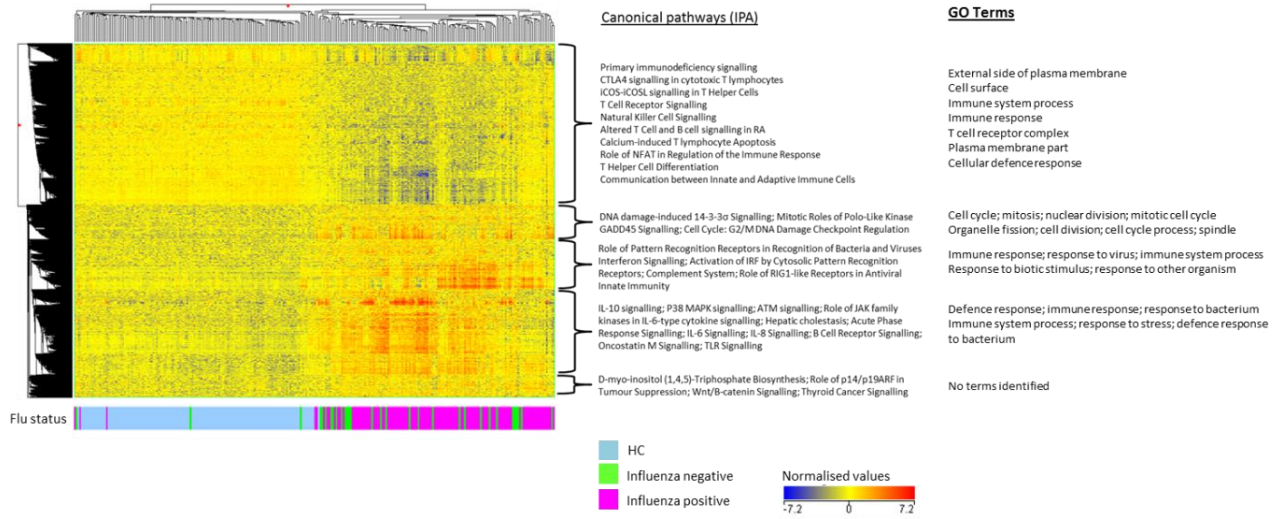


Figure 4.3 Influenza-positive and influenza-negative adult patients cluster separately from healthy controls at T1

For each recruitment period, normalised intensity value transcripts which were significantly detected from background, and filtered for low expression (transcripts retained if >3FC from median normalised intensity value, followed by statistical filtering (Kruskal-Wallis with Bonferroni FWER, p <0.01). Hierarchical clustering was then performed (clustered on transcripts and by individuals; Pearson's Uncentered with average linkage). Listed next to the heat-map are the top IPA canonical (by significance p<0.05 Fishers Exact test) for up-regulated and down-regulated transcripts. Corresponding terms identified by separate Gene Ontology (GO Terms) analysis are shown alongside. Patients were identified according to their influenza status (healthy control; influenza-negative; influenza-positive). HC= healthy control.

Influenza-positive and influenza-negative patients did not form two separate, distinct groups in either cohort, but the dendrograms for both heat-maps suggest that major clusters exist, clusters that contain patients with similar transcriptional profiles from both groups. For the 2009/10 cohort, four major clusters of transcripts were identified by inspection of the heat-map and five major clusters of transcripts were identified for the 2010/11 cohort. The transcripts within each cluster were selected and exported for canonical pathways analysis in Ingenuity Pathway Analysis (IPA). The transcript lists also underwent Gene Ontology (GO) terms analysis. Statistically significant pathway associations are listed alongside the heat-maps. These are not exhaustive lists and only the top pathways are shown, as determined by *p* value sorting (figure 4.3).

Although the constituent canonical pathways identified by IPA for each major cluster differed slightly between the two cohorts, the general pathway categories were similar. IPA analysis of the largest single transcript cluster in each cohort (495 transcripts for 2009/10 patients, 1082 transcripts for the 2010/11 patients), seen in the upper half of each heat-map, demonstrated a relative *under-abundance* of T- and B-cell pathways in the majority of patients but not in the healthy controls. Examples of top canonical pathways that were common to both cohorts include 'T-cell receptor signalling', 'iCOS-iCOSL signalling in T-helper cells' and 'primary immunodeficiency signalling'. GO Terms analysis provides less detail, but also identifies GO terms associated with T- and B-cell gene functions.

The degree of under-abundance is greater for some individuals than others, forming two large sub-groups of patients in each season. Further subdivisions were seen within this 'under-abundant' transcript cluster, as illustrated by sub-trees within the major branches of the horizontal dendrograms. In the 2009/10 cohort, it is notable that the most marked under-abundance, as demonstrated by a block of blue transcripts in the heat-map, is dominated by patients with influenza-negative status.

The remaining major clusters of transcripts for each seasons show relative *over-abundance* in patients but not in healthy controls. For the 2009/10 cohort, three major over-abundant transcript clusters were identified. For the upper-most cluster (93 transcripts), the broad category for the identified canonical pathways can be summarised as 'signalling and inflammation pathways' e.g. 'P38 MAPK Signalling', 'IL-10 Signalling' and 'TLR Signalling'. GO term associations for this cluster include 'innate immune response', 'inflammatory response' and 'defense response'. The middle transcript cluster (86 transcripts) associates with pathways relating to interferon and immune response e.g. 'role of pattern recognition receptors in recognition of bacteria and viruses', 'interferon signalling' and 'activation of IRF by cytosolic pattern recognition receptors'. The same

cluster was associated with GO Terms that include 'immune response', 'response to virus' and 'defense response'. The transcript cluster at the bottom of the heat-map (26 transcripts) is also associated with inflammation and signalling e.g. 'airway pathology in COPD'. This cluster associated with multiple GO Terms, including 'defense response to bacterium', 'response to bacterium' and 'response to other organism'.

The degree of transcript over-abundance for each of these major clusters differed between individuals, but subgroups of patients with similar transcriptional signatures were seen. For example, the most marked over-abundance of transcripts that associate with the interferon and immune response pathways category was seen in a small sub-group of five patients, all of whom were influenza-positive. Interestingly, when compared with patients who do not have such marked over-abundance of the interferon and immune response-associated transcripts, under-abundance of transcripts associated with the T- and B-cell pathways category was also less marked. Similarly, there was a sub-cluster adjacent and to the right of the healthy controls which demonstrates over-abundance of the interferon and immune response pathway, but with less marked under-abundance of T- and B-cell pathway transcripts. In fact, a further sub-cluster of six patients within this cluster had a relative over-abundance of T- and B-cell pathways, similar to that seen in healthy controls. On visual inspection, these findings did not appear to correlate with influenza infection status.

In the 2010/11 heat-map, the first, upper-most 'over-abundant' transcript cluster (239 transcripts) associated with IPA canonical pathways that broadly represent cell cycle and division processes e.g. 'DNA damage-induced 14-3-3σ Signalling', 'mitotic roles of polo-like kinase GADD45 signalling' and Cell Cycle: G2/M DNA Damage Checkpoint Regulation'. Multiple GO terms were identified for this cluster, which are also associated with cell cycle and division processes. The second over-abundant transcript cluster (345 transcripts) was associated with interferon and immune pathways e.g. 'role of pattern recognition receptors in recognition of bacteria and viruses', interferon signalling' and activation of IRF by cytosolic pattern recognition receptors'. GO terms associated with this cluster included 'immune response', 'response to virus', 'response to other organism' and 'defense response'. The third over-abundance cluster (589 transcripts) contained transcripts that associated with pathways falling under the category of signalling and inflammation pathways e.g. 'IL-10 Signalling, 'IL-6 signalling', 'IL-8 signalling' and 'acute phase response signalling'. GO Terms for this cluster included 'defense response', 'immune response', 'response to bacterium' and 'defense response to bacterium'. The fourth over-abundance cluster contained transcripts that associated with pathways linked to a wide range of cellular and metabolic processes. GO analysis did not identify specific GO terms associated with these transcripts at the designated *p* value cut-off.

Influenza-positive and influenza-negative patients from the 2010/11 season fall into two broad groups, based on hierarchical clustering. Patients in the first group, on the right hand side of the heat-map, generally had marked over-abundance of transcripts that associated with interferon and immune pathways, but less marked over-abundance of transcripts that associated with signalling and inflammation pathways. The second group of patients had signatures where the reverse is true and additionally, this group had more marked *under-abundance* of transcripts associating with T- and B-cell pathways. Although further granularity can be seen within each group, with sub-clusters suggested by dendrogram branch divisions, there is no obvious association with influenza infection status i.e. influenza-negative patients are distributed relatively evenly across each of the two groups.

For each recruitment cohort, analysis of the heat-maps suggests the presence of smaller sub-groups of influenza-positive and influenza-negative patients with similar transcriptional profiles (especially in the 2009/10 cohort). Influenza infection status did not explain these sub-clusters, however. Therefore, clustering using different clinical interpretations was also performed on the same entity lists. For convenience, the IPA canonical pathway categories associated with each major transcriptional cluster and which have been described above are summarised alongside each heat-map in all subsequent analyses (the constituent pathways are not listed, but they remain unchanged).

This semi-supervised analysis of results for all adult patients at T1 and HCs demonstrates that, for both periods of recruitment, the majority of patients clustered separately from the healthy controls, although a small number of influenza-positive and influenza-negative patients clustered within the healthy controls. Additionally, in the 2009/10 heat-map, a single healthy control sample clusters within the majority of the patients, albeit at the periphery. RIN scores for the samples from the outlying patients from both seasons were all above 5. There did not appear to be a common, clinical reason to explain these outliers, at least based on the clinical and demographic characteristics that were investigated, although most outliers had mild illness and some may have been sampled in the recovery phase of illness (table 4.3). Other patients with similar clinical, sampling and demographic profiles were not outliers in either season (data not shown).

Outlier	Influenza status	Age (years)	Sex	Ethnicity	Comorbidities	Day of illness at T1	Severity score at T1
1	Positive	25	Male	Other	None	3	1
2	Negative	41	Male	White	Asthma	4	1
3	Negative	55	Female	White	Diabetes; obesity	5	1
4	Positive	45	Female	Black	None	16	1
5	Negative	47	Female	White	Asthma; obesity	7	2
6	Positive	22	Male	Black	Haematological malignancy	3	1
7	Negative	85	Female	Black	Asthma; diabetes	15	1
8	Positive	44	Male	White	None	28	1
9	Negative	48	Male	White	Chronic neurological disease; obesity	14	1

Table 4.3 Selected characteristics of adult patients who cluster with healthy controls
Nine patients were identified as outliers in hierarchical clustering analysis. These patients did not cluster within their respective infection status groups (influenza-positive or influenza-negative). Selected demographic and clinical data for each patient are shown in the table.

4.3.2 Association of transcriptional response with day of illness and severity of illness at T1

Patients from both recruitment periods lacked heterogeneity at the time of T1 sampling: both severity at T1 and the number of days since onset of influenza/influenza-like illness ('day of illness') varied significantly. Therefore, it was important to determine whether the clusters of major transcriptional signatures can be explained by either of these two clinical variables when they are applied to hierarchical clustering.

For the 2009/10 cohort, patients of varying severities were distributed throughout, although the sub-cluster immediately adjacent to the healthy controls contained signatures that appear to be more commonly associated with patients of lower severity, especially grade 1 severity. This small group of patients were at early (0-4 days) and late stages (> 5 days) of illness and generally there was no obvious association of transcriptional clustering with day of illness for all patients at T1. Only a small number of severity 3 patients were recruited during the 2009/10 season, so it is inappropriate to comment on their association with particular signatures (figure 4.4 (A), upper panel).

By contrast, the major transcriptional groups in the 2010/11 cohort, which contained many more patients, did appear to associate with severity grade and day of illness. Patients with transcriptional

signatures demonstrating a marked over-abundance of the interferon and immune pathways category were more commonly severity 1 or severity 2 and also tended to be at an earlier stage of illness (days 0-4), in contrast to those with the signature containing marked over-abundance of signalling/inflammation pathways and generally greater under-abundance of transcripts relating to T- and B-cell pathways. All but one of the severity 3 patients fell within this latter group. Additionally, patients with this signature tended to be at a later stage of illness (figure 4.4 [A], lower panel).

4.3.3 Association of transcriptional response with non-influenza virus detection at T1

To investigate whether influenza-positive patients cluster together with non-influenza patients who were infected with other, non-influenza viruses, the same entity lists from each season were interpreted according to viral detections. In the heat-maps shown in figure 4.5, the non-influenza virus detections in influenza-negative patients are indicated; influenza-positive patients and healthy controls are also identified. For the 2009/10 cohort, 19 non-influenza patients had other, non-influenza viruses detected at T1 and 23 did not; testing was not possible in two patients. Influenza-negative patients infected with non-influenza viruses do not cluster together. Seven different viruses were detected in all patients at T1: human rhinovirus (HRV); respiratory syncytial virus (RSV); adenovirus; 229E coronavirus; OC43 coronavirus; HKU1 coronavirus; human metapneumovirus (HuMPV). HRV was the most commonly detected non-influenza virus, although patients infected with the same non-influenza virus did not cluster together (data not shown). One patient was co-infected with adenovirus and HRV (figure 4.5 [B], upper panel).

In the 2010/11 cohort, 5 different non-influenza viruses were detected in all patients at T1: HRV, RSV, NL63 coronavirus, OC43 coronavirus and HuMPV. HRV was again the most commonly detected non-influenza virus and co-infections were seen. 7 influenza-negative patients had evidence of viral infection, 23 did not and one patient was not tested. Overall, no obvious clustering patterns were seen according to infection with another virus in non-influenza patients (figure 4.5, lower panel) and again, patients infected with the same non-influenza virus did not cluster together (data not shown). In summary, the detection of non-influenza viruses at T1 did not explain why patients with or without influenza virus infection cluster together.

4.3.4 Association of transcriptional response with serum procalcitonin at T1

Serum procalcitonin (PCT) has been used as a surrogate marker of the presence of invasive bacterial infection, bacterial pneumonia and bacterial sepsis. A published systematic review of the role of serum/plasma PCT measurement in identifying bacterial infections in influenza-infected patients

concluded that a low PCT value (< 0.5 ng/mL) has utility as a "rule-out" test (negative predictive value for bacterial infection) (400). As an initial method to investigate whether transcriptional signature patterns may be associated with bacterial sepsis, the T1 entity lists from each season were interpreted according to their serum procalcitonin results. Patients were allocated one of two categories based on PCT value measured at the T1 time point: < 0.5 ng/mL or > 0.5 ng/ml.

In the 2009/10 cohort, there was a heterogeneous distribution of patients with PCT < 0.5 ng/ml, although the heat-map suggests that those patients with the most marked over-abundance of interferon and immune pathway transcripts had PCT < 0.5 ng/ml (figure 4.6, upper panel).

For the 2010/11 patients, there appeared to be a clearer association of clustering according to PCT value. Both influenza-positive and influenza-negative patients with over-abundance of the interferon and immune pathway-associated transcripts most commonly had PCT < 0.5 ng/mL, whereas PCT > 0.5 ng/mL was much more commonly seen in patients with marked over-abundance of the signalling/inflammation pathways and marked under-abundance of T- and B-cell pathway-associated transcripts (figure 4.6, lower panel).

4.3.5 Association of transcriptional response with detection of bacteria in nasopharyngeal aspirate at T1

Respiratory tract bacteria were detected by PCR analysis of T1 nasopharyngeal aspirate (NPA) samples in a total of 19/66 (29%) patients from the 2009/10 cohort; 14 of these detections were in influenza-negative patients. Thirteen patients did not undergo PCR testing. No obvious association between signatures and NPA PCR bacterial detections was seen (figure 4.4 [c], upper panel), irrespective of the bacterial species identified (data not shown). Six influenza-negative patients also had bacteria detected by culture of the same NPA sample, although five of the positive cultures identified the same bacterial species identified by PCR.

Of the 140 patients from the 2010/11 cohort, bacteria were detected by PCR in NPA samples from 46 individuals (33%); 10 of the 31 influenza-negative patients (32%) had bacteria detected (figure 4.7 [C], lower panel). Overall, *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Haemophilus influenzae* were the most commonly detected species. Bacteria were not detected in 37 patients and 7 patients did not undergo testing (of which two were influenza-negative). Similar to the 2009/10 cohort, NPA culture at T1 did not increase the overall yield of detections, with positive cultures occurring predominantly in those who had already had bacteria detected by PCR (data not shown).

4.3.6 Association of transcriptional response with T1 throat swab cultures and blood cultures

Across both seasons, throat swab cultures for respiratory tract pathogens at T1 were positive in three 2009/10 patients (all influenza-negative) and six 2010/11 patients (of which one was influenza-negative). Patients with positive throat swab cultures did not cluster together (data not shown). In terms of the results of NHS blood cultures obtained up to 48h either side of T1, positive detections were infrequent; clinically-significant positive cultures were obtained in five patients from the 2009/10 period and seven patients from the 2010/11 period (one positive culture was from an influenza-negative patient). Blood cultures were taken at the discretion of the attending clinician and many patients did not have a blood culture taken around the time of T1, therefore, bacteraemia and its relation to transcriptional signatures was difficult to interpret. However, inspection of the 2010/11 cohort heat-map revealed that all but one of the patients with known positive blood cultures fell within in the group with over-abundance of transcripts associated with the signalling/inflammation pathway but without over-abundance of transcripts associated with the interferon and immune pathways. Of note, four influenza-positive patients with positive blood cultures clustered together. Two of these had *S. pneumoniae* detected, one had Group A *Streptococcus* detected and one had *S. aureus* detected. One influenza-positive patient had *S. pneumoniae* isolated from blood but had over-abundance of transcripts associated with interferon and immune pathways, rather than transcripts associated with the signalling/inflammation (bacterial response) pathways (figure 4.4 [D]).

4.3.7 Association of transcriptional response with pneumococcal detections at T1

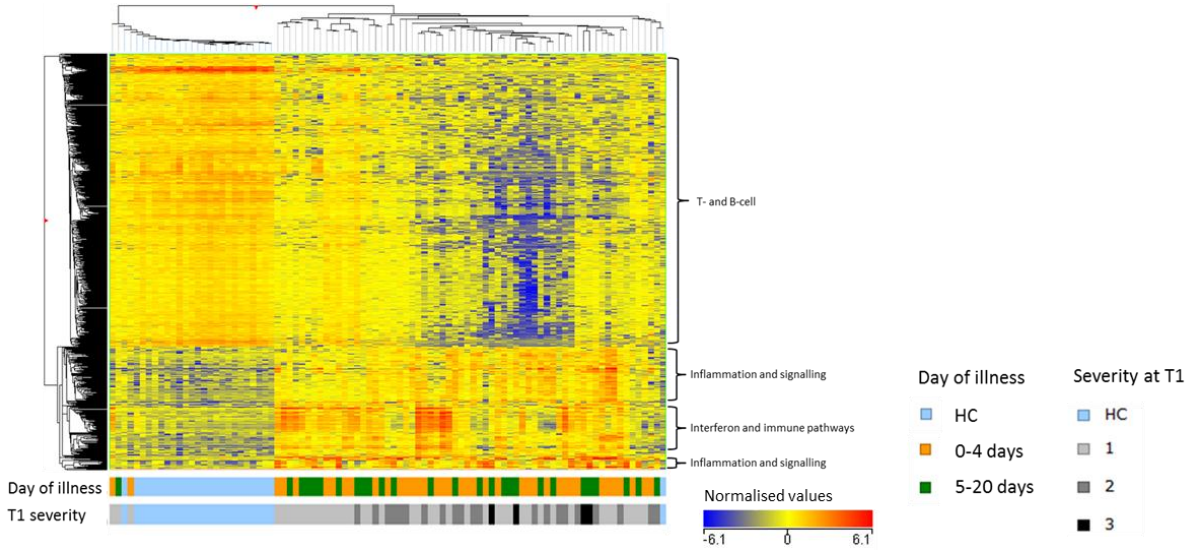
To address whether patients with *S. pneumoniae* infection have similar transcriptional profiles, irrespective of influenza infection status, interpretations of known detections of *S. pneumoniae* were applied to the T1 hierarchical clustering heat-maps from each cohort. Invasive and non-invasive detections were included. Invasive was defined as detection of *S. pneumoniae* in a blood culture obtained +/-48h of T1 and/or a detection of pneumococcal urinary antigen in a MOSAIC-obtained urine sample or an NHS-tested urine sample that was obtained +/-48h of T1. Non-invasive detections were defined as detection of *S. pneumoniae* by PCR or culture in a T1 NPA sample and/or detection of *S. pneumoniae* by culture of a T1 throat swab.

In the 2009/10 cohort, there were six patients who were known to have non-invasive detections and three who were known to have invasive detections. All but one of these infections (an invasive *S. pneumoniae* infection) were in influenza-negative patients. Clustering of patients with *S. pneumoniae* infection was not evident, irrespective of whether the infection was invasive or non-

invasive. It should be noted that patients who were not known to have *S. pneumoniae* may have provided appropriate samples for testing and the results were negative, or appropriate samples may not have been taken (figure 4.4 [E], upper panel).

In the 2010/11 cohort, 21 patients (15%) were known to have *S. pneumoniae* infection, of which 12/21 (57%) were invasive. Four of these infections were in influenza-negative patients, of which two were invasive. Overall, only four patients with *S. pneumoniae* infection fell within the group with marked over-abundance of the 'interferon and immune pathways' associated transcripts; the majority fell within the group that had marked over-abundance of the signalling/inflammation pathway without marked over-abundance of transcripts associated with the interferon and immune pathways. Invasive and non-invasive infections did not form discrete clusters, however (figure 4.4 [E], lower panel).

2009/10 (704 transcripts)



2010/11 (2,426 transcripts)

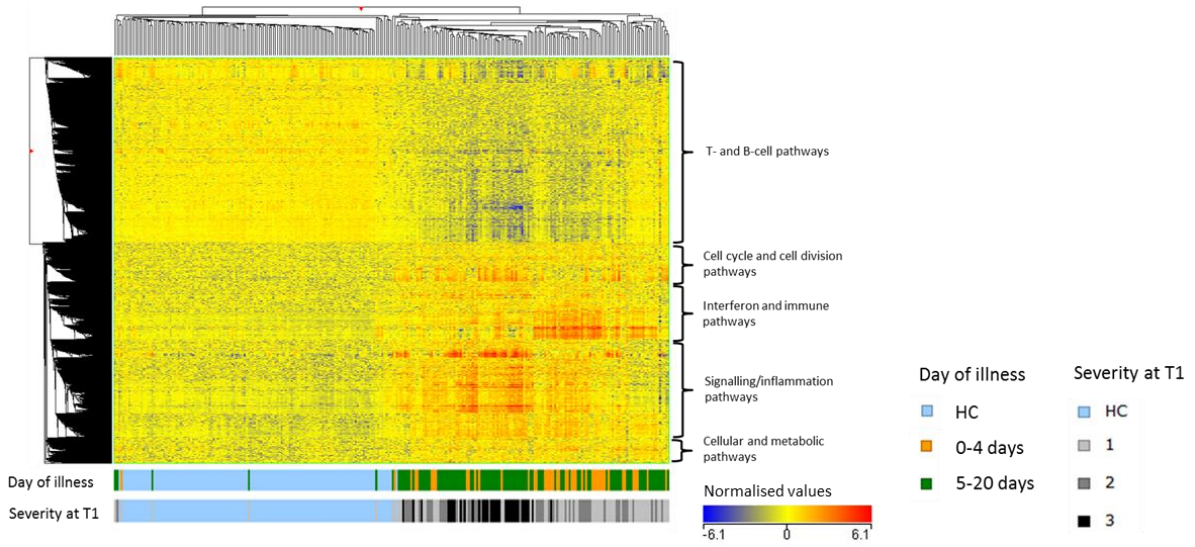
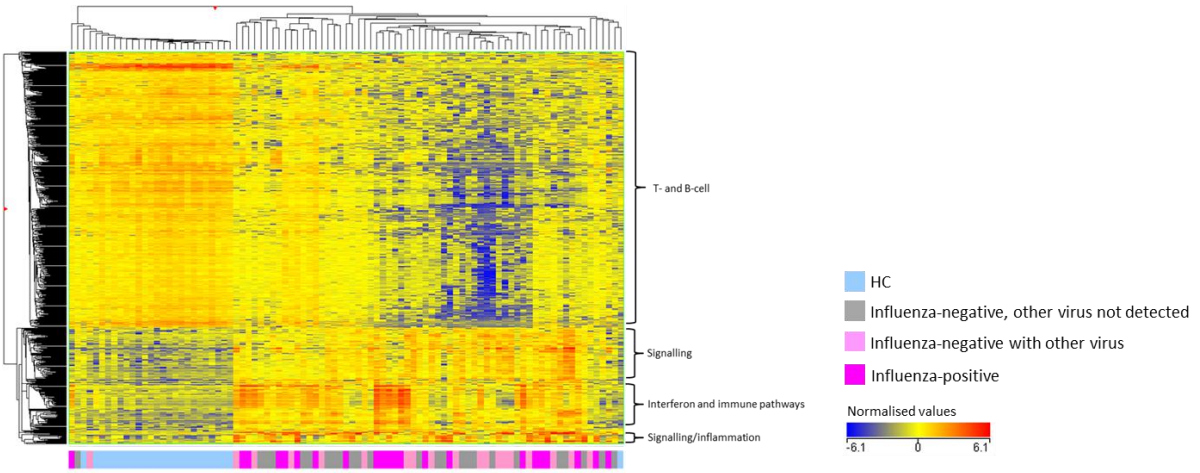


Figure 4.4 (A-F) Influenza-positive and influenza-negative adults at T1 and healthy controls
(A) Dominant clusters of differentially-expressed transcripts and their association with duration of illness and severity of illness at T1
For each recruitment period, normalised intensity value transcripts significantly detected from background were filtered for low expression (transcripts retained if >3FC from median normalised intensity value), followed by statistical filtering (Kruskal-Wallis with Bonferroni FWER, p <0.01). Hierarchical clustering was then performed (clustered on transcripts and by individuals; Pearson's Uncentered with average linkage). Listed next to each heat-map are IPA canonical pathway category summaries (by significance p<0.05 Fishers Exact test) for up-regulated and down-regulated transcripts. Adult participants were identified according to day of illness at T1 (healthy control; 0-4 days; 5-20 days; healthy control) and severity of illness measured at T1 (healthy control; severity 1; severity 2; severity 3). HC = healthy control.

2009/10 (704 transcripts)



2010/11 (2,426 transcripts)

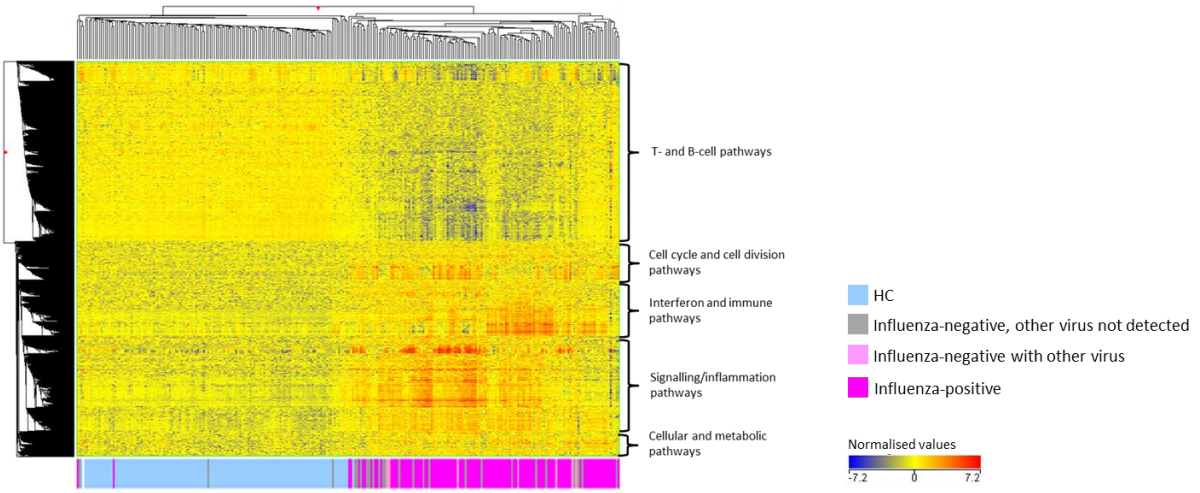
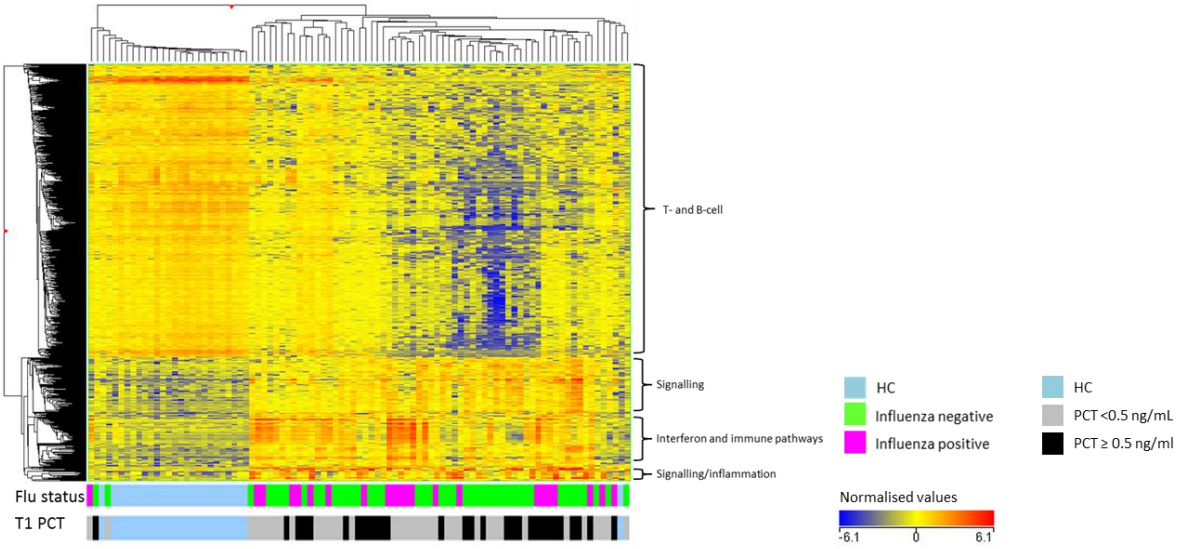


Figure 4.4 (B) Patients with any viral infection do not form distinct clusters, irrespective of underlying influenza infection status

Hierarchical clustering was performed as described for figure 4.4 (A). Adult participants were identified according to day of illness at T1 (healthy control; 0-4 days; 5-20 days; healthy control) and severity of illness measured at T1 (healthy control; severity 1; severity 2; severity 3). HC = healthy control.

2009/10 (704 transcripts)



2010/11 (2,426 transcripts)

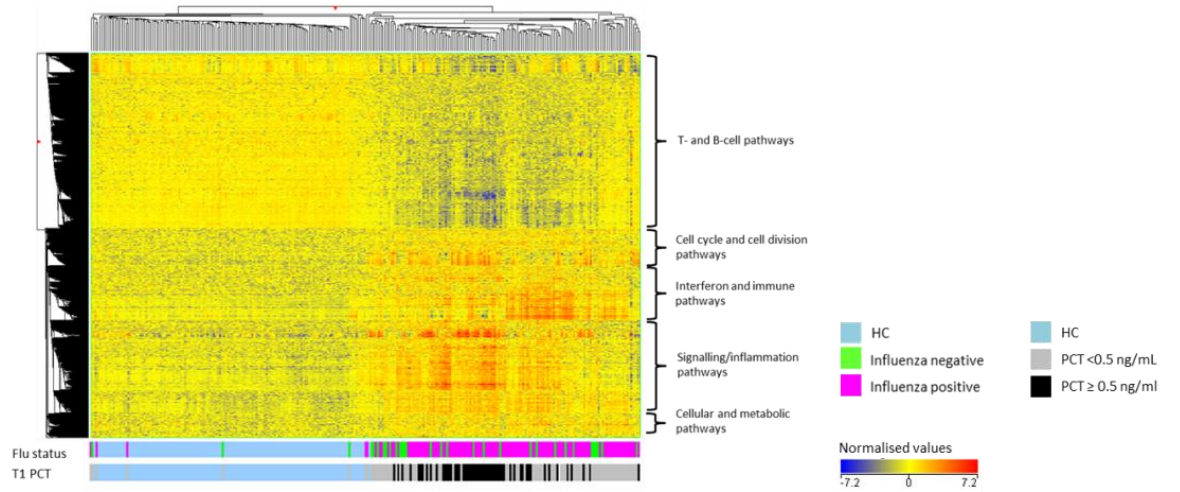
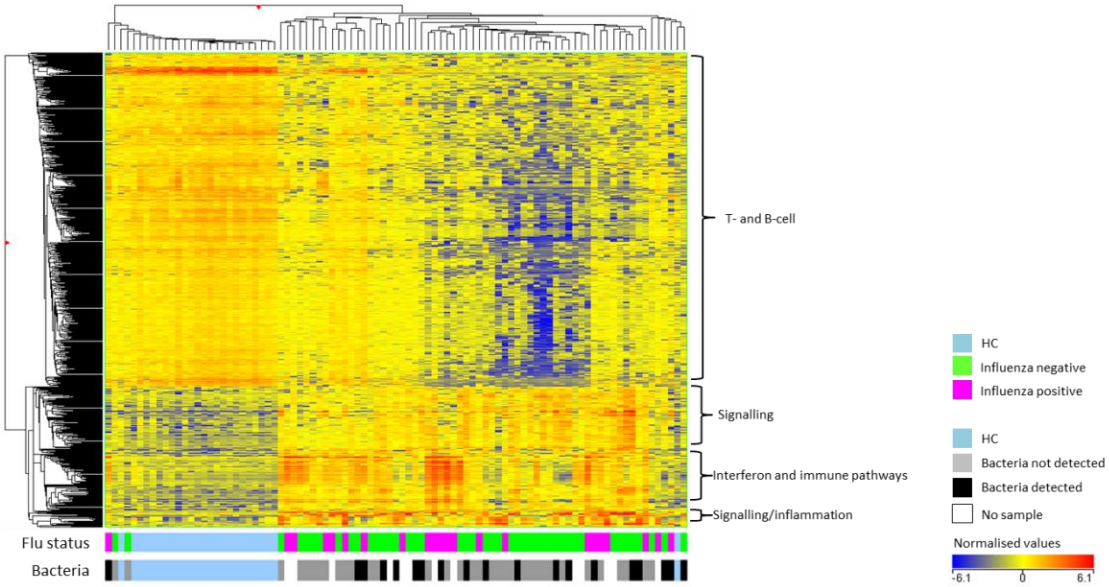


Figure 4.4 (C) Association of transcriptional response with low and elevated serum procalcitonin levels at T1

Hierarchical clustering was performed as described for figure 4.4 (A). Adult participants were identified according to serum procalcitonin measured at T1: <0.5 ng/mL (grey) or ≥0.5 ng/mL (black). HC = healthy control (light blue). Influenza status is shown in the top bar: HC (light blue); influenza-negative (green), influenza-positive (pink).

2009/10 (704 transcripts)



2010/11 (2,426 transcripts)

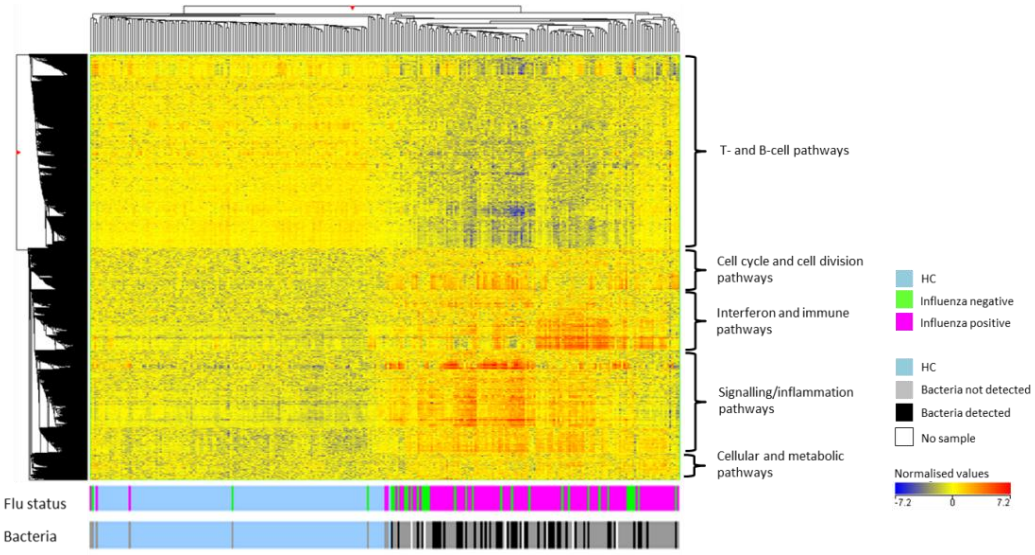


Figure 4.4 (D) Association of transcriptional response at T1 with detection of bacteria by PCR in T1 nasopharyngeal aspirate (NPA) samples

Hierarchical clustering was performed as described for figure 4.4 (A). Adult participants were identified according to bacterial detection in T1 NPA samples: Healthy control (HC, light blue); bacteria not detected (grey); bacteria detected (black). Influenza status of individuals is also shown: HC (light blue); influenza negative (green); influenza-positive (pink).

2010/11 (2,426 transcripts)

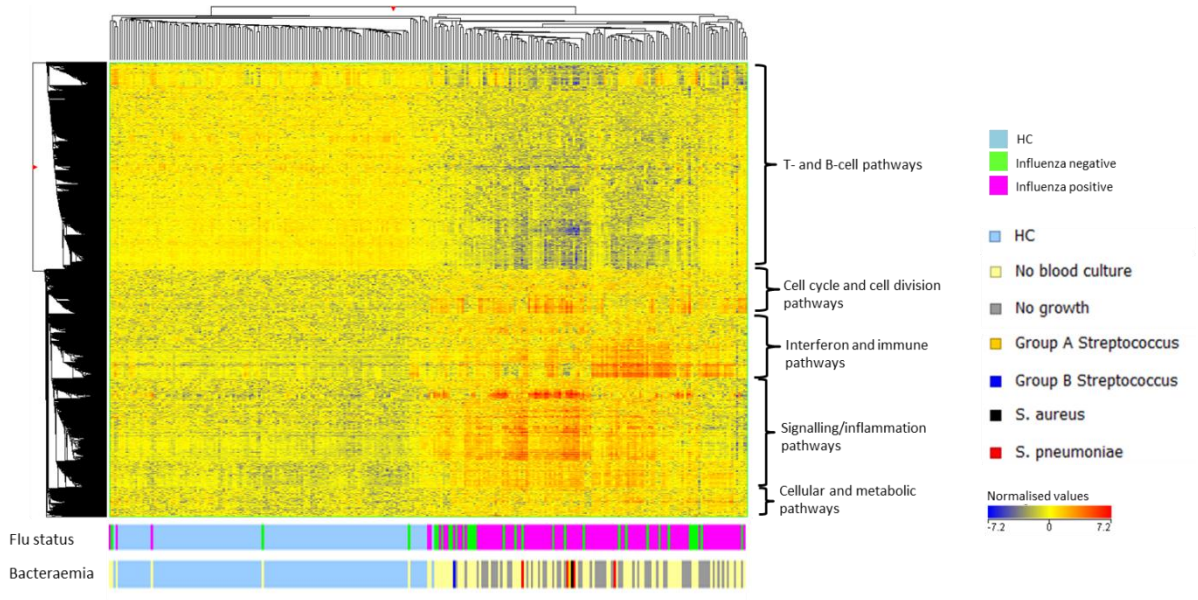
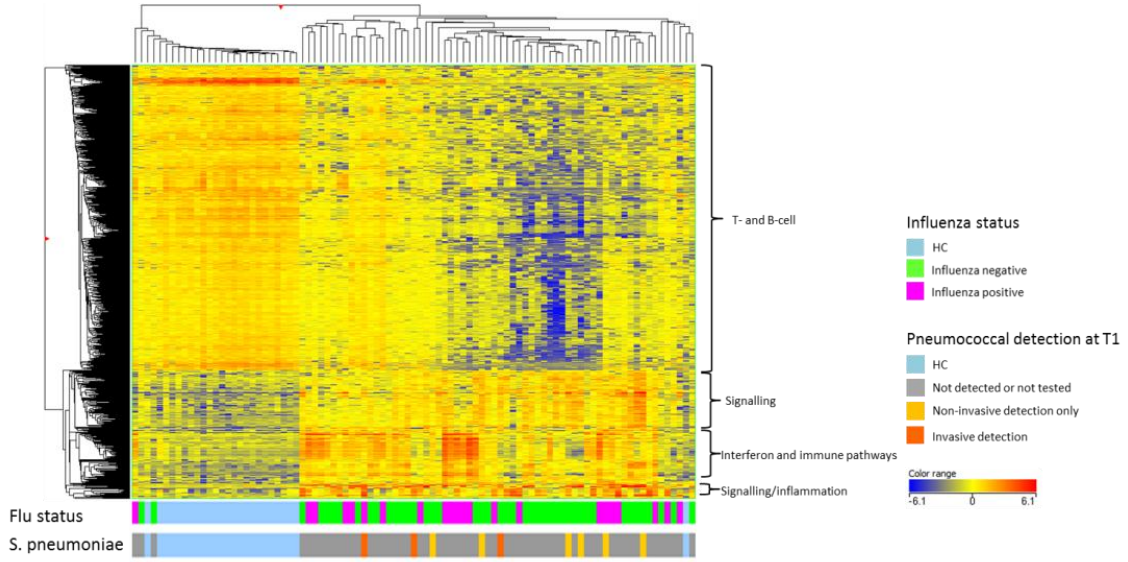


Figure 4.4 (E) The majority of 2010/11 patients with known bacteraemia had a relative over-abundance of transcripts related to signalling/inflammation (bacterial response) pathways
Hierarchical clustering was performed as described for figure 4.4 (A). Adult participants were identified according blood culture (bacteraemia) detections: Healthy control (HC, light blue); no blood culture sample (light yellow); no growth of bacteria (grey); Group A *Streptococcus* (light orange); Group B *Streptococcus* (blue); *S. aureus* (black); *S. pneumoniae* (red). Influenza status of individuals is also shown: HC (light blue); influenza negative (green); influenza-positive (pink).

2009/10 (704 transcripts)



2010/11 (2,426 transcripts)

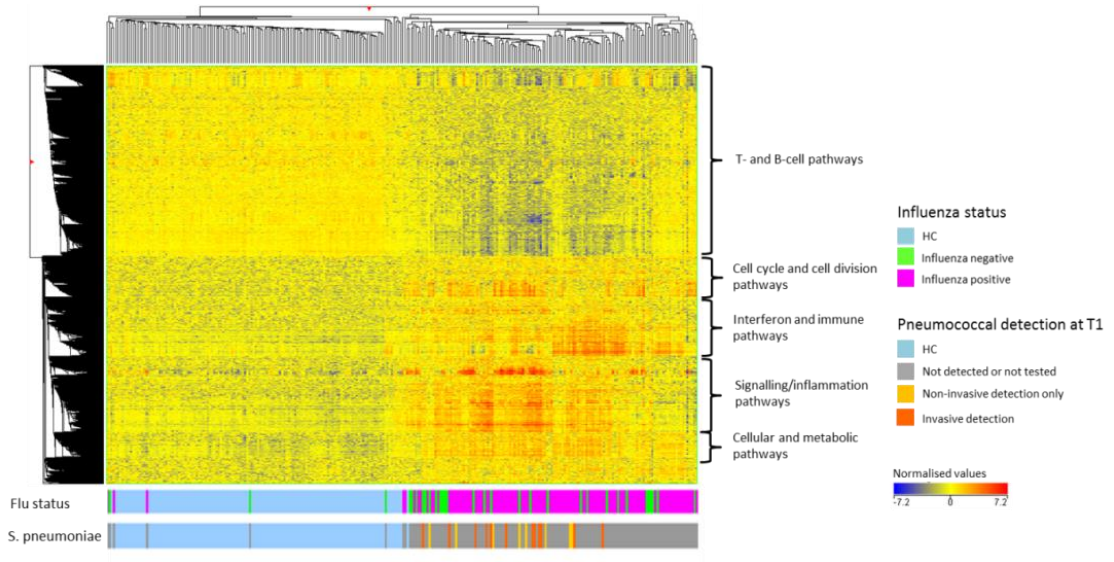


Figure 4.4 (F) Identified pneumococcal infection is more common in patients who have a relative over-abundance of transcripts related to signalling/inflammation (bacterial response) pathways
Hierarchical clustering was performed as described for figure 4.4 (A). Adult participants were identified according to *pneumococcal (S. pneumoniae)* detections: Healthy control (HC, light blue); *pneumococcus* not detected or patient not tested (grey); non-invasive *pneumococcal detection* (light orange); invasive pneumococcal detection (dark orange). Influenza status of individuals is also shown: HC (light blue); influenza negative (green); influenza-positive (pink).

4.4 Analysis of adult patients with influenza virus infection at T1

4.4.1 Approach to analysis

To focus more on the influenza-infected patients, following analysis of all adult patients at T1, we then analysed the transcriptional responses of influenza-positive adults at T1 and healthy controls, without including influenza-negative patients. To analyse transcriptional responses in greater depth, we applied hierarchical clustering analysis and also K-means clustering analysis, with biological interpretation by IPA and GO Terms. Additionally, we also investigated the transcriptional response using BIIR modular transcriptional analysis.

4.4.2 Association of transcriptomic response with influenza infection status

For each of the two periods of recruitment, microarray results from influenza-positive cases sampled at T1 and results from all of the matched healthy controls were analysed. Expression and statistical filtering resulted in lists of differentially expressed transcript containing 1281 transcripts and 2355 transcripts for the 2009/10 and 2010/11 cohorts, respectively. The filtering process is summarised in figure 4.5.

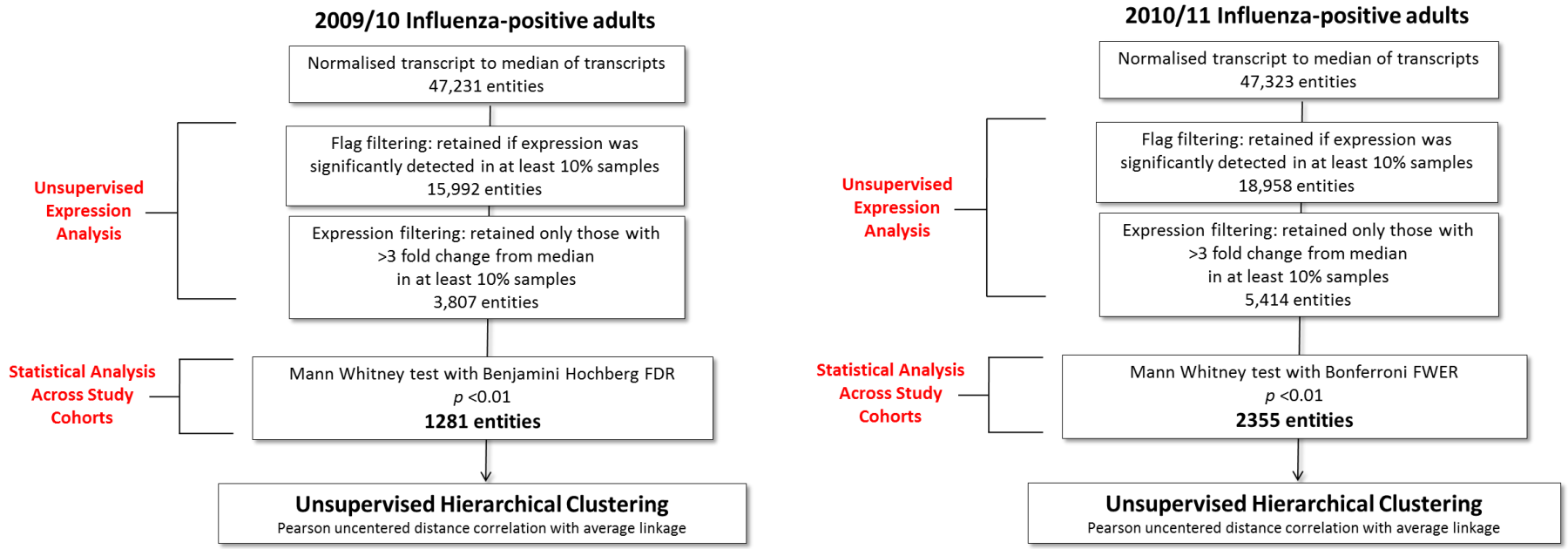


Figure 4.5 Analytical approach used for analysing microarray data in GeneSpring for influenza-positive adults at T1 and healthy controls
Adult patients from 2009/10 and 2010/11 were analysed separately. Each recruitment cohort had its own set of matched healthy controls. Original normalised transcript lists (>47,000 transcripts) underwent background detection (flag) filtering, followed by expression filtering with retention of transcripts that demonstrated >3 fold change from the medain normalised intensity value in at least 10% samples. Statistical testing with mutliple testing corrections were then applied (Mann Whintey unpaired test; Benjamini and Hochberg FDR for 2009/10; Bonferroni FWER for 2010/11). Hierarchcial clustering was performed on the final entity lists (shown in bold).

Although three-fold-change expression filtering was applied to results from both recruitment periods, Benjamini and Hochberg FDR, rather than Bonferroni FWER, was applied to the 2009/10 data during statistical filtering. This was because Bonferroni FWER resulted in a comparatively small transcript list (369 transcripts) for the much smaller 2009/10 cohort (data not shown). Because there were relatively few influenza-positive patients in the 2009/10 cohort, there was a risk of losing true-positive expressed transcripts by accepting the more stringent Bonferroni FWER correction, making it more difficult to compare results the two seasons. The final transcript lists underwent hierarchical clustering using influenza-infection status as the initial interpretation. The presentation format is the same as that used for analysis of all patients at T1. Major transcriptional clusters were identified visually and entity lists underwent IPA canonical pathway analysis and GO Terms analysis.

4.4.2.1 2009/10 influenza-positive cohort at T1

For the 2009/10 cohort, confirmed influenza cases clustered separately from most of the healthy controls. A single healthy control clustered within the patient group. Three major transcriptional clusters were identified. The largest cluster (819 transcripts) contained transcripts that were relatively under-abundant when compared to the per-gene median intensities for all samples. Similar to the findings for all patients at T1, these were transcripts associated with pathways related to T- and B-cell signalling in IPA canonical pathway analysis. T-cell pathway associations were also identified in GO analysis for this transcript cluster, along with metabolic pathway associations (figure 4.6, upper panel).

Two major clusters of transcripts demonstrating relative overabundance were seen. The smaller cluster (148 transcripts) contained transcripts that in IPA associated with various cell-cycle and metabolic pathways and the 'airway pathology in COPD' pathway. The IL-8 signalling pathway was within the *p* value threshold, but was not in the top five canonical pathways. GO terms analysis identified several cell cycle-associated GO terms but also 'defense response to bacterium' and 'response to bacterium' GO Terms. The larger over-abundant transcript cluster (305 transcripts) was associated with interferon signalling, pattern recognition receptor and immune response pathway categories in IPA. GO Terms analysis identified terms including 'response to virus' and 'immune response' (figure 4.6, upper panel).

The influenza-positive patients from the 2009/10 cohort broadly separated into two groups based on over-abundant transcripts: those with marked over-abundance of interferon and immune pathway-associated transcripts and those with marked over-abundance of signalling/inflammation pathway-associated transcripts. Under-abundance of T- and B-cell pathway-associated transcripts can be seen throughout, although this under-abundance was less marked in a sub-cluster of the

patients with over-abundance of signalling/inflammation pathway-associated transcripts (figure 4.6, upper panel).

4.4.2.2 2010/11 influenza-positive cohort at T1

In the 2010/11 cohort, the majority of the influenza-positive patients clustered separately from the healthy controls. One healthy control samples was within the patient cluster and three patients were within the healthy controls. The largest transcriptional cluster (1066 transcripts) contained a relative under-abundance of transcripts associating with T- and B-cell pathways in both IPA and GO Terms analysis, similar to the 2009/10 cohort (figure 4.6, lower panel)

In terms of over-abundant transcript clusters, a greater number of groups were seen compared to the 2009/10 cohort, but the two most prominent clusters were similar to those identified in the 2009/10 cohort. The first over-abundant cluster (323 transcripts) was associated with cell-cycle and division pathways and in IPA and related terms in GO analysis. The second cluster (167 transcripts) contained transcripts that were associated with interferon- and immune-associated pathways in IPA (and response to virus and immune response in GO Terms analysis). The third cluster (664 transcripts) contained transcripts associated with inflammation/signalling pathways in IPA (and defense response, response to bacterium and immune response in GO Terms analysis). A final, small over-abundant transcriptional cluster (147 transcripts) was also identified, containing transcripts that were associated with signalling and metabolic processes e.g. oestrogen receptor signalling and oxidised GTP and dGTP detoxification, but no associations with GO Terms were found and the degree of over-abundance appeared to be uniform throughout all of the patients (figure 4.6, lower panel).

Similar to the 2009/10 cohort, the influenza-positive patients in the 2010/11 cohort could be divided into two broad groups based upon common signatures generated through the semi-supervised clustering algorithm. Patients within the first group had marked over-abundance of inflammation/signalling pathway-associated transcripts, but generally did not have marked over-abundance of interferon and immune pathway-associated transcripts. Additionally, they tended to have marked over-abundance of transcripts that associated with the cell-cycle pathways. In contrast, patients within the second group demonstrate marked over-abundance of interferon and immune pathway-associated transcripts, with less marked over-abundance overall of transcripts associating the inflammation/signalling and cell-cycle pathways (figure 4.6, lower panel).

For each recruitment period and for each major transcript cluster identified, the pathways meeting *p*-value thresholds in IPA canonical pathway analysis and the corresponding *p* values, ratios and component molecules are summarised separately (appendix 7).

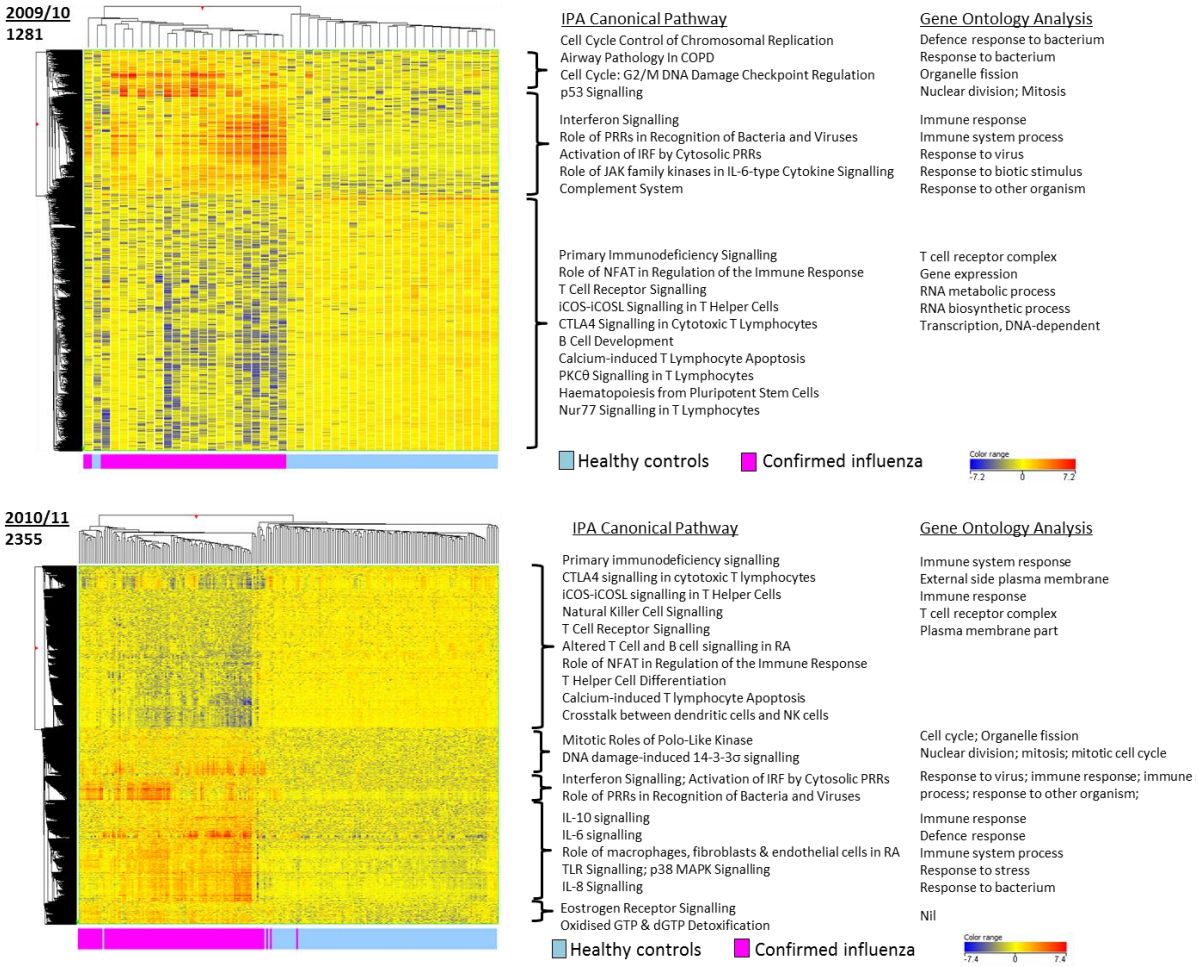


Figure 4.6 Influenza-positive patients cluster separately from healthy controls at T1 and form two major groups defined by differentially-expressed transcriptional clusters
For each recruitment period, normalised intensity value transcripts which were significantly detected from background, and filtered for low expression (transcripts retained if >3FC from median normalised intensity value, followed by statistical filtering (Mann Whitney unpaired test with Benjamini Hochberg FDR, p <0.01, for 2009/10; Mann Whitney unpaired test with Bonferroni FWER, p <0.01 for 2010/11). Hierarchical clustering was then performed (clustered on transcripts and by individuals; Pearson's Uncentered with average linkage). Listed next to the heat-map are the top IPA canonical (by significance p <0.05 Fishers Exact test) for up-regulated and down-regulated transcripts. Corresponding terms identified by separate Gene Ontology (GO Terms) analysis are shown alongside. Patients were identified according to their influenza status (healthy control (HC), light blue; confirmed influenza, pink).

4.4.3 Association of transcriptional response in influenza patients with day of illness at T1 and severity of illness at T1

Relationships between selected clinical variables and hierarchical clustering were seen when influenza-positive and influenza-negative patients were analysed as a whole. Therefore, the same clinical variables were subsequently applied to the separate influenza-positive and healthy control analysis.

For the 2009/10 influenza-positive cohort, patients who had marked over-abundance of the interferon and immune response pathway-associated transcripts were predominantly at an early stage of illness (0-4 days) and had severity grade 1 or 2. Only two of eight patients within this group were at a later stage of illness (>5 days) at the time of T1 sampling. Although patients within the group demonstrating over-abundance of inflammation/signalling and cell-cycle pathways-associated transcripts were of mixed severity, all three of the influenza patients who were severity grade 3 fell within this group (upper panel, figure 4.7 [A])

Associations with the day of illness categories and severity grades were more obvious for the larger 2010/11 cohort. This cohort contained a larger number of severity grade 3 patients, the majority of whom fell within the group containing marked over-abundance of inflammation/signalling and cell-cycle pathways-associated transcripts. Furthermore, almost all of these patients tended to be at the later stage of illness. Very few severity grade 1 or early stage of illness patients were seen in this group; most severity grade 1 patients fell within the interferon and immune pathways-associated group, which also contained most of the patients who were at an early stage of illness (lower panel, figure 4.7 [A]).

4.4.4 Association of transcriptional response in influenza patients with serum procalcitonin levels at T1

Given the marked over-abundance of transcripts that may be associated with response to secondary or concomitant bacterial infection in influenza-positive patients, especially those who have more advanced and severe illness, serum procalcitonin (PCT) was again used as an initial screen to investigate the relationship with bacterial sepsis. The results for the 2009/10 demonstrate that patients with marked over-abundance of interferon and immune pathways-associated transcripts but less marked over-abundance of inflammation/signalling pathways-associated transcripts tended to have low PCT values (<0.5 ng/mL); only two of eight patients falling within the discrete cluster had elevated PCT (>0.5 ng/mL). By contrast, in the group of patients where the opposite transcriptional profile was seen, only 5 of the 12 patients had low PCT levels (upper panel, figure 4.7 [B])

In the larger 2010/11 cohort, the clustering suggested a stronger association between the signature dominated by marked over-abundance of interferon and immune pathways-associated transcripts, less abundance of the inflammation/signalling and cell-cycle pathway transcripts and low PCT. In the group where over-abundance of inflammation/signalling and cell-cycle pathway-associated transcripts (identified as bacterial response in GO Terms analysis) dominate the signature, many patients had elevated PCT. Furthermore, as the over-abundance becomes more marked, a sub-cluster can be seen where all of the patients have PCT > 0.5 ng/mL (lower panel, figure 4.7 [B]). Gradation of PCT results into low (< 0.5 ng/mL), moderate (0.5-2.0 ng/mL) and high (> 2.0 ng/mL) suggests that as the over-abundance of inflammation/signalling pathways-associated transcripts increases in the 2010/11 patients, so does the PCT level (figure 4.7 [C])

4.4.5 Association of transcriptional response in influenza patients with detection of bacteria in nasopharyngeal aspirate at T1

Bacteria associated with respiratory tract infections were detected in NPA samples from just five of the twenty-two influenza-positive patients recruited during 2009/10. Although the results should be interpreted with caution, since eight patients did not have samples tested by PCR, all but one of the patients with detectable bacteria were in the group with marked over-abundance of the inflammation and signalling pathway-associated transcripts. This transcriptional cluster also associated with “bacterial response” in GO Terms analysis (upper panel, figure 4.7 [D])

In the 2010/11 cohort, most of the patients had undergone PCR testing and positive detections appeared to be more common in those who lacked overabundance of the interferon and immune pathway-associated transcripts, but who had marked over-abundance of inflammation/signalling and cell cycle pathway-associated transcripts (associated with “bacterial response” in GO Terms analysis) (lower panel, figure 4.7 [D]). *S. pneumoniae*, *S. aureus* and *H. influenzae* were the most commonly-detected bacteria, but individual bacterial species did not cluster together (data not shown).

4.4.6 Association of transcription response with pneumococcal infection in influenza patients

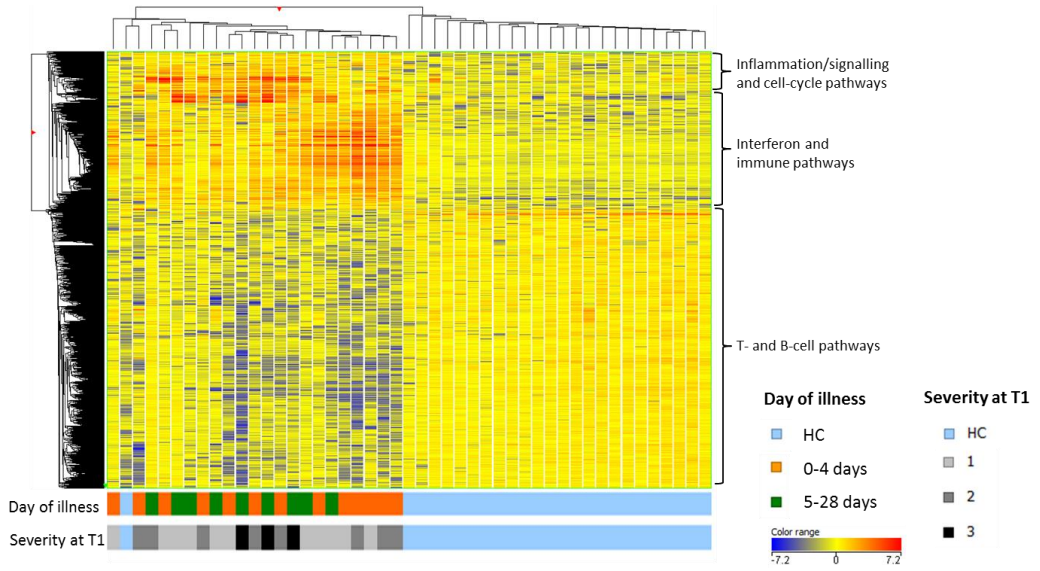
Only two of the twenty-two influenza-positive patients in the 2009/10 cohort had pneumococcal infection detected (identified using the same methods as described for all patients at T1). Both of these patients fell within the group with marked over-abundance of inflammation/signalling pathways-associated transcripts (upper panel, figure 4.7 [E]).

Of the 109 influenza-positive patients from 2010/11, *S. pneumoniae* infection was identified in sixteen individuals (14.7%), of which 10 had evidence of invasive infection. Twelve of the patients who were known to have *S. pneumoniae* infection were within the group with marked over-abundance of inflammation/signalling and cell cycle pathway-associated transcripts (associated with bacterial response in GO Terms analysis), although invasive infections did not form a distinct cluster (lower panel, figure 4.7 [E]).

4.4.7 Association of transcriptional response with absolute white cell counts

To investigate whether changes in the absolute number of neutrophils and lymphocytes in blood are associated with changes in transcriptional signatures, we performed an initial analysis using hierarchical clustering of transcripts, grouped according to changes in differential white cell counts at T1 (clustering on transcripts, supervised analysis). Heatmap analysis suggested that patients who had neutrophil counts above the normal range also appeared to have an over-abundance of transcripts associated with signalling and inflammation (bacterial response) pathways, although a relative under-abundance of transcripts associated with T- and B-cell pathways also appears to be common (figure 4.8, upper panel). A relative under-abundance of transcripts associated with T- and B-cell associated pathways also appeared to be common in patients with low lymphocyte counts (figure 4.8, lower panel).

2009/10 (1281 transcripts)



2010/11 (2355 transcripts)

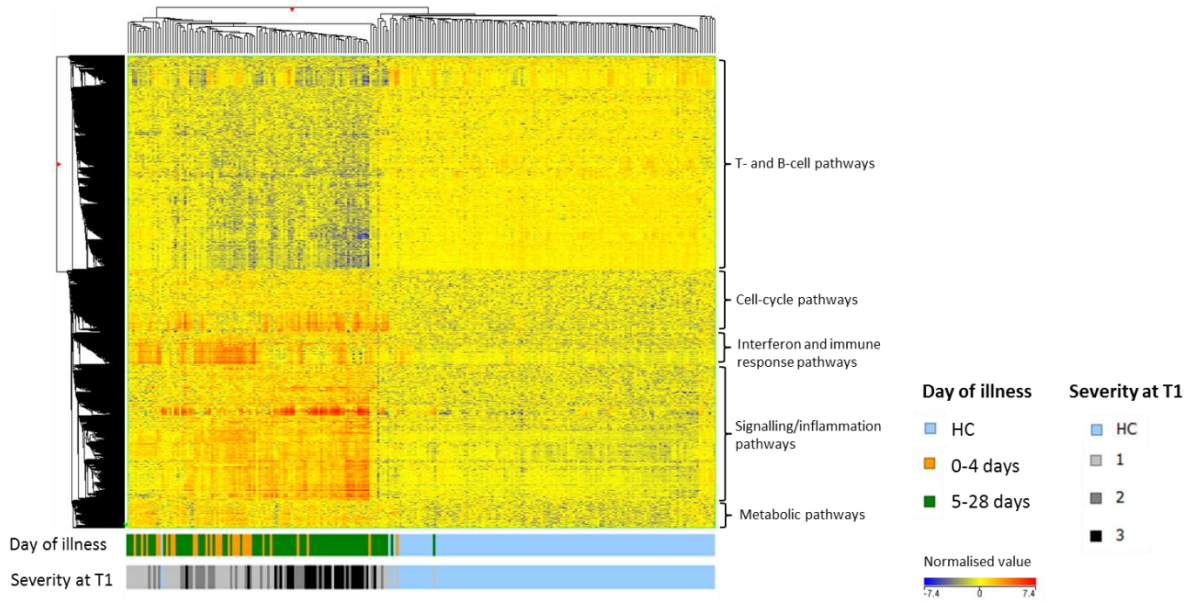
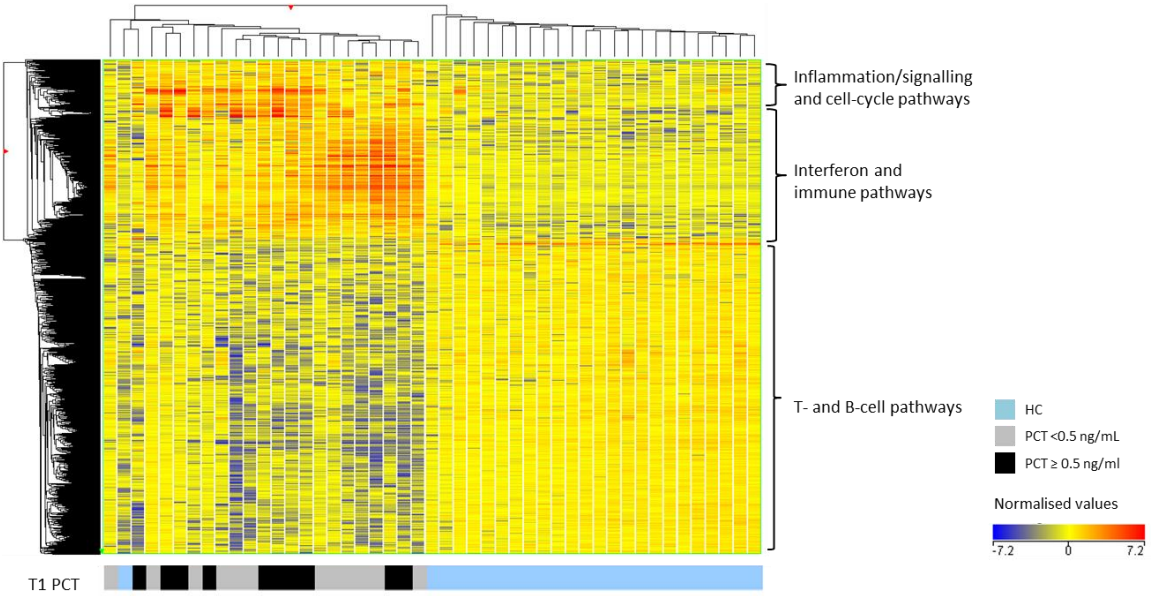


Figure 4.7 (A-E) Influenza-positive adults and healthy controls at T1

(A) Dominant clusters of differentially expressed transcripts and their association with duration of illness and severity of illness at T1 in influenza-positive adult patients

For each recruitment period, normalised intensity value transcripts which were significantly detected from background, and filtered for low expression (transcripts retained if >3FC from median normalised intensity value, followed by statistical filtering (Mann Whitney unpaired test with Benjamini Hochberg FDR, p <0.01, for 2009/10; Mann Whitney unpaired test with Bonferroni FWER, p <0.01 for 2010/11). Hierarchical clustering was then performed (clustered on transcripts and by individuals; Pearson's Uncentered with average linkage). Listed next to the heat-map are summaries of identified associated IPA pathways (by significance p <0.05 Fishers Exact test) for up-regulated and down-regulated transcripts. Adult participants were identified according to day of illness at T1 (healthy control, light blue; 0-4 days, orange; 5-20 days, green) and severity of illness measured at T1 (healthy control, light blue; severity 1, light grey; severity 2, grey; severity 3, black). HC = healthy control.

2009/10 (1281 transcripts)



2010/11 (2355 transcripts)

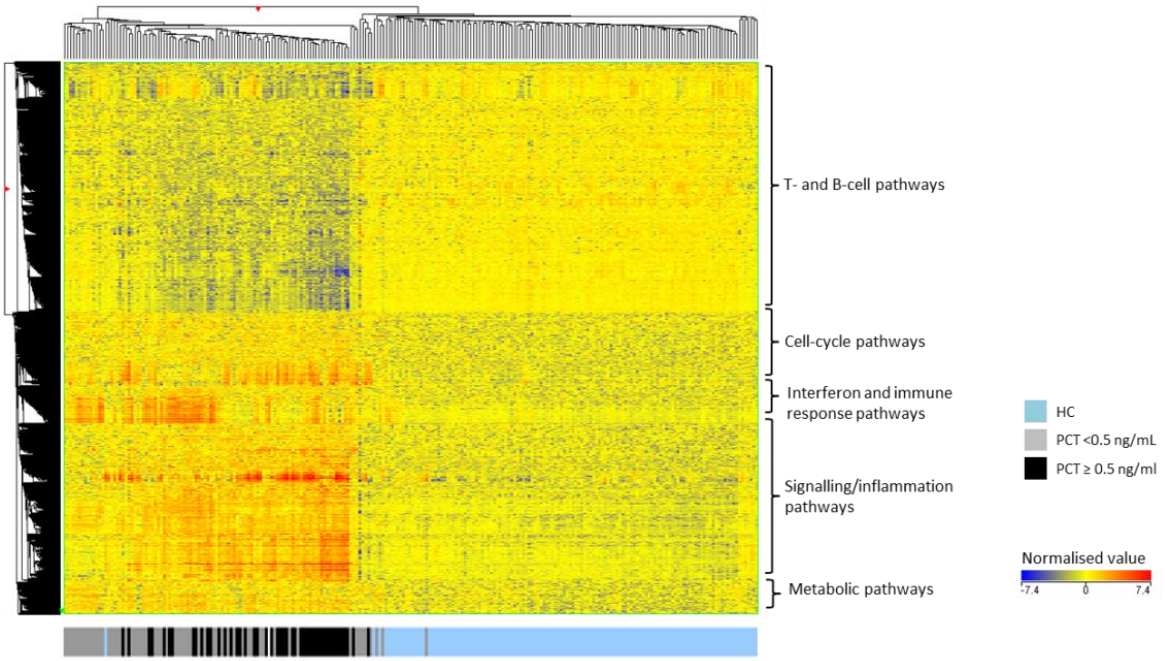


Figure 4.7 (B) Low procalcitonin is more commonly associated with overabundance of interferon-related transcripts and decreased abundance of signalling/inflammation-related (bacterial response) transcripts
Hierarchical clustering was performed as described for figure 4.7 A. Adult participants were identified according to serum procalcitonin measured at T1: PCT <0.5 ng/mL (grey); PCT ≥0.5 ng/mL (black). HC = healthy controls (light blue).

2010/11 (2355 transcripts)

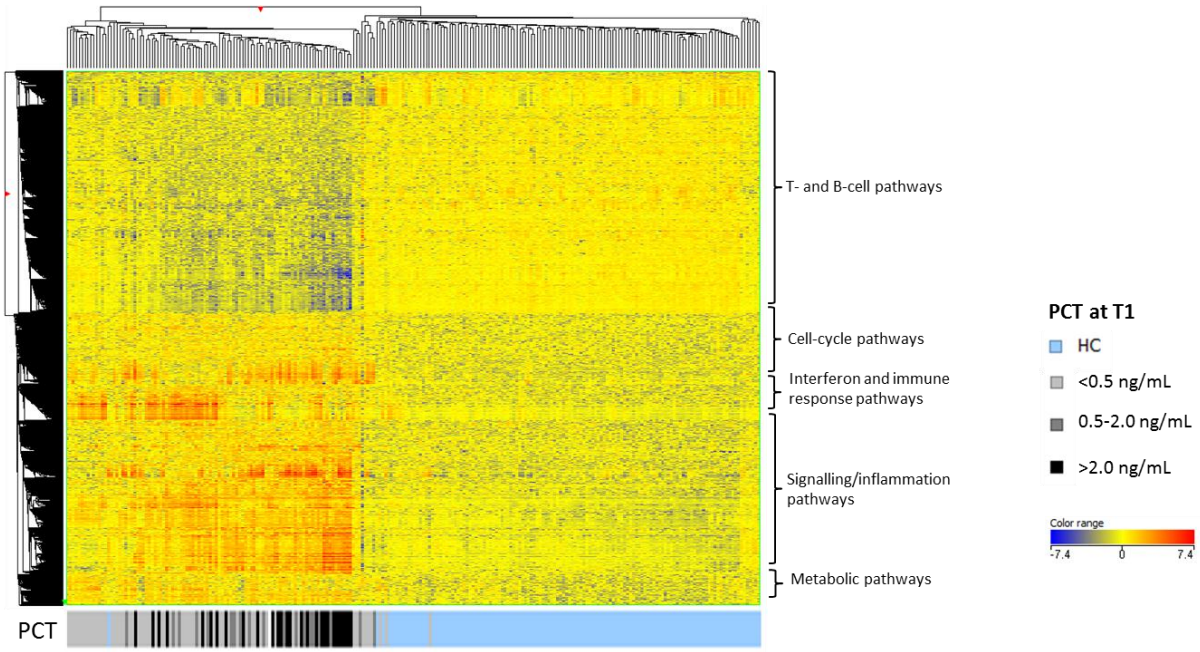
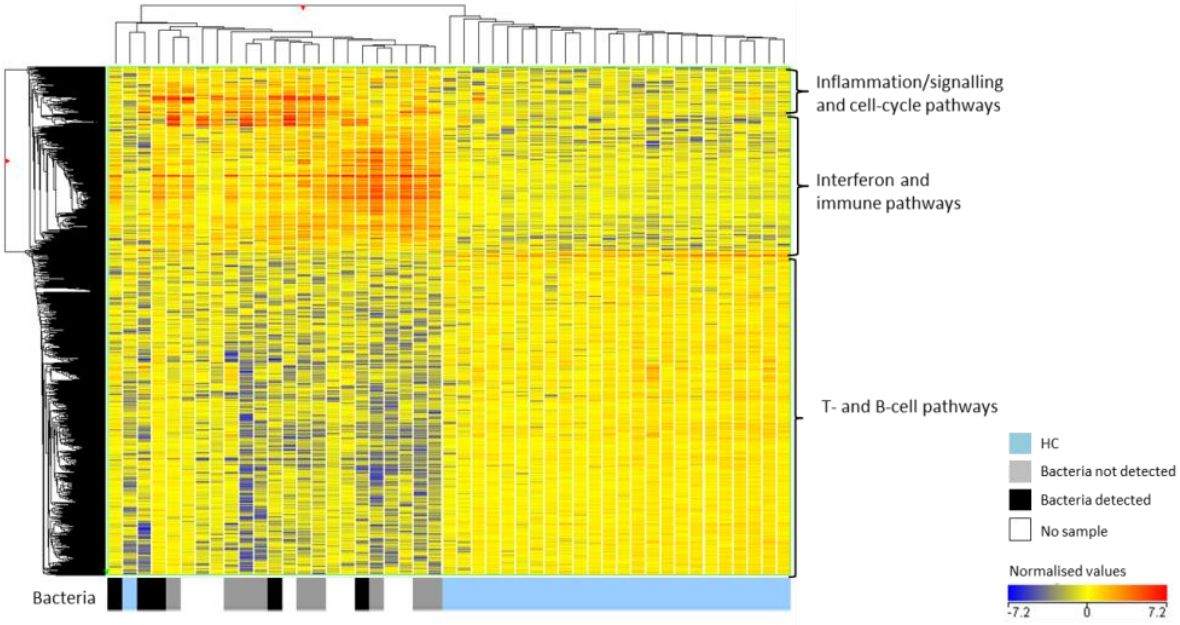


Figure 4.7 (C) High procalcitonin is more commonly associated with influenza over-abundance of signalling/inflammation (bacterial response) transcripts and less abundant interferon-associated transcripts

Hierarchical clustering was performed as described for figure 4.7 A. Adult participants from 2010/11 season only were identified according to serum procalcitonin measured at T1: <0.5 ng/mL (light grey); 0.5-2.0 ng/mL (grey) >2.0 ng/mL (black). HC = healthy controls (light blue).

2009/10 (1281 transcripts)



2010/11 (2355 transcripts)

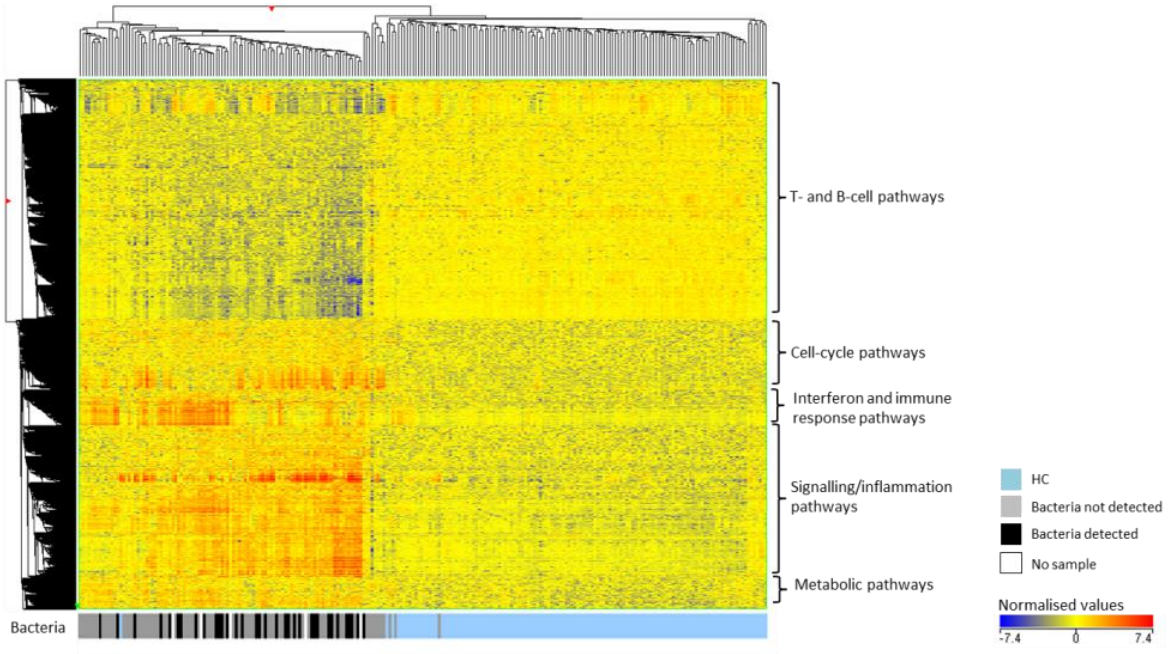
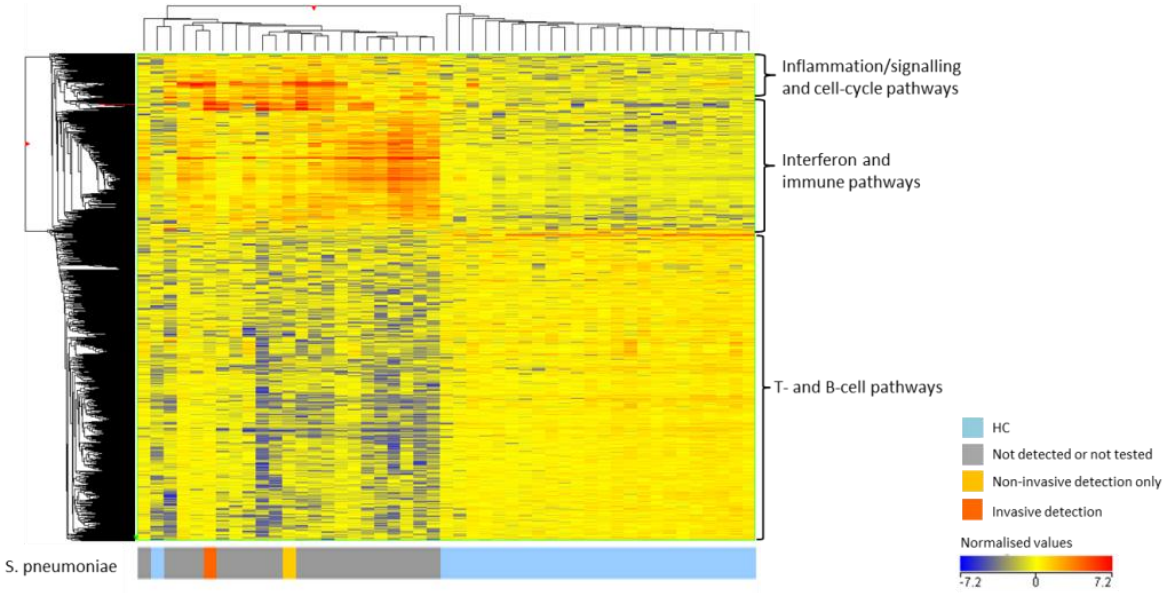


Figure 4.7 (D) Association of transcriptional response with detection of bacteria in T1 nasopharyngeal aspirate samples in influenza patients.
Hierarchical clustering was performed as described for figure 4.7 A Adult participants were identified according to detection of bacteria by PCR in T1 NPA samples: Bacteria not detected (grey); bacteria detected (black); no NPA sample available for PCR testing (white). HC = healthy controls (blue).

2009/10 (1281 transcripts)



2010/11 (2355 transcripts)

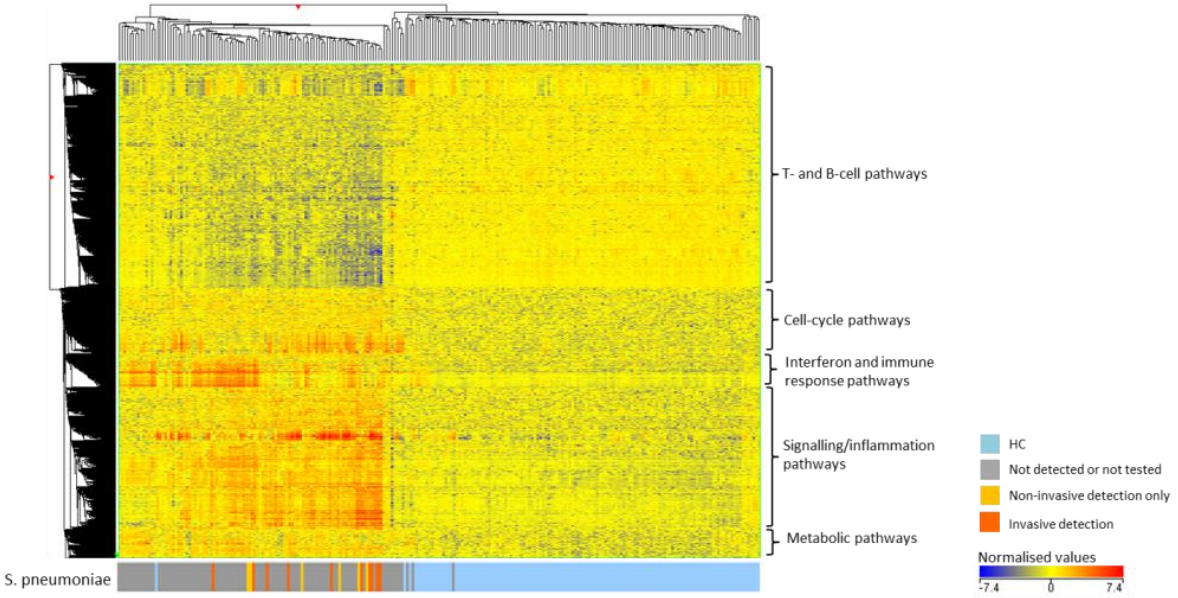


Figure 4.7 (E) Known pneumococcal infections are more common in influenza patients with over-abundance of signalling/inflammation (bacterial response)-associated transcripts

Hierarchical clustering was performed as described for figure 4.7 A. Adult participants were identified according to detection of pneumococcus (*S. pneumoniae*) by PCR in T1 NPA samples: pneumococcus not detected or testing not performed (grey); non-invasive pneumococcus detected (light orange); invasive pneumococcus detected (dark orange). HC = healthy controls (light blue).

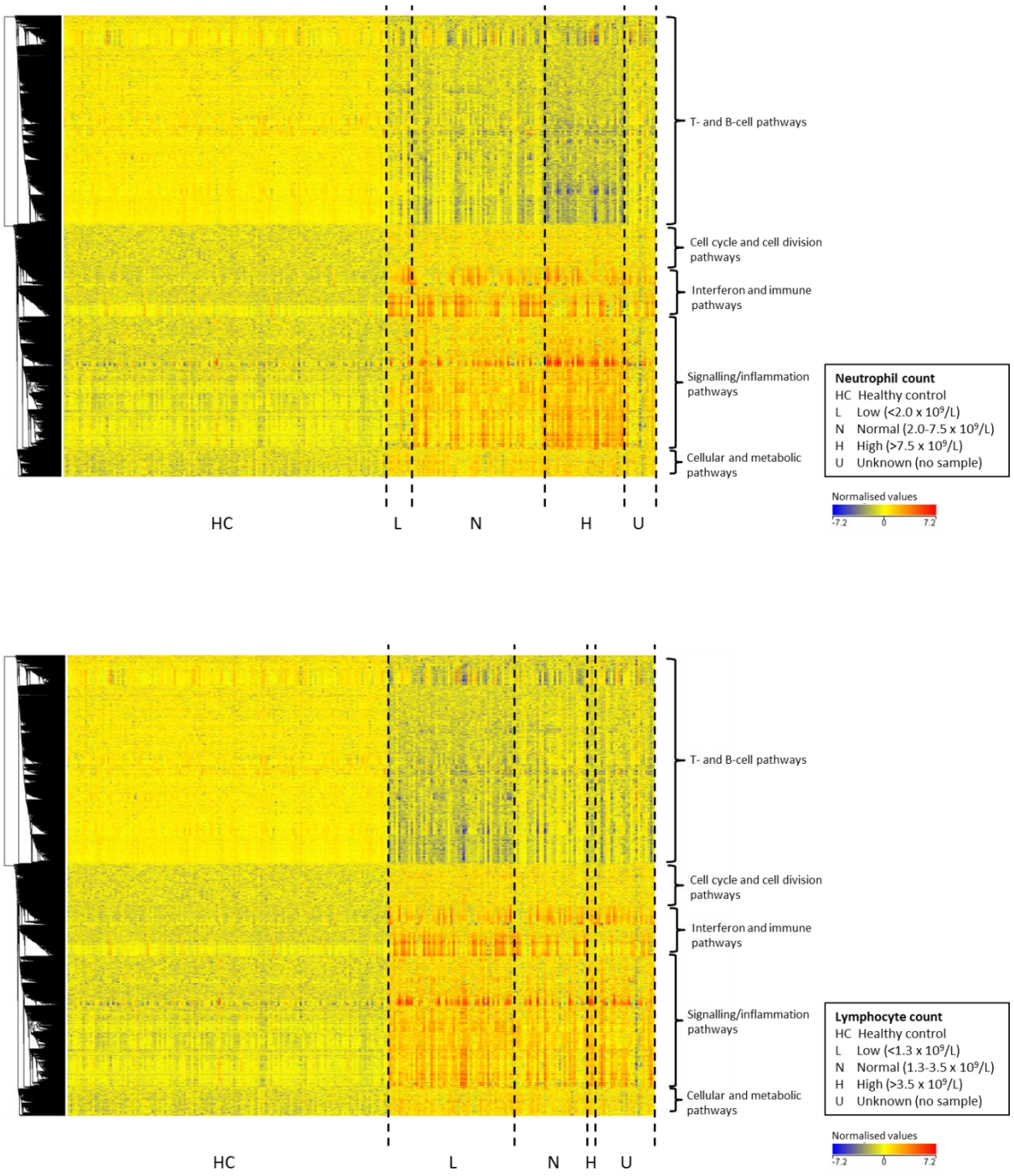


Figure 4.8 Relationship between transcriptional signatures and absolute neutrophil and lymphocyte counts in influenza-positive patients

Transcriptional signatures for the 2010/11 influenza-positive adult patients at T1 (2355 transcripts, obtained as described previously) were grouped according to patients' absolute blood neutrophil count (upper panel) and lymphocyte count (lower panel). Hierarchical clustering was performed on entities only (supervised analysis; Pearson's uncentered clustering with average linkage). Reference ranges for neutrophils and lymphocytes were provided by the clinical laboratory that performed the differential cell count assays.

4.4.8 K-means clustering of transcripts from influenza-positive patients and healthy controls

4.4.8.1 Analytical approach for K-means clustering

To validate the results of hierarchical clustering on severity, a separate clustering method (K means clustering) was performed. For the 2009/10 and 2010/11 cohorts, the lists of differentially expressed transcripts for influenza-positive patients at T1 and healthy controls, derived as previously described (1281 and 2355 transcripts, respectively), underwent K-means clustering. The algorithm was instructed to create 5 clusters for the 2009/10 cohort list and 10 clusters for the 2010/11 cohort list. Clustering was performed on transcripts only, using severity at T1 as the interpretation (HC, severity 1, severity 2 and severity 3). The transcript lists that underwent K-means clustering were generated by statistical comparisons comparing all influenza-positive patients against healthy controls using Mann Whitney unpaired, rather than paired-condition analysis of healthy controls with each severity group using the Kruskal Wallis test. However, for the 2010/11 cohort, both statistical methods were applied initially and these were shown to produce transcript lists of similar size (2355 and 2315 transcripts, respectively). Furthermore, each method produced lists that shared 2207 common transcripts following Venn analysis. Similar agreement between the two methods was achieved when 2009/10 cohort lists were analysed. Therefore, the original transcript lists i.e. those that were used in hierarchical clustering, were also used for K-means clustering.

4.4.8.2 K-means clustering results for the 2009/10 influenza-positive cohort are consistent with hierarchical clustering results

For the 2009/10 influenza-positive cohort at T1, expression profiles were examined for each of the five clusters generated using K-means clustering. Three clusters were selected where the expression profiles indicated a marked difference in transcript abundance between HCs and patients of different severity grades (figure 4.9).

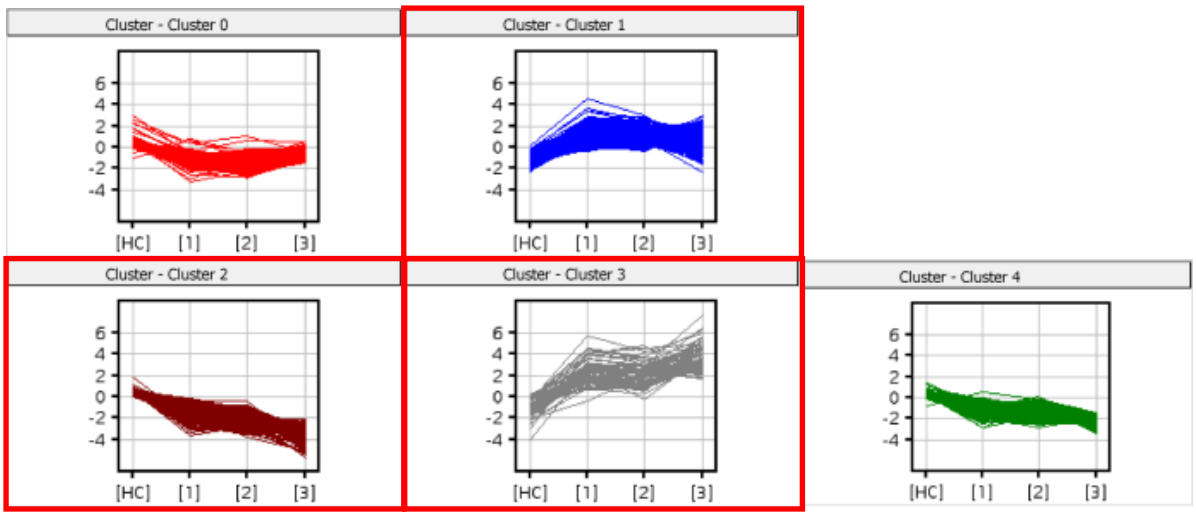


Figure 4.9 K-means clustering of identifies three of five clusters where transcript expression changes with influenza severity (2009/10 cohort)

Normalised intensity value transcripts significantly detected from background were filtered for low expression (transcripts retained if >3FC from median normalised intensity value), followed by statistical filtering (Mann Whitney unpaired test with Benjamini Hochberg FDR, p <0.01). The resulting 1281 transcripts underwent K-means clustering using severity score at T1 as the condition with *K* set to 5 and maximum permutations selected. For each of the five cluster plots, average normalised intensity (log scale) is shown on the Y axis and severity categories at T1 are shown on the X axis (HC = healthy controls; 1 = severity grade 1; 2 = severity grade 2; 3 = severity grade 3). Clusters selected for further analysis (1, 2 and 3) are shown in red boxes.

Cluster 1 contained 387 transcripts that were over-abundant in all patients relative to healthy controls, with the most marked over-abundance seen in patients who were severity grade 1 or 2. IPA analysis indicated an association of transcriptional response with interferon and immune pathways. The top three canonical pathways based on *p*-value for association were 'interferon signalling', 'role of pattern recognition receptors in recognition of bacteria and viruses' and 'activation of IRF by cytosolic pattern recognition receptors'. Thirteen GO Terms were associated and included the terms 'immune response', 'response to virus', 'defense response', 'innate immune response' and 'response to bacterium' (figure 4.10).

Cluster 3 demonstrated over-abundance of 73 transcripts in the patients. However, abundance appears similar in severity grade 1 and 2 patients, but then increases in patients who had grade 3 severity of illness. Transcripts for interferon-associated genes were included in the cluster, but additionally, transcripts for genes involved in bacterial response were identified e.g. defensins. Associated IPA canonical pathways were relatively diverse, related to signalling, inflammation, metabolic and cell-cycle processes and PRRs involved in recognition of bacteria and viruses. Associated GO Terms were in general agreement with the IPA findings and also specifically identified 'response to bacterium' and 'defense response to bacterium' (figure 4.11).

Cluster 2 contained 183 transcripts that were under-abundant in all patients, with the under-abundance becoming more marked as severity increased. Marked under-abundance was seen in severity grade 3 patients. Many of the associated canonical pathways identified by IPA are related to T-cell signalling. GO Terms analysis only identified two terms that met the *p*-value cut-off, both related to calcium-mediated signalling (figure 4.12).



Figure 4.10 K-means clustering-identified transcripts in influenza-positive adults from 2009/10 that decrease in abundance as severity increases (cluster 1 of 5, 387 transcripts)

Changes in the expression of transcripts identified by K-means clustering are represented as an expression profile graph (top left) and heat-map (bottom left). Entity names are not displayed for technical reasons. HC = healthy controls; 1 = severity grade 1; 2 = severity grade 2; 3 = severity grade 3. GO Terms and top IPA Canonical pathways associated with identified transcripts are also shown (middle and right-hand panels).

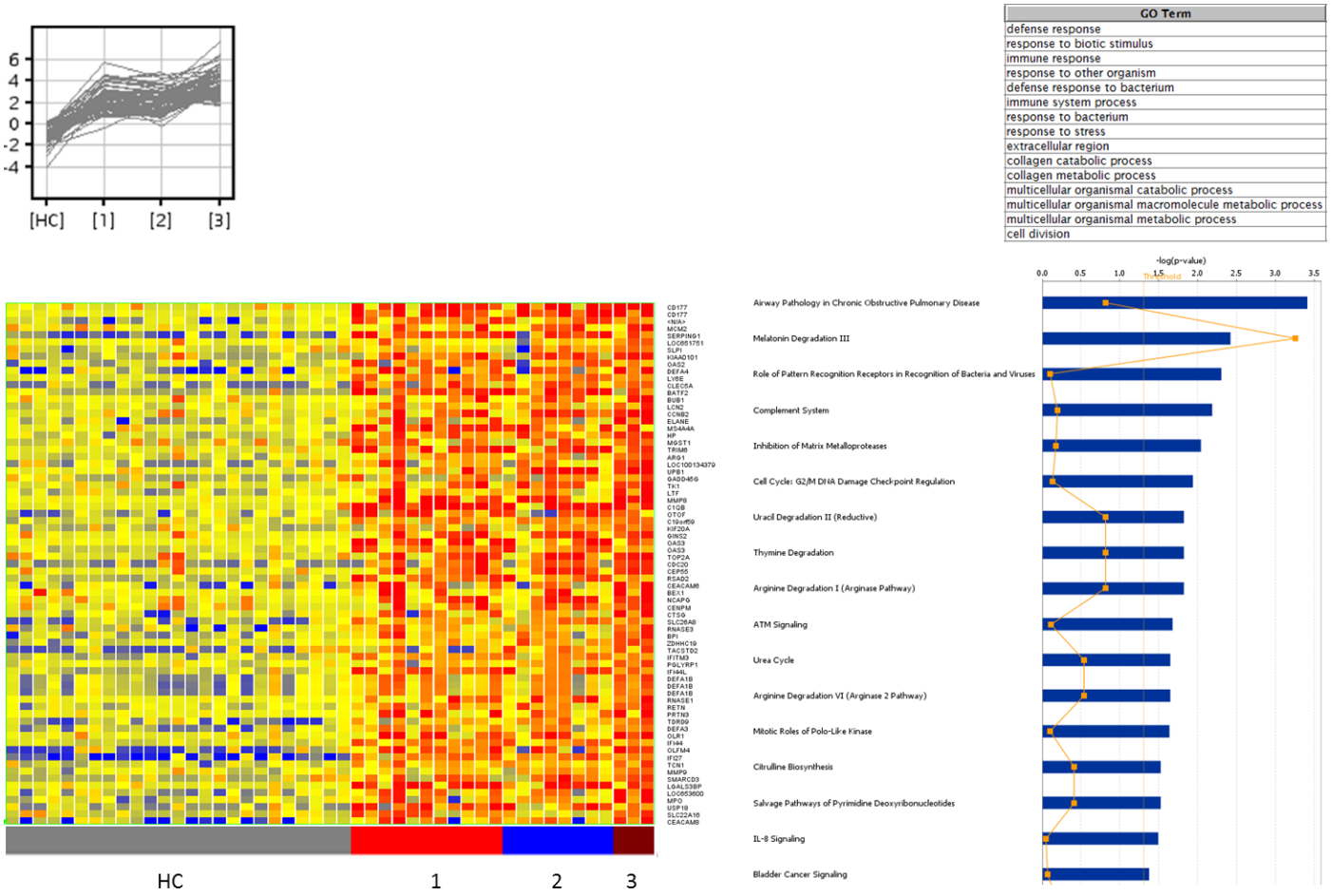


Figure 4.11 K-means clustering-identified transcripts that are most abundant in influenza-positive adults from 2009/10 with severity 3 illness (cluster 3 of 5, 73 transcripts)

Changes in the expression of transcripts identified by K-means clustering are represented as an expression profile graph (top left) and heat-map (bottom left). Entity names are displayed adjacent to the heat-map. HC = healthy controls; 1 = severity grade 1; 2 = severity grade 2; 3 = severity grade 3. GO Terms and top IPA Canonical pathways associated with identified transcripts are also shown (right-hand panels).

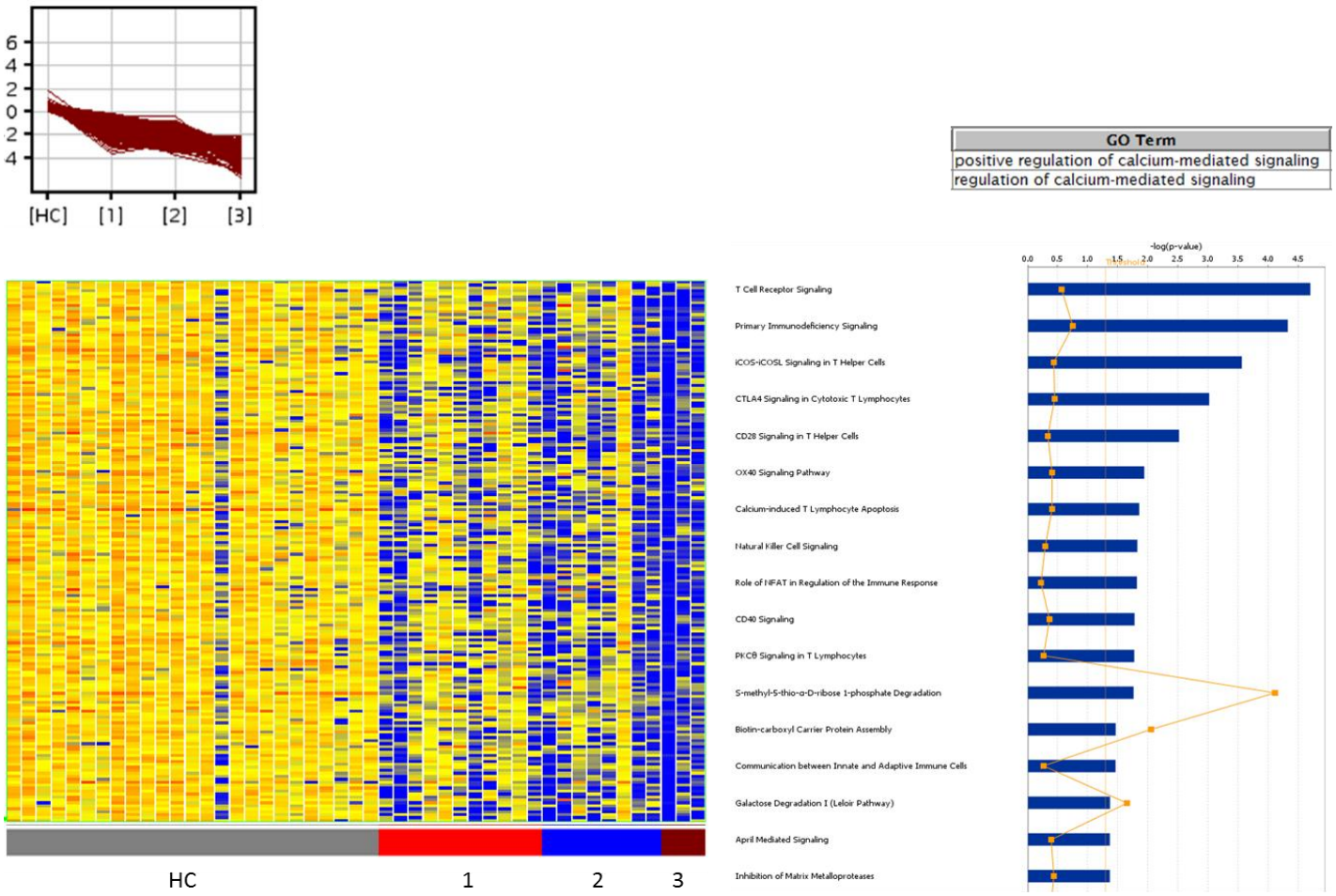


Figure 4.12 K-means clustering-identified transcripts that are least abundant in influenza-positive adults from 2009/10 with severity 3 illness (cluster 2 of 5, 183 transcripts)

Changes in the expression of transcripts identified by K-means clustering are represented as an expression profile graph (top left) and heat-map (bottom left). Entity names are displayed adjacent to the heat-map. HC = healthy controls; 1 = severity grade 1; 2 = severity grade 2; 3 = severity grade 3. GO Terms and top IPA Canonical pathways associated with identified transcripts are also shown (right-hand panels).

4.4.8.3 K-means clustering results for the 2010/11 influenza-positive cohort

Of the ten clusters created in K-means clustering, five had expression profiles that suggested a difference in transcript abundance between healthy controls and patients of different severity grades (figure 4.13). Three clusters (6, 7 and 9) demonstrated an over-abundance of selected transcripts that increased as the severity grade increased. Cluster 5 contained over-abundant transcripts that were less abundant in critically-ill patients (grade 3 severity) compared to those with milder illness (grades 1 and 2 severity). Cluster 8 contained transcripts in influenza patients that were less abundant relative to healthy controls, and these became less abundant as severity of illness increased.

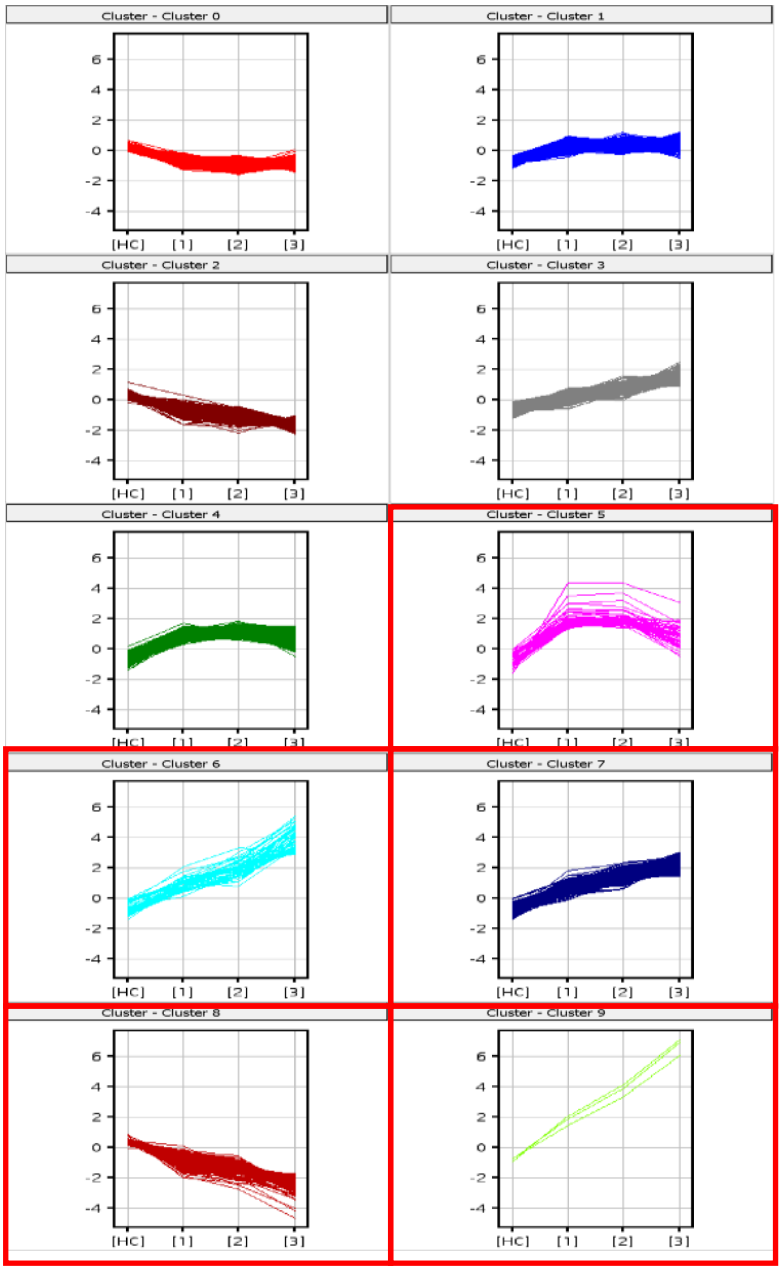


Figure 4.13 K-means clustering identifies five of ten clusters where transcript expression changes with influenza severity (2010/11 cohort)

Normalised intensity value transcripts significantly detected from background were filtered for low expression (transcripts retained if >3FC from median normalised intensity value), followed by statistical filtering (Mann Whitney unpaired test with Bonferroni FWER, p <0.01). The resulting 2355 transcripts underwent K-means clustering using severity score at T1 as the condition, with K set to 10 and maximum permutations selected. For each of the five cluster plots, average normalised

intensity (log scale) is shown on the y axis and severity categories at T1 are shown on the x axis (HC = healthy controls; 1 = severity grade 1; 2 = severity grade 2; 3 = severity grade 3).

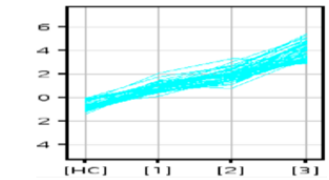
Cluster 6 contained 54 transcripts that associate with metabolic, cell cycle and inflammatory pathways in IPA. Intense over-abundance can be seen in the severity 3 group. Identified genes include defensins and others that are related to neutrophil activity and response to bacterial infection. Additionally, associated GO terms include 'response to bacterium' and 'defense response to bacterium' (figure 4.14).

Cluster 7 contained 183 transcripts that were associated with signalling, inflammation and cell cycle pathways in IPA. IL-10 signalling and TLR signalling were the two top canonical pathways. Additionally, 'role of PKR in interferon induction and antiviral response' was identified. Associated GO Terms included those relating to cell cycle, immune response and bacterial defense response (figure 4.15).

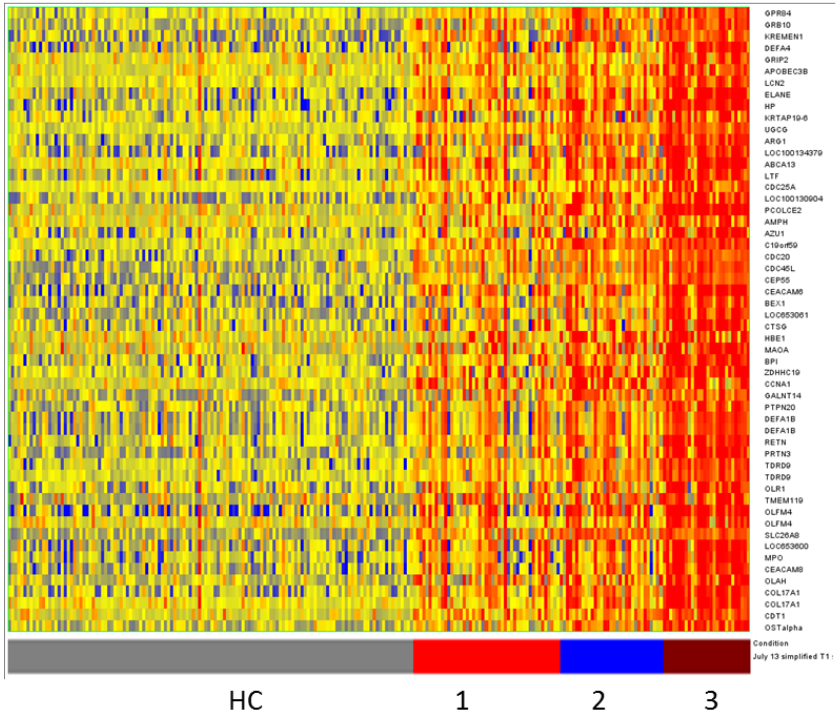
Cluster 9 contained just 3 transcripts, but their abundance increased markedly as severity also increased. Two transcripts were for the gene CD177, the product of which is neutrophil-associated and is over-abundant in a number of inflammatory conditions including bacterial infection. The third transcript was matrix metalloproteinase-8 (MMP8) (figure 4.16).

By contrast, cluster 5 contained 53 transcripts that were markedly over-abundant in patients with severity grades 1 or 2, but that were less over-abundant in patients with severity grade 3. Numerous interferon-associated genes were identified e.g. IFIT3, IFIT1, MX1, and IFITM3. An exception was IFI27, which remained markedly over-abundant in all three groups. IPA analysis identified 'role of pattern recognition receptors in recognition of bacteria and viruses', 'interferon signalling' and 'complement system' as the top three canonical pathways. Associated GO Terms included 'immune response ', 'immune system processes and 'response to virus' (figure 4.17).

Finally, cluster 8 contains 179 transcripts that are relatively under-abundant in influenza-positive patients and that become relatively less abundant as severity of illness increases. The transcripts were associated with T- and B-cell pathways in IPA, and the top canonical pathway was 'natural killer cell signalling'. Associated GO Terms were mainly related to cell signalling (figure 4.18).



GO Term
serine hydrolase activity
serine-type peptidase activity
defense response to bacterium
serine-type endopeptidase activity
DNA replication checkpoint
response to bacterium



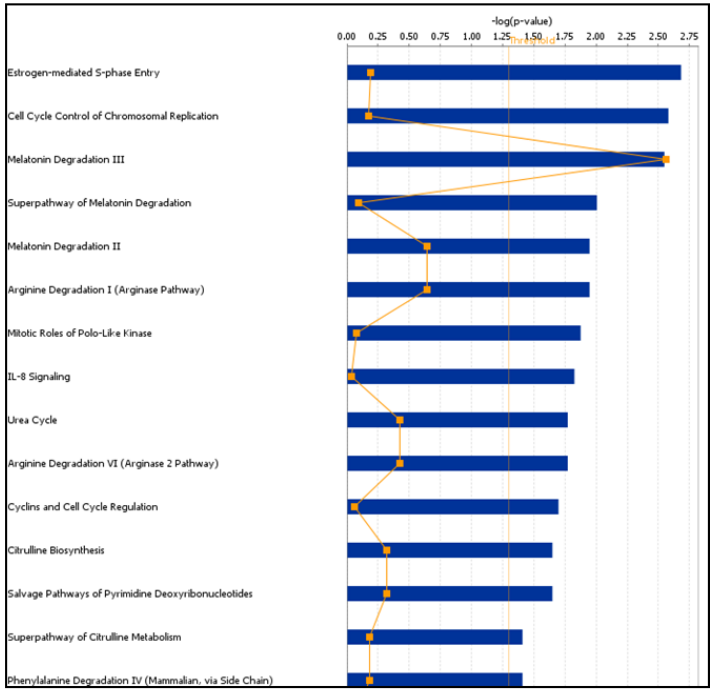
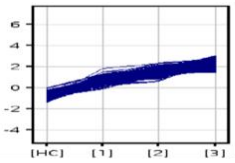
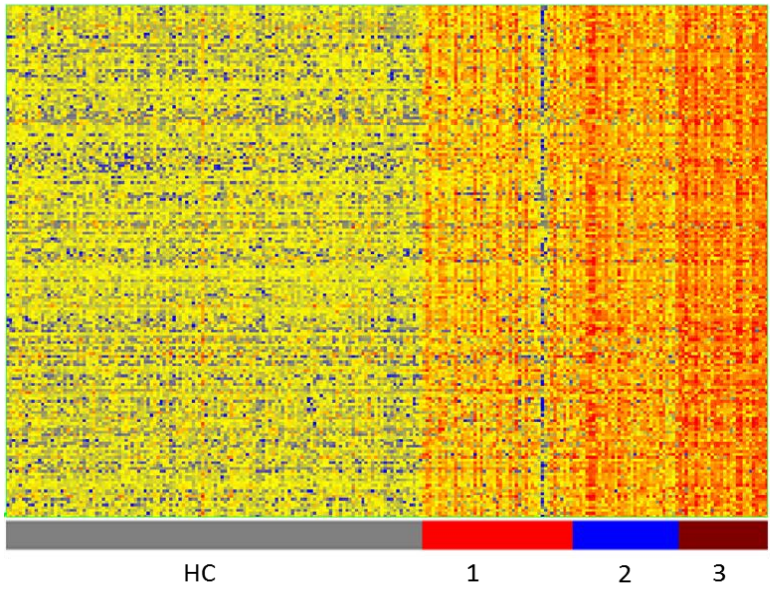


Figure 4.14 K-means clustering-identified signalling and bacterial response-associated transcripts that are most abundant in influenza-positive adults from 2010/11 with severity 3 illness (cluster 6 of 10, 54 transcripts)

Changes in the expression of transcripts identified by K-means clustering are represented as an expression profile graph (top left) and heat-map (bottom left). Entity names are displayed adjacent to the heat-map. HC = healthy controls; 1 = severity grade 1; 2 = severity grade 2; 3 = severity grade 3. GO Terms and top IPA Cannonical pathways associated with identified transcripts are also shown (right-hand panels).



GO Term
mitosis
nuclear division
organelle fission
immune response
defense response
mitotic cell cycle
cell cycle process
mitotic sister chromatid segregation
sister chromatid segregation
cell division
chromosome segregation
response to stimulus
response to stress
immune system process
regulation of nuclear division
regulation of mitosis
microtubule cytoskeleton
immunoglobulin binding
defense response to bacterium
cell cycle



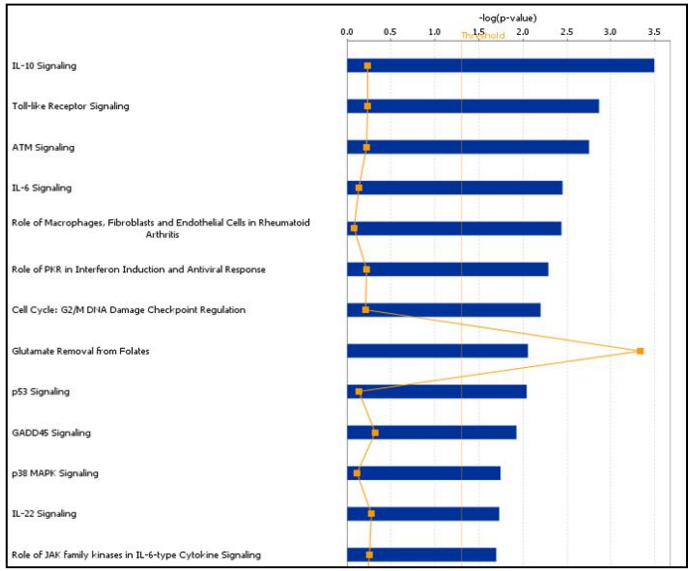


Figure 4.15 K-means clustering-identified pro- and anti-inflammatory signalling transcripts that are most abundant in influenza-positive adults from 2010/11 with severity 3 illness (cluster 7 of 10, 183 transcripts)

Changes in the expression of transcripts identified by K-means clustering are represented as an expression profile graph (top left) and heat-map (bottom left). Entity names are displayed adjacent to the heat-map. HC = healthy controls; 1 = severity grade 1; 2 = severity grade 2; 3 = severity grade 3. GO Terms and top IPA Cannonical pathways associated with identified transcripts are also shown (right-hand panels).

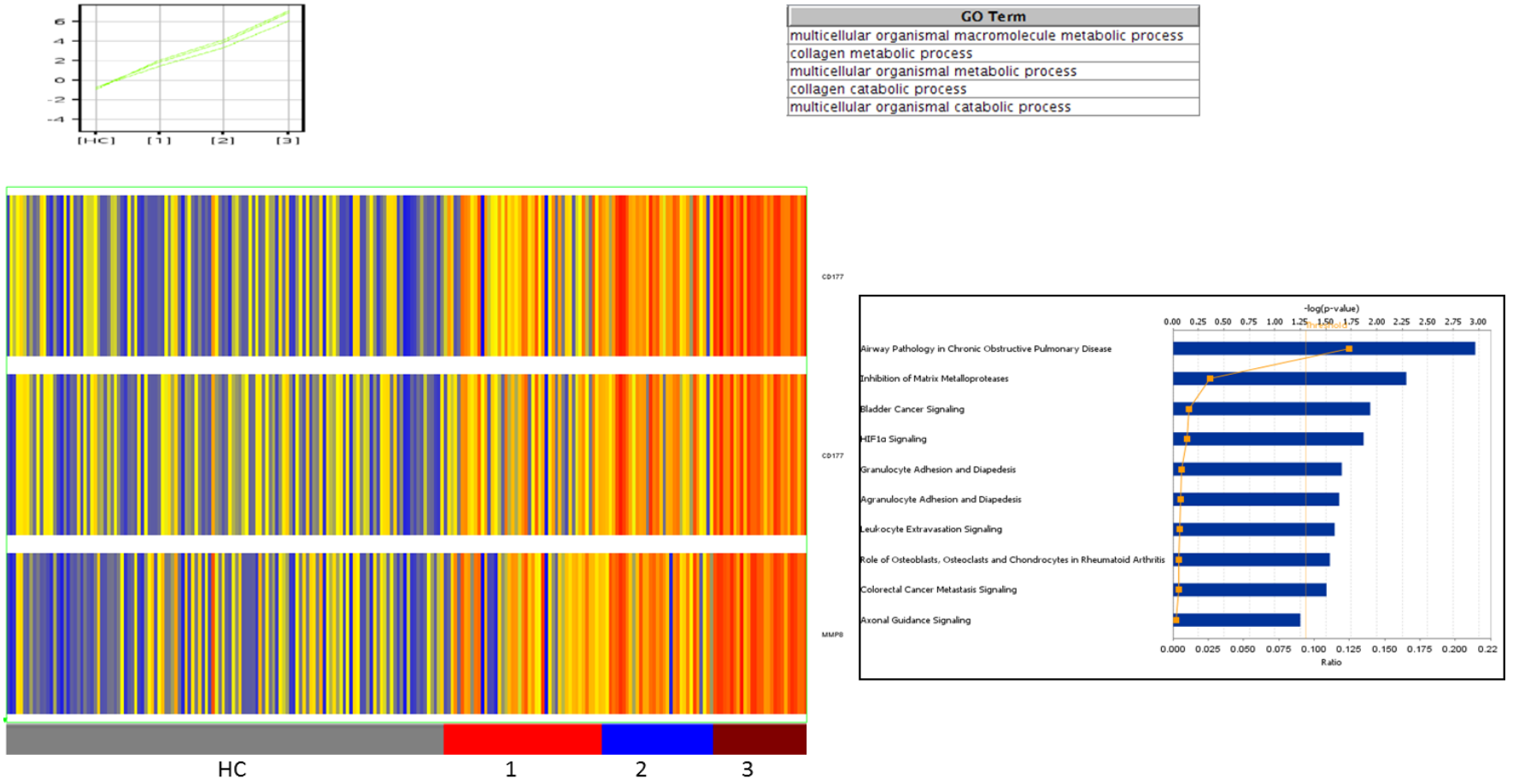
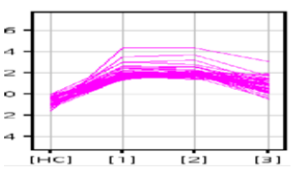
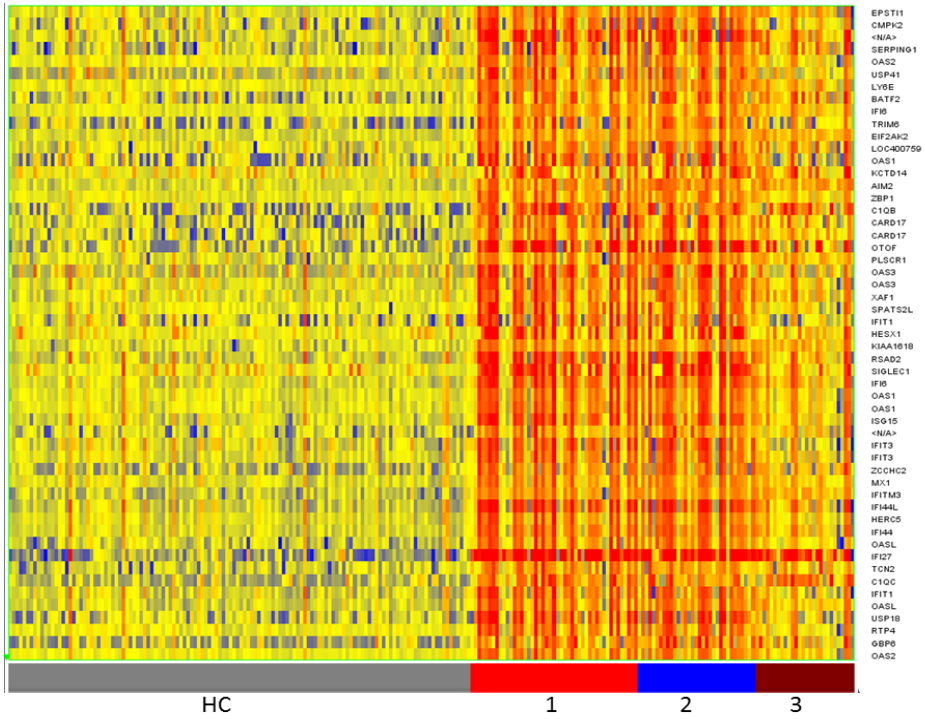


Figure 4.16 K-means clustering identified an increase in abundance of CD177 and MMP8 transcripts as severity increases, in influenza-positive adults from 2010/11 (cluster 9 of 10, 3 transcripts)

Changes in the expression of transcripts identified by K-means clustering are represented as an expression profile graph (top left) and heat-map (bottom left). Three transcripts for two genes (CD177 and MMP8) are displayed adjacent to the heat-map. HC = healthy controls; 1 = severity grade 1; 2 = severity grade 2; 3 = severity grade 3. GO Terms and top IPA Cannonical pathways associated with identified transcripts are also shown (right-hand panels).



GO Term
immune response
immune system process
response to virus
response to biotic stimulus
response to other organism



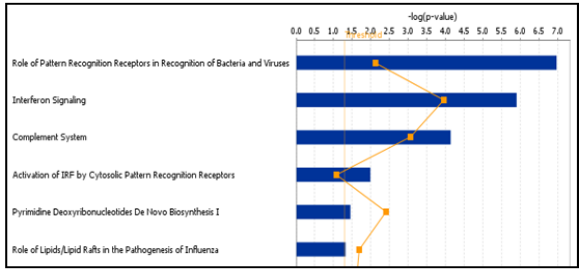


Figure 4.17 K-means clustering identified decreased abundance of interferon-associated transcripts in severity 3 illness, in influenza-positive adults from 2010/11 (cluster 5 of 10, 53 transcripts)

Changes in the expression of transcripts identified by K-means clustering are represented as an expression profile graph (top left) and heat-map (bottom left). Transcript names are displayed adjacent to the heat-map. HC = healthy controls; 1 = severity grade 1; 2 = severity grade 2; 3 = severity grade 3. GO Terms and top IPA Cannonical pathways associated with identified transcripts are also shown (right-hand panels).

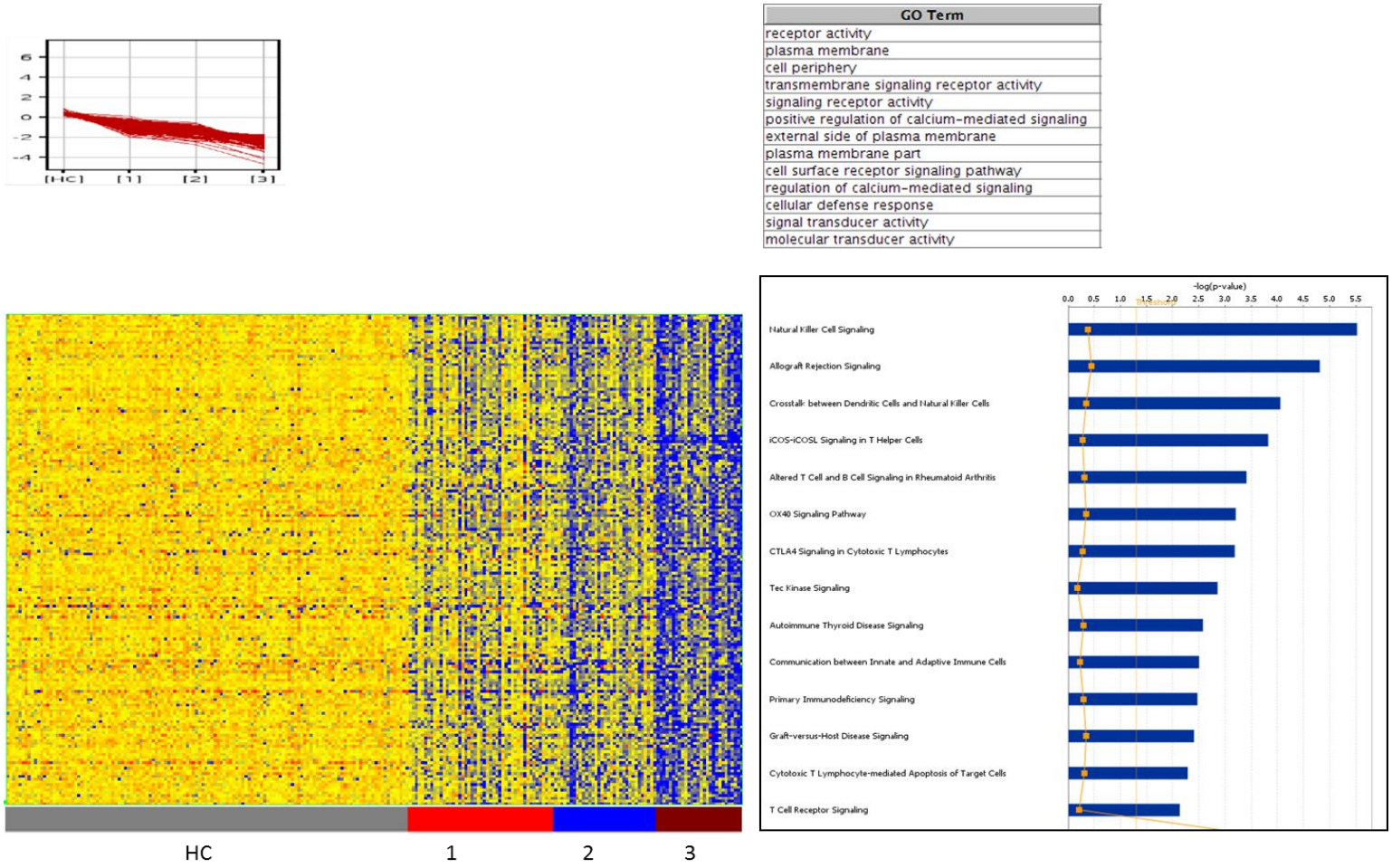


Figure 4.18 K-means clustering identified transcripts associated with NK cell, T- and B-cell pathways which become progressively less abundant as severity increases in influenza-positive adults from 2010/11 (cluster 8 of 10, 179 transcripts)

Changes in the expression of transcripts identified by K-means clustering are represented as an expression profile graph (top left) and heat-map (bottom left). Entity names are displayed adjacent to the heat-map. HC = healthy controls; 1 = severity grade 1; 2 = severity grade 2; 3 = severity grade 3. GO Terms and top IPA Cannonical pathways associated with identified transcripts are also shown (right-hand panels).

4.5 Modular analysis of the transcriptional response

4.5.1 Modular analysis methodology

The raw expression levels of all transcripts significantly detected from background hybridisation were compared between each patient sample from the group of interest and of the healthy control samples. These data were analysed by the Baylor Institute for Immunology Research (BIIR) modular analysis tool (384) and the proportion of significantly expressed genes in each module, expressed as a percentage, were represented by colour intensity, with statistical filtering applied (student t-test, $p < 0.05$). Each module corresponds to a set of co-regulated genes that were assigned biological functions by unbiased literature profiling (385). For each module, red indicated over-abundance and blue indicated under-abundance. Due to the smaller number of influenza-positive patients in 2009/10, this analysis was only performed for the 2010/11 cohort.

4.5.2 Comparison of influenza-positive and influenza-negative ILI adults at T1, 2010/11 cohort

Modular analysis of all adult samples obtained at T1 revealed similar module activity between influenza-positive and influenza-negative adults. Marked over-expression of the three interferon modules (M1.2, M3.4 and M5.12) was prominent in the majority of influenza-positive adults; only 8/109 (7.3%) of the influenza-positive patients demonstrated under-expression in these modules, relative to healthy controls. Overall, relative interferon over-expression was less marked in the influenza-negative ILI patients, compared to the influenza-positive patients, although some individuals did appear to have marked over-expression in all three individual modules (figure 4.19).

Intense over-expression of the neutrophil module (M5.15) was also seen in some, but not all patients at T1, irrespective of their influenza-infection status. Inflammation modules M3.2 and M4.2 were generally over-expressed, regardless of influenza infection status. Whereas cell cycle M3.3 genes were over-expressed in many patients from both groups, cell cycle M3.5 genes tended to be under-expressed relative to healthy controls. B- and T-cell associated genes (M4.10 and M4.1 modules, respectively) tended to be under-expressed in both groups.

Overall, marked differences in modular expression between these non-stratified influenza-positive and influenza-negative ILI patients were not apparent. Marked over-expression of interferon- and neutrophil-associated genes was seen in a large number of patients from both groups. Intense over-expression of genes in one of these two pathways was often associated with less intense expression of genes in the other pathway, although a small proportion of patients demonstrated intense expression of both interferon- and neutrophil-associated genes (figure 4.19).

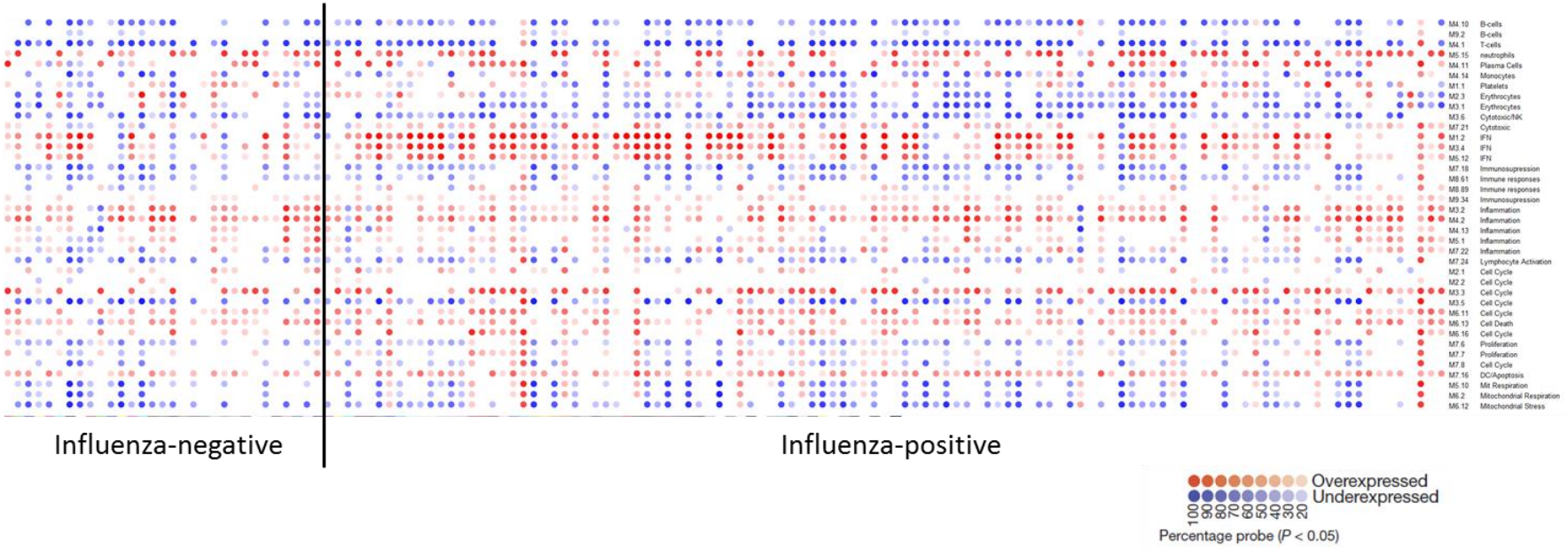


Figure 4.19 Modular analysis of transcriptional response in influenza-negative and influenza-positive patients in the 2010/11 cohort
Gene expression levels of all transcripts that were significantly detected compared to background hybridisation in at least 10% of samples (p <0.01) were compared between healthy controls and each of the following patient groups: influenza-negative adults and influenza-positive adults. Each column is an individual patient T1 sample. Red indicates over-abundance of transcripts in a particular module, whereas blue indicated under-expression relative to healthy controls (p <0.05). The colour intensity correlates with the percentage of genes in that module that are significantly differentially expressed.

4.5.3 Correlation of modular response with severity of illness at T1, 2010/11 influenza-positive cohort

Modular analysis was performed using the same methodology described above, although for this analysis, only influenza-positive adults and healthy controls were included. Intensity of gene expression relative to healthy controls was then displayed in the modular map, with patients arranged into groups according to their severity of illness at T1 (figure 4.20).

The genes associated with the interferon modules (M1.2, M3.4 and M5.12) were over-expressed in the majority of influenza-positive patients, but the intensity of over-expression was much less marked in those with severity 3 illness, compared to those with severity 1 or severity 2 illness. By contrast, almost all patients with severity 3 illness had marked over-expression of genes in the neutrophil module, whereas a greater proportion of those with less severe illness (especially those with severity 1 illness) did not demonstrate such over-expression of neutrophil response-associated genes. This is keeping with the results of hierarchical and K-means clustering, suggesting that those with less severe illness (who also tended to be at an earlier stage of illness when recruited) had an interferon pathway-dominated transcriptional response, whereas those with the most severe illness (who tended to be later in the course of illness) showed a neutrophil pathway-dominated transcriptional response.

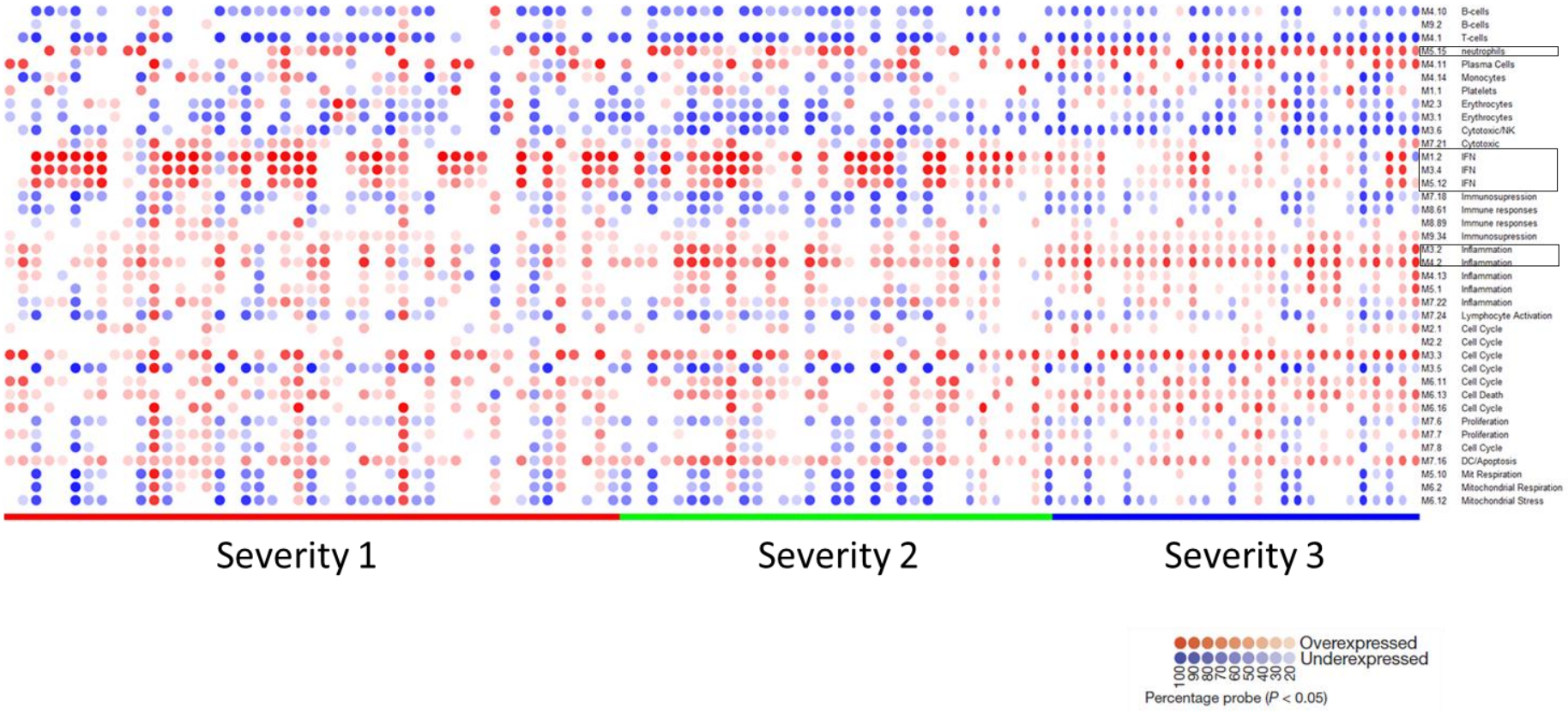


Figure 4.20 Modular analysis of transcriptional response in influenza-positive patients from the 2010/11 cohort

Gene expression levels of all transcripts that were significantly detected compared to background hybridisation in at least 10% of samples (p <0.01) were compared between healthy controls and influenza-positive adults. Each column is an individual patient T1 sample. Red indicates over-abundance of transcripts in a particular module, whereas blue indicated under-expression relative to healthy controls (p<0.05). The colour intensity correlates with the percentage of genes in that module that are significantly differentially expressed.

4.6 Discussion

4.6.1 Summary

We have shown that in the context of a novel influenza outbreak, whole blood microarray analysis of RNA transcripts from a large number of patients is achievable. We initially analysed transcriptional responses in adult patients with confirmed influenza and those who presented with influenza-like illness but did not have evidence of active influenza infection. Hierarchical clustering of differentially expressed transcript lists was performed, with Ingenuity Pathway Analysis (IPA) and Gene Ontology (GO) Terms analysis to assist with biological interpretation of the changes in expression that were seen. We then focussed on adults with confirmed influenza, again using hierarchical clustering and pathway interpretations, but also analysing the data in greater depth through additional K-means clustering analysis and Baylor Institute of Immunity (BIIR) modular analysis.

Analysis of transcriptomic profiles obtained from adults at a single, acute time point revealed that patients who had been admitted to hospital with suspected influenza had markedly different gene expression from asymptomatic, matched healthy controls, regardless of influenza-infection status. This was an expected finding, but confirms that whole blood transcriptional profiling can differentiate acute illness from a normal state of health, even when studying diseases that primarily affect the respiratory tract. Influenza-positive and influenza-negative adults (with status determined by detection of influenza virus by PCR) did not form two distinct groups in hierarchical clustering when all patients were analysed together. Possible explanations include similarities in expression, irrespective of the pathogen causing influenza-like illness (ILI), or an inability to see differences due to the comparison of a specific disease (influenza) with a syndrome that may be caused by many different pathogens. Furthermore, the possibility of preceding influenza could not be confidently excluded in the influenza-negative patients. Additional, detailed characterisation of diagnoses in influenza-negative patients may identify more appropriate sub-groups of patients for comparison with influenza patients.

Semi-supervised analysis of transcriptomic profiles from influenza-positive adults, comparing them with transcriptomic profiles of matched healthy controls, has revealed two major groups of patients:

1. Those with a marked over-abundance of transcripts associated with interferon-related pathways but less abundance of transcripts associated with inflammatory and bacterial-response pathways

2. Those with a marked over-abundance of transcripts associated with inflammatory and bacterial-response pathways but less abundance of interferon-related pathways

Application of clinical characteristics, through semi-supervised hierarchical clustering supported by secondary, supervised K-means clustering and BIIR modular analysis, revealed that influenza patients who had less severe illness and who were earlier in the course of illness tended to have the interferon-dominated signature. By contrast, those who had more severe illness and were generally later in the course of illness tended to have the neutrophil/bacterial response-dominated signature. In terms of relation to day of illness alone, the signature change appeared to occur at around day five of illness.

The majority of patients with the most severe illness also tended to be at a later stage of illness at the time of sampling. This reflects the reported natural history of A(H1N1)pdm09 virus infection i.e. adult patients with the most severe illness tend to present to hospital later and are admitted to critical care units at or shortly after admission. It also means that the relative contribution of duration of illness and severity of illness could not be separated.

It is possible that neutrophil-associated transcription may be up-regulated in late and severe illness without the presence of bacterial co-infection, especially in those with established ARDS (429). However, our results suggest that invasive infection with *S. pneumoniae*, a well-recognised co-pathogen in influenza patients, was more common in the late/severe group, compared to those with earlier and less severe illness. Furthermore, hierarchical clustering correlation with a surrogate marker for invasive bacterial infection, serum procalcitonin, suggested that an absence of bacterial sepsis or bacterial pneumonia was much more likely in those with early and mild illness. Taken together, these results suggest that severe illness in those with the neutrophil/bacterial response signature may be explained, at least in part, by bacterial co-infection, even if bacteria were not identified by culture-based or molecular methods in these patients. It is possible that influenza infection causes a robust antiviral response which subsequently renders individuals more sensitive to secondary infections by pathogenic bacteria (342). These findings require further exploration and if confirmed, this observation supports the use of empirical antibiotics in addition to antivirals, in hospitalised patients with severe influenza who require higher levels of care.

4.6.2 Comparison of influenza-positive, influenza-negative patients and healthy controls

4.6.2.1 Approach to analysis

All patients recruited to MOSAIC had clinician-suspected influenza infection that resulted in a diagnostic PCR test for influenza being performed. At the time of recruitment, a clinical diagnostic algorithm was in use to assist clinicians in identifying patients who may have influenza on the basis of the presenting symptoms. Although patients with certain underlying conditions may have had

atypical presentations e.g. those with immunodeficiency, it can be assumed that the majority of patients will initially have had the same clinical syndrome i.e. influenza-like illness. Therefore, it was reasonable to ask initially whether underlying biological mechanisms, particularly those relating to the immune response, may be similar, irrespective of the underlying cause e.g. ILI caused by influenza versus ILI caused by infection with another respiratory virus, and analyse all T1 samples together. Alternatively, it may be the case that influenza virus infection is associated with distinct immunological responses, which may be detectable at a transcriptional level.

Whole blood transcriptomics has proven utility in investigating disease-specific transcriptomic changes. Therefore, for each period of recruitment, the initial approach was to compare and contrast T1 transcriptional profiles from patients with PCR-confirmed influenza with those from patients who had influenza PCR-negative ILI, and with the transcriptional profiles from matched healthy controls. For each cohort, because the individual condition groups were of similar size to each other, per-gene normalisation was applied by dividing each messenger RNA transcript by the median intensity of all the samples. Applying the same, relatively strict expression and statistical filters to each cohort produced entity (transcript) lists that differed in size, with the 2010/11 cohort list containing considerably more transcripts than the list for the 2009/10 cohort. This may be explained by the larger number of patients recruited during 2010/11, a cohort that exhibits a greater diversity of illness as well as greater diversity of other factors, such as underlying comorbidities. Furthermore, the 2010/11 cohort was dominated by influenza-positive patients, whereas the 2009/10 cohort was dominated by influenza-negative patients.

4.6.2.2 Influenza-positive and influenza-negative adult patients have different transcriptional responses compared to healthy controls

For both seasons, it was clear that the vast majority of patients with ILI of any cause had transcriptional signatures that clustered separately from the healthy controls. Of those patients who clustered within the group of healthy controls, their outlier status could not be fully explained by comorbidities, day of illness or demographics, since other patients with similar characteristics were not outliers. Of note, all but one of the outliers was beyond 5 days of illness at the time of sampling and some were sampled between day 14 and day 28 of illness, so perhaps these later patients – if they were already recovering – may be returning to a transcriptional profile that is associated with a normal state of health. Alternatively, they may have had an acute viral infection that had resolved. Other explanations, perhaps relating to host or external factors, may also explain these outliers.

4.6.2.3 Transcriptional profiling does not differentiate influenza-positive from influenza-negative patients

In hierarchical clustering analysis, patients did not group together according to influenza infection status. However, sub-clusters of patients did group together according to related transcriptional profiles, with relative over- or under-abundance of transcripts that are associated with specific biological pathways. This transcriptional-driven sub-clustering was evident in both seasons, but appears to be clearer for the 2010/11 cohort, probably because a greater number of patients were recruited in 2010/11.

4.6.2.4 Common transcriptional responses can be seen in influenza-positive and influenza negative patients

Using IPA canonical pathway analysis and GO Terms analysis, pathway categories were identified that were common to sub-groups in both cohorts, although some of the constituent pathways differed between the 2009/10 and 2010/11 cohorts. Of the IPA associations identified, three were of significant interest: a relative under-abundance of T- and B-cell pathways; a relative over-abundance of interferon and immune pathways; a relative over-abundance of signalling/inflammation pathways. GO Term analysis generally supports these broad categories although additionally, bacterial response/defense-associated terms were identified for the transcriptional clusters associated with signalling/inflammation pathways in IPA.

Further major transcriptional clusters were identified, although their pathways categories are more difficult to interpret, with a mixture of signalling, cell-cycle and disease-specific pathways identified. This may be explained by the clinical heterogeneity of the patients recruited in each, or perhaps different stages of illness and therefore different stages of response and recovery to infection. A greater number of robust transcriptional clusters were identified in the 2010/11 cohort, which may be due to a greater number of patients overall and greater clinical heterogeneity than the 2009/10 cohort.

Many of the recruited patients recruited in both seasons had influenza virus infection confirmed by PCR at the time of diagnosis, or had common respiratory viruses known to cause ILI detected by PCR analysis of their T1 NPA samples. Since most patients were recruited soon after ILI was suspected or diagnosed, it is reasonable to assume that the non-influenza viruses detected at T1 may be the cause of ILI in non-influenza patients who had positive multiplex PCR results. On this basis, since common respiratory virus infections are known to elicit an early, innate immune response involving interferon-associated pathways, we initially questioned whether those patients with any known

virus infection – influenza or otherwise - were more likely to cluster together and associate with the 'interferon and immune pathways signature'.

4.6.2.5 Interferon-associated transcriptional response was variable in influenza-negative patients with other respiratory virus infections

In the 2009/10 cohort, the heat-map suggested that some of the patients infected with non-influenza viruses do appear to have over-abundance of the interferon and immune pathways-associated transcripts, but there were also patients who did not, with some even demonstrating a relative under-abundance. This was not explained by the identification of the infecting virus. The same finding was seen with the 2010/11 cohort, although overall there were fewer influenza-negative patients recruited during this season, of which only a small number had non-influenza viruses detected at T1.

4.6.2.6 Severity of illness and duration of illness are associated with differences in transcriptional responses in combined analysis of influenza and non-influenza patients

Because it is not known whether the non-influenza viruses detected at T1 were the cause of the presenting illness and since detectable viral RNA may not necessarily occur at the same time as transcriptional changes, and because not all patients underwent multiplex PCR testing for viruses, other factors had to be explored. Following the same assumption that transcription of interferon-related genes is more likely to occur earlier in the course of influenza/influenza-like illness, the next question was whether earlier day of illness was more likely to be associated with this signature.

The time periods for early illness (0-4 days) and for later illness (5-20 days) were chosen based on what is known about the natural history of influenza infection from published experimental data and also median day of illness and interquartile range of day of illness at admission to hospital for patients who were recruited to the Flu-CIN study (171). For the 2009/10 cohort, an obvious pattern was not seen, although heat-map analysis suggested that the influenza-positive patients in the sub-cluster with the most marked over-abundance of the interferon and immune pathway-associated transcripts tended to be at an earlier stage of illness. By contrast, those with the most marked over-abundance of signalling/inflammation (bacterial response) pathway-associated transcripts tended to have less marked over-abundance of interferon and immune pathway-associated transcripts and tended to be at a later stage of illness. No obvious association could be seen between a marked under-abundance of T- and B-cell associated pathways and early illness. Such under-abundance of T- and B-cell transcripts has been described in studies of other respiratory tract infections (378, 430).

Severity of illness at T1 was over-laid on the same 2009/10 cohort heat-map. Although there was a sub-group of patients with similar transcriptional profiles who were all grade 1 severity, patients of different severity grades were distributed throughout. Furthermore, only a small proportion of patients had the highest severity grade in this cohort, limiting the ability to look for transcriptional associations with severity of illness.

In the larger, influenza-dominated 2010/11 cohort, there was a clearer association with both the day of illness and severity of illness. The group where over-abundance of the interferon and immune pathway-associated transcripts dominates the signatures contained patients where the majority were at an earlier stage of illness and were severity grade 1 or 2. By contrast, most patients in the group with signatures dominated by over-abundance of signalling-inflammation pathway-associated transcripts and under-abundance of T- and B-cell pathway-associated transcripts were at a later stage of illness, with a severity grade 2 or 3. In fact, all but one of the patients who had grade 3 severity of illness fell within this group. This suggests that patients with the most severe illness and who were later in the course of disease may have been experiencing a common process, perhaps driven by a host response to bacterial infection that manifests as marked over-abundance of signalling/inflammation pathway-transcripts. Although IPA canonical analysis did not specifically identify pathway categories for bacterial response, it did identify component pathways that are of importance in the host-response to bacterial infection e.g. IL-6 and IL-8 signalling. Furthermore, GO Terms analysis identified pathways associated with bacterial response for these transcripts.

4.6.2.7 Low serum procalcitonin is associated with less marked abundance of bacterial response-associated transcripts in combined analysis of influenza-negative and influenza-positive patients

To investigate further whether the transcriptional responses seen in severe, late disease were associated with bacterial infection in influenza-positive and influenza-negative patients, a number of bacterial-associated variables were applied to the heat-maps from each recruitment period. Procalcitonin (PCT) is a blood biomarker has been shown in published studies to have a good negative predictive value for bacterial sepsis (defined as systemic inflammatory response syndrome due to bacterial infection) when the serum/plasma level is <0.5 ng/mL (104, 399, 400). When the T1 PCT results were applied to the hierarchical clustering results from the 2009/10 cohort, distinct patterns were not seen, although there was a suggestion that some of the patients who had marked over-abundance of interferon and immune pathways-associated transcripts but a less marked over-abundance of the signalling/inflammation pathway-associated transcripts also had low PCT. A clearer pattern was visible in the larger 2010/11 cohort, with low PCT levels being infrequent in those patients who patients who fell within the group with marked over-abundance of the signalling-

inflammation pathways-associated transcripts. This suggests that bacterial sepsis cannot be confidently excluded in this group of patients. In contrast, low PCT was relatively common in the group with marked over-abundance of interferon and immune pathways-associated transcripts, implying that many of the patients with this transcriptional pattern did not have bacterial sepsis.

While serum/plasma PCT has been shown to have a good negative predictive value, it performs less well in identifying those who have bacterial sepsis (400). Therefore, other evidence of bacterial infection needs to be applied to investigate the relationship. However, it is interesting to note that in the 2010/11, most of the patients with PCT >2.0 ng/mL appear to aggregate in the group with marked over-abundance of signalling/inflammation pathways-associated transcripts and less marked interferon and immune pathways-associated transcripts. In studies of patients without influenza, PCT serum/plasma >2.0 ng/mL has been shown to be associated with bacterial sepsis, including those with critical illness. However, PCT may also be elevated in response to prolonged or severe cardiogenic shock and prolonged severe organ perfusion anomalies (399).

4.6.2.8 Bacterial detections and their relationship to transcriptomic responses in influenza-negative and influenza-positive adult patients

The most complete set of bacteriology results available for analysis in this work was the T1 NPA bacterial PCR data-set. Application of these data to hierarchical clustering for each cohort revealed that detections of bacteria that commonly cause respiratory tract infections e.g. *S. pneumoniae, H. influenzae and S. aureus*, were frequently detected in the nasopharyngeal secretions of influenza-positive and influenza-negative patients. In some patients, more than one species was detected in an individual sample using this sensitive technique. Bacterial detections were seen throughout the cohorts, with no discrete clustering pattern visible. Furthermore, bacterial detections in NPA secretions do not necessarily imply a causative pathological role for these bacteria; some of the detections may simply reflect harmless carriage of bacteria. Therefore, association with confirmed *invasive* bacterial infections may be more informative when interpreting our results.

Since there were limited numbers of patients with positive blood cultures or who had blood cultures taken around the time of T1, the application of these data to hierarchical clustering was not particularly informative. However, since *S. pneumoniae* is the most commonly identified co-infecting bacterial species in patients with A(H1N1)pdm09 virus infection in published series and since *S. pneumoniae* may itself present as ILI in the absence of viral infection, it was logical to look for associations with known *S. pneumoniae* infection, both invasive (positive blood culture or urine antigen) and non-invasive (detection in upper respiratory tract secretions). Although a distinct pattern was not seen in the 2009/10 cohort, some but not all of the patients with *S. pneumoniae*

infections appear to have marked over-abundance of the signalling/inflammation pathway-associated transcripts. In the 2010/11 cohort, the majority of *S. pneumoniae* patients were within the group with marked over-abundance of signalling/inflammation pathways-associated transcripts (identified as bacterial response transcripts in GO Terms analysis).

4.6.2.9 Combined analysis of influenza-negative and influenza-positive adult patients: summary

Hierarchical clustering of transcriptomic profiles from all adults at T1 did not separate influenza-positive and influenza-negative patients into distinct groups. This may be expected, since there are multiple factors other than the underlying infectious aetiology that could explain the major transcriptional sub-clusters that were seen. We have demonstrated that, irrespective of influenza infection status, there are two major groups of signatures that are of particular interest, as revealed by pathway analysis of differentially expressed genes: one suggesting an interferon/viral-response-dominated, early transcriptional response to infection, that appears to be more common in those with early and milder illness, and another that is associated with a signalling/inflammation response, possibly directed against bacteria, which is more common in those with later and more severe illness. These emerging patterns appear to be more obvious in the larger, influenza-dominated 2010/11 cohort.

The influenza-negative group represents a relatively heterogeneous collection of ILI cases with underlying infections that may have been bacterial or viral (or mixed, or even non-infectious) in origin. This makes direct comparison with influenza-positive cases more difficult. Therefore, we undertook an in-depth study of differentially expressed genes in influenza-positive cases and healthy controls, excluding the influenza-negative cases.

4.6.3 Comparison of influenza-positive patients and healthy controls

4.6.3.1 Influenza-positive patients have different transcriptional responses to healthy controls

Semi-supervised hierarchical clustering of differentially expressed transcripts revealed that the majority of influenza-positive patients in each season clustered separately from the healthy controls. The influenza sub-types detected in 2010/11 patients did not influence clustering, suggesting that different subtypes of influenza may trigger similar host responses at the transcriptional level. It should be noted, however, that only a relatively small number of H3N2 and influenza B viruses were detected in MOSAIC (and most of these were in the 2010/11 cohort).

4.6.3.2 Two major groups of influenza patients are identified by transcriptional profiling

Similar to analysis of all adult patients (influenza-positive and influenza-negative) at T1, major transcriptional clusters dominated the heat-maps for each season, broadly dividing the patients into two groups. The first group was identified by marked over-abundance of transcripts that are related to interferon and immune pathways. The second group did not have such marked over-abundance of these transcripts, but instead demonstrated marked over-abundance of transcripts associated with inflammation/signalling and cell-cycle pathways in IPA. Additionally, GO Terms analysis suggested that these transcripts have a role in the response to bacterial infection.

4.6.3.3 Severity of illness and duration of illness is associated with changes in transcriptional response in influenza-positive patients

For selected clinical variables, the clustering trends seen for all patients at T1 were also apparent for influenza-positive patients alone at T1. Focussing on influenza-positive patients and healthy controls without influenza-negative patients provided greater clarity, however, revealing stronger clustering patterns, especially for the larger 2010/11 cohort. The most striking patterns seen were those relating to severity of illness and day of illness at the time of sampling. Patients with signatures that were dominated by over-abundance of transcripts associating with interferon and immune pathway tended to be at an early stage of illness, typically with less severe illness. In contrast, those who lacked this marked over-abundance and instead had marked overabundance of transcripts associated with inflammation/signalling and cell-cycle pathways, generally had illness that was of longer duration and that was more severe, particularly those with critical illness.

4.6.3.4 Interferon-associated responses dominate transcriptional profiles in those with early and less severe illness

In both seasons, influenza-positive patients demonstrated abundance of transcripts associated with 'interferon signalling' pathways and 'role of PRRs in recognition of bacteria and viruses' pathways, following canonical pathways analysis in IPA.

Four genes were common to the IFN-associated pathways in both seasons: *OAS1, IFIT3, IFIT1* and *Mx1*. The *IFI35* gene was also identified in the 2009/10 analysis. The 2'-5'-oligoadenylate synthetase-1 (OAS1) protein is type I IFN-inducible and activates RNase L, which inhibits virus replication by cleaving viral and cellular single-stranded RNAs (316). Production of OAS1 is seen in several respiratory virus infections and mice deficient in RNase L have been shown to have increased susceptibility to infection with influenza virus (317). *OAS1* along with *OAS2* and *OAS3* were also identified in the 'role of PRRs in recognition of bacteria and viruses' pathways in both seasons.

The interferon-induced proteins with tetratricopeptide repeats (IFIT) family consists of four proteins in humans (IFIT1, IFIT2, IFIT3 and IFIT5), which are involved in regulation of transcription, translation, antiproliferative effects and also induction of type I interferon (431). IFIT1 binds directly to 5'-triphosphorylated RNA (5' PPP-RNA), which is found in the cytosol during the life-cycle of negative-strand RNA viruses, including influenza, and binds to RIG-I. IFIT3 binds with RNA by forming a complex with the other IFIT proteins. Small-interfering RNA-induced knockdown of *IFIT1*, *IFIT2* and *IFIT3* in HeLa cells has been shown to be associated with increased replication of influenza virus *in vitro* (432). *IFIT1* is known to be one of the most commonly induced genes following IFNαβ signalling and production of its protein appears to have a central role in antiviral defense, including response to influenza virus infection (432).

The orthomyxovirus resistance gene *Mx1* (human analogue *MxA*) is an ISG that encodes proteins that belong to a family of GTPases that directly target nucleocapsid-like structures of influenza viruses, interfering with virus replication. When expressed transgenically in IFNαβ-receptor-deficient mice, human *MxA* has been shown to confer complete protection against otherwise fatal influenza infection (314).

For both seasons, *IRF7* was also identified in the 'role of PRRs in recognition of bacteria and viruses' pathways. This gene encode interferon regulatory factor 7 (*IRF7*), which is a member of the interferon regulatory transcription factor (IRF) family of proteins. *IRF7* is a transcriptional regulator of several virus-inducible genes, including type I interferon-associated genes. *IRF7* is induced by several respiratory viruses, including influenza virus and its own expression is enhanced via signalling triggered by the type I interferons it induces, resulting in amplification of antiviral response (433, 434).

The engagement of PRRs by influenza and other viruses initiates signalling that results in the secretion of interferons, especially type I interferons. Activation of a multitude of antivirals genes is subsequently triggered by interferons interacting with their respective receptors e.g. IFNαβR. Interferons are generally released early in the course of infection, as part of the innate immune response, promoting antiviral effects and inhibiting various stages of virus infection, in infected cells and also in neighbouring, uninfected cells (435). Interferons are not typically detected in blood and respiratory tract samples from hospitalised patients with respiratory tract infections. By contrast, assessment of interferon-related activity at the transcriptional level reveals that the effects of interferons – as measured by expression of interferon-stimulated gene transcripts – can be seen in hospitalised patients presenting several days after the onset of symptoms, as demonstrated in this study.

In addition to antiviral effects, interferons may also exert an immunomodulatory effect, influencing the production of pro-inflammatory mediators that may contribute to pathogenesis (128, 436, 437). Furthermore, it has been suggested that the immunomodulatory effects of type I interferons may influence subsequent, secondary infection, in addition to the initial response to viral infection.(108, 109, 438) Although not studied in detail in human influenza, it has been shown in murine models that type I IFN production following viral infection is associated with the suppression of innate responses to a number of bacterial infections, including *Listeria sp.* and *Mycobacterium tuberculosis* (307, 439, 440). It may be that some patients infected with influenza have an interferon response that effectively inhibits virus replication, but through immunomodulation, the same response may encourage secondary bacterial infection, especially if their respiratory tracts are already colonised by bacteria with pathogenic potential, such as *S. pneumoniae*. However, to address this further in human would require longitudinal studies of host immune responses from the point of infection through to symptomatic illness and the development of complications in each individual. Because those with severe illness and bacterial complications tended to be at this stage of illness on admission to hospital, it was not possible to investigate such evolution of illness in this study.

4.6.3.5 The dominant transcriptional response changes from antiviral-associated to bacterial-associated after 5 days of illness, but a transitional signature is not present

In a study of gene expression patterns in blood leukocytes from children with acute infections classifier genes were identified to discriminate influenza A from bacterial infections (*S. pneumoniae, S. aureus* and *E. coli*) (380). Interestingly, approximately one third of the patients with bacterial infection demonstrated elevated levels of IFN-related transcripts, albeit at lower levels than in those with influenza. Although this didn't impact on disease classification outcomes in this study, the authors were unable to explain the interferon-associated response, suggesting that it could be part of the response to the bacterial infection itself, or perhaps represent a preceding, undiagnosed viral infection (380). The same authors subsequently proposed that the patients with a mixed signature consisting of over-abundant interferon-related and neutrophil-related transcripts may have concurrent viral and bacterial infections, although they were unable to demonstrate this through microbiological and virological investigations (441, 442). In the analysis of MOSAIC T1 results, a similar 'overlap' or 'transitional' signature was not seen, at least when all influenza-positive adults from either season were analysed without further stratification based on clinical variables. Rather, the major signature pattern appears to switch from interferon-associated to bacterial-response/neutrophil-associated at around day 5 of illness, suggesting that the pathways of genes may negatively regulate the reciprocal transcripts.

4.6.3.6 Low serum procalcitonin levels suggest that significant bacterial infection is absent in influenza patients with an interferon-dominated transcriptional response

To test the hypothesis that bacterial co-infection or secondary infection is associated with more severe and later-stage illness, association of transcriptional signatures with bacterial infection was investigated using clustering analysis. While common respiratory tract bacteria could be detected by PCR in T1 NPA samples from patients within both of these transcriptionally-defined groups, it is noteworthy that for the 2010/11 patients, most of the patients with an over-abundance of interferon and immune pathways-associated transcripts also had low PCT values, indicating that invasive bacterial infection was unlikely to be present. This contrasts with the group with over-abundance of transcripts associating with inflammation/signalling and cell-cycle pathways (bacterial response in GO Terms analysis), where PCT values were generally above the 0.5 ng/mL threshold for excluding bacterial sepsis.

4.6.3.7 Invasive pneumococcal infection was most common in influenza-positive patients with bacterial response-dominated transcriptional profiles

In terms of detection of the most commonly identified bacterial species capable of causing respiratory tract infections, the majority of *S. pneumoniae* infections in the 2010/11 cohort were in patients who had marked over-abundance of transcripts associating with inflammation/signalling and cell-cycle pathways (and associated with bacterial response in GO Terms analysis). Many of these infections were known to be invasive, based on culture of the organism in blood and/or detection of a positive urine antigen. Given that over-abundant transcripts in this group are associated with response to bacterial infection and that many of these patients did have positive detections of bacteria, it may be the case that we underestimate the incidence of bacterial infection in hospitalised patients with A(H1N1)pdm09 virus infection (especially critically ill patients), due to the limitations of bacteriological detection methods. Furthermore, patients who are at an earlier stage of illness may have less severe illness because they do not have concomitant or secondary invasive bacterial infection. Analysis of immune responses – or components of the immune response identified through transcriptional studies – may reveal the true burden of secondary bacterial infection in influenza patients.

Application of the bacteriological data has limitations, however. Appropriate samples for testing, either through research sampling or routine clinical sampling, were not available for all patients (refer to chapter 3 for additional detail). Standard microscopy and culture is limited in its ability to identify bacterial infections, especially in patients who may have commenced antibiotics. Although MOSAIC utilised highly-sensitive PCR detection of bacteria, it cannot distinguish between harmless

carriage of bacteria and invasive infection that may trigger a systemic immune response. Even when focussing on the species known to common cause secondary or concomitant infections in influenza patients, the samples required to demonstrate invasive infection may not have been obtained or results may have been influenced by prior antibiotic use. Although identified inflammation/signalling canonical pathways contain genes that are known to be involved in host response to bacteria e.g. defensins and metalloproteinases, these may not be unique to bacterial response; they may also be associated with innate response to viral infections including influenza (443, 444). However, it is of interest that activation of these pathways was generally observed when the interferon-associated pathways were extinguished, suggesting that the course of illness in some patients does not reflect the classical antiviral response to influenza (445).

4.6.3.8 Association of transcriptional changes with absolute neutrophil and lymphocyte counts

The transcriptional changes associated with signalling and inflammation (bacterial response), interferon and immune pathways (response to virus), and T- and B-cell pathways could represented altered cell composition of changes in gene expression in discrete cellular populations in whole blood. As an initial investigation for potential associations, we performed supervised hierarchical clustering of results from the 2010/11 influenza-positive cohort. Heat-map analysis suggested that patients with neutrophil counts above the normal range also appeared to have an over-abundance of transcripts associated with signalling and inflammation (bacterial response) pathways. A relative under-abundance of transcripts associated with T- and B-cell pathways was also seen in those with high neutrophil counts. A relative under-abundance of transcripts associated with T- and B-cell associated pathways also appeared to be common in patients with low lymphocyte counts. The associations seen in this initial, supervised analysis require further investigation using correlation analysis.

4.6.3.9 K-means clustering analysis identifies severity-associated transcriptional response patterns in influenza-positive patients

Since the most striking association identified relates to severity and duration of illness and since exploration of mechanisms of severity was a key objective of the MOSAIC study, the next step was to identify clusters of differentially expressed transcripts in influenza-positive patients that differ according to severity grade.

In addition to hierarchical clustering, a second method of clustering, K-means clustering, identified over- and under-abundant transcripts in influenza-positive patients from both seasons of recruitment. Because hierarchical clustering suggested that different major transcriptional

signatures in influenza-positive patients were strongly associated with differences in severity of illness, severity was also used as the interpretation for K-means clustering. Although K-means clustering is a supervised analysis, this second method identified transcripts that are associated with pathways that are similar to those identified through hierarchical clustering, using IPA and GO Terms analysis. Additionally, the relative abundance patterns were in agreement for transcriptional responses identified by hierarchical clustering and by K-means clustering.

Overall, transcripts relating to innate immune response and response to viral infection were most over-abundant in patients who were at a lower severity of illness, with less marked abundance in those who had the most severe illness. By contrast, transcripts associated with inflammation and bacterial response pathways tended to increase as severity increased, being most abundant in those with the most severe grade of illness (severity 3) i.e. critically-ill patients. In both seasons, the relative under-abundance of transcripts associated with T-cells pathways and to a lesser extent, B-cell pathways, became more obvious as severity increased. The under-abundance of these transcripts has been seen in cohorts of patients with other infections, including tuberculosis and bacterial infections, such as invasive *S. aureus* infection (378, 442, 446, 447). Flow-cytometry studies of invasive *S. aureus* infections have demonstrated that a decrease in T- and B-cell transcripts was not associated with decreases in absolute numbers of total T- and B-cells, although decreases in sub-populations e.g. central memory T-cells have been seen, suggesting that there may be 'reorganization' of the peripheral blood T-cell compartment, which is reflected in the decreased transcript abundance (446). Alternative explanations for under-expression include migration or death of T- and B-cells or a relative increase in other cell populations.

It cannot be assumed, however, that the patterns seen are only related to severity. Since hierarchical clustering revealed a relationship between the major transcriptional signature groups and day-of-illness at time of sampling, severity and day of illness may not be independent variables; the transcriptional changes seen may also reflect changes that occur with the natural course of infection, for example inflammatory processes that may follow the initial, infectious insult that triggered an innate immune response.

The results of K-means clustering were similar to those of hierarchical clustering and provide further evidence that the major transcriptional groups and their associated pathways, identified through IPA and GO Terms analysis, are likely to be genuine. Again, the patterns seen with K-means clustering were stronger for the 2010/11 cohort than for the 2009/10 cohort, probably because the 2010/11 cohort was significantly larger and contained a greater number of patients of differing severities, especially those with severity grade 3 illness.

4.6.3.10 Modular analysis reveals that transcriptional profiles for critical influenza illness are dominated by inflammatory and neutrophil-associated genes, rather than interferon-inducible genes

The modular transcriptional analysis of the 2010/11 cohort supports the findings of clustering analysis, but also provided additional insights with regards to the relationship between severity of illness in influenza-positive patients and the components of the immune response that are up-regulated. The interferon modules (M1.2, M3.4 and M5.12) includes interferon-inducible genes that encode antiviral molecules (e.g. OAS1-3 and OAS-L; GBP1, G1P2, EIF2AK2/PKR; MX1; PML), chemokines (e.g. CXCL10/IP-10) and signalling modules (e.g. STAT1, STAT2, IRF7 and ISGF3G). In addition to demonstrating more marked abundance of interferon-associated genes in patients with less severe illness, the modular approach identified changes in neutrophil-associated genes in those with more severe illness; this had been suggested but not specifically identified as neutrophil-associated in the inflammation pathways and bacterial response pathways identified by IPA and GO Terms analyses. The neutrophil module (M2.2) includes innate molecules found in neutrophil granules, including lactotransferrin, deformed epidermal autoregulatory factor 1 (*DEAF1)*, bacterial permeability increasing protein (BPI) and cathelicidin antimicrobial protein (CAMP).

Greater over-abundance of neutrophil-associated transcripts was seen in influenza-positive patients with severity 3 illness, in addition to an over-abundance of transcripts in the inflammation modules, compared to those with severity 1 or severity 2 illness. These findings of neutrophil intensity are consistent with the modular 'fingerprints' of patients with community acquired pneumonia (defined according to British Thoracic Society criteria) that have been described by others (447).

Although a neutrophil-dominated response is expected in bacterial infection, with neutrophils performing important phagocytic and bacteria-trapping functions (448-450), neutrophils have also been shown to be part response to influenza virus infection in mice infected with highly pathogenic avian influenza A(H5N1) and 1918 A(H1N1) viruses, detectable in the pulmonary alveolus 24 hours after infection (339). Furthermore, mice depleted of neutrophils have increased levels of virus in the lungs and also in non-respiratory sites, compared to mice not depleted of neutrophils (451). Neutrophils may contribute to pathogenesis of influenza-induced acute lung injury in the absence of bacterial co-infection, damaging the epithelial-endothelial barrier through the production of reactive oxygen species, by producing pro-inflammatory soluble mediators e.g. CXCL-10 (IP-10) and IL-8 (452). However, it is difficult to prove that the response seen is not due to infection with residing bacteria that has an opportunity to become invasive following influenza virus infection.

4.6.4 Comparison with findings of other transcriptomic analyses of influenza patients

There have been few transcriptomics studies of hospitalised patients with influenza, especially adults and children infected with A(H1N1)pdm09 virus infection. In a proof of concept study, Ramilo *et al.* (442) compared gene expression profiles in blood leukocytes in predominantly paediatric patients infected with pre-pandemic, seasonal influenza A infection, *E. coli* infection, *S. aureus* infection and *S. pneumoniae* infections associated a diverse range of clinical syndromes. The authors hypothesised that each infection would have its own transcriptional signature that could, in turn, be used to identify the different infections in an independent cohort. They used a training- and test-set approach and included healthy controls. 36 patients with influenza A were included and, in contrast to MOSAIC, all age groups were analysed together (median age, 1.2 years; range 2 weeks to 36 years). The mean day of illness (7 days) was greater than the mean day of illness in MOSAIC and detailed information concerning differences in severity of illness was not provided. Pathogen-based diagnoses were established by using relatively limited methods: by bacterial culture of a variety of different sample types, and by direct fluorescent antigen testing and culture for the detection of viruses. Unlike MOSAIC, patients were excluded if they had chronic disease, immunodeficiency, had received corticosteroids or immunomodulators, or had suspected or confirmed polymicrobial infection. In this respect, their study population may not be representative of patients who are most at risk of influenza and its complications.

The authors identified 35 genes that could discriminate patients with influenza A virus infection from patients with either *E. coli* or *S. pneumoniae* infection. These genes were shown to be capable of classifying an independent set of patients with either influenza A, *E. coli*, or *S. pneumoniae* infection. Consistent with the clinical observation that blood white cell counts and differential cell counts cannot differentiate between viral and bacterial infections, they did not find significant differences in these parameters in patients with different infections in this study. This suggests that the differences in gene expression levels reflected changes in transcriptional activity, rather than altered cellular composition in whole blood. By comparing profiles of samples from patients infected with pathogens associated with respiratory tract infections, they identified 4 transcriptional signatures: healthy state (uninfected), influenza-A infection-associated with interferon-inducible genes; bacterial infection-associated with neutrophil-associated genes (*S. aureus or S. pneumoniae*); mixed pattern (bacterial- and viral-like). Three of the patients with the mixed signature had confirmed influenza and clinical - but not microbiological - evidence of bacterial co-infection (radiographic consolidation and marked neutrophil predominance). Although the analysed performed to date did not identify a similar 'mixed' signature in MOSAIC adults at T1, the detailed microbiological and

virological investigations demonstrate that we do have patients with co-infections. Application of their classifier gene lists and their 'mixed viral/bacterial' gene list to the much larger MOSAIC cohort should be performed in future work, to see if these known co-infected patients can be identified.

Ioannidis *et al.* (453) compared PBMC transcriptomic signatures in children with acute influenza illness or RSV bronchioloitis with transcriptomic signatures derived from human airway epithelial cells (AECs) infected with corresponding viruses. A common type I interferon transcriptional signature was seen in influenza- and RSV-infected AECs, with IRF-7 at the centre of expression networks. The contributing transcripts in this response were greater in number and degree of change in influenza-infected AECs than in RSV-infected AECs. The authors used a similar (more limited) BIIR transcriptional modular analysis approach to the method we have used and showed that the interferon module contained up-regulated transcripts for both viruses, but greater intensity was seen with influenza infection. Influenza-specific genes that were differentially expressed by infected AECs included those encoding interferon B1, type III interferons, interleukins (IL-6, IL-1a, IL-1b, IL-23a, IL-17c, and IL-32) and chemokines (MCP-1 [CCL2], MCP-2 [CCL8], and ENA-78 [CXCL5]). The antiviral transcriptional response of influenza or RSV-infected AECs correlated with the transcriptomic responses of PBMCs obtained from influenza or RSV-infected children, respectively. However, IFI27 was the only common DEG induced in response to RSV and influenza virus infection in both AECs and PBMCs and the authors suggest that IFI27 overexpression may be a biomarker of viral infection in the upper airways and peripheral blood. Virus-specific transcriptional responses of PBMCs were seen, but a common interferon-inducible signature was also identified and this appeared to be more robust in influenza patients that in RSV patients. The authors also assessed the association between a composite respiratory severity score and changes in transcriptional response in PBMCs. Molecular perturbation (of overexpressed DEGs) was shown to increase as severity of illness increased.

The findings from this group's study support the notion that blood transcriptional profiling can be used to assess transcriptional responses to infections where viruses infect and replicate in a different compartment i.e. the respiratory tract. Furthermore, they showed an association between clinical severity and changes in PBMC transcriptional response in children, supporting our observation that clinical severity is associated with changes in transcriptional signatures in adults. However, their use of average perturbation of overexpressed DEGs and a lack of stratified group analysis means that severity grade-specific signatures are not apparent, as suggested by our study. Furthermore, they excluded patients with evidence of polymicrobial infections and therefore were unable to assess the

contribution of bacterial co-infection, a common feature of complicated influenza illness, to the transcriptional response.

Zaas *et al.* (445) also identified whole-blood transcriptomic signatures for bacterial and viral acute respiratory infections, including H3N2 influenza A infection, but in human viral challenge studies using healthy adult subjects. Subjects with symptomatic but uncomplicated illness caused by influenza, rhinovirus or RSV had common signatures that differentiated infected from uninfected and also asymptomatic individuals. The 27-gene signature for symptomatic acute respiratory viral illness, associated with peak infection, included up-regulation of many ISGs, including *MX1*, *IFIT1-3*, *OAS1* and *OAS3*. Following Venn analysis, 15 of 27 genes were common to RSV, HRV and influenza. *TNFAIP1*, *SEPT4*, and *IFI27* were 'unique' to influenza. *OAS2*, *CXCL10*, and *SOCS1* were 'unique' to HRV and. *FCGR1A*, *GBP1*, *LAP3*, *ETV7*, and *FCGR1B* were 'unique' to RSV. The authors concluded that the generic viral response dominates over responses specific to the individual viruses, at least at the time of peak symptoms in this human challenge model. They also identified genes with the greatest discriminating power and found that different infections/conditions were associated with specific genes e.g. *ISG15* for influenza and *IFI27, RSAD2, IFI6, CXCL10, FLJ20035, GBP1* and *SIGLEC1* for viral infections versus bacterial infection. Assuming that the virus detections in influenza-negative adult patients in MOSAIC explain the episodes of ILI, this may explain why patients with influenza did not form discrete clusters, separate from patients with other respiratory viral infections, following hierarchical clustering. It will be interesting to apply the generated gene lists from the Zaas *et al*. study to the MOSAIC transcriptomic cohort, to test whether they have discriminatory power in actual patients with varying clinical phenotypes and underlying health conditions, who have presented at different days of illness.

The same research group has already performed a separate human challenge study, comparing whole blood transcriptional signatures in healthy individuals infected with either seasonal H3N2 or seasonal (pre-pandemic) H1N1 influenza A viruses. Samples were taken every eight hours following inoculation, for 7 days and 18/41 (44%) subjects developed symptomatic infection. An influenza-specific gene signature, capable of identifying symptomatic patients, could be seen as early as 29 hours following infection, with maximal predictive accuracy seen at 43 hours and 38 hours before the peak of symptoms, for H1N1 and H3N2, respectively. The infecting influenza virus appeared not to affect the composite genes and the up-regulated genes (relative to healthy controls) were similar to those reported in the previous study and those seen in MOSAIC influenza-infected adults with early and less severe infection i.e. ISGs and genes involved in the anti-viral innate immune response. The researchers went on to apply the pre-symptomatic influenza gene list to a cohort of 36 adult

patients presenting to an emergency department with confirmed A(H1N1)pdm09 virus infection. Although information on clinical phenotypes and severity of illness were not supplied, 33/36 (92%) of patients were correctly identified (454).

Accepting the caveat that the challenge volunteers were healthy young adults, if the discriminatory signatures are confirmed as being consistent with gene lists identified in the MOSAIC adult influenza-positive cohort at T1, then combining the longitudinal results from the challenge studies with those from MOSAIC could provide a more complete picture of transcriptional responses in human influenza infection.

Herberg *et al.* (455) recruited 75 hospitalised paediatric subjects with confirmed A(H1N1)pdm09 virus infection (n=25), RSV infection (n=34) and bacterial infection (n=18), along with 33 healthy controls. Eighteen of the children were infected with at least one additional virus and RSV-infected infants were considerably younger than influenza-infected infants. Through comparison with controls, they identified 1,267 significantly differentially expressed transcripts (p<0.001), with a class-prediction sensitivity of 96% identified using Support Vector Machine analysis with leave-one-out validation. IPA canonical pathway analysis of genes identified in the influenza cohort revealed that 'protein synthesis'-associated pathways were most significantly over-represented, especially genes related protein translation, with a relative under-abundance compared to healthy controls. Under-abundance of genes associated with protein synthesis was also prominent when comparing influenza with RSV and bacterial infections. Similar to MOSAIC adult analysis, the top increased abundance pathways included 'signalling from pattern recognition receptors' (top increased genes *OAS3*, *C1QB*, *C1QC*) and interferon signalling (*IFI35*, *IFIT1* and *IFIT3*). They also reduced abundance of T- and B-cell-associated transcripts, similar to analysis of MOSAIC adults. Although differences in blood lymphocytes were seen between influenza-infected children and controls (ratio 2.1:1), the minimum fold change of the top 800 significantly differentially-expressed genes was 2.3 (median 4.5), suggesting that differences in lymphocyte proportion did not explain the observed differences.

4.6.5 Limitations and caveats

This analysis has only addressed transcriptomic responses in T1 samples from adult patients and adult healthy controls. Initial, longitudinal analysis of gene expression profiles for influenza-positive adults at T1, T2 and T3 suggests that global changes in expression can be seen as early as T2, approximately 48h after T1 (data not shown). It will be important to compare the major signature patterns seen at T1 with those seen at T2, and then with either convalescence or on-going (critical) illness at T3, to obtain a more complete picture of the course of illness in hospitalised patients.

Children were specifically excluded from this analysis, since they may have different immune and transcriptional responses to adults, especially young infants.

Day of illness at T1 varied between patients. The majority of influenza-infected patients with the most severe grade of illness were also at a later stage of illness, making it difficult to separate severity and duration of illness. Separate analysis of patients with early (0-4 days) and late illness (5 days and over) did not alter the major transcriptional signatures and associations seen when all patients were analysed together (data not shown). This demonstrates the unbiased nature of the approach used, however, since the transcriptomic signatures generated by semi-supervised analysis were subsequently explained by application of clinical variables, especially severity of illness and day of illness. Furthermore, because the vast majority of critically-ill patients had already reached maximum severity shortly after admission and at T1, there is little scope to monitor evolution from mild/moderate illness to severe illness in any subsequent, longitudinal analyses. However, this is representative of the natural history of A(H1N1)pdm09 virus infection in published series of hospitalised patients.

Most patients had underlying comorbidities, often with two or more comorbidities being present in an individual. Given the sample size and the presence of multiple comorbidities, I did not attempt to perform sub-group experiments according to the presence or absence of specific comorbidities e.g. asthma, obesity, no comorbidities. Clustering analysis of the entire cohort with application of the top five comorbidities and non-comorbid status did not reveal distinct clusters, however (data not shown). Additionally, some patients may have received medications that influence host transcriptional response e.g. corticosteroids. Medication data are being collated and these will need applying to analyses in due course.

For identification of disease-specific transcriptional signatures, influenza-positive patients were compared with influenza-negative ILI patients (with status determined by PCR testing). Although all patients had clinical features consistent with influenza infection that lead to diagnostic testing, the latter group represents a mixture of different viral, bacterial and mixed infections, and some patients do not yet have a microbiological or virological diagnosis. Additionally, the possibility of influenza infection preceding presentation cannot be excluded. However, a high incidence of biphasic symptomatic illness was not detected in the influenza-negative group, based on data recorded in the Flu-CIN form.

Although PCR analysis of T1 NPA samples was available for most patients, urine samples for pneumococcal antigen testing and blood culture samples were not obtained in a significant

proportion of patients. Additionally, some patients had received antibiotics at the time of sampling. Other patients may have experienced invasive pneumococcal infections but did not undergo appropriate testing, or had negative culture results because of antibiotic inhibition.

Although the major transcriptional patterns seen in the 2009/10 and 2010/11 adult cohorts were similar, formal validation of transcriptional signatures is required. I originally proposed to use the 2009/10 cohort as a training set and then test the derived signatures on the 2010/11 cohort (the 'test set') and perform class prediction analyses. However, due to the aforementioned difficulties in recruiting adequate numbers of influenza-positive patients in 2009/10, especially those with the most severe level of illness, this was not possible. Validation steps will need to be included in future work, either through obtaining samples from an independent cohort, or through grouping of all MOSAIC adult patients and then randomly allocating patient samples into a training and test sets.

In assessing biological significance of transcriptomic changes, IPA and GO Terms analyses rely on a knowledge base and interpretation may be limited by the assigned biological pathway categories. For genes of particular interest, further independent literature searches may enhance the findings and can be performed as part of future work. Finally, a frequent criticism of whole blood transcriptomics is that the changes seen may reflect changes in the composition of blood cell populations. Although the relevant clinical laboratory data are still being collated and correlations will be performed in future work, studies by others (described in section 4.7.5) found that transcriptional changes could not be explained by relative changes in populations of certain cells such as lymphocytes or neutrophils.

4.6.6 Conclusions

Through microarray analysis of whole blood samples obtained at a single acute time point from two influenza seasons, we have demonstrated that transcriptional profiling can be performed successfully in the context of an outbreak caused by a novel pandemic influenza strain. Symptomatic adult patients have been shown to have markedly different transcriptomic signatures compared to matched healthy controls, irrespective of severity of illness and the presence of underlying comorbidities. Overall, semi-supervised hierarchical clustering did not clearly differentiate those with confirmed influenza and those who had other causes of influenza-like illness. This may reflect similarities in transcriptional response between the two conditions, especially for non-influenza viruses causing ILI. Further characterisation of non-influenza cases may allow more appropriate comparisons, along with attempts to perform class prediction using pathogen-specific derived signatures.

Unbiased hierarchical clustering demonstrated that different transcriptomic signatures of influenza-infected adults are associated with severity of illness and time since onset of symptoms. Patients with less severe illness who are also at an earlier stage of illness have an interferon/antiviral-response dominated signature and the top identified genes and pathways are consistent with findings from transcriptomic studies of seasonal influenza and human viral challenge models. By contrast, patients with the most severe illness who are later in the course of illness have a neutrophil/bacterial-response dominated signature, with much less marked abundance of interferon/antiviral-associated transcripts. T- and B-cell pathways tended to be down-regulated compared to healthy controls and this was most marked in those who were at a later stage of more severe illness.

Although it is possible that some of the up-regulation seen in the later/more severe illness patients may be caused by inflammatory processes that occur in fulminant viral pneumonia or ARDS, correlation with evidence of bacterial infection – both invasive and non-invasive – in unbiased hierarchical clustering suggests that bacterial infection was more frequently detected in this group. This is further supported by evidence of higher serum procalcitonin levels in the same patients. Concurrent or secondary infection with common pathogenic bacterial species may be more common in severe influenza – particularly in those with critical illness – than appreciated through observational studies. Given the limitations of bacterial detection techniques (even molecular detection methods), the signature identified in patients with later and more severe illness may be capable of identifying bacterial infection in influenza patients and provides direction for future work. Although this study has not been able to monitor mechanisms associated with progression from mild/moderate illness through to more severe illness, our findings support the use of empirical antibiotics in addition to antiviral agents, in adult patients hospitalised with influenza, especially in those with critical illness.

Chapter Five

5 Soluble immune mediator profiling

5.1 Introduction

5.1.1 The role of soluble immune mediators in response to infection

The immunological response to influenza virus infection in humans involves innate and adaptive responses. Soluble immune mediators including many different cytokines and chemokines are fundamental to the production and regulation of the innate response and the subsequent, adaptive response to infection. These signalling proteins are released by and exert effects on a variety of different cells, through interactions with specific receptors. Release of mediators in different compartments occurs at different times during the course of infection, and measured levels in body fluids are the result of both production and consumption or destruction.

Soluble immune mediators and their receptors are components of often complex signalling pathways and cascades, which may involve both positive and negative feedback loops. The effector functions of different mediators demonstrate considerable and often act synergistically, whereas other mediators may have directly opposing actions, for example promoting a pro-inflammatory response versus an anti-inflammatory response. Although the primary purpose of soluble mediators and their associated immunological responses are to facilitate eradication of an infecting pathogen and the development of protective immunity against subsequent infections with the same pathogen, the release of mediators may also contribute to pathology and tissue damage. Similarly, while an anti-inflammatory state is an important part of recovery and repair following infection, it may leave the host open other infections.

The Th1 response is an important component of the pro-inflammatory immune state, whereby the transcription of numerous cytokines, such as interferon-gamma (IFN-γ), tissue necrosis factor (TNF) and interleukin-2 (IL-2), is increased in type 1 T-helper (Th1) cells in response to infection. Th1-associated cytokines are then released by effector cells, including endothelial cells and macrophages, inducing lymphocyte activation and infiltration of lymphocytes into infected tissues. The Th2 response is an important component of the subsequent switch to anti-inflammatory state, with secretion of mediators such as interleukin-4 (IL-4) and interleukin-10 (IL-10) by activated Th2 cells. These two pathways are interlinked and do not operate in isolation of each other in infection. Furthermore, the mediators produced are involved in positive, negative and auto-regulatory feedback loops that can influence the persistence or abrogation of each response.

5.1.2 Soluble immune mediators in influenza virus infection

Host responses to influenza virus infection involve innate, cell-mediated and humoral immune pathways. Data from human and animal studies suggest that exuberant and dysregulated cytokine and chemokine responses contribute to some of the clinical manifestations and pathology seen in severe influenza illness, particularly the progressive lung inflammation and extra-pulmonary organ dysfunction (456) that may continue once suppression of viral replication has been achieved. These responses may be influenced by the characteristics of the infecting virus, including mechanisms that have evolved to evade antiviral response, or by predisposing factors in the host, or perhaps by a combination of the two.

The term “cytokine storm” is often used to describe an exuberant soluble mediator response, which can occur rapidly in severe influenza illness, especially HPAI A(H5N1) virus infection (295, 457, 458). More specifically, cytokine storm refers to a soluble mediator milieu (typically described in blood) that is dominated by pro-inflammatory mediators, with little evidence of effective regulation by anti-inflammatory mediators, such as transforming growth factor-beta (TGF-β) and IL-10. Cytokine storm is also seen in non-viral infections, such as invasive group A Streptococcus disease (459), and also in non-infectious conditions, such as graft-versus-host disease (460). A proposed model for initiation of cytokine storm is that super-antigens or super-agonists stimulate excessive receptor chronic-linking, leading to profound stimulation of the inflammatory response. A notable example of this phenomenon was the administration of a super-agonist monoclonal antibody against CD28 (TGN1412), which lead rapidly to SIRS, critical illness, and hypercytokinaemia consistent with cytokine storm, during a first-in-human clinical trial of a proposed treatment for B cell chronic lymphocytic leukaemia (461). After the pro-inflammatory cytokine response has been triggered, secondary cascades follow that serve to amplify and maintain the pro-inflammatory state, including production of mediators that are not induced directly by the invading pathogen e.g. TNF (136, 462). However, the soluble immune mediator response to influenza virus is complex and may be influenced by many factors, including the type of infecting virus and the host species (or mouse strain) infected. Furthermore, inhibition of specific mediators e.g. TNF-α, IL-6, or MCP-1 (CCL2), or the administration of broadly acting immunosuppressants such as corticosteroids, fails to protect mice from morbidity and mortality following HPAI A(H5N1) virus infection (463). This suggests that attempts to prevent the cytokine storm from becoming established, for example through the use of early antivirals, may be a more effective approach to preventing severe influenza in humans. However, most critically-ill influenza patients present with established lung injury (39, 66) and since early and effective antiviral therapy is difficult to deliver. Therefore, the identification of effective

immunomodulatory targets and adjunct therapies, following an initial exploration of immune responses to infection, remains an important goal.

Although deaths during the 1918 pandemic were often attributed to untreated secondary bacterial infection, infection of animals with reconstructed isolates of 1918 H1N1 virus have demonstrated marked pathology and dysregulated innate and cellular immune responses (339, 464). It has not been possible to perform very detailed studies of the immunopathogenesis of severe influenza, but elevated levels of interleukin-6 (IL-6), interleukin-8 (IL-8), TNF-α, IFN-α, IFN-γ, and IL-10 in serum/plasma and nasal secretions have been described in patients with uncomplicated influenza illness (465, 466). Levels of IL-6 and IL-8 have also been shown to correlate with symptom severity in mild illness (331). In a small study of hospitalised patients with seasonal influenza A(H1N1) virus infection in Chinese patients (mean age 57 years), increased levels of plasma IL-6, IL-8, IP-10, MIG, MCP-1 were seen during acute influenza illness. Hypercytokinaemia occurred in patients who were of advanced age, had major comorbidities and developed cardiac/respiratory complications. Additionally, elevated levels of these mediators were shown to correlate with high viral loads in upper respiratory tract secretions. No significant changes in IFN-γ, IL-1β, IL-10, IL-12p70 and TNF-α concentrations during the acute phase of illness have been reported (467).

Although an uncommon infection in humans, HPAI A(H5N1) virus infection has been associated with severe pneumonitis and variable multi-organ dysfunction in the majority of hospitalised patients. The mortality rate is approximately 60% and remains high despite access to advance medical care and early antiviral therapy (39). HPAI H5N1 virus infection in humans serves as an important model of mediator responses and inflammatory cascades in severe influenza. Animal and *in vitro* experiments demonstrate that virus replication in macrophages and alveolar epithelium leads to the release of cytokines and chemokines, which trigger autocrine and paracrine pro-inflammatory cascades, which may then be amplified through interaction with adjacent, uninfected cells. Further amplification is provided by the subsequent infiltration of inflammatory cells into alveolar spaces. Replication of virus in myeloid dendritic cells may facilitate spread of infection to extra-pulmonary sites, but the systemic activity of soluble mediators may also be responsible for complications such as myocarditis and encephalopathy (295).

Increased plasma levels of IL-10, IL-6, IFN-γ, TNF-a, sIL-2r, IP-10, MCP-1, and MIG have been described in HPAI A(H5N1) virus infection and levels of IL-6, IL-8, IL-1β, MCP-1 appear to be higher in fatal cases than in those who survive (135, 468). Although certain mediators are elevated in samples from patients infected with seasonal influenza viruses and those infected with HPAI A(H5N1) virus, levels tend to be much higher in the latter group, suggesting that magnitude of response may be

more important than strain/subtype-specific cytokine profiles. This difference may be explained by virus characteristics, because HPAI H5N1 virus is known to have a different internal gene constellation (e.g. NS1) that may result in more marked cytokine induction in the infected host (116, 469).

There are similarities between the illness caused be HPAI A(H5N1) virus infection illness and severe illness caused by A(H1N1)pdm09 virus infection e.g. rapidly progressive pneumonitis with ARDS, multi-organ dysfunction and extra-pulmonary disease. Therefore, one may expect mediator responses to be dysfunctional and exuberant in both diseases. Certain responses - and the pathways that control them - may be virus subtype-dependent, whereas others may be common to a number of influenza infections. IL-8, IP-10 and growth-related oncogene-α (GRO-α) have been shown to be predominantly induced in virus-infected human normal respiratory epithelial cells, irrespective of the infecting virus subtype. TLR responses to infection appear to differ, however, with TLR-5 being predominantly induced in cells infected with seasonal A(H1N1) virus, A(H1N1)pdm09 virus, or HPAI A(H5N1) virus, and TLR-3 being predominantly induced in cells infected with seasonal A(H3N2) virus (470).

A small number of published studies report soluble mediator levels in patients with differing severities of A(H1N1)pdm09 virus infection, but patterns of innate and adaptive immune responses remain only partially characterised (248, 333, 334). These studies recruited relatively small numbers of patients and did not have complimentary data on cellular immune responses or host transcriptomic profiles, restricting the biological interpretation of the soluble mediator findings. Furthermore, published comparisons of soluble mediator profiles from patients presenting with influenza-like illness and/or sepsis caused by other infections have been limited, or have only included paediatric populations (471).

Increased plasma levels of IL-6, IL-8, IL-12p70 and IL-15 have been found in patients infected with A(H1N1)pdm09 virus causing critical illness and have been shown to correlate with the extent of lung field involvement on chest imaging (333). Th1 and T-helped cell 17 (Th17)-associated responses have also been found in severe, but not mild/moderate illness (334). Patients with A(H1N1)pdm09 virus infection who developed ARDS and/or died also had increased plasma levels of IL-6, IL-10 and IL-15, and increased levels of G-CSF, IL-1a, IL-8, IP-10 and TNF-α in the later stages of illness (248, 333). Again, it is had not been demonstrated whether these patterns are specific to A(H1N1)pdm09 virus infection.

In addition to describing patterns of soluble mediator responses in humans, the biological implications of such responses need to be investigated. Some cytokines, such as the interferons, are known to have an antiviral effect, but the overall effect of exuberant cytokine cascades might contribute to pathogenesis. Conflicting results between in vitro, animal and human studies of infection with different HPAI A(H5N1) viruses suggest that the specificity of host-pathogen response needs to be considered.

Although the complex nature of immune responses and signalling pathways suggests that changes in the levels of soluble mediators alone are unlikely to explain the pathology seen in severe influenza virus infection, the quantification of key mediators in different biological compartments is an important step in understanding the mechanisms of pathogenesis. By quantifying levels of many different mediators, it is possible to form mediator profiles that have the potential to reveal which immune responses predominate in individuals or groups of individuals during the course of infection. By combining immune mediator findings with detailed information on clinical characteristics, cellular immune responses, virus characteristics, co-infections, host genetic factors and changes at the level of transcription, the MOSAIC study has the potential to form an integrated model of the pathogenesis of severe influenza in humans.

5.1.3 Hypotheses and aims

This initial analysis addresses the following hypotheses:

1. Soluble immune mediator/biomarker profiles of patients with influenza virus infection are distinct from those seen in patients with influenza-like illness
2. In patients with influenza virus infection, specific soluble immune mediator/biomarkers are associated with different severities of illness

The aims of this initial analysis was to quantify soluble immune mediators and biomarkers in samples of blood and different respiratory tract secretions obtained from adult healthy controls, and from influenza-positive and influenza-negative hospitalised adult patients in the acute phase of illness. Levels of mediators in T1 samples were compared according to infection status and, for influenza-positive adults, according to the severity of illness at T1. Analyses of results from other time points and other, more complete analyses will follow later.

5.2 Summary of assay techniques

Soluble immune mediators were measured using MSD electrochemiluminescence and ELISA assays. 28 mediators were quantified using five different MSD assay plates. MSD assay panels were selected

based on our experience of mediator responses in viral challenge and observational studies of asthma and COPD patients, and also studies of mediator responses in influenza and respiratory infection studies that have been conducted by others. Although it was not possible or practical to quantify all known cytokines and chemokines, the mediators selected for analysis are components of the major immune responses to respiratory viral infections, including pro-inflammatory, Th1, Th2 and anti-inflammatory pathways. When the study was designed, MSD assays for certain mediators were not available. Therefore, ELISA was used to measure four mediators: sTNFR1, sTNFR2, neopterin and RANTES (CCL5).

CRP and vitamin D were measured using clinically validated, commercial assays, which were performed by the Chemical Pathology Department at Imperial College Health Care NHS Trust. For blood samples, most mediator analysis was performed using serum. Although some patients only had plasma samples available for analysis, the MSD and ELISA results have not been combined since our own initial experiments and work performed by others have shown that soluble immune mediator levels in plasma and serum are not directly comparable for many mediators. For the quantification of PCT, CRP and vitamin D, plasma samples were used for the majority of the assays for the 2009/10 season (for logistical reasons), whereas serum samples were used for all of the 2010/11 season assays. The results of these particular assays are not significantly affected by the sample type used (plasma versus serum) and therefore the results were pooled for combined analysis.

Although specimens were obtained from adult and paediatric patients at all three time points, only results for T1 serum (and plasma), T1 NPA, T1 SAM from adults are presented here. Adult BAL samples are also included, but these were not linked to a specific time point. Results from adult healthy controls are also presented, where available; samples for analysis or comparable results from paediatric healthy controls are still being sought.

5.3 Assay performance

5.3.1 MSD electrochemiluminescence

For all sample types obtained at all time points, twenty-nine soluble immune mediators (described in materials and methods) were measured successfully using the MSD multiplex electrochemiluminescence assay. Standard curve plots for individual mediators analysed on each of the five assay plate types (total 186 plates) matched the expected distributions. Quality control analysis demonstrated that the results for samples analysed in duplicate (all samples except SAM eluates) were consistent between duplicates, with satisfactory co-efficient of variation (cv) percentages. IL-8 was measured twice, on two separate assay plates. Correlation and linear

regression analysis showed that results were consistent between the two plates (figures 5.1 and 5.2). Therefore, only one set of results (9-plex IL-8) was included in the final analysis.

A conservative approach was taken to determine the lower limit of detection (LLOD) for each mediator, ignoring the software-generated LLOD and only accepting values for samples that were above the value for the lowest detected standard with a cv of >20% (this was typically the seventh of eight standards). LLOD values for individual mediators were generally consistent across all plates, although there were exceptions. For example, the per-plate LLOD for eotaxin-1 varied quite markedly between plates. For this reason and to facilitate interpretation of pooled data, the geometric mean was calculated for each mediator's LLOD and half the value of the geometric mean of the lower limit of detection (GM-LLOD) was then applied universally.

Zero values were observed for a small minority of samples, in addition to observed values below the LLOD. These may represent a true absence of specific mediators in samples obtained from certain individuals, or they could represent assay failure. However, after discussion with the manufacturer, the latter was deemed to be unlikely, since for an individual sample of concern, the results were not accompanied by zero value results for all of the other mediators included in the multiplex assay well. Although failure of a single assay 'spot' within a well cannot be excluded, this phenomenon was seen across the assays plates that were run. This suggests that if failure to measure an individual mediator did occur, it will have occurred at a similar rate across all assays, avoiding bias because there was no apparent 'batch effect'. Furthermore, influenza-positive and influenza-negative samples were analysed together on the same plates following random allocation, without any prior knowledge of patient characteristics.

One set of results was unexpected. Levels of eotaxin-1 in serum obtained from the 36 adult healthy controls were unexpectedly high, based on comparison with healthy control levels of reported in the literature (measured using a variety of assay techniques). Other mediators measured by the same multiplex assay were as expected. To investigate this, a pristine set of HC serum aliquots was re-analysed, using an assay plate from a different production batch and with the manufacturer's technician in attendance. The results from the repeated assay were almost identical to those from the original assay (the raw signal intensities and calculated values were similar between experiments). In the absence of an obvious explanation for the discrepancy, these HC results have been used in analyses.

For certain mediators measured in specific sample types e.g. IL-8 measured in NPA and SAM, many results were above the pre-defined ULOD for that mediator. Due to the large number of samples to

assess, limited remaining sample volumes, the significant cost of the assays and the risk of diluting-out other mediators that occur at low levels, it was not possible to dilute samples and re-run the assays simply to re-measure mediators such as IL-8. A decision was made not to re-analyse samples using a different method e.g. ELISA, for some of the reasons already mentioned. All MSD results are reported as pg/ml. The GM-LLOD value for each mediator is shown in table 5.1.

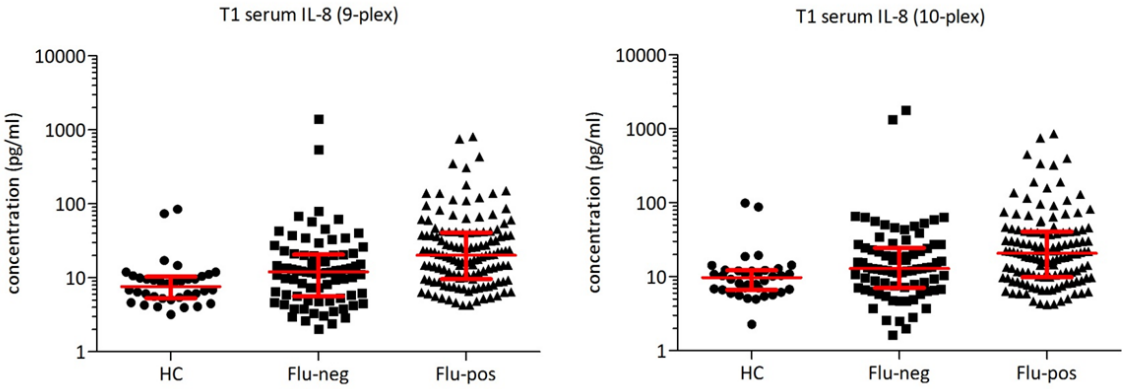


Figure 5.1 IL-8 concentrations in T1 serum measured using two separate MSD multiplex assays
Levels of IL-8 (pg/mL, log scale) were determined in aliquots of the same serum obtained from individual subjects added to two separate types of MSD multiplex mediator assay plates (9-plex and 10-plex plates). Results were grouped according to infection status: HC=healthy controls (•); Flu-neg=influenza-negative (▪); Flu-pos=influenza-positive (▲).

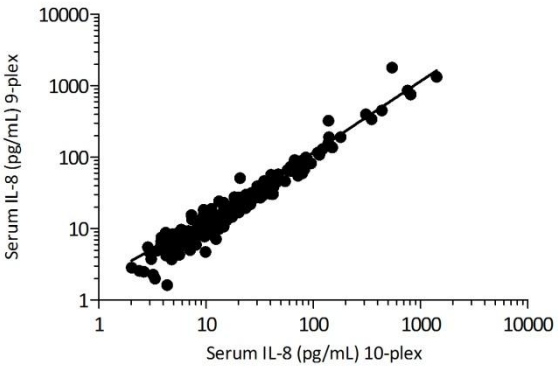


Figure 5.2 Concentrations of IL-8 in serum are consistent across two different types of MSD multiplex assay
Levels of Il-8 in serum from healthy adult controls, and influenza-positive and influenza-negative adults at T1 were measured using two different MSD multiplex mediator assays: 9-plex (Y axis) and 10-plex (X axis). Spearman correlation r=0.962 (p<0.0001); linear regression r^2=0.769

	Upper limit of detection (ULOD; pg/mL)	Geometric mean of lower limit of detection (GM-LLOD; pg/mL)	Half GM-LLOD (pg/mL)
IFN-β	100000	23.3	11.65
IFN-α2a	10000	2.6	1.3
IFN-λ (IL-29)	10000	24.0	12.0
IFN-γ	2500	0.6	0.3
IL-13	2500	0.6	0.3
IL-15	2500	0.6	0.3
IL-17	2500	0.5	0.25
I-TAC (CXCL11)	2500	0.6	0.3
MIG (CXCL9)	2500	0.6	0.3
MIP-1α (CCL3)	2500	2.7	1.35
Eotaxin-1 (CCL11)	40000	30.0	15.0
Eotaxin-3 (CCL26)	10000	5.0	2.5
IL-8	2500	0.6	0.3
IP-10 (CXCL-10)	10000	8.7	4.35
MCP-1 (CCL2)	10000	3.4	1.7
MCP-4 (CCL13)	10000	5.9	2.95
MDC (CCL22)	400000	108.8	54.4
MIP-1β (CCL4)	40000	10.5	5.25
TARC (CCL17)	40000	10.0	5.0
GM-CSF	2500	0.6	0.3
IL-10	2500	0.6	0.3
IL-12 p70	2500	0.6	0.3
IL-1β	2500	0.7	0.35
IL-2	2500	1.2	0.6
IL-4	2500	0.8	0.4
IL-5	2500	0.6	0.3
IL-6	2500	0.7	0.35
IL-8	2500	0.6	0.3
TNF-α	2500	0.6	0.3

Table 5.1 Limits of detection for MSD electrochemiluminescence multiplex assays
Assays are displayed in their corresponding multiplex groups (single plex, 2-plex, 7-plex, 9-plex, and 10-plex). For each mediator, half the value of GM-LLOD was assigned to results that were below the GM-LLOD, including zero results.

5.3.2 Enzyme-linked immunosorbent assays (ELISAs)

RANTES, neopterin and sTNFR1 and sTNFR2 were measured using ELISA. Assays were successfully completed for serum samples from adult patients and adult healthy controls, but analyses of NPA, SAM eluates and BAL fluid are on-going. Therefore, only T1 adult serum results are presented here.

5.3.3 Procalcitonin, c-reactive protein and vitamin D assays

Procalcitonin (PCT), c-reactive protein (CRP) and vitamin D were successfully measured in the majority of samples provided to the collaborating laboratory. The most common reason for failure was inadequate sample volume (predominantly low volume paediatric samples).

PCT could not be measured in seven samples due to an assay error; attempts were not made to re-measure PCT in these samples. For samples that reached the ULOD (100 ng/mL), we decided not to dilute the samples and perform a repeat analysis. The reported LLOD was 0.02 ng/mL and QC measures (target mean, target SD and CV percentage) were satisfactory. Although the clinical laboratory releases only those results that are above 5 ng/mL, the assay LLOD is 0.2 ng/mL; results above the LLOD but <5 ng/mL have been included along with other results.

Vitamin D (25-hydroxyvitamin D) was measured using different assays for the 2009/10 and 2010/11 seasons. For the 2009/10 cohort, the Diassorin Liaison chemiluminescence assay was performed, whereas samples from the 2010/11 cohort were measured using the Waters MassTrak 25 ultra-performance liquid chromatography mass spectroscopy (UP-LCMS) assay. The latter method was adopted by the collaborating clinical laboratory because it was considered to be more accurate (the chemiluminescence assay may slightly over/underestimate high and/or low vitamin D values). However, results from both seasons have been combined for presentation here. The reported LLOD for both assays was 15 nmol/L.

5.4 Approach to initial analysis of soluble immune mediator data

The T1 mediator data for serum, NPA and SAM were separated into two main groups. The first compared results for influenza-positive adult patients, influenza-negative adult patients and adult healthy controls. The second group contained T1 results from healthy controls and influenza-positive patients only, with the influenza patients being divided according to their severity of illness at T1: grade 1 severity, grade 2 severity, and grade 3 severity (see chapter 3 for further details on severity grading). Thus, median values for each of the three severity groups were compared against healthy controls and then against each other.

Histogram analysis and D'Agostino's test demonstrated that levels of many mediators did not follow a normal distribution. Therefore, non-parametric statistical tests (Kruskal-Wallis with Dunn's post-

test) were performed on non-transformed data using GraphPad Prism. Scatter-plots were then created for selected mediators.

5.5 Comparison of MSD electrochemiluminescence soluble immune mediator results for influenza-positive, influenza-negative and healthy controls at T1

For each mediator measured using electrochemiluminescence multiplex assay, the median values were calculated for influenza-positive, influenza-negative and healthy controls in T1 serum, T1 NPA and T1 SAM eluate. Kruskal-Wallis *p* value and Dunn's post-test *p* values (HC versus influenza positive, HC versus influenza-negative and influenza-negative versus influenza-positive) were calculated. If the Kruskal Wallis *p* value was >0.05, Dunn's post-test was not performed (automatically assigned non-significance).

5.5.1 IL-8 and IP-10 in T1 serum from influenza-positive and influenza-negative adult patients, and healthy control serum

In serum, median IL-8 and IP-10 concentrations were significantly higher in influenza-negative and influenza-positive patients compared to healthy controls, and the highest levels were seen in influenza-positive patients (figure 5.3). Some mediators were elevated in patients compared to healthy controls, but the median values for influenza-negative and influenza-positive patients did not differ significantly (IL-15, IL-12p70, IL-4, IL-13, IL-17, IL-6, IL-10, MIG, I-TAC, MDC, TARC, eotaxin-1, eotaxin-3, MIP-1β, MCP-4). Compared to healthy controls, MIP-1α was significantly elevated in influenza-negative patients relative to healthy controls, but not in influenza-positive patients (figure 5.4). Significant differences were not observed when comparing median values in healthy controls, influenza-negative and influenza-positive patients, for the following mediators: IFN-λ, TNF-α, IL-5, IL-1β and GM-CSF. Although the applied statistical test suggested significance for IFN-β results, the median values were identical; although the mean ranks were different, the medians are equal and Kruskal-Wallis does not test the null hypothesis that the populations have equal medians. Therefore 'NA' was entered in the respective *p* value columns for this mediator (table 5.2).

	Mediator	Healthy control (n=36) Median (IQR) pg/mL	Influenza-negative (n=79) Median (IQR) pg/mL	Influenza-positive (n=128) Median (IQR) pg/mL	Kruskal-Wallis *p* value	HC vs. Flu-Neg *p* value	HC vs. Flu-Pos *p* value	Flu neg vs. Flu-pos *p* value
Types I and III IFNs	IFN-α2a	5.3 (3.5-6.2)	3.6 (1.3-6.7)	5.8 (1.3-10.6)	0.0369	NS	NS	NA
	IFN-β	11.7 (11.7-11.7)	11.7 (11.7-11.7)	11.7 (11.7-36.7)	N/A	NA	N/A	N/A
	IFN-λ (IL-29)	26.8 (12.0-42.2)	12.0 (12.0-39.4)	12.0 (12.0-42.3)	NS	N/A	N/A	N/A
Th1-secreted/associated cytokines	IFN-γ	0.3 (0.3-0.3)	0.8 (0.3-5.6)	2.3 (0.3-6.1)	0.0024	NS	<0.001	NS
	TNF-α	5.2 (4.6-5.9)	5.1 (3.7-7.9)	6.0 (4.5-9.0)	NS	N/A	N/A	N/A
	IL-2	4.7 (2.8-7.7)	2.1 (0.6-5.6)	5.8 (3.0-9.4)	<0.0001	<0.01	NS	N/A
	IL-15	1.6 (1.5-1.9)	3.8 (2.6-6.2)	4.6 (2.8-7.6)	<0.0001	<0.001	<0.001	NS
	IL-12p70	4.0 (1.9-9.1)	2.0 (1.0-3.7)	2.0 (0.9-4.6)	0.0140	<0.05	<0.05	NS
Th2-secreted cytokines	IL-4	1.7 (1.2 – 2.0)	0.9 (0.4-1.7)	1.0 (0.4-2.0)	0.0002	<0.001	<0.01	NS
	IL-5	1.1 (0.6-0.7)	1.3 (0.7-2.6)	1.2 (0.7-2.6)	NS	N/A	N/A	N/A
	IL-13	1.2 (0.4-1.9)	2.6 (1.1-4.5)	2.7 (1.4-4.5)	<0.0001	<0.001	<0.001	NS
Th17-secreted cytokines	IL-17	3.2 (2.2-3.8)	19.2 (0.3-269.4)	20.8 (0.3-518.7)	<0.0001	<0.001	<0.001	NS
Other inflammatory chemokines	IL-6	3.8 (2.9-6.4)	23.8 (7.5-64.3)	28.5 (7.4-96.8)	<0.0001	<0.001	<0.001	NS
	IL-8	**7.6 (5.3-10.3)**	**12.1 (5.7-20.7)**	**20.3 (9.6-40.9)**	**<0.0001**	**<0.05**	**<0.001**	**<0.001**
	IL-1β	0.8 (0.3-1.2)	0.3 (0.3-0.9)	0.3 (0.3-1.2)	NS	N/A	N/A	N/A
	GM-CSF	1.1 (0.4-1.6)	0.3 (0.3-1.1)	0.8 (0.3-1.7)	NS	N/A	N/A	N/A
Anti-inflammatory cytokine	IL-10	1.5 (1.1-2.8)	3.4 (1.9-9.3)	6.0 (3.2-13.3)	<0.0001	<0.001	<0.001	NS
IFN-associated/Th1-chemokines	MIG (CXCL9)	44.6 (33.2-57.7)	119.7 (62.8-412.9)	108.6 (61.5-250.7)	<0.0001	<0.001	<0.001	NS
	IP-10 (CXCL10)	**167.1 (145.1-183.3)**	**400.3 (196.6-1137)**	**1181 (584.8-2673)**	**<0.0001**	**<0.001**	**<0.001**	**<0.001**
	I-TAC (CXCL11)	60.98 (53.44-74.34)	178.7 (119.3-517)	242.7 (135.4-404.6)	<0.0001	<0.001	<0.001	NS
Th2-associated chemokines	MDC (CCL22)	4011 (3197-4584)	1407 (894.6-1039)	1685 (1039-2675)	<0.0001	<0.001	<0.001	NS
	TARC (CCL17)	412.8 (297.2-660.3)	260.4 (115.3-393.8)	261.8 (103.5-437.3)	0.0005	<0.01	<0.001	NS
	Eotaxin-1 (CCL11)	1251 (729-1535)	456.3 (283.2-797.5)	590.4 (356.6-957.6)	<0.0001	<0.001	<0.001	NS
	Eotaxin-3 (CCL26)	2.5 (2.5-11.07)	12.4 (2.5-20)	17.4 (10.8-31)	<0.0001	<0.001	<0.001	NS
Other inflammatory chemokines	**MIP-1α (CCL3)**	**11 (9.3-14)**	**25.9 (11-43.7)**	**13.6 (9.8-25.2)**	**<0.0001**	**<0.001**	**NS**	**<0.01**
	MIP-1β (CCL4)	165.3 (130.5-236.2)	115.6 (64.7-164.2)	132.8 (99.5-200.7)	0.0004	<0.001	<0.05	NS
	MCP-1 (CCL2)	415.8 (320-502.6)	328.2 (190.7-526.8)	496.2 (288.3-1095)	<0.0001	NS	NS	N/A
	MCP-4 (CCL13)	641 (439.5-797.2)	415.4 (247.9-730.8)	381.7 (218.1-606.1)	0.0014	<0.05	<0.001	NS

Table 5.2 Summary statistics for 28 soluble immune mediators in T1 serum measured by MSD

Serum was obtained from adult patients at T1 (2009/10 and 2010/11 seasons combined). Kruskal-Wallis (KW) one-way analysis of variance by ranks test was performed to results for adult healthy controls (HC), influenza-negative (flu-neg) and influenza-positive (flu-pos) patients. Following KW, to compare influenza-negative (flu-neg) and influenza-positive (flu-pos), Dunn's post-test was applied. Mediators highlighted in bold demonstrate significant difference between medians of the influenza-negative and influenza-positive and where at least one group's median is significantly different from that of the healthy controls. Dunn's post-test was not performed if the medians did not differ on Kruskal-Wallis testing (significance cut-off $p<0.05$). Flu negative vs. flu positive significance was not tested if both influenza-positive and influenza-negative medians did not differ from the healthy control median. NS = no sample obtained. N/A = not applicable.

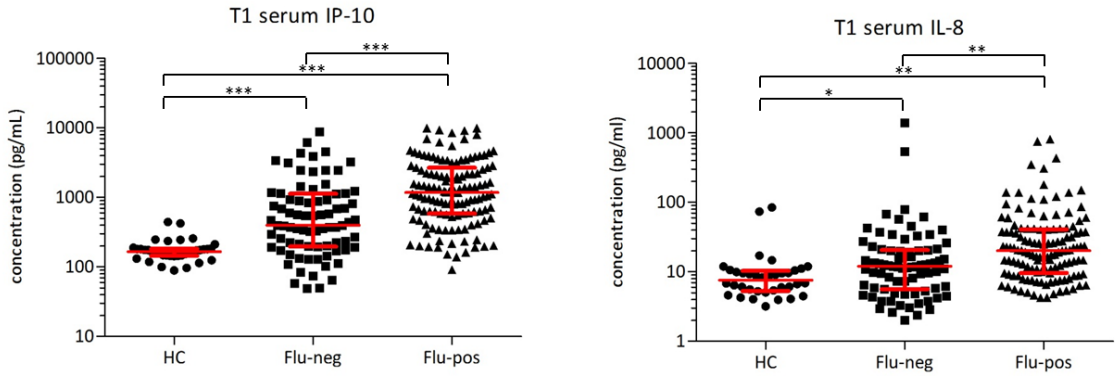


Figure 5.3 IP-10 and IL-8 levels in T1 serum are significantly higher in influenza-positive patients than in influenza-negative patients and healthy controls

Adult T1 serum from both seasons (2009/10 and 2010/11). Calculated concentrated means (pg/ml) have been plotted (log scale) and for each group the median and interquartile ranges were overlaid in red. Kruskal-Wallis test with Dunn's post-test was used to assess significance (***p <0.001; **p <0.01; *p<0.05). Sample groups: HC=healthy controls (•); Flu-neg=influenza-negative (▪); Flu-pos=influenza-positive (▲).

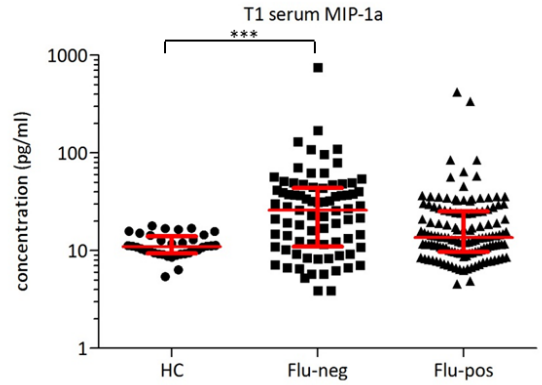


Figure 5.4 MIP-1α in T1 serum is elevated in influenza-negative patients, but not in influenza-positive patients

Calculated concentrated means are plotted (log scale) and for each group the median and interquartile ranges are overlaid in red. Kruskal-Wallis test with Dunn's post-test was used to assess significance (*** p<0.001). Sample groups: HC=healthy controls (•); Flu-neg=influenza-negative (▪); Flu-pos=influenza-positive (▲).

5.5.2 Levels of IP-10 and IL-8 in T1 nasopharyngeal aspirate (NPA) from influenza-positive and influenza-negative adult patients, and healthy control NPA

Similar to the serum results, median levels of IL-8 and IP-10 in T1 NPA were significantly elevated in patients compared to healthy controls, and a significantly higher level was seen in influenza-positive patients compared to influenza-negative patients. Influenza-positive patients had an approximately four-fold greater median concentration of IL-8 and an approximately 20-fold higher median concentration of IP-10, compared to influenza-negative patients. Median levels of IL-8 and IP-10 in NPA were also considerably higher than the median levels measured in serum samples (20.3pg/mL vs. 994.1 pg/mL and 1181 pg/mL vs. 3964 pg/mL, respectively) (figure 5.5).

5.5.3 Levels of Th1, Th2, and inflammatory chemokines in T1 NPA from influenza-positive and influenza-negative patients, and healthy control NPA

In contrast to serum, statistically significant differences in NPA median values for all three groups, accompanied by a significant increase in influenza-positive patients compared to influenza-negative patients, were observed for two other IFN-associated/Th1-attracting chemokines (MIG, I-TAC), certain Th2-associated chemokines (MDC, TARC, eotaxin-1) and other inflammatory chemokines (MIP-1α, MIP-1β, MCP-1 and MCP-4). Note that in contrast to serum results, MIP-1α in NPA was higher in influenza-positive patients than in influenza negative patients (figure 5.5).

5.5.4 Levels of interferons, Th1, Th2, and Th-17 and anti-inflammatory cytokines in NPA from influenza-positive and influenza-negative patients, and healthy control NPA

The median concentrations of the cytokines IFN-α2a, IFN-β, IFN-λ (IL-29) and IFN-γ did not differ, being equal to half the respective GM-LLOD for all groups (reflecting the fact that the interferons were not detected or were at the lower limit of detection in NPA samples from most patients and healthy controls). TNF-α was significantly elevated in NPA from influenza-positive patients compared to healthy controls, but not in influenza-negative patients. No difference was seen in the levels of IL-15, IL-12p70 or Il-17. Several mediators were increased in patients compared to healthy controls, but a difference was not seen between median values for influenza-negative and influenza-positive patients: IL-2, IL-13, IL-6, IL-1β and eotaxin-3. Increased levels of IL-10 in influenza-positive patients compared to influenza-negative patients were observed, but the median values for all three groups were very low (table 5.3).

	Mediator	Healthy control (n=36) Median (IQR) pg/mL	Influenza-negative (n=79) Median (IQR) pg/mL	Influenza-positive (n=128) Median (IQR) pg/mL	Kruskal-Wallis *p* value	HC vs. Flu Neg *p* value	HC vs. Flu Pos *p* value	Flu neg vs. Flu-pos *p* value
Types I and III IFNs	IFN-α2a	1.3 (1.3-1.3)	1.3 (1.3-1.3)	1.3 (1.3-1.3)	N/A	N/A	N/A	N/A
	IFN-β	11.7 (11.7-60.1)	11.7 (11.7-28.4)	11.7 (11.7-94.5)	N/A	N/A	N/A	N/A
	IFN-λ (IL-29)	12.0 (12.0-12.0)	12.0 (12.0-12.0)	12.0 (12.0-12.0)	N/A	N/A	N/A	N/A
Th1-secreted/associated cytokines	IFN-γ	0.3 (0.3-0.3)	0.3 (0.3-0.3)	0.3 (0.3-4.5)	N/A	N/A	N/A	N/A
	TNF-α	0.3 (0.3-0.3)	0.6 (0.3-5.5)	7.0 (0.3-68.2)	<0.0001	NS	<0.001	<0.001
	IL-2	0.6 (0.6-1.8)	6.9 (1.7-24.1)	5.0 (1.3-21.0)	<0.0001	<0.001	<0.001	NS
	IL-15	0.3 (0.3-0.3)	0.3 (0.3-1.2)	0.6 (0.3-2.6)	NS	NS	NS	NS
	IL-12p70	0.3 (0.3-0.3)	0.7 (0.3-2.3)	1.2 (0.3-6.3)	NS	NS	NS	NS
Th2-secreted cytokines	IL-4	0.4 (0.4-0.4)	0.4 (0.4-1.5)	1.1 (0.4-3.8)	0.0224	NS	NS	N/A
	IL-5	0.3 (0.3-0.3)	0.3 (0.3-0.72)	0.7 (0.3-4.5)	0.0016	<0.05	NS	<0.01
	IL-13	0.8 (0.3-1.3)	1.0 (0.3-3.6)	1.9 (0.9-4.0)	<0.0001	<0.05	<0.001	NS
Th17-secreted cytokines	IL-17	2.2 (0.3-20.9)	2.3 (0.3-8.3)	5.4 (0.8-22.4)	0.0311	NS	NS	NS
Other inflammatory chemokines	IL-6	0.3 (0.3-0.3)	4.9 (1.5-51.0)	32.4 (1.1-309.6)	<0.0001	<0.001	<0.001	NS
	IL-8	**10.4 (1.4-205.6)**	**234.1 (19.8-1531)**	**994.1 (118.7-2500)**	**<0.0001**	**<0.01**	**<0.001**	**<0.05**
	IL-1β	0.3 (0.3-2.7)	3.3 (0.8-19.5)	3.3 (0.3-87.2)	<0.0001	0.001	0.001	NS
	GM-CSF	1.1 (0.4-1.6)	1.5 (0.3-3.2)	2.4 (0.3-11.7)	0.0004	NS	<0.001	NS
Anti-inflammatory cytokine	IL-10	0.3 (0.3-0.3)	0.3 (0.3-0.9)	0.8 (0.3-8.2)	**<0.001**	**<0.05**	**<0.001**	**<0.01**
IFN-associated/Th1-chemokines	**MIG (CXCL9)**	**2.5 (0.3-17.0)**	**51.8 (6.5-391.9)**	**233.9 (42.2-865.0)**	**<0.0001**	**<0.001**	**<0.001**	**<0.05**
	IP-10 (CXCL10)	**12.0 (4.3-60.2)**	**210.2 (26.9-1176)**	**3964 (552.5-10000)**	**<0.0001**	**<0.001**	**<0.001**	**<0.001**
	I-TAC (CXCL11)	**0.8 (0.3-3.7)**	**13.1 (1.4-52.5)**	**134.2 (19.0-485.3)**	**<0.0001**	**<0.01**	**<0.001**	**<0.001**
Th2-associated chemokines	**MDC (CCL22)**	**54.4 (54.4-140.1)**	**248.5 (132.9-382.2)**	**391.2 (185.8-767.8)**	**<0.0001**	**<0.001**	**<0.001**	**<0.05**
	TARC (CCL17)	**5.0 (5.0-5.0)**	**11.2 (5.0-24.6)**	**33.5 (17.2-58.5)**	**<0.0001**	**<0.01**	**<0.001**	**<0.001**
	Eotaxin-1 (CCL11)	**15.0 (15.0-116.9)**	**98.3 (15.0-698.8)**	**648.8 (191.8-1389)**	**<0.0001**	**<0.01**	**<0.001**	**<0.001**
	Eotaxin-3 (CCL26)	2.5 (2.5-2.5)	8.9 (2.5-49.2)	22.0 (2.5-96.3)	<0.0001	<0.001	<0.0001	NS
Other inflammatory chemokines	**MIP-1α (CCL3)**	**1.4 (1.4-3.1)**	**13.4 (1.4-41.9)**	**27.2 (6.1-151.0)**	**<0.0001**	**<0.001**	**<0.001**	**<0.05**
	MIP-1β (CCL4)	**5.3 (5.3-5.3)**	**14.6 (5.3-52.0)**	**158.2 (5.3-1581)**	**<0.0001**	**<0.01**	**<0.001**	**<0.001**
	MCP-1 (CCL2)	**1.7 (1.7-7.1)**	**25.1 (4.2-57.9)**	**113.9 (27.0-1278)**	**<0.0001**	**<0.001**	**<0.001**	**<0.001**
	MCP-4 (CCL13)	**2.9 (2.9-12.3)**	**19.0 (8.5-67.3)**	**38.2 (18.1-100.7)**	**<0.0001**	**<0.01**	**<0.001**	**<0.01**

Table 5.3 Summary statistics for 28 soluble immune mediators in T1 NPA

Samples of NPA were obtained from adult patients at T1 (2009/10 and 2010/11 seasons combined). Kruskal-Wallis (KW) one-way analysis of variance by ranks test was performed to results for adult healthy controls (HC), influenza-negative (flu-neg) and influenza-positive (flu-pos) patients. Following KW, to compare influenza-negative (flu-neg) and influenza-positive (flu-pos), Dunn's post-test was applied. Mediators highlighted in bold demonstrate significant difference between medians of the influenza-negative and influenza-positive and where at least one group's median is significantly different from that of the healthy controls. Dunn's post-test was not performed if the medians did not differ on Kruskal-Wallis testing (significance cut-off *p* 0.05). Flu negative vs. flu positive significance was not tested if both influenza-positive and influenza-negative medians did not differ from the healthy control median. NS = no sample obtained. N/A = not applicable.

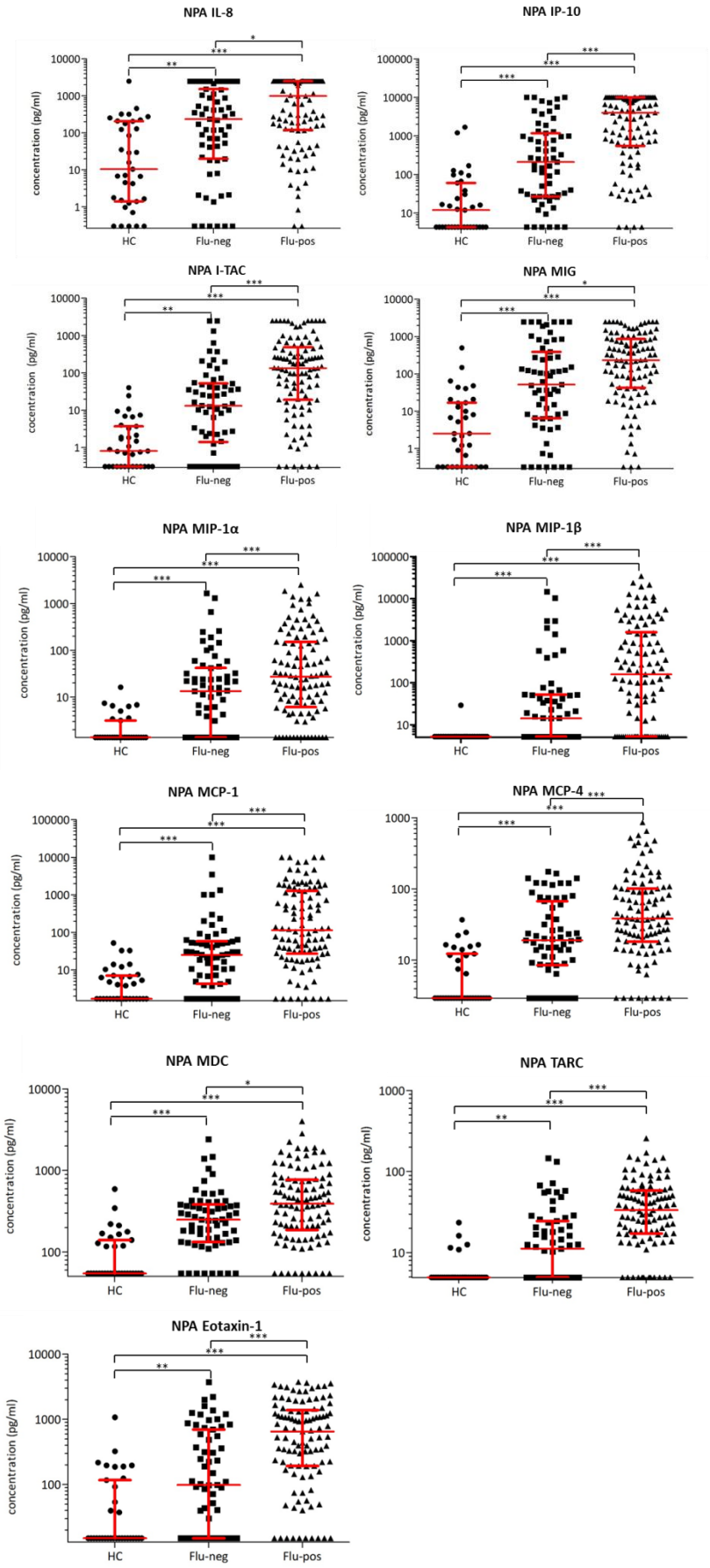


Figure 5.5 Inflammatory and interferon/Th1-associated cytokines, Th2 and other inflammatory chemokines in T1 NPA are higher in influenza-positive patients that in influenza-negative patients and healthy controls

Calculated concentrated means are plotted (log scale) and for each group the median and interquartile ranges are overlaid in red. Kruskal-Wallis test with Dunn's post-test was used to assess significance (***p<0.001; **p<0.01; *p<0.05). Sample groups: HC=healthy controls (•); Flu-neg=influenza-negative (▪); Flu-pos=influenza-positive (▲).

5.5.5 Mediators in T1 SAM eluate from influenza-positive and influenza-negative adult patients, and healthy control SAM eluate

In addition to NPA sampling, the anterior compartment of the nose was sampled using the SAM nasosorption technique. Some but not all of the mediators which were present at high levels in NPA from influenza-positive patients were also significantly higher in SAM eluates from influenza-positive patients than in SAM eluates from influenza-negative cases: IP-10, I-TAC, MDC, TARC, MIP1α and MIP1β and MCP-1 (figure 5.6). However, other mediators that followed this trend in NPA did not demonstrate the same trend in SAM eluates: MIG, eotaxin-1 and MCP-4 (table 5.4).

In contrast to levels in NPA and serum, IFN-β was detectable in a large proportion of SAM eluate samples from patients and healthy controls. Median levels were greatest in healthy controls (66.1 pg/ml), however. The median for influenza-negative patients was equivalent to the half the GM-LLOD (11.7 pg/mL), but the median for influenza-positive patients was 46.9 pg/ml. In contrast to results for NPA and serum, IFN-γ and TNF-α were elevated in SAM eluate samples from influenza-positive patients, albeit at a low level (figure 5.7).

Median levels of IL-6 were modestly increased in NPA from both influenza-negative and influenza-positive patients, and higher levels were seen in SAM eluate samples compared to NPA, with significantly higher levels seen in SAM eluate samples influenza-positive patients compared to levels in influenza-negative patients and healthy controls. Of all sample-types tested, the highest median levels of IL-8 for each group were observed in the SAM eluate samples. Again, there was a suggestion that influenza-positive patients demonstrated the greatest levels, and influenza-negative patients had lower median levels than those seen in healthy controls. However, the results were non-significant (Kruskal-Wallis p=0.902). Of note, many of the observed IL-8 values in all groups were at the upper limit of detection for the IL-8 assay. Overall, IP-10 appeared to be the most consistent and statistically significant discriminator of influenza-positive status in all sample types (figure 5.8).

	Mediator	Healthy control (n=36) Median (IQR) pg/mL	Influenza-negative (n=78) Median (IQR) pg/mL	Influenza-positive (n=133) Median (IQR) pg/mL	Kruskal-Wallis *p* value	HC vs. Flu Neg *p* value	HC vs. Flu Pos *p* value	Flu neg vs. Flu-pos *p* value
Types I and III IFNs	IFN-α2a	1.3 (1.3-1.3)	1.3 (1.3-1.3)	1.3 (1.3-1.3)	N/A	N/A	N/A	N/A
	IFN-β	**66.1 (32.0-151.5)**	**11.7 (11.7-47.6)**	**46.9 (11.7-132.0)**	**<0.0001**	**<0.001**	**NS**	**<0.01**
	IFN-λ (IL-29)	12.0 (12.0-12.0)	12.0 (12.0-12.0)	12.0 (12.0-46.5)	N/A	N/A	N/A	N/A
Th1-secreted/associated cytokines	IFN-γ	**0.3 (0.3-0.3)**	**0.3 (0.3-1.3)**	**1.5 (0.3-8.9)**	**<0.0001**	**<0.05**	**<0.001**	**<0.001**
	TNF-α	**1.3 (0.3-2.4)**	**1.5 (0.3-5.4)**	**2.9 (0.9-11.7)**	**0.0001**	**NS**	**<0.001**	**<0.05**
	IL-2	1.9 (0.6-4.5)	3.9 (0.6-12.6)	3.1 (0.6-10.8)	0.0358	<0.05	NS	NS
	IL-15	**1.2 (0.9-1.8)**	**1.3 (0.7-3.0)**	**2.0 (1.0-5.4)**	**0.0054**	**NS**	**<0.05**	**<0.05**
	IL-12p70	1.5 (0.6-2.6)	1.2 (0.5-3.3)	1.3 (0.4-3.0)	NS	N/A	N/A	N/A
Th2-secreted cytokines	IL-4	0.4 (0.4-1.0)	0.4 (0.4-0.4)	0.4 (0.4-0.4)	NS	N/A	N/A	N/A
	IL-5	1.0 (0.3-1.7)	0.7 (0.3-1.6)	0.9 (0.3-2.5)	NS	N/A	N/A	N/A
	IL-13	0.8 (0.3-1.9)	1.6 (0.3-4.6)	2.7 (0.8-6.7)	0.0010	NS	<0.001	NS
Th17-secreted cytokines	IL-17	7.1 (4.7-9.5)	5.5 (2.4-19.7)	9.4 (3.8-23.8)	NS	NS	NS	NS
Other inflammatory chemokines	**IL-6**	**12.3 (7.5-21.1)**	**41.6 (13.5-134.5)**	**118.1 (26.0-384.2)**	**<0.0001**	**<0.001**	**<0.001**	**<0.05**
	IL-8	1880 (597.2-2500)	675.8 (37.6-2500)	2436 (819.6-2500)	NS	NS	NS	NS
	IL-1β	31.1 (14.0-63.4)	28.4 (7.3-80.9)	29.3 (7.7-121.3)	NS	N/A	N/A	N/A
	GM-CSF	1.2 (0.6-2.0)	1.9 (0.9-3.7)	2.0 (0.3-9.3)	NS	N/A	N/A	N/A
Anti-inflammatory cytokine	IL-10	0.8 (0.3-1.5)	1.1 (0.3-2.6)	1.3 (0.4-3.1)	0.0037	NS	<0.01	NS
IFN-associated/Th1-chemokines	MIG (CXCL9)	212.3 (140.0-307.5)	338.5 (133.9-1803)	511.8 (153.4-1645)	0.0329	NS	<0.05	NS
	IP-10 (CXCL10)	**773.8 (462.9-1435)**	**1762 (659.4-5064)**	**4840 (1511-10000)**	**<0.0001**	**NS**	**<0.001**	**<0.001**
	I-TAC (CXCL11)	**11.65 (8.6-23.6)**	**19.2 (5.0-120.5)**	**89.5 (11.0-607.0)**	**<0.0001**	**NS**	**<0.001**	**<0.01**
Th2-associated chemokines	**MDC (CCL22)**	**115.9 (54.4-198.1)**	**127.6 (54.4-180.5)**	**158.8 (116.6-225.0)**	**0.0006**	**NS**	**<0.05**	**<0.01**
	TARC (CCL17)	**5.0 (5.0-12.8)**	**7.5 (5.0-17.2)**	**14.7 (5.0-23.4)**	**<0.0001**	**NS**	**<0.001**	**<0.05**
	Eotaxin-1 (CCL11)	138.5 (66.6-191.6)	234.8 (120.7-384.6)	242.8 (147.9-362.8)	<0.0001	<0.001	<0.001	NS
	Eotaxin-3 (CCL26)	30.9 (12.7-57.5)	45.0 (20.1-79.2)	45.1 (22.9-61.5)	NS	NS	NS	NS
Other inflammatory chemokines	**MIP-1α (CCL3)**	**8.7 (6.3-11.2)**	**20.0 (9.4-39.4)**	**29.2 (14.9-64.7)**	**<0.0001**	**<0.001**	**<0.001**	**<0.05**
	MIP-1β (CCL4)	**23.3 (5.3-61.0)**	**50.8 (24.8-155.8)**	**132.9 (36.8-445.4)**	**<0.0001**	**<0.05**	**<0.001**	**<0.01**
	MCP-1 (CCL2)	**49.2 (31.8-87.4)**	**85.8 (44.6-183.7)**	**166.0 (54.8-411.0)**	**<0.0001**	**<0.05**	**<0.001**	**<0.01**
	MCP-4 (CCL13)	12.4 (2.9-17.5)	10.8 (7.7-17.4)	12.9 (8.6-19.3)	0.3321	NS	NS	NS

Table 5.4 Summary statistics for 28 soluble immune mediators measured in T1 SAM eluates from adult patients and from healthy controls
Kruskal-Wallis test was performed, with Dunn's post-test to compare influenza-negative (flu-neg) and influenza-positive. Mediators highlighted in bold demonstrate significant differences between medians of the influenza-negative and influenza-positive and where at least one group's median is significantly different from that of the healthy controls. Dunn's post-test was not performed if the medians did not differ on Kruskal-Wallis testing (significance cut-off *p* 0.05). Flu negative vs. flu positive significance was not reported if both influenza-positive and influenza-negative medians did not differ from the healthy control median. NS = no sample obtained. N/A = not applicable.

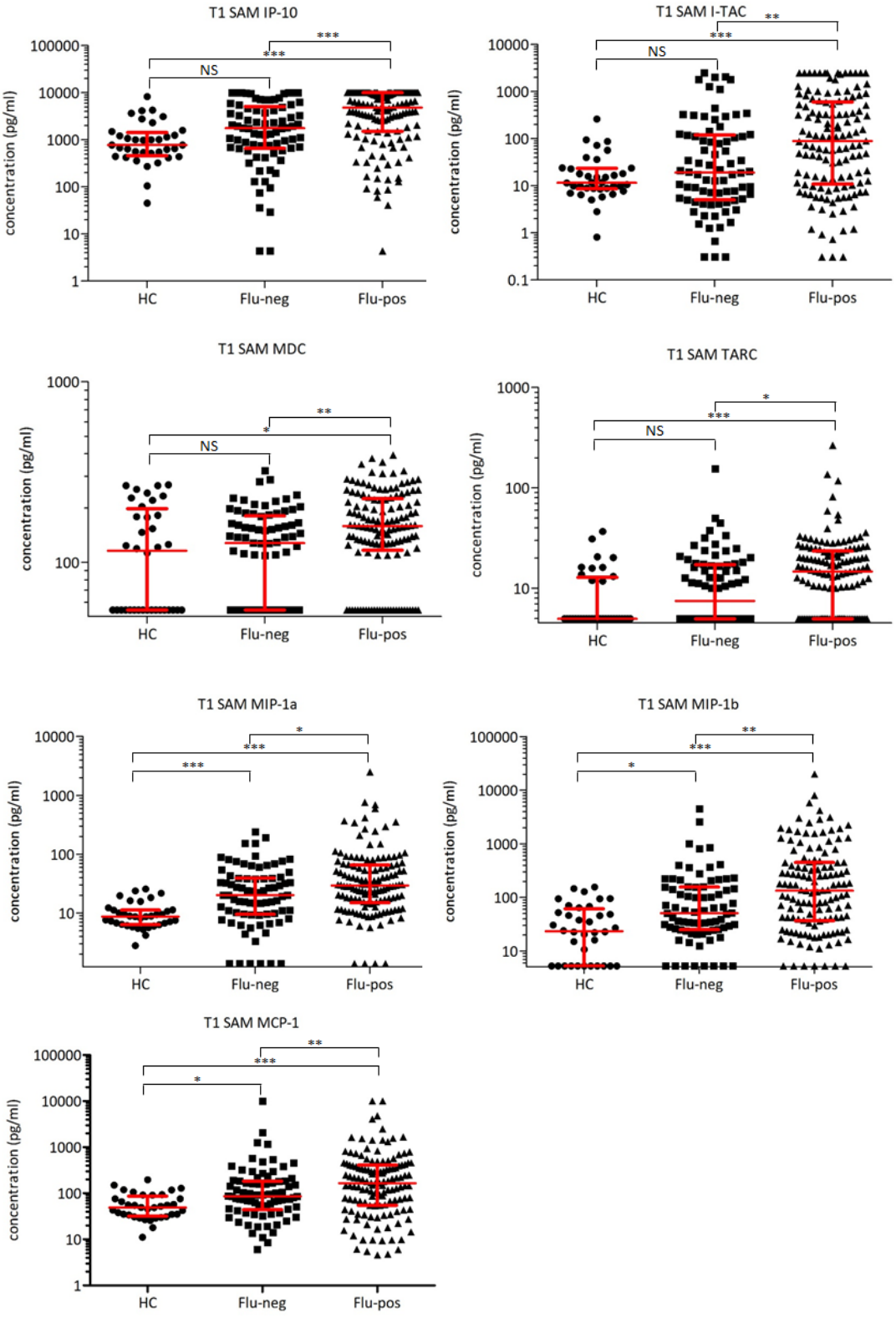


Figure 5.6 Levels of Th1, Th2 and other inflammatory chemokines in T1 NPA are higher in influenza-positive patients than in influenza-negative patients and healthy controls
Calculated concentrated means are plotted (log scale) and for each group the median and interquartile ranges are overlaid in red. Kruskal-Wallis test with Dunn's post-test was used to assess significance (***p<0.001; **p<0.01; *p<0.05; NS=non-significant). Sample groups: HC=healthy controls (•); Flu-neg=influenza-negative (▪); Flu-pos=influenza-positive (▲).

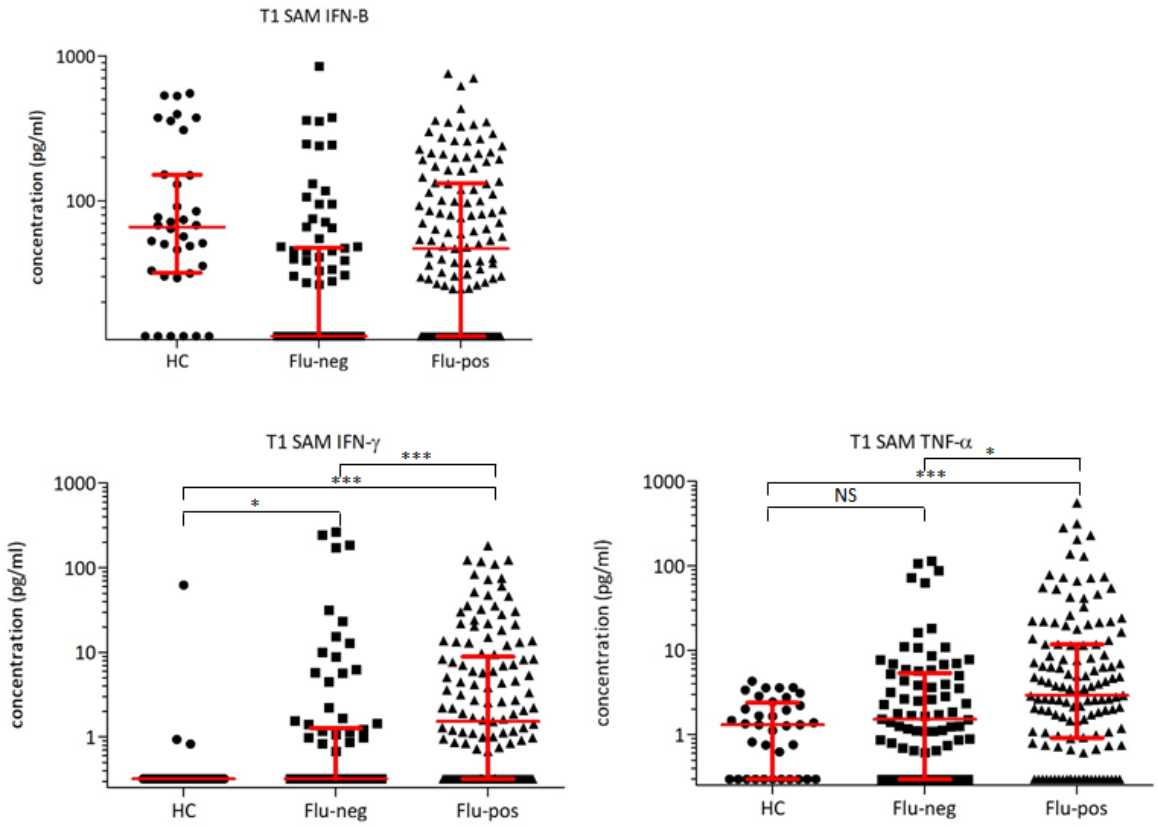


Figure 5.7 Levels of IFN-γ and TNF-α, but not IFN-β, are higher in T1 SAM from influenza-positive adults than in SAM from influenza-negative patients and healthy controls

Calculated concentrated means are plotted (log scale) and for each group the median and interquartile ranges are overlaid in red. Kruskal-Wallis test with Dunn's post-test was used to assess significance (***p<0.001; *p<0.05; NS=non-significant). Sample groups: HC=healthy controls (•); Flu-neg=influenza-negative (▪); Flu-pos=influenza-positive (▲).

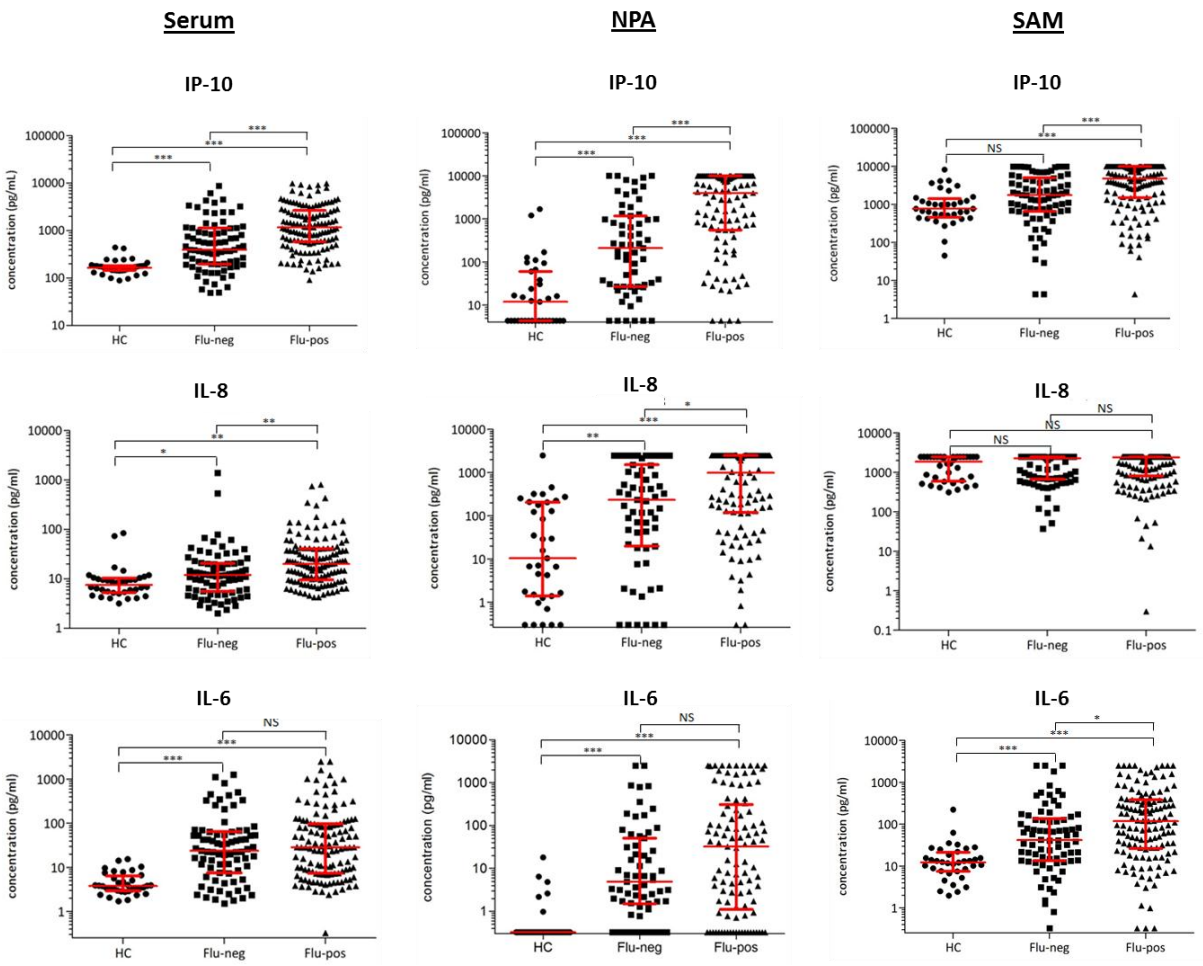


Figure 5.8 IP-10 is higher in influenza-positive patients compared to influenza-negative patients regardless of sample type

Levels of IL-6, IL-8 and IP-10 were measured in serum, NPA and SAM eluate obtained from patients at T1 and from healthy controls. Calculated concentrated means are plotted (log scale) and for each group the median and interquartile ranges are overlaid in red. Kruskal-Wallis test with Dunn's post-test was used to assess significance (***p<0.001; **p<0.01; *p<0.05; NS=non-significant). Sample groups: HC=healthy controls (•); Flu-neg=influenza-negative (▪); Flu-pos=influenza-positive (▲).

5.6 ELISA results for RANTES, neopterin, sTNFR1 and sTNFR2 in T1 serum from influenza-positive and influenza-negative patients, and healthy controls

In T1 serum, neopterin was significantly higher in influenza-positive patients than in influenza-negative patients and healthy controls. RANTES was significantly elevated in influenza-negative patients, but not in influenza-positive patients. TNFR1 and TNFR2 were elevated to the same degree in both influenza-negative and influenza-positive patients (figure 5.9).

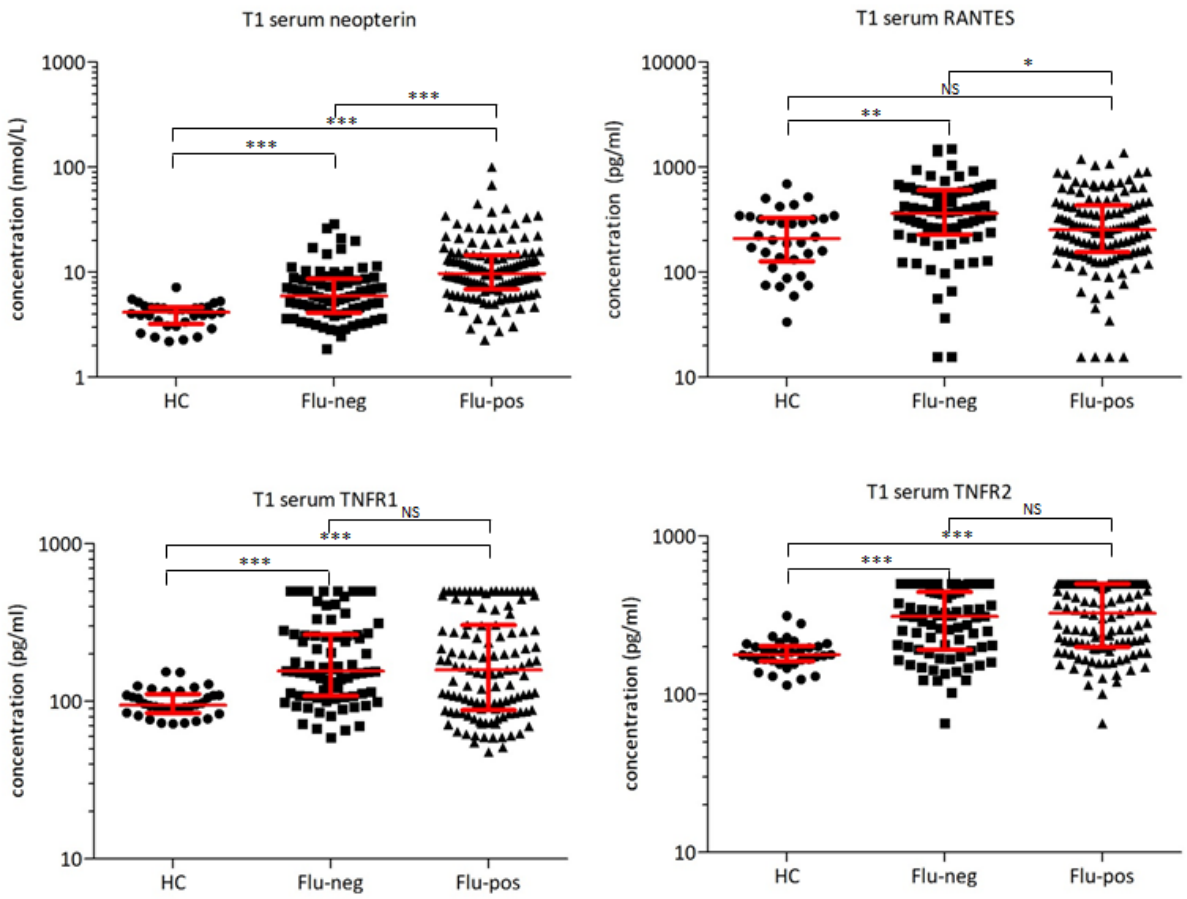


Figure 5.9 Levels of T1 serum neopterin, but not RANTES, TNFR1 or TNFR2, are higher in influenza-positive patients than in T1 serum form influenza-negative patients and serum from healthy controls

ELISA assays were performed to quantify each mediator. Calculated concentrated means are plotted (log scale) and for each group the median and interquartile ranges are overlaid in red. Kruskal-Wallis test with Dunn's post-test was used to assess significance (***p<0.001; *p<0.05; NS=non-significant). Sample groups: HC=healthy controls (•); Flu-neg=influenza-negative (▪); Flu-pos=influenza-positive (▲).

5.7 Investigation of soluble immune mediator trends in influenza-negative and influenza-positive patients using hierarchical clustering

The pooled analyses comparing patients based on their influenza infection status suggested that many mediators are detectable in a proportion of patients, but there are others who have very low or undetectable levels. Traditional approaches to data analysis and presentation make it difficult to identify such sub-populations. There were a large number of mediators measured in each sample, and given the different sample types and the number of clinical variables that may be used to investigate the mediator data, a hierarchical clustering approach was utilised subsequently, to help identify soluble mediator patterns and trends.

Following application of limits of detection (as described in section 5.3.1), data obtained by MSD assays were imported into GeneSpring and log-transformed, before being converted into Z scores. Baseline transformation and normalisation were not required. Non-parametric statistical filtering was then applied (Kruskal Wallis or Mann Whitney unpaired tests against healthy controls, as appropriate), with Benjamini and Hochberg FDR multiple testing correction, and corrected *p* value cut-off = 0.05. Following hierarchical clustering using Pearson's uncentered (cosine) method with the average linkage rule, heat-maps were generated. Clustering on both mediators and patient groups provided an semi-supervised assessment of relationships e.g. when assessing the relationship between mediator levels and the severity of illness grade.

5.7.1 T1 serum mediators hierarchical clustering results

Hierarchical clustering of serum results using influenza infection-status as the interpretation (influenza-positive, influenza-negative and healthy controls) and *without* statistical filtering revealed that 31/36 healthy controls formed a consecutive cluster and have similar levels of the 28 mediators that were measured. Based on major branch division, two major mediator groups can be seen. Firstly, a group where levels tend to be low in the majority of healthy control samples but that are generally higher in large proportion of patients (IL-10, IL-12, IL-5, MIP-1α, IL-15, IL-17, IL-6, TNF-α, IL-8, MCP-1, MIG, IFN-γ, IP-10, IL-13) and a group where levels are generally similar or lower in patients compared with healthy controls (IL-4, GM-CSF, IL-1β, IFN-β, eotaxin-3, IL-2, IFNa2a, IFN-λ (IL-29), MIP-1β, MDC, TARC, eotaxin-1, MCP-4). Although these results were not statistically filtered, there was a suggestion that combinations of certain mediators – both relatively increased and relatively decreased – may associate with specific groups of patients, but there was also a suggestion that this may not be explained by influenza infection status, Furthermore, sub-groups of patients were apparent, for example, a group with marked relative increases in IL-10, IL12p70 and IL-5 (figure 5.10)

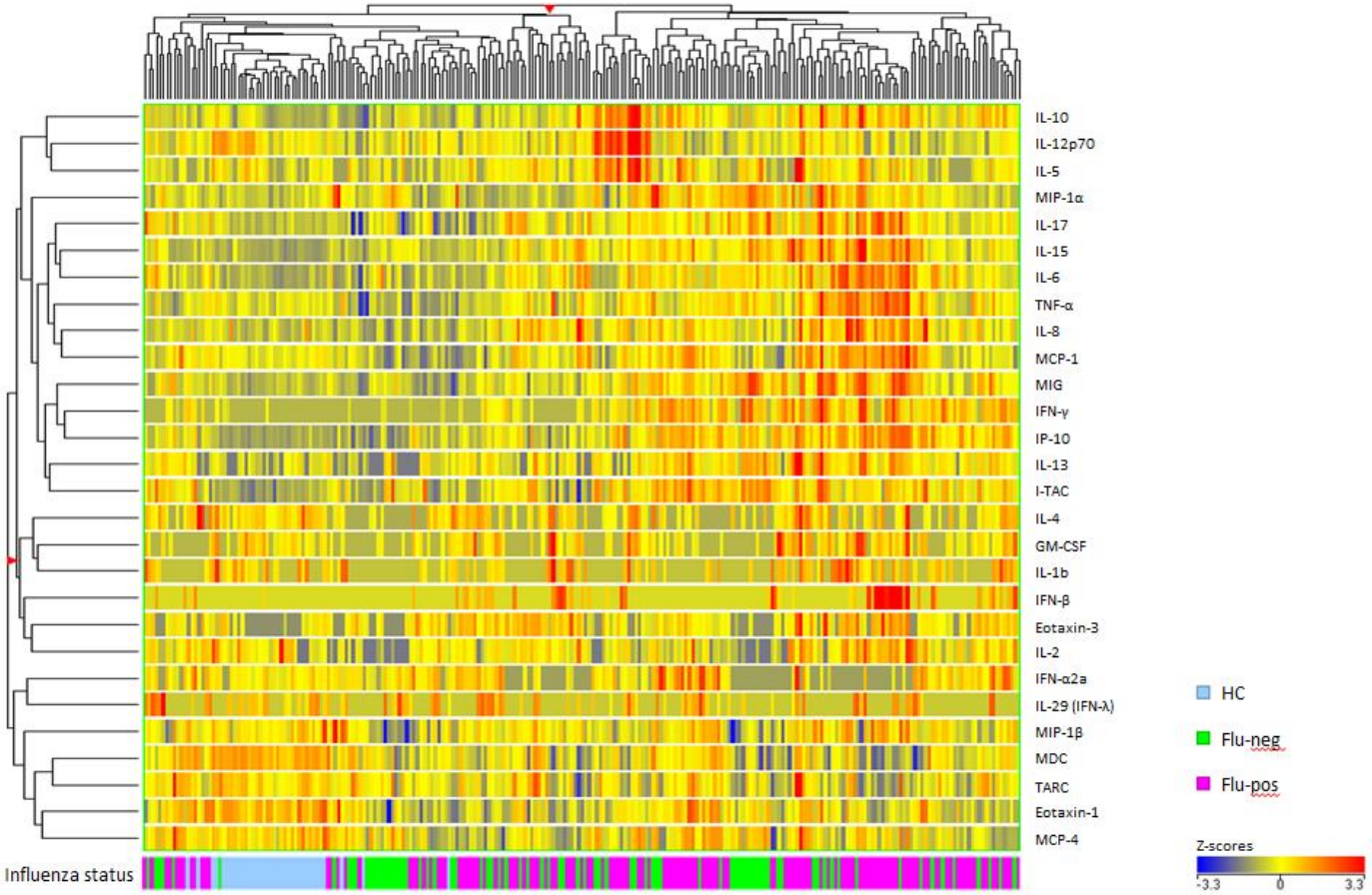


Figure 5.10 Hierarchical clustering analysis of 28 mediators measured by MSD in serum without statistical filtering

For each mediator, values were log-transformed and then transformed to Z-scores. Statistical filtering and multiple testing correction was not performed. Hierarchical clustering (on entities and influenza status) was performed using Pearson's uncentered (cosine) method with the average linkage rule and a heat-map was generated. Z-scores are represented by colour intensity spectrum (lowest z-score is dark blue, zero value is yellow, highest z-score is red). Sample groups: HC = healthy controls (pale blue); Flu-neg = influenza-negative patients (green); Flu-pos = influenza-positive patients (pink).

The same T1 serum results were then subjected to statistical filtering. Four mediators did not reach significance (IL-29, IL-1β, IL-5 and TNF-α), leaving 24 mediators for clustering analysis. Again, the majority of the healthy controls clustered together. There was a suggestion of sub-groups of patients with similar mediator profiles, but these sub-groups contained both influenza-positive and influenza-negative patients. This suggests that certain mediator responses are not specific to influenza and may reflect responses that are common to hospitalised cases of influenza-like illness *per se*. However, it can be seen that one subgroup appears to be defined by most patients having relatively high levels of certain mediators (IL-8, MCP-1, MIG, IFN-γ, IP-10, IL-17, IL-15, IL-6, MIP-1α, IL-13, eotaxin- 3 and IL-2) and relatively low levels of others (MDC, TARC and IFN-α2a). A proportion of patients within this latter group also had high levels of IFN-β. There was another sub-group of patients with high IFN-β, but these patients do not have marked elevations of the mediators that were seen in the other group with raised IFN-β. Additionally, they did not have such marked relative depression of MDC or TARC, but did appear to have marked relative depression of eotaxin-1 and MCP-4. Another subgroup of patients, who were predominantly influenza-positive, appeared to have marked elevation of IFN-α2a, eotaxin-1 and I-TAC, and more modest elevation of MCP-4, IFN-γ and IP-10 (figure 5.11).

Some mediators that are related by their immunological functions generally followed the same patterns of relative elevation or depression. For example, increases in IFN-γ were accompanied by increases in the IFN-associated chemokines IP-10 and MIG in most patients. In contrast, the most marked increases in each of the type I interferons (IFN-α2a and IFN-β) did not appear to occur in the same patients (figure 5.11).

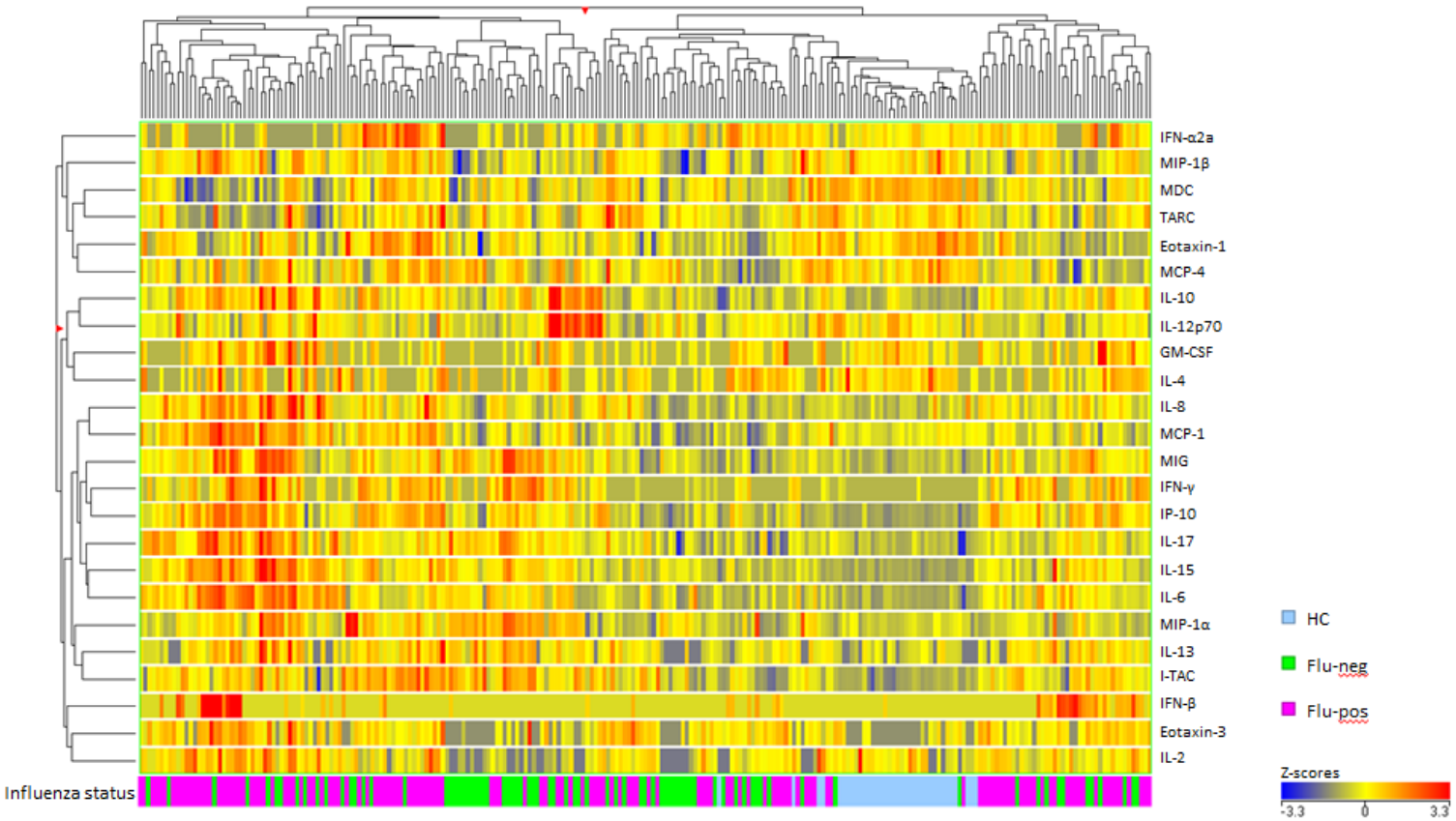


Figure 5.11 Hierarchical clustering analysis of 24 mediators in T1 serum following statistical filtering

For each mediator, values were log-transformed and then transformed to Z-scores. Results for 28 mediators measured by MSD were subjected to statistial filtering and multiple testing correction (Kruskal Wallis test with Benjamini and Hochberg FDR, correct *p* value cut-off 0.05). Differences in IL-29, IL-1β, IL-5 and TNF-α did not meet the statistical filtering cut-off. Hierarchical clustering (on entities and influenza status) was performed on the remaining 24 mediator results using Pearson's uncentered (cosine) method with the average linkage rule, and a heat-map was generated. Z-scores are represented by a colour intensity spectrum (lowest z-score is dark blue, zero value is yellow, highest z-score is red). Sample groups: HC = healthy controls (pale blue); Flu-neg = influenza-negative patients (green); Flu-pos = influenza-positive patients (pink).

5.7.2 T1 NPA mediators hierarchical clustering results

For T1 NPA samples, 25 mediators remained following statistical filtering: IL-4, IL-2, IFN-γ, IL-13, IL-15, TARC, IL-8, MIG, I-TAC, IP-10, GM-CSF, TNF-α, IL-10, IL-1β, IL-12p70, IL-5, eotaxin-3, IL-6, MCP-4, MDC, IL-17, MIP-1α, eotaxin, MCP-1 and MIP-1β. Differences in the levels of IFN-α2a, IFN-β and IFN-λ did not reach statistical significance. Levels of all mediators were low in almost all of the samples obtained from healthy controls, which tended to cluster together. The patients appeared to divide into two broad groups, based on the major division of the horizontal dendrogram: those with levels of mediators that were as low as those seen in the patients or were not markedly elevated, and those with more marked elevation of all mediators in the majority of patients within that group. The latter group – especially those with the highest levels – tended to be influenza-positive. Within this group, there was a sub-cluster with marked elevation of IFN-γ and elevation of IL-13 and IL-15 in some, but not all. Overall, the majority of patients with higher levels of all mediators appeared to be influenza-positive (figure 5.12).

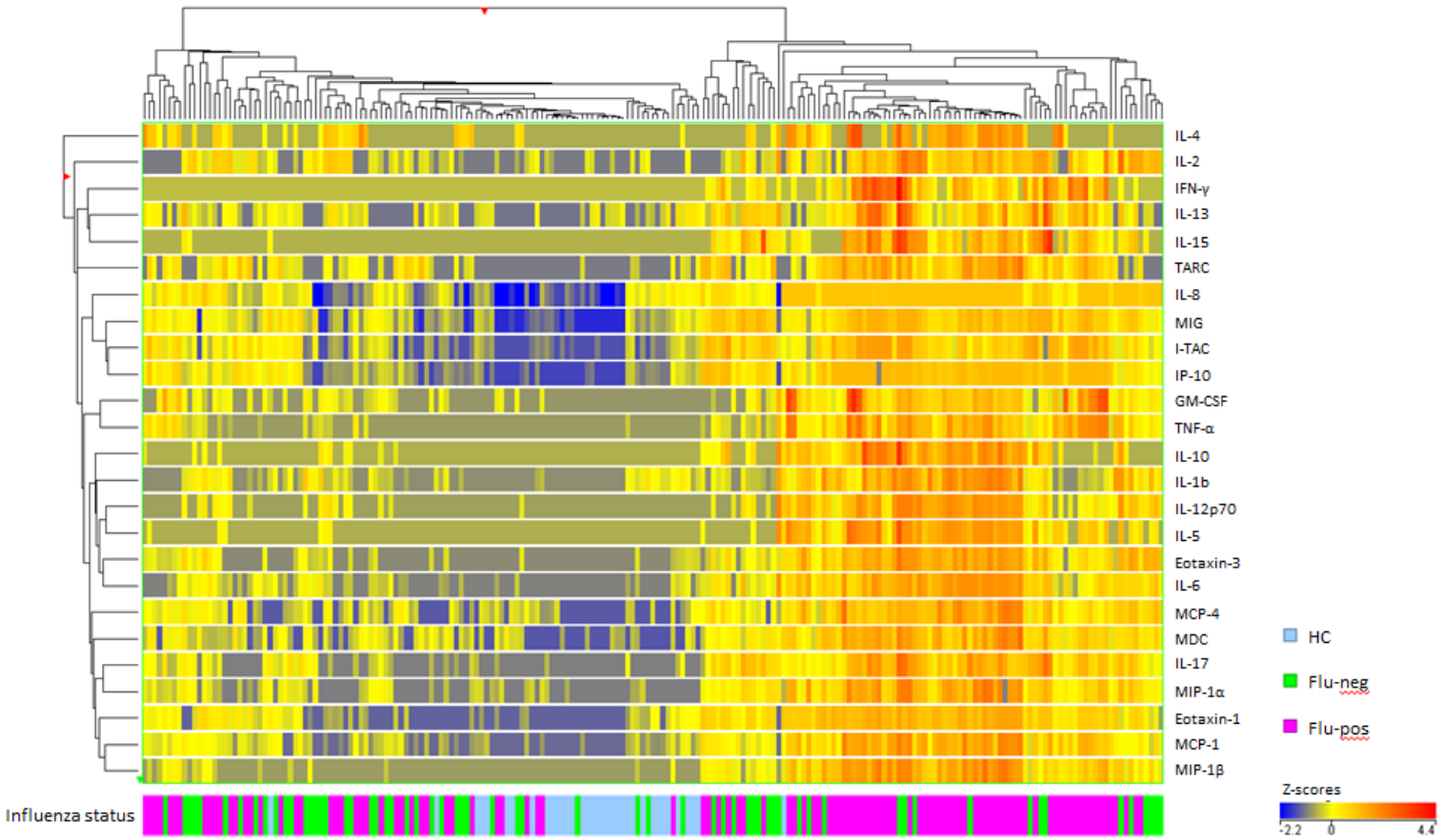


Figure 5.12 Hierarchical clustering analysis of 25 mediators in T1 NPA

For each mediator, values were log-transformed and then transformed to Z-scores. Results for 28 mediators measured by MSD were subjected to statistial filtering and multiple testing correction (Kruskal Wallis test with Benjamini and Hochberg FDR, correct *p* value cut-off 0.05). Differences in IFN-α2a, IFN-β and IFN-λ did not meet the statistical filtering cut-off. Hierarchical clustering (on entities and influenza status) was performed on the remaining 24 mediator results using Pearson's uncentered (cosine) method with the average linkage rule, and a heat-map was generated. Z-scores are represented by a colour intensity spectrum (lowest z-score is dark blue, zero value is yellow, highest z-score is red). Sample groups: HC = healthy controls (pale blue); Flu-neg = influenza-negative patients (green); Flu-pos = influenza-positive patients (pink).

5.7.3 T1 SAM mediators hierarchical clustering results

For the T1 SAM eluate samples, 16 mediators remained following statistical filtering: IFNβ, IL-29, MDC, eotaxin-1, MIP-1α, MCP-1, IL-10, IL-6, MIP-1b, TNF-α, TARC, IL-13, IFN-γ, IL-15, I-TAC, and IP-10. The following mediators did not reach significance: IFNα2a, IL-17, MIG, eotaxin-3, IL-8, MCP-4, GM-CSF, IL-12p70, IL-1β, IL-2, IL-4 and IL-5. Relative to values for patients, healthy control values were generally low for all mediators, although there was more variability than was observed for NPA and serum obtained from healthy controls. Additionally healthy controls did not cluster together, being interspersed with patients who also tended to have low levels of most mediators; this formed one major group based on similarity of mediator profiles. The second major group mainly consisted of patients, both influenza-positive and influenza-negative, with a small number of healthy controls. Most patients within this group tended to have relatively higher levels of most of the mediators (figure 5.13).

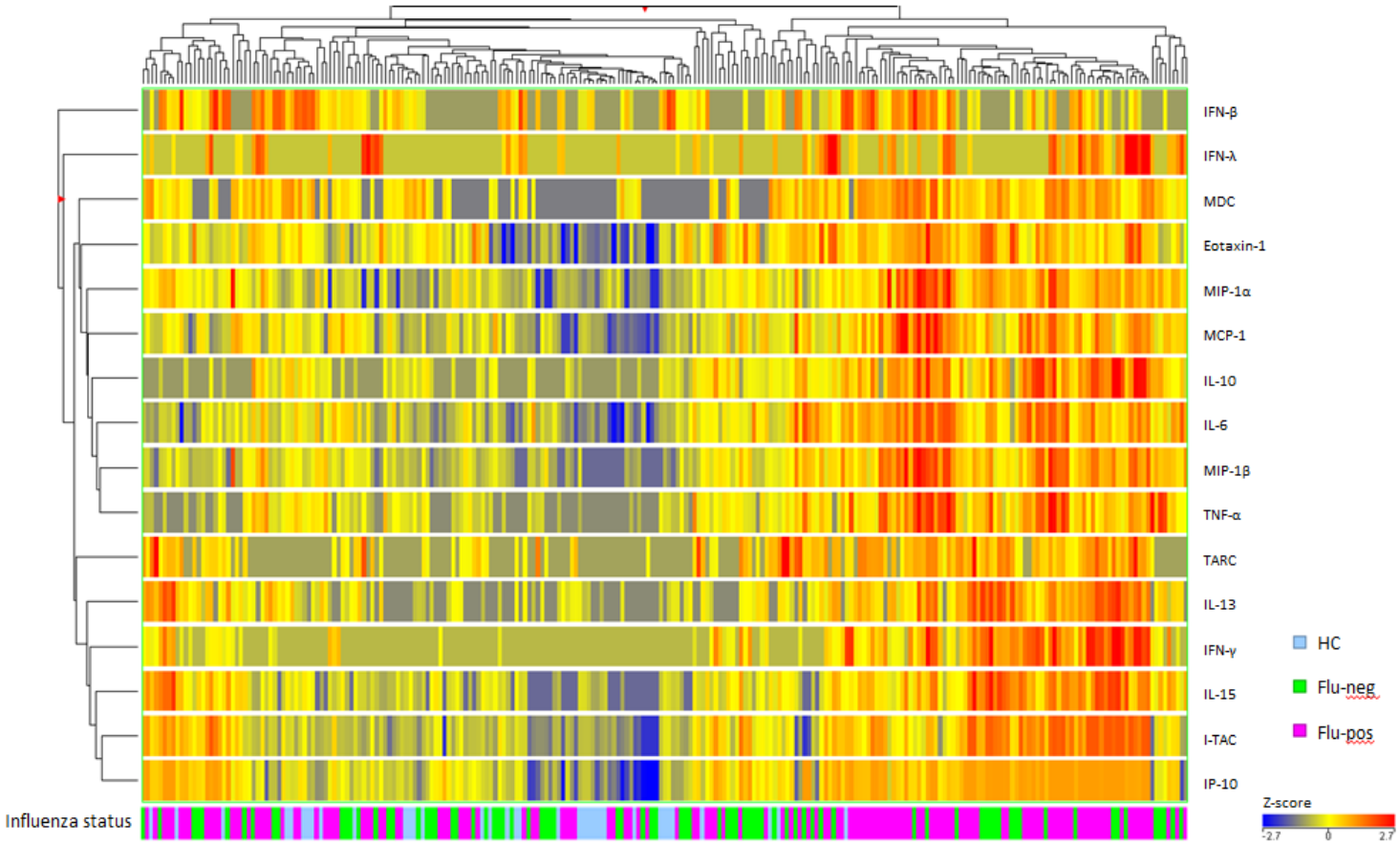


Figure 5.13 Hierarchical clustering analysis of 25 mediators in T1 SAM eluate

For each mediator, values were log-transformed and then transformed to Z-scores. Results for 28 mediators measured by MSD were subjected to statistial filtering and multiple testing correction (Kruskal Wallis test with Benjamini and Hochberg FDR, correct *p* value cut-off 0.05). Differences in IFNα2a, IL-17, MIG, eotaxin-3, IL-8, MCP-4, GM-CSF, IL-12p70, IL-1β, IL-2, IL-4 and IL-5 did not meet the statistical filtering cut-off. Hierarchical clustering (on entities and influenza status) was performed on the remaining 16 mediator results using Pearson's uncentered (cosine) method with the average linkage rule, and a heat-map was generated. Z-scores are represented by a colour intensity spectrum (lowest z-score is dark blue, zero value is yellow, highest z-score is red). Sample groups: HC = healthy controls (pale blue); Flu-neg = influenza-negative patients (green); Flu-pos = influenza-positive patients (pink).

5.8 Comparison of soluble immune mediator results in T1 samples from influenza-positive patients with differing severities of illness

5.8.1 Approach to analysis of MSD results

MSD results for influenza-positive adult patients from both seasons were combined for each sample type obtained at T1 (serum, NPA and SAM eluate). Patients were divided according to their severity score at T1 and differences in median values were compared, using Kruskal-Wallis test to measure statistical significance. If median values differed then Dunn's post-test was also applied to compare conditions. Results for mediators grouped according to major immunological function are shown in tables 5.5, 5.6 and 5.7.

	Mediator	Healthy control (n=36) Median (IQR) pg/mL	Influenza-positive Severity 1 (n=58) Median (IQR) pg/mL	Influenza-positive Severity 2 (43) Median (IQR) pg/mL	Influenza-positive Severity 3 (31) Median (IQR) pg/mL	Kruskal-Wallis *p* value	Severity 1 vs. 2 *p* value	Severity 1 vs. 3 *p* value	Severity 2 vs. 3 *p* value	Severity group with exclusive increase over HC (*p* value)
Types I and III IFNs	**IFN-α2a**	**5.3 (3.5-6.2)**	**8.0 (4.2-14.6)**	**6.8 (3.9-11.0)**	**1.3 (1.3-3.6)**	**<0.0001**	**NS**	**<0.001**	**<0.001**	-
	IFN-β	**11.7 (11.7-11.7)**	**11.7 (11.7-11.7)**	**11.7 (11.7-14.9)**	**60.11 (11.7-260.5)**	**0.0006**	**NS**	**<0.001**	**<0.001**	**Severity 3 (<0.001)**
	IFN-λ (IL-29)	26.8 (12.0-42.2)	12.0 (12.0-39.5)	12.0 (12.0-58.2)	12.0 (12.0-39.7)	0.5343	N/A	N/A	N/A	-
Th1-secreted/associated cytokines	IFN-γ	0.3 (0.3-0.3)	2.1 (0.3-4.8)	2.4 (0.3-9.0)	2.1 (0.3-5.7)	<0.0001	NS	NS	NS	-
	TNF-α	**5.2 (4.6-5.9)**	**5.2 (4.2-6.9)**	**5.5 (4.2-8.0)**	**10.1 (6.4-19.0)**	**<0.0001**	**NS**	**<0.001**	**<0.01**	**Severity 3 (<0.001)**
	IL-2	4.7 (2.8-7.7)	5.0 (2.7-6.5)	6.1 (2.7-9.5)	8.4 (5.0-17.9)	0.0024	NS	**<0.01**	NS	**Severity 3 (<0.01)**
	IL-15	**1.6 (1.5-1.9)**	**3.2 (2.2-4.8)**	**4.5 (2.8-7.5)**	**8.3 (6.3-10.9)**	**<0.0001**	**NS**	**<0.001**	**<0.05**	-
	IL-12p70	4.0 (1.9-9.1)	2.1 (1.1-4.4)	1.6 (0.9-4.7)	2.1 (0.9-4.7)	0.0319	NS	NS	NS	-
Th2-secreted cytokines	IL-4	1.7 (1.2 – 2.0)	1.0 (0.4-1.8)	1.0 (0.4-2.2)	1.0 (0.4-2.0)	0.0105	NS	NS	NS	-
	IL-5	1.1 (0.6-0.7)	1.2 (0.7-2.6)	1.1 (0.3-2.3)	1.6 (0.7-3.7)	NS	N/A	N/A	N/A	-
	IL-13	1.2 (0.4-1.9)	2.8 (1.5-4.1)	2.7 (1.2-6.0)	2.7 (1.5-5.0)	<0.0001	NS	NS	NS	-
Th17-secreted cytokines	**IL-17**	**3.2 (2.2-3.8)**	**6.1 (3.0-8.6)**	**5.9 (4.1-16.8)**	**18.4 (8.2-41.2)**	**<0.0001**	**NS**	**<0.001**	**<0.05**	-
Other inflammatory cytokines	**IL-6**	**3.8 (2.9-6.4)**	**9.0 (4.6-31.6)**	**28.7 (8.9-82.0)**	**125.3 (44.6-343.6)**	**<0.0001**	**<0.05**	**<0.001**	**<0.05**	-
	IL-8	**7.6 (5.3-10.3)**	**13.4 (7.5-22.9)**	**17.8 (8.9-27.3)**	**41.9 (27.5-110.2)**	**<0.0001**	**NS**	**<0.001**	**<0.001**	-
	IL-1β	0.8 (0.3-1.2)	0.3 (0.3-1.0)	0.3 (0.3-1.3)	0.7 (0.3-1.7)	NS	N/A	N/A	N/A	-
	GM-CSF	**1.1 (0.4-1.6)**	**0.3 (0.3-1.1)**	**0.8 (0.3-1.7)**	**1.7 (0.3-3.3)**	**0.0016**	**NS**	**<0.001**	**NS**	-
Anti-inflammatory cytokine	**IL-10**	**1.5 (1.1-2.8)**	**3.9 (2.3-6.7)**	**6.4 (3.6-19.0)**	**10.8 (7.5-19.7)**	**<0.0001**	**NS**	**<0.001**	**NS**	-
IFN-associated/Th1-attracting chemokines	**MIG (CXCL9)**	**44.6 (33.2-57.7)**	**78.6 (52.5-139.0)**	**125.6 (61.4-236.7)**	**241.0 (102.4-466.5)**	**<0.0001**	**NS**	**<0.001**	**NS**	-
	IP-10 (CXCL10)	**167.1 (145.1-183.3)**	**930.1 (349.7-1793)**	**1234 (589.6-3720)**	**2116 (962.4-4422)**	**<0.0001**	**NS**	**<0.05**	**NS**	-
	I-TAC (CXCL11)	60.98 (53.44-74.34)	232.0 (124.2-456.1)	201.2 (137.2-328.0)	310.7 (169.2-461.2)	<0.0001	NS	NS	NS	-
Th2-associated chemokines	MDC (CCL22)	4011 (3197-4584)	2112 (1267-2870)	1706 (1145-2352)	971.3 (546.0-1488)	<0.0001	NS	**<0.001**	NS	-
	TARC (CCL17)	412.8 (297.2-660.3)	299.34 (175.9-588.7)	283.0 (177.4-452.5)	93.82 (63.0-207.4)	<0.0001	**NS**	**<0.001**	**<0.01**	-
	Eotaxin-1 (CCL11)	1251 (729-1535)	626.1 (340.4-957.6)	681.1 (448.1-1164)	483.9 (350.7-750.8)	<0.0001	NS	NS	NS	-
	Eotaxin-3 (CCL26)	2.5 (2.5-11.07)	17.1 (9.9-28.8)	14.6 (10.0-32.0)	27.7 (11.5-46.8)	<0.0001	NS	NS	NS	-
Other inflammatory chemokines	MIP-1α (CCL3)	11 (9.3-14)	13.1 (8.6-21.0)	13.8 (9.8-32.6)	16.1 (10.8-25.1)	0.0203	NS	NS	NS	-
	MIP-1β (CCL4)	165.3 (130.5-236.2)	119.8 (92.1-191.9)	136.3 (99.5-223.8)	143.2 (111.1-215.1)	0.0327	NS	NS	NS	-
	MCP-1 (CCL2)	**415.8 (320-502.6)**	**419.4 (209.1-560.0)**	**547.0 (286.9-1213)**	**1104 (660.4-1931)**	**<0.0001**	**NS**	**<0.001**	**<0.05**	**Severity 3 (<0.001)**
	MCP-4 (CCL13)	641 (439.5-797.2)	367.9 (215.6-636.1)	425.7 (293.7-687.8)	381.2 (205.3-552.1)	0.0022	NS	NS	NS	-

Table 5.5 Summary statistics for 28 soluble immune mediators measured in T1 serum obtained from influenza-positive patients of differing severities of illness, and from healthy controls

Kruskal-Wallis test was performed to compare results from healthy controls (HCs), grade 1 severity, grade 2 severity, and grade 3 severity influenza-positive adult patients. Dunn's post-test was used to compare severity pairs with each other (severity 1 vs. 2, 1 vs. 3, and 2 vs. 3) and each severity group with HCs (increases in a single severity group over HCs are highlighted in the final column). Mediators highlighted in bold demonstrate significant differences severity 3 and at least one other severity group and where at least one group's median was significantly different from that of the healthy controls. Dunn's post-test was not performed if the medians did not differ on Kruskal-Wallis testing (significance cut-off *p* 0.05). NS = no sample obtained. N/A = not applicable.

	Mediator	Healthy control (n=35) Median (IQR) pg/mL	Influenza-positive Severity 1 (n=50) Median (IQR) pg/mL	Influenza-positive Severity 2 (n=32) Median (IQR) pg/mL	Influenza-positive Severity 3 (n=27) Median (IQR) pg/mL	Kruskal-Wallis *p* value	Severity 1 vs. 2 *p* value	Severity 1 vs. 3 *p* value	Severity 2 vs. 3 *p* value	Severity group with exclusive increase over HC (*p* value)
Types I and III IFNs	IFN-α2a	1.3 (1.3-1.3)	1.3 (1.3-5.2)	1.3 (1.3-1.3)	1.3 (1.3-3.1)	N/A	N/A	N/A	N/A	-
	IFN-β	11.7 (11.7-60.1)	11.7 (11.7-29.2)	11.7 (11.7-68.0)	121.8 (11.7-304.9)	N/A	N/A	N/A	N/A	**Severity 3 (<0.01)**
	IFN-λ (IL-29)	12.0 (12.0-12.0)	12.0 (12.0-18.8)	12.0 (12.0-12.0)	12.0 (12.0-12.0)	N/A	N/A	N/A	N/A	
Th1-secreted/associated cytokines	IFN-γ	0.3 (0.3-0.3)	0.3 (0.3-6.2)	0.3 (0.3-2.0)	1.1 (0.3-5.8)	<0.0001	NS	NS	<0.05	-
	TNF-α	**0.3 (0.3-0.3)**	**1.5 (0.3-21.6)**	**3.6 (0.3-26.2)**	**50.9 (23.5-320.1)**	**<0.0001**	**NS**	**<0.001**	**<0.01**	-
	IL-2	**0.6 (0.6-1.8)**	**2.9 (0.6-20.3)**	**4.2 (0.6-15.6)**	**28.0 (13.1-40.5)**	**<0.0001**	**NS**	**<0.001**	**<0.01**	-
	IL-15	**0.3 (0.3-0.3)**	**0.3 (0.3-1.6)**	**0.3 (0.3-1.4)**	**2.6 (1.3-5.2)**	**<0.0001**	**NS**	**<0.001**	**<0.001**	-
	IL-12p70	**0.3 (0.3-0.3)**	**0.5 (0.3-1.8)**	**0.7 (0.3-1.7)**	**10.8 (4.9-12.2)**	**<0.0001**	**NS**	**<0.001**	**<0.001**	-
Th2-secreted cytokines	**IL-4**	**0.4 (0.4-0.4)**	**0.4 (0.4-2.3)**	**0.4 (0.4-1.6)**	**25.7 (2.1-7.6)**	**<0.0001**	**NS**	**<0.001**	**<0.001**	-
	IL-5	**0.3 (0.3-0.3)**	**0.3 (0.3-1.6)**	**0.3 (0.3-0.9)**	**5.8 (4.5-6.8)**	**<0.0001**	**NS**	**<0.001**	**<0.001**	-
	IL-13	0.8 (0.3-1.3)	1.8 (0.7-4.7)	1.2 (0.4-3.3)	3.0 (1.3-4.8)	<0.0001	NS	NS	NS	-
Th17-secreted cytokines	**IL-17**	**2.2 (0.3-20.9)**	**3.4 (0.3-9.5)**	**19.3 (7.6-36.0)**	**0.3 (0.3-0.3)**	**<0.0001**	**<0.01**	**<0.001**	**<0.001**	-
Other inflammatory cytokines	**IL-6**	**0.3 (0.3-0.3)**	**4.8 (0.3-101.5)**	**4.9 (0.4-62.7)**	**1552 (75.5-2500)**	**<0.0001**	**NS**	**<0.001**	**<0.001**	-
	IL-8	**10.4 (1.4-205.6)**	**196.1 (23.6-2431)**	**284.8 (40.2-1800)**	**2500 (2500-2500)**	**<0.0001**	**NS**	**<0.001**	**<0.01**	-
	IL-1β	**0.3 (0.3-2.7)**	**1.0 (0.3-12.4)**	**1.3 (0.3-24.2)**	**352.1 (425.1-1107)**	**<0.0001**	**NS**	**<0.001**	**<0.001**	**Severity 3 (<0.001)**
	GM-CSF	**1.1 (0.4-1.6)**	**0.3 (0.3-1.1)**	**1.8 (0.3-1.7)**	**1.7 (0.3-3.3)**	**0.0016**	**NS**	**<0.001**	**NS**	-
Anti-inflammatory cytokine	**IL-10**	**0.3 (0.3-0.3)**	**0.3 (0.3-4.1)**	**0.3 (0.3-1.4)**	**11.6 (5.8-18.6)**	**<0.0001**	**NS**	**<0.001**	**<0.001**	-
IFN-associated/Th1-attracting chemokines	**MIG (CXCL9)**	**2.5 (0.3-17.0)**	**96.6 (16.5-650.6)**	**168.4 (32.5-478.3)**	**747.9 (243.4-1276)**	**<0.0001**	**NS**	**<0.05**	**NS**	-
	IP-10 (CXCL10)	12.0 (4.3-60.2)	1426 (84.8-9683)	1141 (429.0-7433)	8694 (4257-10000)	<0.0001	NS	NS	NS	-
	I-TAC (CXCL11)	0.8 (0.3-3.7)	105.4 (3.6-944.7)	82.2 (17.0-442.9)	175.9 (38.9-268.8)	<0.0001	NS	NS	NS	-
Th2-associated chemokines	**MDC (CCL22)**	**54.4 (54.4-140.1)**	**251.5 (128.0-497.0)**	**291.8 (158.9-464.0)**	**786.0 (577.2-1707)**	**<0.0001**	**NS**	**<0.001**	**<0.01**	-
	TARC (CCL17)	5.0 (5.0-5.0)	24.8 (13.5-58.1)	26.8 (16.9-42.8)	50.7 (32.6-89.6)	<0.0001	NS	NS	NS	-
	Eotaxin-1 (CCL11)	**15.0 (15.0-116.9)**	**326.4 (15.0-117.0)**	**344.0 (93.9-1003)**	**1333 (1071-2996)**	**<0.0001**	**NS**	**<0.001**	**<0.01**	-
	Eotaxin-3 (CCL26)	**2.5 (2.5-2.5)**	**10.5 (2.5-49.1)**	**8.2 (2.5-29.9)**	**136.1 (58.8-214.1)**	**<0.0001**	**NS**	**<0.001**	**<0.001**	-
Other inflammatory chemokines	**MIP-1α (CCL3)**	**1.4 (1.4-3.1)**	**10.8 (3.0-36.4)**	**17.4 (4.7-48.6)**	**285.8 (139.7-780.4)**	**<0.0001**	**NS**	**<0.001**	**<0.001**	-
	MIP-1β (CCL4)	**5.3 (5.3-5.3)**	**48.8 (5.3-325.9)**	**37.7 (5.3-320.2)**	**3088 (698.4-6609)**	**<0.0001**	**NS**	**<0.001**	**<0.001**	-
	MCP-1 (CCL2)	**1.7 (1.7-7.1)**	**38.9 (9.0-299.7)**	**52.4 (20.8-208.5)**	**1858 (828.3-4706)**	**<0.0001**	**NS**	**<0.001**	**<0.001**	-
	MCP-4 (CCL13)	**2.9 (2.9-12.3)**	**23.2 (13.9-65.3)**	**33.2 (12.7-57.7)**	**170 (89.9-424.7)**	**<0.0001**	**NS**	**<0.001**	**<0.001**	-

Table 5.6 Summary statistics for 28 soluble immune mediators measured in T1 NPA obtained from influenza-positive patients of differing severities of illness, and from healthy controls

Kruskal-Wallis test was performed to compare results from healthy controls, grade 1 severity, grade 2 severity, and grade 3 severity influenza-positive adult patients. Dunn's post-test was used to compare severity pairs (severity 1 vs. 2, 1 vs. 3, and 2 vs. 3). Mediators highlighted in bold demonstrate significant differences severity 3 and at least one other severity group and where at least one group's median was significantly different from that of the healthy controls. Dunn's post-test was not performed if the medians did not differ on Kruskal-Wallis testing (significance cut-off *p* 0.05). NS = no sample obtained. N/A = not applicable.

	Mediator	Healthy control (n=36) Median (IQR) pg/mL	Influenza-positive Severity 1 (n=60) Median (IQR) pg/mL	Influenza-positive Severity 2 (n=43) Median (IQR) pg/mL	Influenza-positive Severity 3 (n=30) Median (IQR) pg/mL	Kruskal-Wallis *p* value	Severity 1 vs. 2 *p* value	Severity 1 vs. 3 *p* value	Severity 2 vs. 3 *p* value	Severity group with exclusive increase over HC (*p* value)
Types I and III IFNs	IFN-α2a	1.3 (1.3-1.3)	1.3 (1.3-8.4)	1.3 (1.3-1.3)	1.3 (1.3-1.3)	<0.0001	NS	<0.001	NS	-
	IFN-β	66.1 (32.0-151.5)	34.1 (11.7-108.1)	37.3 (11.7-99.7)	113.8 (11.7-213.0)	0.0572	N/A	N/A	N/A	-
	IFN-λ (IL-29)	12.0 (12.0-12.0)	12.0 (12.0-45.8)	12.0 (12.0-39.5)	12.0 (12.0-45.4)	N/A	N/A	N/A	N/A	-
Th1-secreted/associated cytokines	IFN-γ	0.3 (0.3-0.3)	2.5 (0.3-17.1)	1.1 (0.3-8.3)	0.9 (0.3-4.5)	<0.0001	NS	NS	NS	-
	TNF-α	**1.3 (0.3-2.4)**	**2.4 (0.7-8.4)**	**2.6 (0.7-6.4)**	**7.0 (2.6-57.5)**	**<0.0001**	**NS**	**<0.05**	**<0.05**	-
	IL-2	1.9 (0.6-4.5)	2.8 (0.6-11.0)	3.1 (0.6-8.8)	5.8 (0.6-18.0)	0.1320	NS	NS	NS	-
	IL-15	1.2 (0.9-1.8)	2.4 (1.2-5.6)	1.7 (1.0-5.8)	1.7 (0.8-3.7)	0.0167	NS	NS	NS	-
	IL-12p70	1.5 (0.6-2.6)	1.2 (0.3-2.4)	1.1 (0.3-2.0)	3.4 (1.1-6.4)	0.0005	NS	<0.01	<0.001	**Severity 3 (<0.001)**
Th2-secreted cytokines	IL-4	0.4 (0.4-1.0)	0.4 (0.4-0.4)	0.4 (0.4-0.4)	0.4 (0.4-4.0)	N/A	N/A	N/A	N/A	-
	IL-5	1.0 (0.3-1.7)	0.9 (0.3-2.1)	0.6 (0.3-1.6)	2.1 (0.3-3.8)	0.0639	NS	NS	NS	-
	IL-13	0.8 (0.3-1.9)	3.1 (1.4-8.6)	1.8 (0.3-6.0)	3.0 (1.5-5.1)	<0.0001	NS	NS	NS	-
Th17-secreted cytokines	IL-17	7.1 (4.7-9.5)	11.7 (5.3-28.6)	6.6 (3.0-23.3)	7.7 (3.7-17.6)	0.1507	NS	NS	NS	-
Other inflammatory cytokines	IL-6	12.3 (7.5-21.1)	79.4 (18.4-327.9)	92.7 (27.1-249.2)	335.5 (58.1-1441)	<0.0001	NS	NS	NS	-
	IL-8	**1880 (597.2-2500)**	**2158 (599.8-2500)**	**1284 (712.4-2500)**	**2500 (2500-2500)**	**0.0215**	**NS**	**NS**	**<0.05**	-
	IL-1β	**31.1 (14.0-63.4)**	**30.0 (5.3-66.5)**	**23.9 (6.8-46.0)**	**136.6 (18.1-468.6)**	**0.0040**	**NS**	**<0.05**	**<0.01**	-
	GM-CSF	**1.2 (0.6-2.0)**	**1.5 (0.8-2.5)**	**1.5 (0.7-2.7)**	**4.8 (1.8-7.3)**	**0.0001**	**NS**	**<0.01**	**<0.01**	**Severity 3 (<0.001)**
Anti-inflammatory cytokine	IL-10	0.8 (0.3-1.5)	2.0 (0.3-9.3)	1.5 (0.3-10.4)	3.1 (0.8-8.8)	0.0125	NS	NS	NS	-
IFN-associated/Th1-attracting chemokines	**MIG (CXCL9)**	**212.3 (140.0-307.5)**	**783.6 (243.2-2039)**	**413.4 (120.5-803.2)**	**268.5 (103.7-653.0)**	**0.0015**	**NS**	**<0.01**	**NS**	**Severity 1 (<0.01)**
	IP-10 (CXCL10)	773.8 (462.9-1435)	8261 (2047-10000)	3867 (833.4-9055)	2990 (927.2-10000)	<0.0001	NS	NS	NS	-
	I-TAC (CXCL11)	**11.65 (8.6-23.6)**	**321.4 (34.0-1056)**	**116.2 (11.5-528.2)**	**19.03 (5.6-72.3)**	**<0.0001**	**NS**	**<0.001**	**NS**	-
Th2-associated chemokines	MDC (CCL22)	115.9 (54.4-198.1)	167.7 (113.2-219.4)	139.6 (109.1-184.0)	163.0 (121.6-285.8)	0.0190	NS	NS	NS	-
	TARC (CCL17)	5.0 (5.0-12.8)	16.6 (5.0-25.8)	11.3 (5.0-20.3)	11.8 (5.0-21.3)	0.0005	NS	NS	NS	-
	Eotaxin-1 (CCL11)	138.5 (66.6-191.6)	230.8 (154.0-329.1)	203.3 (121.5-334.6)	313.4 (192.2-449.3)	<0.0001	NS	NS	NS	-
	Eotaxin-3 (CCL26)	30.9 (12.7-57.5)	48.2 (26.0-61.7)	30.5 (15.2-61.0)	44.7 (27.2-66.5)	0.2575	NS	NS	NS	-
Other inflammatory chemokines	**MIP-1α (CCL3)**	**8.7 (6.3-11.2)**	**28.9 (16.4-53.0)**	**18.2 (11.4-45.0)**	**62.6 (19.1-251.2)**	**<0.0001**	**NS**	**NS**	**<0.05**	-
	MIP-1β (CCL4)	**23.3 (5.3-61.0)**	**113.2 (36.5-427.2)**	**77.4 (21.6-267.9)**	**272.4 (86.9-1747)**	**<0.0001**	**NS**	**NS**	**<0.05**	-
	MCP-1 (CCL2)	**49.2 (31.8-87.4)**	**147.5 (47.3-356.0)**	**88.8 (34.1-295.5)**	**292.7 (156.3-961.0)**	**<0.0001**	**NS**	**NS**	**<0.0001**	-
	MCP-4 (CCL13)	12.4 (2.9-17.5)	13.02 (10.0-17.1)	12.2 (7.5-19.2)	15.7 (10.8-26.3)	0.0723	N/A	N/A	N/A	-

Table 5.7 Summary statistics for 28 soluble immune mediators measured in T1 SAM eluate obtained from influenza-positive patients of differing severities of illness, and from healthy controls

Kruskal-Wallis test was performed to compare results from healthy controls, grade 1 severity, grade 2 severity, and grade 3 severity influenza-positive adult patients. Dunn's post-test was used to compare severity pairs (severity 1 vs. 2, 1 vs. 3, and 2 vs. 3). Mediators highlighted in bold demonstrate significant differences severity 3 and at least one other severity group and where at least one group's median was significantly different from that of the healthy controls. Dunn's post-test was not performed if the medians did not differ on Kruskal-Wallis testing (significance cut-off *p* 0.05). NS = no sample obtained. N/A = not applicable.

5.8.2 Interferons in T1 serum, nasopharyngeal aspirate, and SAM eluate from influenza patients with differing severities of illness

Serum IFN-α2a was significantly reduced in patients of grade 3 severity, but in contrast, serum IFN-β was significantly increased in influenza-positive patients with the most severe illness. These trends were not seen in NPA or SAM eluates. Median IFN-λ levels were equivalent to the GM-LLOD of the assay in all sample types, irrespective of severity score. IFN-λ was not detectable in any of the patients with the most severe illness. IFN-γ was detectable in serum and SAM eluates in most patients, but this was not true for NPA and levels did not change with severity (figure 5.14).

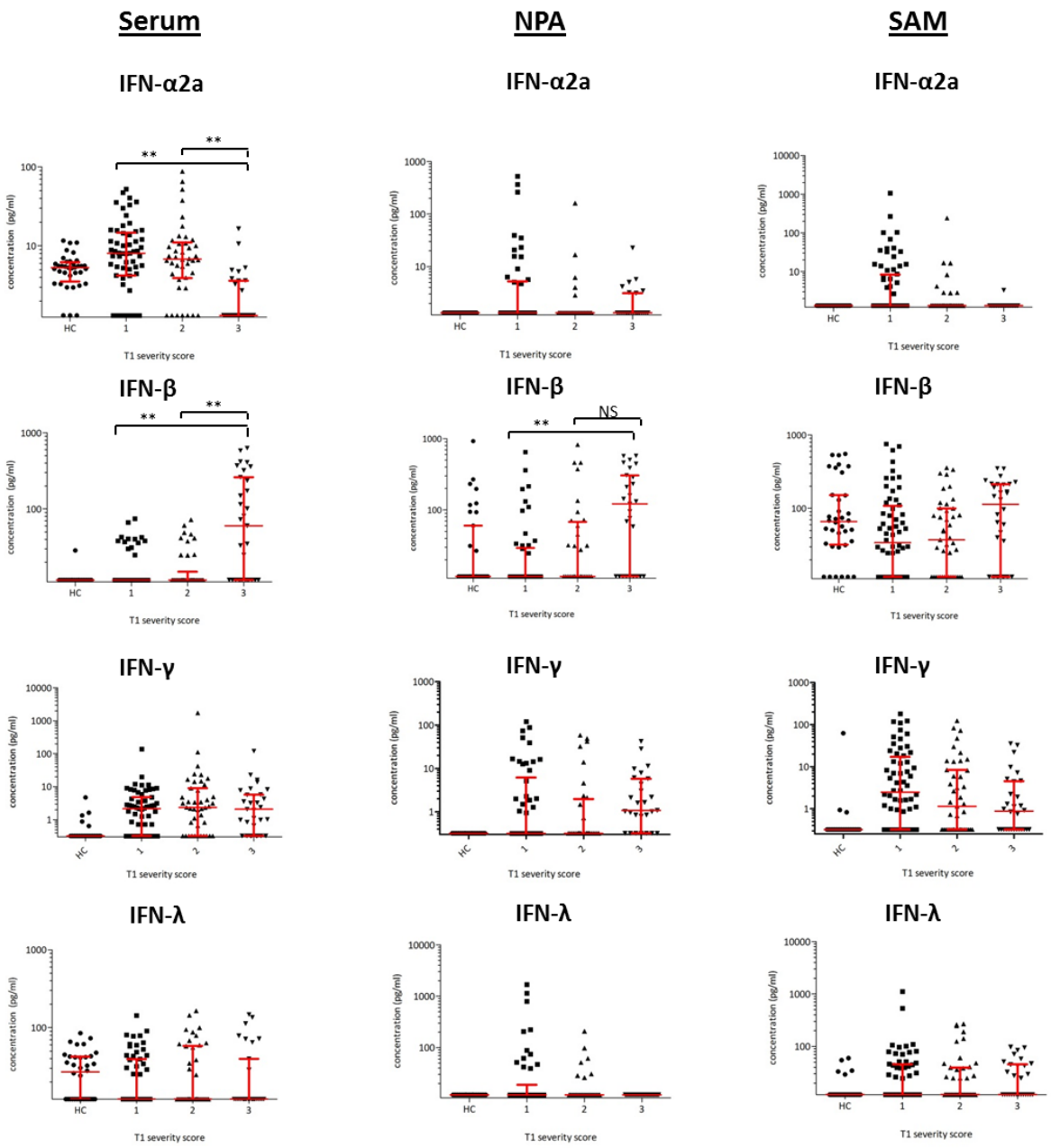


Figure 5.14 Interferon-α is significantly reduced in T1 serum from critically ill influenza-positive patients, but interferon-β is increased in serum and in nasopharyngeal aspirate
IFN-α2a, IFN-β, IFN-γ, and IFN-λ in serum, NPA and SAM eluate obtained from influenza-positive adults at T1 and from healthy controls were measured by MSD. Calculated concentrated means were plotted (pg/mL, log scale) and for each group the median and interquartile ranges were overlaid (in red). Kruskal-Wallis test with Dunn's post-test was used to assess significance (**p<0.01; NS=non-significant). Severity was assessed at T1. Sample groups: HC=healthy controls (•); 1 = grade 1 severity (▪); 2 = grade 2 severity (▲); 3 = grade 3 severity (▼).

5.8.3 Th2-associated and eosinophil-associated mediators in T1 nasopharyngeal aspirate from influenza patients with differing severities of illness

In NPA samples, both IL-4 and IL-5 were significantly elevated in severity 3 patients, compared to patients of lower severity and healthy controls. This trend was not seen in SAM eluate or serum samples. Significant elevations in levels of the chemokines eotaxin-1 and eotaxin-3 were also seen in NPA samples from influenza patients compared to healthy controls. Median levels in severity 1 and severity 2 patients were similar, but significantly higher levels were seen in the most severe patients (figure 5.15). The same trends were not seen in serum or in SAM samples (table 5.5 and table 5.7).

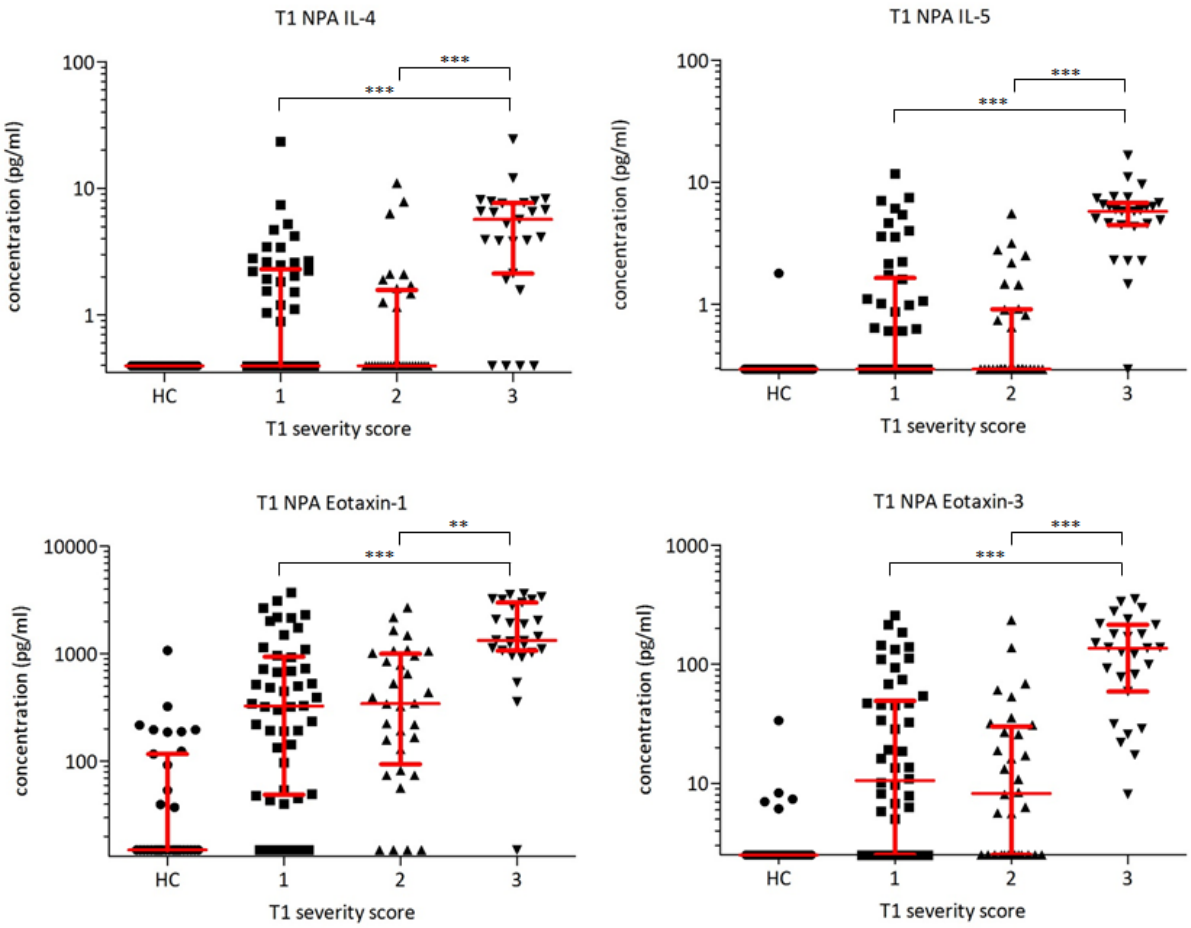


Figure 5.15 Levels of IL-4, IL-5, eotaxin-1, and eotaxin-3 in T1 NPA are greatest in influenza patients with grade 3 severity of illness

IL-4, IL-5, eotaxin-1 and eotaxin-3 in serum and NPA obtained from influenza-positive adults at T1 and from healthy controls were measured by MSD. Calculated concentrated means were plotted (pg/mL, log scale) and for each group the median and interquartile ranges were overlaid (in red). For each mediator, zero values and values below the LLOD were assigned half the GM-LLOD. Kruskal-Wallis test with Dunn's post-test was used to assess significance (***p<0.001; **p<0.01). Severity was assessed at T1. Sample groups: HC=healthy controls (•); 1 = grade 1 severity (▪); 2 = grade 2 severity (▲); 3 = grade 3 severity (▼).

The chemokines MDC and TARC demonstrated opposing trends according to the compartment sampled. In serum, the highest levels were observed in healthy controls, with a progressive decrease being observed as the severity score increased. In NPA, the lowest levels were in healthy controls; similar median levels were observed in severity 1 and severity 2 patients, but the highest levels were seen in the patients with the most severe illness (figure 5.15). However, the differences seen in NPA TARC did not reach statistical significance.

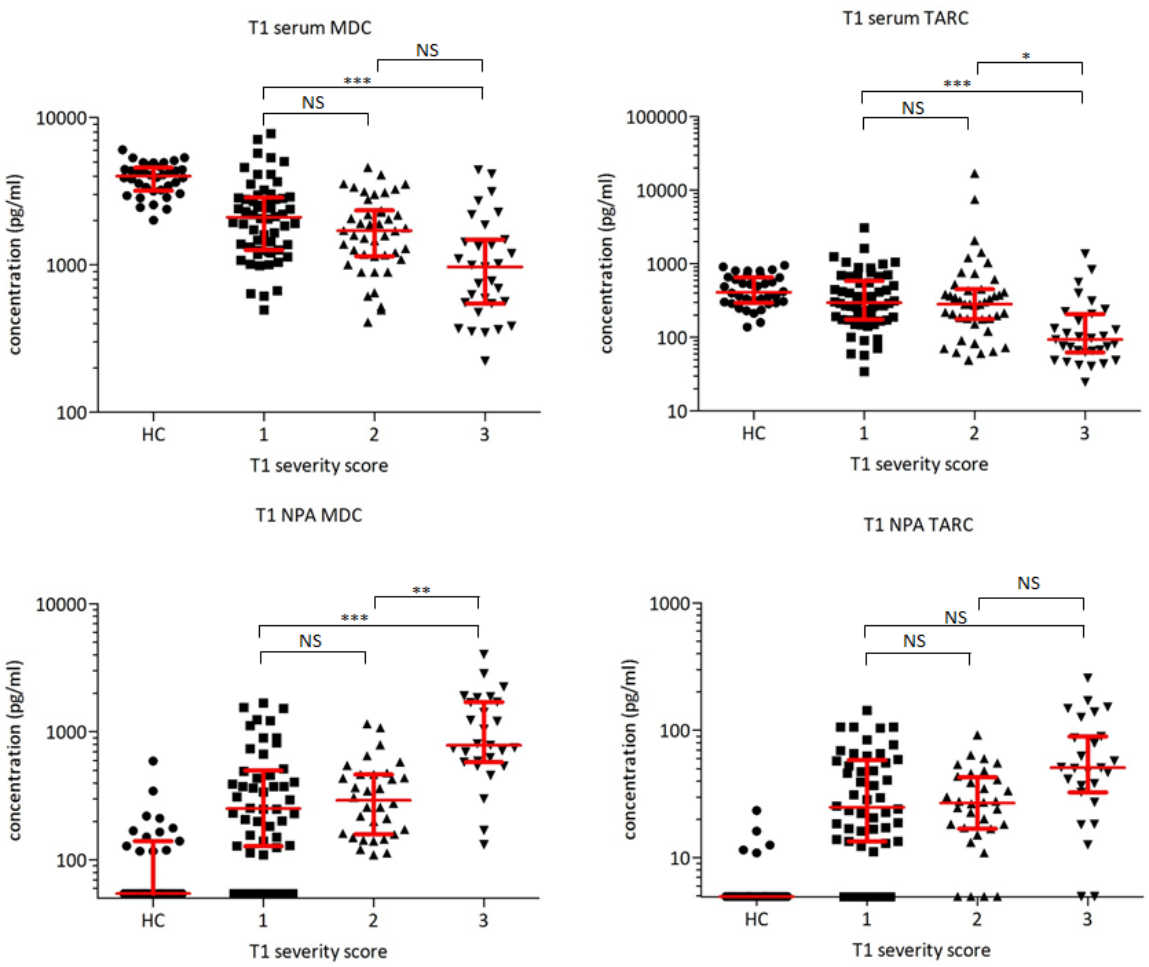


Figure 5.16 Levels of MDC and TARC in T1 serum decrease as severity increases
MDC and TARC serum and NPA obtained from influenza-positive adults at T1 and from healthy controls were measured by MSD. Calculated concentrated means were plotted (pg/mL, log scale) and for each group the median and interquartile ranges were overlaid (in red). For each mediator, zero values and values below the LLOD were assigned half the GM-LLOD. Kruskal-Wallis test with Dunn's post-test was used to assess significance (***p<0.001; **p<0.01; *p<0.05; NS=non-significant). Severity was assessed at T1. Sample groups: HC=healthy controls (•); 1 = grade 1 severity (▪); 2 = grade 2 severity (▲); 3 = grade 3 severity (▼).

5.8.4 IL-6 and IL-8 in T1 serum, nasopharyngeal aspirate and SAM eluate from influenza patients with differing severities of illness

In serum, median IL-6 levels were elevated in all three severity groups compared to healthy controls (p<0.001). Additionally, median IL-6 levels progressively increased as severity increased, with statistically significant differences seen when comparing all severity groups. In NPA, IL-6 was again raised in all patients, but median values for severity 1 and severity 2 patients did not differ and were similar to those seen in serum. However, NPA IL-6 in severity group 3 was markedly elevated and was almost ten-fold higher than the median value seen in serum from severity group 3 patients. In SAM eluates samples, median IL-6 levels were similar in groups 1 and 2 and higher in group 3, but not to the extent seen in NPA samples (figure 5.17).

Serum IL-8 was significantly increased in all severity groups compared to healthy controls. Median values were similar in severity 1 and severity 2 patients, but were higher in severity 3 patients. The same trend was seen in NPA samples, although the levels were much higher and almost all severity 3 patients had values at the upper limit of detection. Median Il-8 levels in SAM eluate samples were very high in all groups, including healthy controls (figure 5.17).

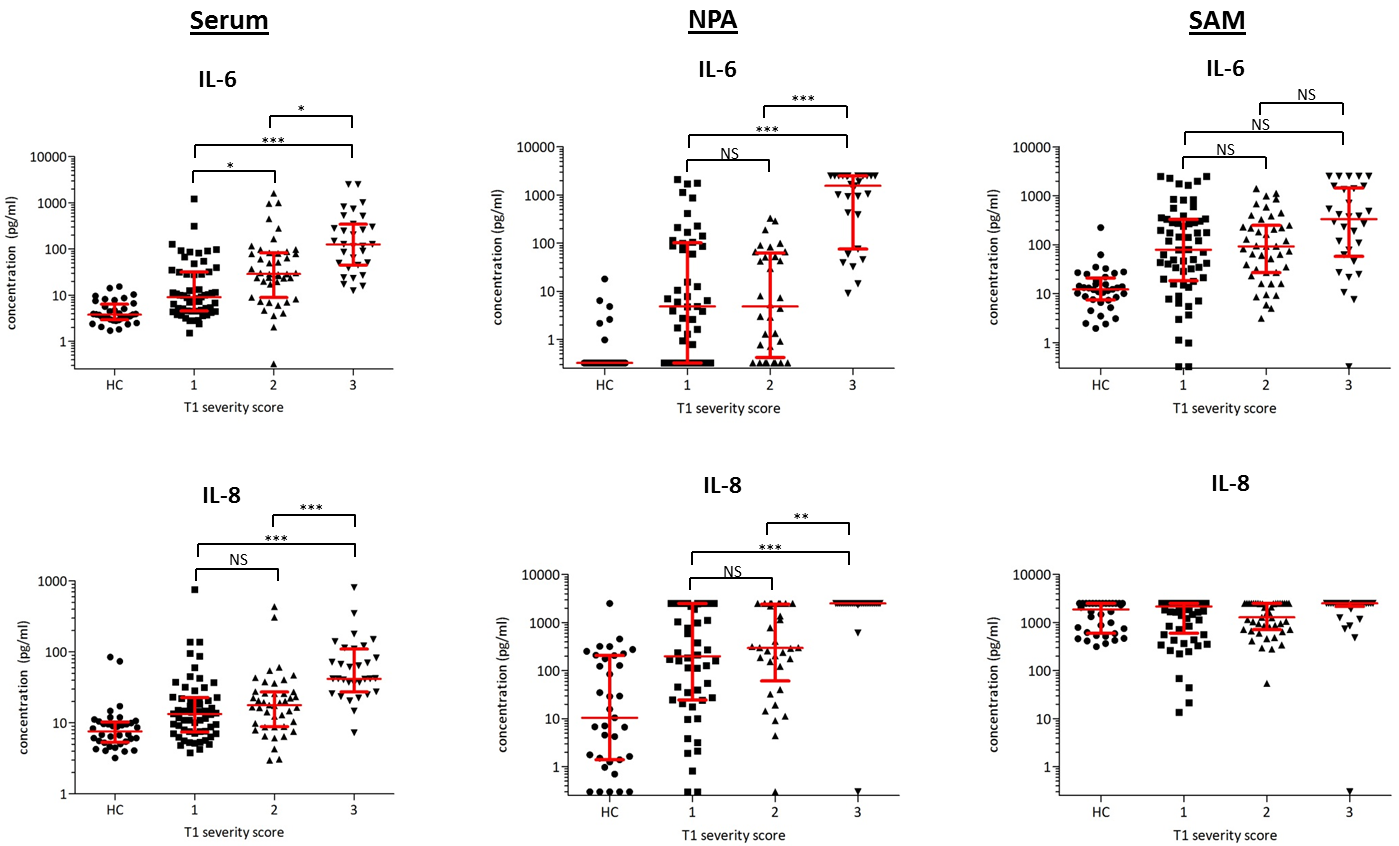


Figure 5.17 IL-6 and IL-8 increase in T1 serum and NPA as influenza severity increases

Levels of IL-6 and IL-8 in serum, T1 NPA and T1 SAM eluate obtained from influenza-positive adults at T1 and from healthy controls were measured by MSD. Calculated concentrated means were plotted (pg/mL, log scale) and for each group the median and interquartile ranges were overlaid (in red). For each mediator, zero values and values below the LLOD were assigned half the GM-LLOD. Kruskal-Wallis test with Dunn's post-test was used to assess significance (****p*<0.001; ***p*<0.01; *p<0.05; NS=non-significant). Severity was assessed at T1. Sample groups: HC=healthy controls (●); 1 = grade 1 severity (■); 2 = grade 2 severity (▲); 3 = grade 3 severity (▼).

5.8.5 IL-15 and IL-17 in T1 serum, nasopharyngeal aspirate and SAM eluate

Modest increases in median IL-15 and IL-17 levels were observed in T1 serum samples from severity 3 influenza-positive patients, when compared to median levels in healthy controls and in with lower severities of illness (figure 5.18). These trends were not observed in NPA or SAM eluate samples (tables 5.6 and 5.7).

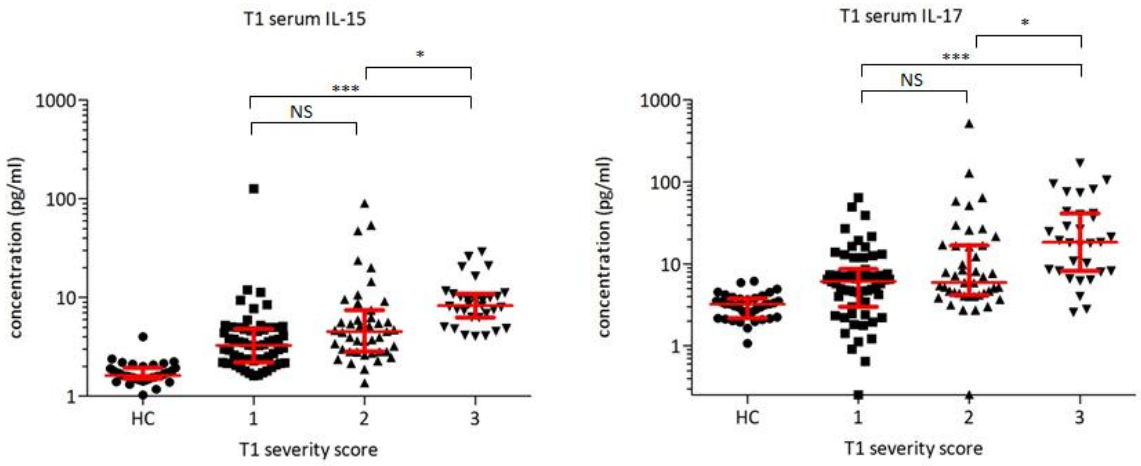


Figure 5.18 Levels of IL-15 and IL-17 in T1 serum increase as influenza illness severity increases
IL-15 and IL-17 in serum obtained from influenza-positive adults at T1 and from healthy controls were measured by MSD. Calculated concentrated means were plotted (pg/mL, log scale) and for each group the median and interquartile ranges were overlaid (in red). For each mediator, zero values and values below the LLOD were assigned half the GM-LLOD. Kruskal-Wallis test with Dunn's post-test was used to assess significance (****p*<0.001; *p<0.05; NS=non-significant). Severity was assessed at T1. Sample groups: HC=healthy controls (•); 1 = grade 1 severity (▪); 2 = grade 2 severity (▲); 3 = grade 3 severity (▼).

5.8.6 MIG and IP-10 in T1 serum, nasopharyngeal aspirate and SAM eluate

Serum MIG was higher in influenza cases compared to healthy controls, especially in those with the most severe illness. Similar trends were seen for MIG measured in NPA samples. Serum IP-10 levels showed very marked increases in patients with influenza compared to healthy controls, especially in those with the most severe illness. A similar trend was seen for IP-10 measured in NPA samples, but levels often clustered at the upper limit of detection for the assay. This effect was also seen, to a greater extent, in SAM samples (figure 5.19).

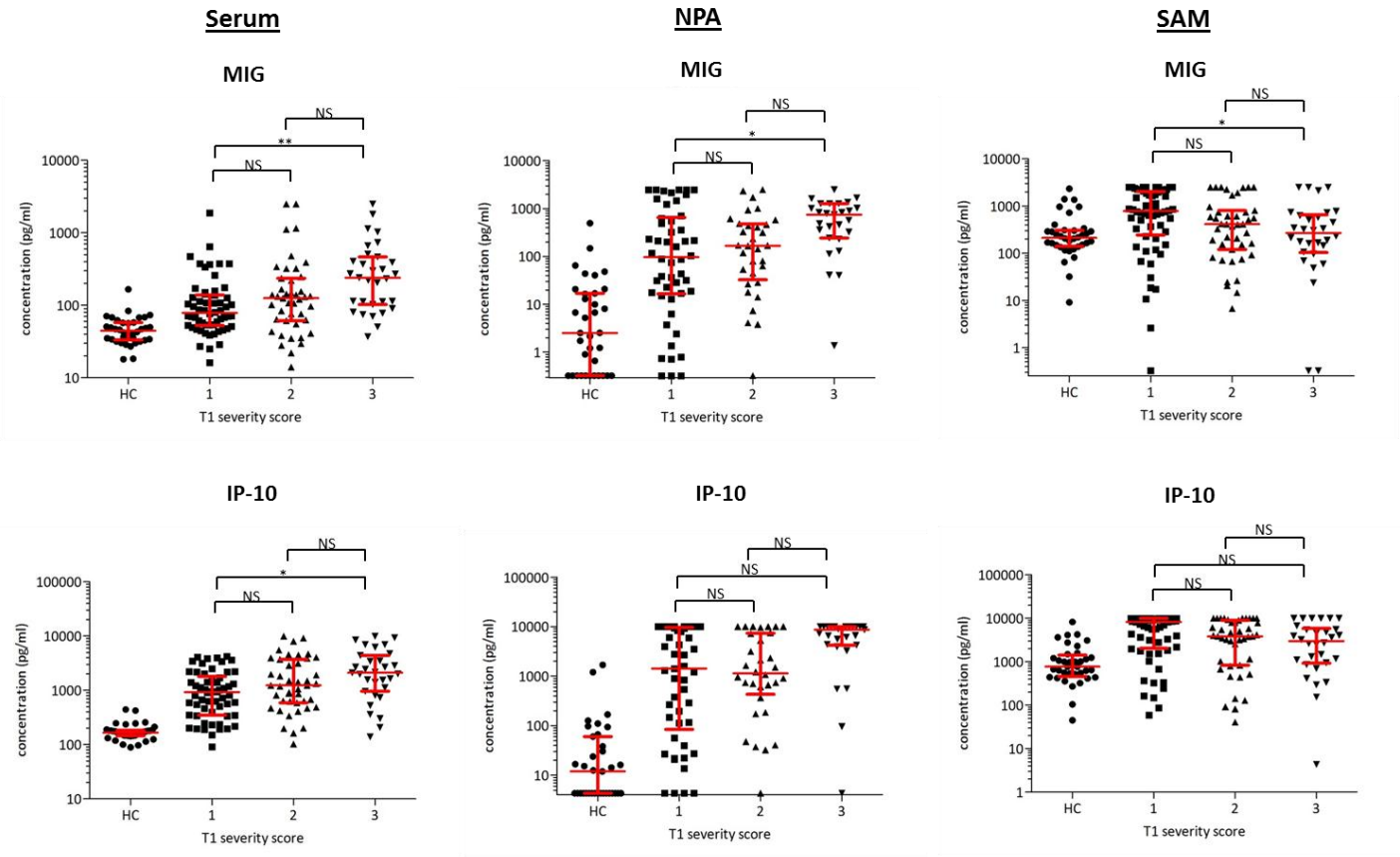


Figure 5.19 MIG and IP-10 show variability according to severity of influenza illness and compartment sampled

Levels of MIG and IP-10 in serum, NPA and SAM eluate obtained from influenza-positive adults at T1 and from healthy controls were measured by MSD. Calculated concentrated means were plotted (pg/mL, log scale) and for each group the median and interquartile ranges were overlaid (in red). For each mediator, zero values and values below the LLOD were assigned half the GM-LLOD. Kruskal-Wallis test with Dunn's post-test was used to assess significance (**p<0.01; *p<0.05; NS=non-significant). Severity was assessed at T1. Sample groups: HC=healthy controls (•); 1 = grade 1 severity (▪); 2 = grade 2 severity (▲); 3 = grade 3 severity (▼).

5.8.7 MCP-1, MIP-1α and MIP-1β in T1 serum, nasopharyngeal aspirate and SAM eluate

MCP-1, MIP-1α and MIP-1B were significantly higher in NPA from severity 3 patients compared to NPA samples from patients with lower severities of illness. Median levels were similar for severity 1 and severity 2 patients (figure 5.20)

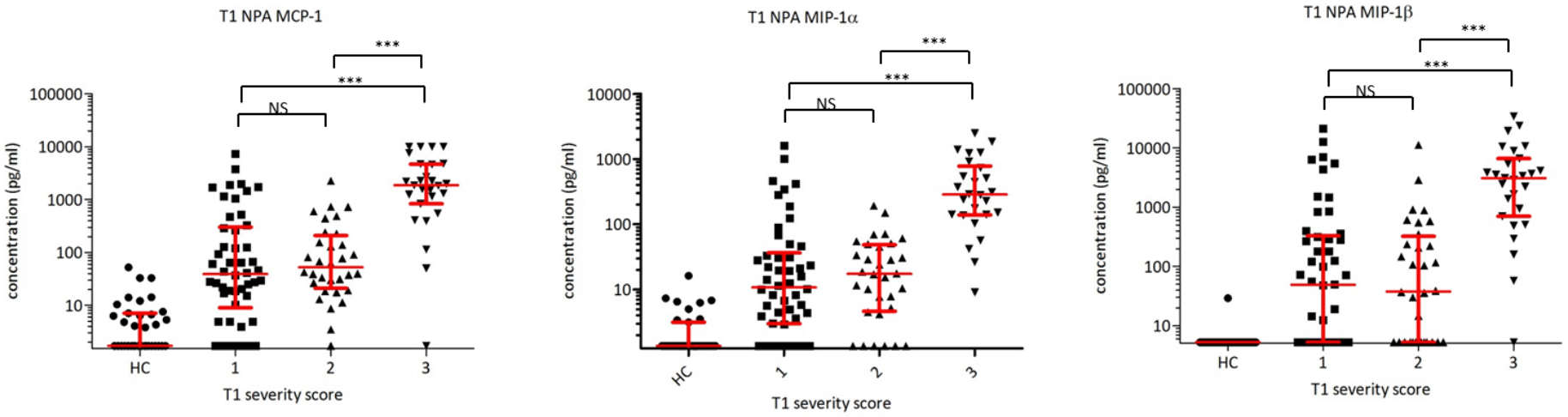


Figure 5.20 Levels of MCP-1, MIP-1α and MIP-1β in T1 NPA are higher in critically ill influenza patients than in patients with less severe illness
MCP-1, MIP-1α and MIP-1β in NPA obtained from influenza-positive adults at T1 and from healthy controls were measured by MSD. Calculated concentrated means were plotted (pg/mL, log scale) and for each group the median and interquartile ranges were overlaid (in red). For each mediator, zero values and values below the LLOD were assigned half the GM-LLOD. Kruskal-Wallis test with Dunn's post-test was used to assess significance (***p<0.001; NS=non-significant). Severity was assessed at T1. Sample groups: HC=healthy controls (•); 1 = grade 1 severity (▪); 2 = grade 2 severity (▲); 3 = grade 3 severity (▼).

5.8.8 Other mediators of interest measured by MSD in T1 serum, nasopharyngeal aspirate and SAM eluate

IL-1β in NPA was markedly raised in severity 3 patients, but not in severity 1 or severity 2 patients, compared to healthy controls. The same pattern was seen in SAM samples, but not in serum. The median concentration of IL-2 in NPA was also highest in severity 3 patients, but the increase over median levels in severity 1 and 3 patients was less marked that for IL-1β. IL-2 levels in serum did not differ significantly between the groups. MCP-4 in NPA was elevated in all severity groups and was markedly elevated in patients with the most severe illness. In contrast to levels in NPA, MCP-4 in serum and SAM samples did not differ significantly between the groups. Severity 3 patients also had the highest NPA IL-12p70 levels. In SAM samples, IL-12p70 levels were similar in healthy controls, severity 1 and severity 2 patients, with the greatest elevation seen in severity 3 patients. In serum, median IL-12p70 levels did not vary significantly between any of the groups (figure 5.21).

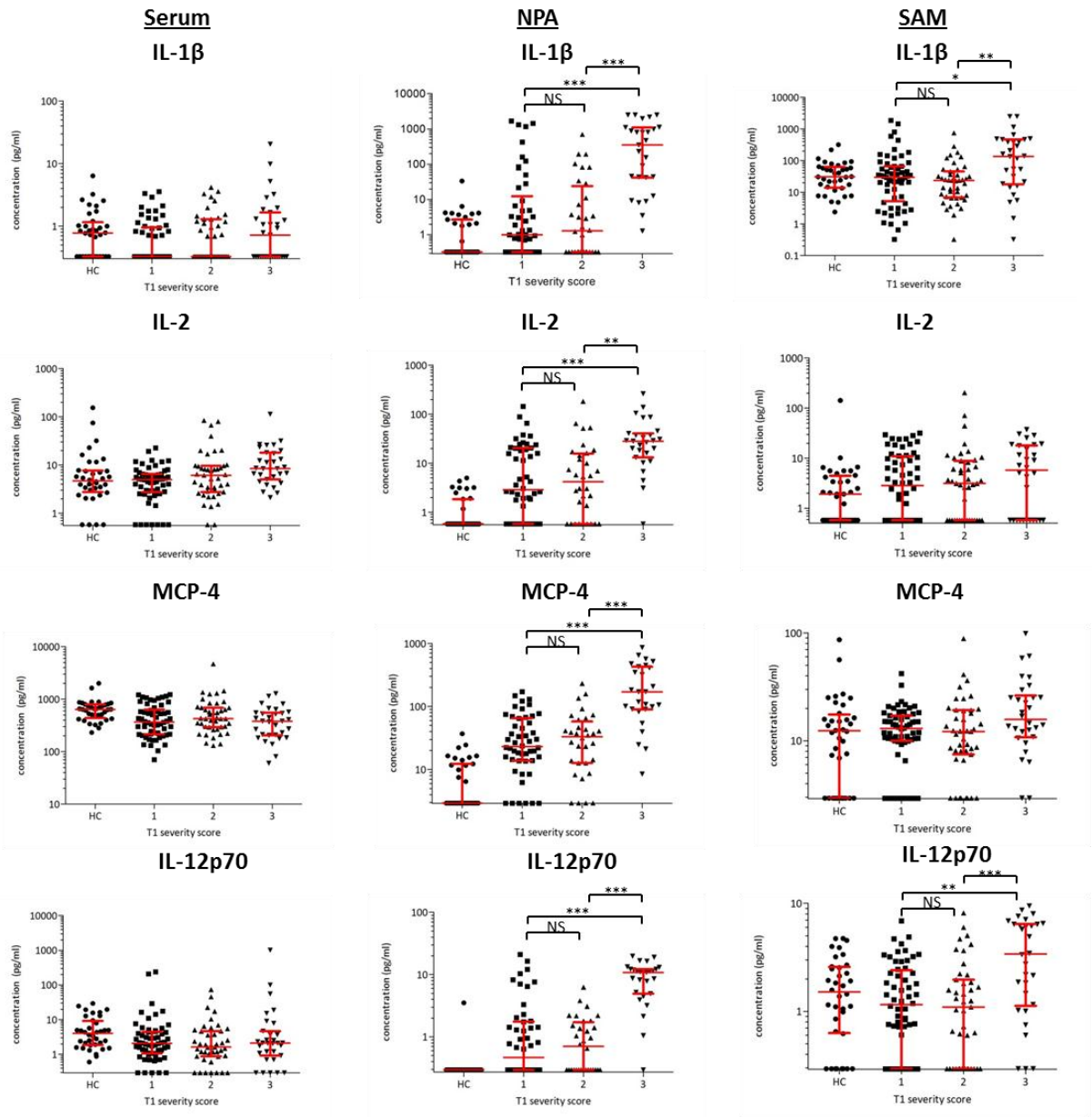


Figure 5.21 Levels of IL-1β, IL-2, MCP-4, and IL-12p70 vary according to severity of influenza illness and biological compartment sampled

IL-1β, IL-2, MCP-4 and IL-12p70 in T1 serum, NPA and SAM eluate obtained from influenza-positive adults at T1 and from healthy controls were measured by MSD. Calculated concentrated means were plotted (pg/mL, log scale) and for each group the median and interquartile ranges were overlaid (in red). For each mediator, zero values and values below the LLOD were assigned half the GM-LLOD. Kruskal-Wallis test with Dunn's post-test was used to assess significance (****p*<0.001; ***p*<0.01; *p<0.05; NS=non-significant). Severity was assessed at T1. Sample groups: HC=healthy controls (•); 1 = grade 1 severity (▪); 2 = grade 2 severity (▲); 3 = grade 3 severity (▼).

5.8.9 Neopterin, RANTES, sTNFR1 and sTNFR2 in T1 serum from influenza

Neopterin, RANTES and sTNFR1 and sTNFR2 were measured in T1 serum only, by individual ELISA. Median levels of neopterin were significantly higher in all three severity groups relative to healthy controls and a significant increase was seen in severity 3 patients compared to severity 1 patients. Levels of RANTES were similar in all three severity groups and did not differ significantly to levels measured in healthy controls. Median levels of sTNFR1 and sTNFR2 in patients with grade 3 severity of illness were markedly elevated compared to levels in patients with milder illness (figure 5.22).

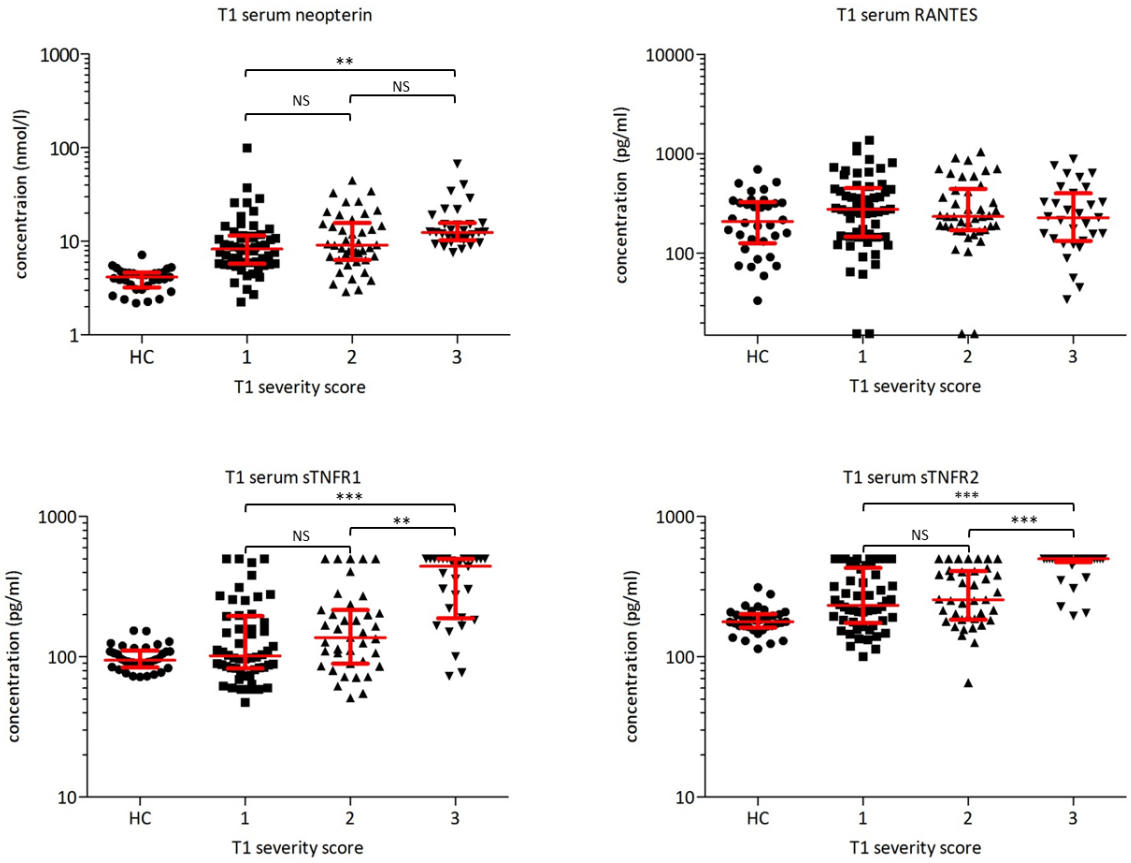


Figure 5.22 Neopterin, sTNFR1 and sTNFR2, but not neopterin, are markedly elevated in critically-ill patients with influenza virus infection

Neopterin, RANTES, sTNFR1 and sTNFR2 in serum obtained from influenza-positive adults at T1 and from healthy controls were measured by ELISA. Calculated concentrated means were plotted (pg/mL or nmol/L, log scale) and for each group the median and interquartile ranges were overlaid (in red). For each mediator, zero values and values below the LLOD were assigned half the GM-LLOD. Kruskal-Wallis test with Dunn's post-test was used to assess significance (***p<0.001; **p<0.01; NS=non-significant). Severity was assessed at T1. Sample groups: HC=healthy controls (●); 1 = grade 1 severity (■); 2 = grade 2 severity (▲); 3 = grade 3 severity (▼).

5.8.10 Mediators that are increased in single severity groups relative to healthy controls

Certain mediators were increased in a single severity group when each group was compared to healthy controls. In serum, significantly increased levels of IFN-β, TNF-α, IL-2 and MCP-1 were seen in patients with grade 3 severity of illness, but not in those with grade 1 or grade 2 severity of illness (table 5.5). In NPA, IFN-β and IL-1β were elevated in patients with grade 3 severity of illness, but not in patients with grade 1 or grade 2 severity of illness (table 5.6). In SAM eluates, only severity 3 patients had significantly elevated levels of IL-12p70 and GM-CSF, whereas only severity 1 patients had a significant elevation in MIG (CXCL9), relative to levels in healthy controls (table 5.7).

5.9 Hierarchical clustering of soluble immune mediator trends in influenza-positive adult patients

5.9.1 Approach to hierarchical clustering analysis

Hierarchical clustering allows patterns of multiple mediator levels to be observed more easily and can identify sub-groups of patients with similar mediator levels. The semi-supervised hierarchical clustering approach used to analyse mediator trends for all patients at T1 was also applied to z score-transformed values for the 28 mediators measured by MSD electrochemiluminescence in samples of serum, NPA and SAM eluate from influenza-positive patients and healthy controls (without influenza-negative cases). Associations between gene expression profiles and severity of illness and day of illness at T1 were seen in hierarchical clustering in transcriptomics analysis (see chapter 4); therefore, severity of illness at T1 was also applied to hierarchical clustering analysis of mediator results for influenza-positive cases and healthy controls. Although it is accepted that changes in gene expression and changes in levels of associated proteins may not occur at the same time, in order to facilitate comparison, the same day of illness groups were used for transcriptomics and mediator clustering (0-4 days and 5-28 days).

5.9.2 Hierarchical clustering analysis of T1 serum results for influenza-positive adults

In serum, 22 of 28 mediators remained following statistical filtering with multiple testing correction. IFN-α2a, IFN-λ, IL-1β, IL-2, IL-5 and GM-CSF did not reach statistical significance (Kruskal-Wallis test with Benjamini and Hochberg FDR, corrected *p* value cut-off 0.05). Related mediators were seen to increase or decrease together, being closely related on the vertical dendrogram. For example, the Th1-associated cytokines and chemokines IFN-γ, IP-10, IL-15 and MIG clustered together within a sub-branch of the vertical dendrogram. All but one of the healthy controls clustered together. Many of the patients with grade 3 severity of illness clustered together and they tended to have the most

marked increase in levels of many mediators, with the exception of MDC, TARC, eotaxin-1, MCP-4, IL-12p70 and IL-4; these mediators were either relatively depressed (especially MDC and TARC) or not so markedly elevated. A small number of patients with grade 1 or grade 2 severity of illness could also be seen within this cluster. A sub-cluster of severity 3 patients appeared to be defined by having marked elevation of IFN-β, a mediator which generally was not elevated in the majority of influenza patients, relative to healthy controls. Most patients with grade 3 severity of illness were at a later point in the course of illness when recruited, but for the patients overall, mediator profiles appeared to correlate with severity score rather than day of illness. Seven patients with severity 3 illness (all ≥ 5 days of illness) did not cluster with the other severity 3 patients (figure 5.22).

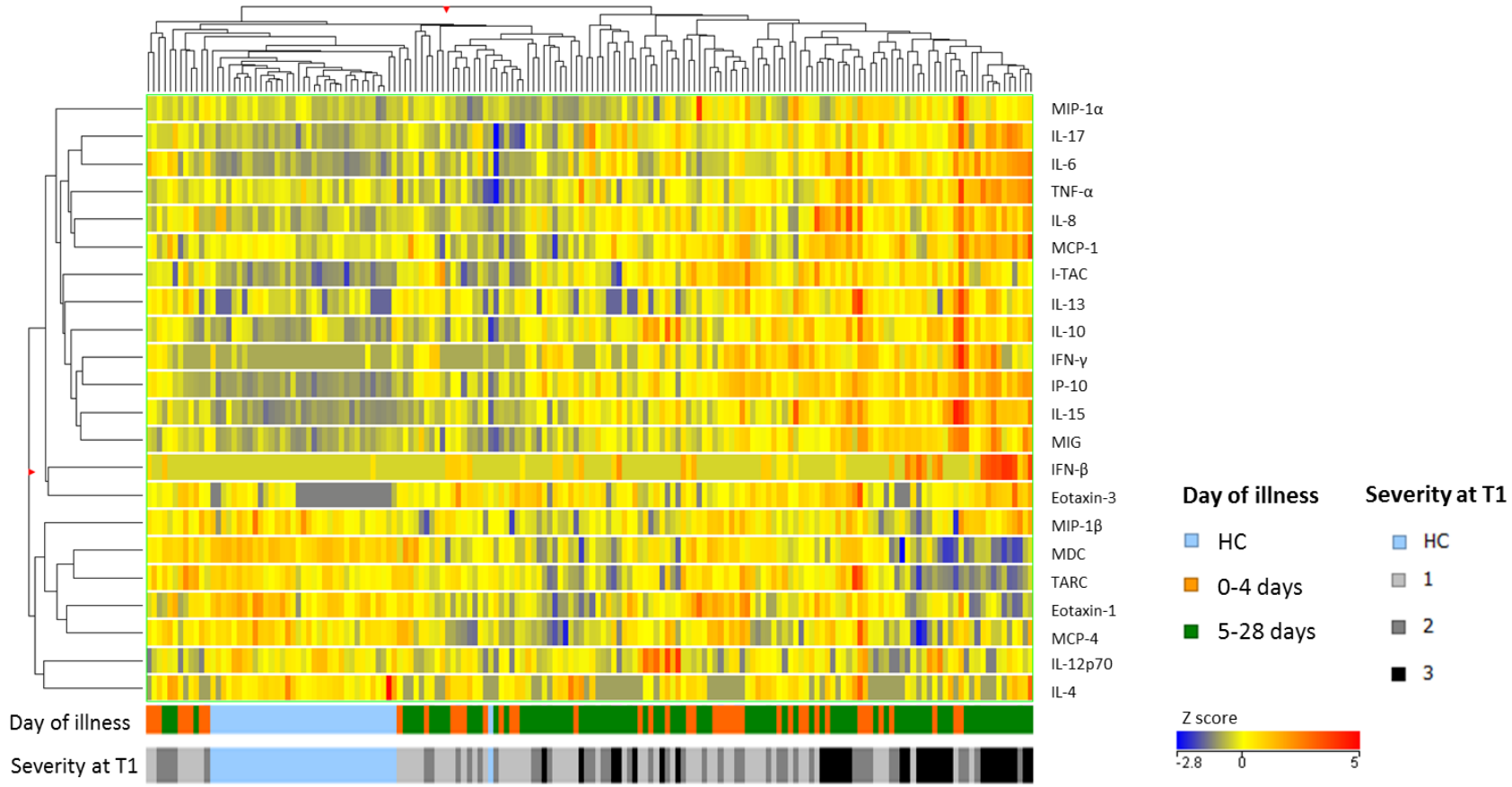


Figure 5.23 Association of severity of influenza illness at T1 with levels of 22 mediators in serum

Mediator values for T1 serum obtained from influenza-positive adults were log-transformed and then converted to Z-scores. Results for 28 mediators measured by MSD were subjected to statistial filtering and multiple testing correction (Kruskal Wallis test with Benjamini and Hochberg FDR, correct *p* value cut-off 0.05). Differences in IFN-α2a, IFN-λ, IL-1β, IL-2, IL-5 and GM-CSF did not meet the statistical filtering cut-off. Hierarchical clustering (on entities, day of illness and severity at T1) was performed on the remaining 22 mediator results using Pearson's uncentered (cosine) method with the average linkage rule, and a heat-map was generated. Z-scores are represented by a colour intensity spectrum (lowest z-score is dark blue, zero value is yellow, highest z-score is red). HC = healthy controls (pale blue).

5.9.3 Hierarchical clustering analysis of T1 NPA results for influenza-positive adults

In T1 NPA, IFN-β did not pass statistical filtering and multiple testing correction, leaving 27 mediators for hierarchical clustering analysis (Kruskal-Wallis test with Benjamini and Hochberg FDR, corrected *p* value cut-off 0.05). The removal of IFN-β durin statistical filtering was unexpected, since the earlier analyses of individual mediators suggested that the median level of IFN-β in NPA was markedly elevated in patients with grade 3 severity, but not those with grade 1 or grade 2 severity, relative to healthy controls.

The horizontal dendrogram split the subjects into two broad groups: those who have low or depressed levels of mediators, mediator signatures that look more similar to the those of the healthy controls, and those who tend to have increased levels of most mediators. All but one of the patients with the most severe illness could be found in this latter group and the majority of the severity 3 patients clustered together, demonstrating increased levels of all mediators except IFN-α2a and IFN-λ. Some severity 1 and severity 2 patients in the second group had marked increases in IFN-α2a and IFN-λ; some of these patients were at an earlier stage of illness, but others were not (figure 5.23).

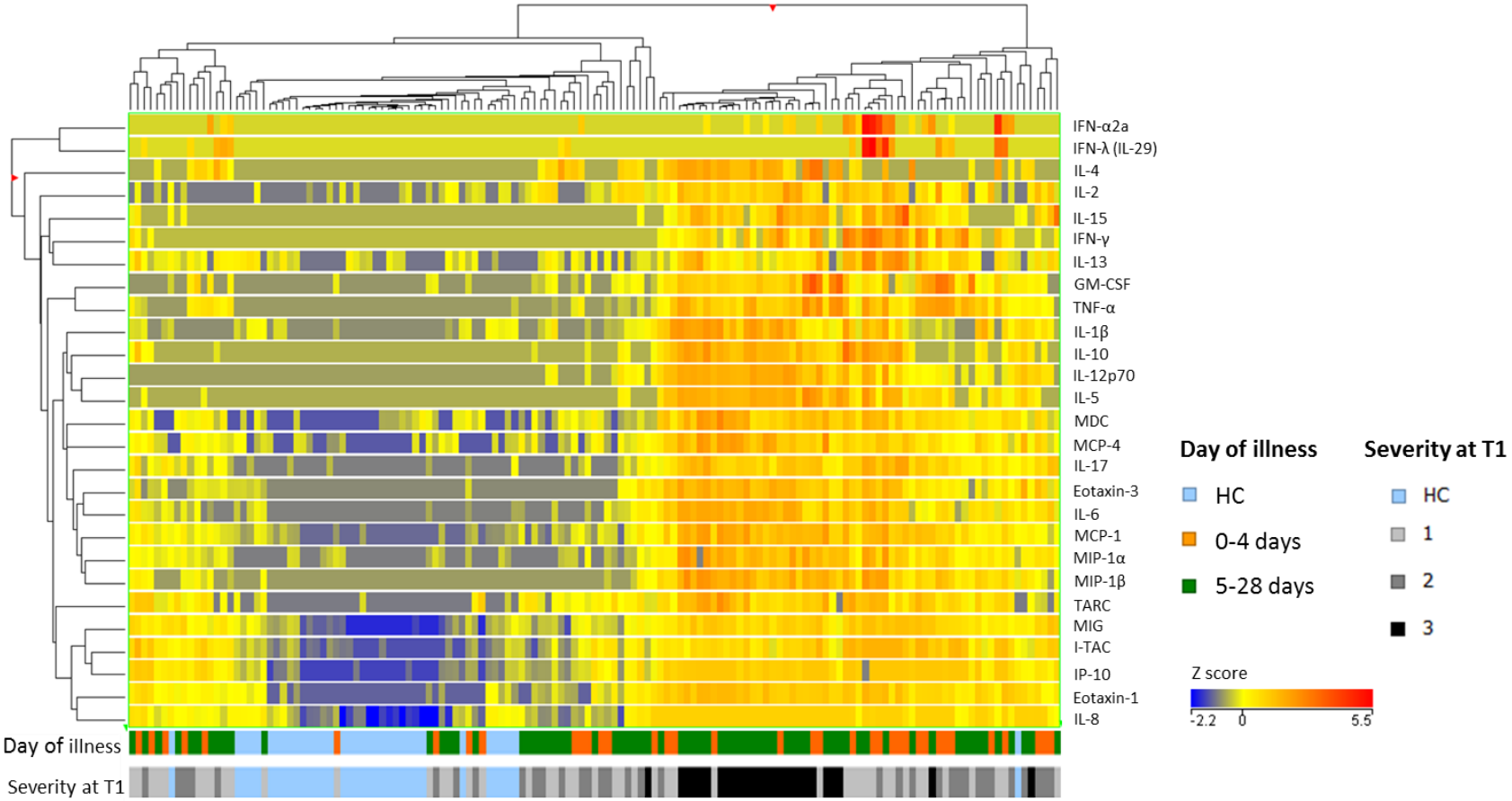


Figure 5.24 Association of severity of influenza illness at T1 with levels of 27 mediators in nasopharyngeal aspirate

Mediator values for T1 NPA obtained from influenza-positive adults were log-transformed and then converted to Z-scores. Results for 28 mediators measured by MSD were subjected to statistial filtering and multiple testing correction (Kruskal Wallis test with Benjamini and Hochberg FDR, correct *p* value cut-off 0.05). Differences in IFN-β did not meet the statistical filtering cut-off. Hierarchical clustering (on entities, day of illness and severity at T1) was performed on the remaining 27 mediator results using Pearson's uncentered (cosine) method with the average linkage rule, and a heat-map was generated. Z-scores are represented by a colour intensity spectrum (lowest z-score is dark blue, zero value is yellow, highest z-score is red). HC = healthy controls (pale blue).

5.9.4 Hierarchical clustering analysis of T1 SAM eluate results

In contrast to NPA and serum, fewer mediators remained following statistical filtering and multiple testing corrections. Healthy controls did not form tight clusters as were seen with serum and NPA analyses, but again, the horizontal dendrogram divides the subjects into two groups: patients and the majority of the healthy controls, who tended to have low or depressed levels of most mediators, and patients and just a few healthy controls who tended to have increased levels of most mediators. The majority of the severity 3 patients formed a sub-cluster within the latter group, with marked increases in MDC, GM-CSF, MIP-1α, MCP-1, IL-6, MIP-1β and TNF-α in most. Again, most were at a later stage in their illness and they also had less marked increases in IFN-γ, I-TAC, IP-10 and MIG compared to other patients demonstrating increased levels relative to healthy controls.

Similar to the NPA results, a sub-cluster of patients – many of whom tended to have the mildest illness and were earlier in the course of illness – appeared to be defined by a marked increase in IFN-α2a. Marked increases in IFN-λ and IFN-γ were also seen in this sub-cluster (figure 5.24).

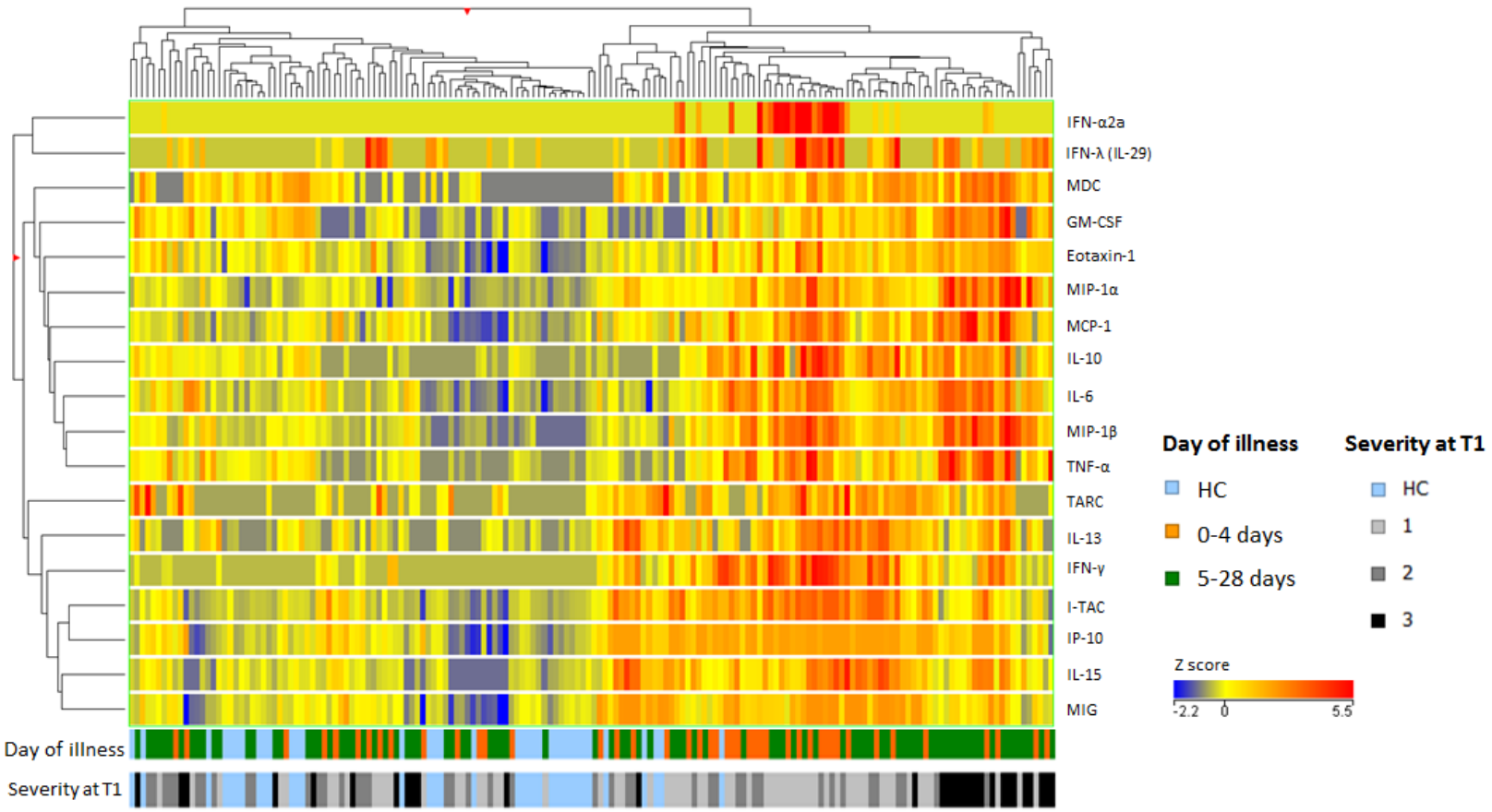


Figure 5.25 Association of severity of influenza illness at T1 with levels of 18 mediators in SAM eluate

Mediator values for T1 SAM eluate obtained from influenza-positive adults were log-transformed and then converted to Z-scores. Results for 28 mediators measured by MSD were subjected to statistial filtering and multiple testing correction (Kruskal Wallis test with Benjamini and Hochberg FDR, correct *p* value cut-off 0.05). Differences in IFN-β, IFN-γ, IL-1β, IL-2, IL-4, IL-5, MCP-4. IL-12p70, IL-17and eotaxin-3 did not meet the statistical filtering cut-off. Hierarchical clustering (on entities, day of illness and severity at T1) was performed on the remaining 18 mediator results using Pearson's uncentered (cosine) method with the average linkage rule, and a heat-map was generated. Z-scores are represented by a colour intensity spectrum (lowest z-score is dark blue, zero value is yellow, highest z-score is red). HC = healthy controls (pale blue).

5.10 Soluble immune mediators levels in BAL fluid

BAL fluid from eleven severity 3 patients was analysed using MSD electrochemiluminescence. These samples were not connected to a particular sampling time point, with BAL having being performed for clinical reasons. Results for 11 healthy controls (with assays having been performed using the same technique) were obtained from a collaborator. Mann Whitney unpaired test was used to compared medians. Most mediators were significantly elevated in BAL fluid from patients compared to BAL fluid from healthy controls. Median levels of IFN-α2a, IFN-λ and IL-4 levels in patients' BAL fluid did not differ to levels in healthy controls, being at the lower limit of detection in most patients. When a mediator was elevated in an upper respiratory tract sample, it was also elevated in BAL fluid. In some cases, mediator levels were similar between BAL fluid and NPA or BAL fluid and SAM, but levels were not consistently similar across all three respiratory tract sample types. IL-17 levels were greater in BAL fluid compared to other sample types, but the difference between the medians of all sample types was not significant. However, MCP-1 was significantly higher in BAL fluid than in all of the other sample types (table 5.8).

Additionally, 8 of the 11 patients had complete sets of NPA, SAM and serum samples available for comparison with BAL fluid. Median IL-17 and MCP-1 levels did not differ significantly between sample types in this sub-group (figure 5.25).

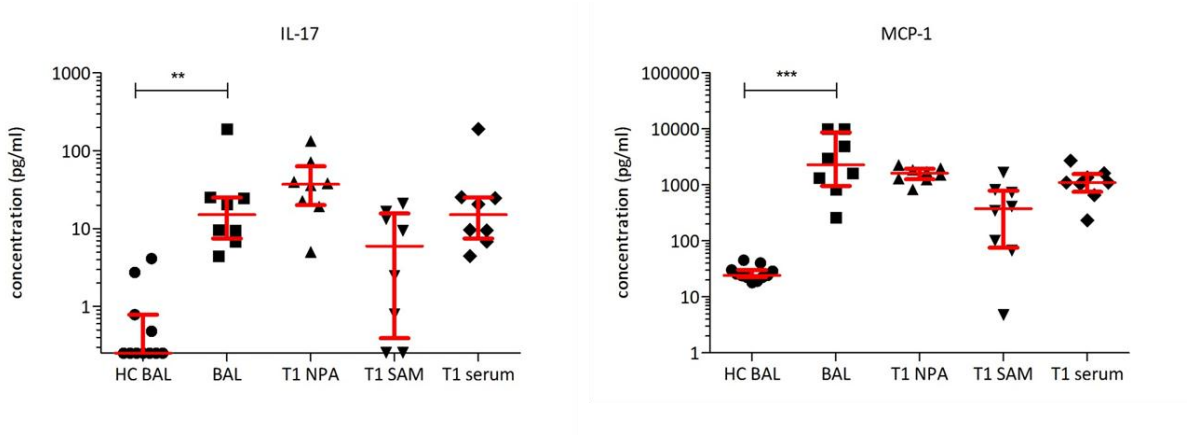


Figure 5.26 In critically ill patients with influenza, elevated levels of MCP-1 and IL-17 in BAL are similar to levels in T1 NPA, SAM and serum from the same patients

Eight critically-ill influenza patients had BAL available for mediator analysis in addition to T1 NPA, SAM and serum. Calculated concentrated means were plotted (pg/mL, log scale) and for each group the median and interquartile ranges were overlaid (in red). For each mediator, zero values and values below the LLOD were assigned half the GM-LLOD. Kruskal-Wallis test with Dunn's post-test was used to assess significance (***p<0.001; **p<0.01). Significant differences in median levels of IL-17 and MCP-1 were not detected for comparisons of BAL with NPA, SAM, and serum. HC BAL = BAL from 11 healthy controls (•)

	Mediator	Healthy control BAL (n=11) Median (IQR) pg/mL	Influenza-positive Severity 3 BAL (n=11) Median (IQR) pg/mL	Influenza-positive Severity 3 NPA (n=27) Median (IQR) pg/mL	Influenza-positive Severity 3 SAM (n=30) Median (IQR) pg/mL	Influenza-positive Severity 3 Serum (n=31) Median (IQR) pg/mL
Types I and III IFNs	IFN-α2a	1.3 (1.3-1.3)	1.3 (1.3-1.3)	1.3 (1.3-3.1)	1.3 (1.3-1.3)	1.3 (1.3-3.6)
	IFN-β	11.7 (11.7-11.7)	117.3 (27.45-298.5)	121.8 (11.7-304.9)	113.8 (11.7-213.0)	60.11 (11.7-260.5)
	IFN-λ (IL-29)	12.0 (12.0-12.0)	12.0 (12.0-12.0)	12.0 (12.0-12.0)	12.0 (12.0-45.4)	12.0 (12.0-39.7)
Th1- secreted/associated cytokines	IFN-γ	0.3 (0.3-0.3)	2.4 (0.3-39.0)	1.1 (0.3-5.8)	0.9 (0.3-4.5)	2.1 (0.3-5.7)
	TNF-α	0.3 (0.3-0.3)	11.0 (4.0-18.9)	50.9 (23.5-320.1)	7.0 (2.6-57.5)	10.1 (6.4-19.0)
	IL-2	0.6 (0.6-0.8)	19.0 (6.8-33.8)	28.0 (13.1-40.5)	5.8 (0.6-18.0)	8.4 (5.0-17.9)
	IL-15	0.6 (0.3-0.9)	2.2 (1.0-8.4)	2.6 (1.3-5.2)	1.7 (0.8-3.7)	8.3 (6.3-10.9)
	IL-12p70	0.3 (0.3-0.3)	3.1 (1.5-9.5)	10.8 (4.9-12.2)	3.4 (1.1-6.4)	2.1 (0.9-4.7)
Th2-secreted cytokines	IL-4	0.4 (0.4-0.4)	1.0 (0.4-4.7)	25.7 (2.1-7.6)	0.4 (0.4-4.0)	1.0 (0.4-2.0)
	IL-5	0.3 (0.3-0.3)	2.7 (0.8-5.9)	5.8 (4.5-6.8)	2.1 (0.3-3.8)	1.6 (0.7-3.7)
	IL-13	0.3 (0.3-0.3)	2.4 (0.3-42.9)	3.0 (1.3-4.8)	3.0 (1.5-5.1)	2.7 (1.5-5.0)
Th17-secreted cytokines	**IL-17**	**0.3 (0.3-0.3)**	**20.6 (9.4-63.4)**	**0.3 (0.3-0.3)**	**7.7 (3.7-17.6)**	**18.4 (8.2-41.2)**
Other inflammatory cytokines	IL-6	10.3 (4.0-11.9)	459.3 (282.1 -2500)	1552 (75.5-2500)	335.5 (58.1-1441)	125.3 (44.6-343.6)
	IL-8	68.6 (57.4-90.8)	2500 (1878-2500)	2500 (2500-2500)	2500 (2500-2500)	41.9 (27.5-110.2)
	IL-1β	2.1 (0.3-3.0)	33.2 (10.1-103.0)	352.1 (425.1-1107)	136.6 (18.1-468.6)	0.7 (0.3-1.7)
	GM-CSF	0.9 (0.3-1.2)	9.1 (3.9-12.1)	1.7 (0.3-3.3)	4.8 (1.8-7.3)	1.7 (0.3-3.3)
Anti-inflammatory cytokine	IL-10	0.3 (0.3-0.3)	6.9 (1.3-27.3)	11.6 (5.8-18.6)	3.1 (0.8-8.8)	10.8 (7.5-19.7)
IFN-associated/Th1-attracting chemokines	MIG (CXCL9)	Unavailable	538.5 (276.8-2500)	747.9 (243.4-1276)	268.5 (103.7-653.0)	241.0 (102.4-466.5)
	IP-10 (CXCL10)	204.9 (148.7-326.0)	5756 (1053-10000)	8694 (4257-10000)	2990 (927.2-10000)	2116 (962.4-4422)
	I-TAC (CXCL11)	2.5 (1.2-8.9)	92.0 (22.1-181.5)	175.9 (38.9-268.8)	19.03 (5.6-72.3)	310.7 (169.2-461.2)
Th2-associated chemokines	MDC (CCL22)	54.4 (54.4-54.4)	275 (246-634.6)	786.0 (577.2-1707)	163.0 (121.6-285.8)	971.3 (546.0-1488)
	TARC (CCL17)	5.0 (5.0-5.0)	23.9 (10.5-39.1)	50.7 (32.6-89.6)	11.8 (5.0-21.3)	93.82 (63.0-207.4)
	Eotaxin-1 (CCL11)	15.0 (15.0-15.0)	573.7 (339.0-804.1)	1333 (1071-2996)	313.4 (192.2-449.3)	483.9 (350.7-750.8)
	Eotaxin-3 (CCL26)	2.5 (2.5-5.1)	87.3 (36.8-187.3)	136.1 (58.8-214.1)	44.7 (27.2-66.5)	27.7 (11.5-46.8)
Other inflammatory chemokines	MIP-1α (CCL3)	2.1 (1.4-2.4)	47.9 (22.7-216.0)	285.8 (139.7-780.4)	62.6 (19.1-251.2)	16.1 (10.8-25.1)
	MIP-1β (CCL4)	12.3 (5.3-13.7)	325.2 (163.8-1763)	3088 (698.4-6609)	272.4 (86.9-1747)	143.2 (111.1-215.1)
	MCP-1 (CCL2)	**24.3 (22.5-30.3)**	**3937 (1209-10000)**	**1858 (828.3-4706)**	**292.7 (156.3-961.0)**	**1104 (660.4-1931)**
	MCP-4 (CCL13)	3.0 (3.0-3.0)	109.4 (89.4-670.8)	170 (89.9-424.7)	15.7 (10.8-26.3)	381.2 (205.3-552.1)

Table 5.8 Comparison of 28 soluble mediators in BAL with T1 NPA, SAM and T1 serum obtained from critically-ill patients with influenza

BAL was obtained from 11 influenza-positive adult patients with grade 3 severity of illness and 28 mediators were measured by MSD. Healthy control BAL reference ranges for 27 mediators (MIG unavailable) derived using MSD assay were obtained from 11 healthy control adult subjects recruited to another study. Levels of the same 28 mediators in T1 NPA, SAM eluate and serum obtained from adult influenza-positive patients with grade 3 severity of illness are shown for comparison. Note that not all patients provided complete sample sets, therefore *n* values differ for each sample type.

5.11 Clinical biomarker assays: Serum/plasma procalcitonin, CRP and vitamin D

5.11.1 Comparison of healthy controls, influenza-negative and influenza-positive patients

Median levels of T1 serum procalcitonin were significantly elevated in influenza-positive and influenza-negative patients compared to median levels in samples from healthy controls (0.01 ng/mL, 0.2 ng/mL and 0.28 ng/mL, respectively; p <0.001 against HCs). The same trend was seen for T1 CRP median values (0.4 mg/L, 103.0 mg/L and 89.7 mg/L, respectively; p <0.001 against HCs). However, median levels did not differ significantly between influenza-negative and influenza-positive patients. Median vitamin D levels were lower in patients than in healthy controls, although again, no difference was seen between influenza-negative and influenza positive patients (55.0 nmol/L, 26.2 nmol/L and 28.2 nmol/L, respectively; p <0.001 vs. HCs) (figure 5.26).

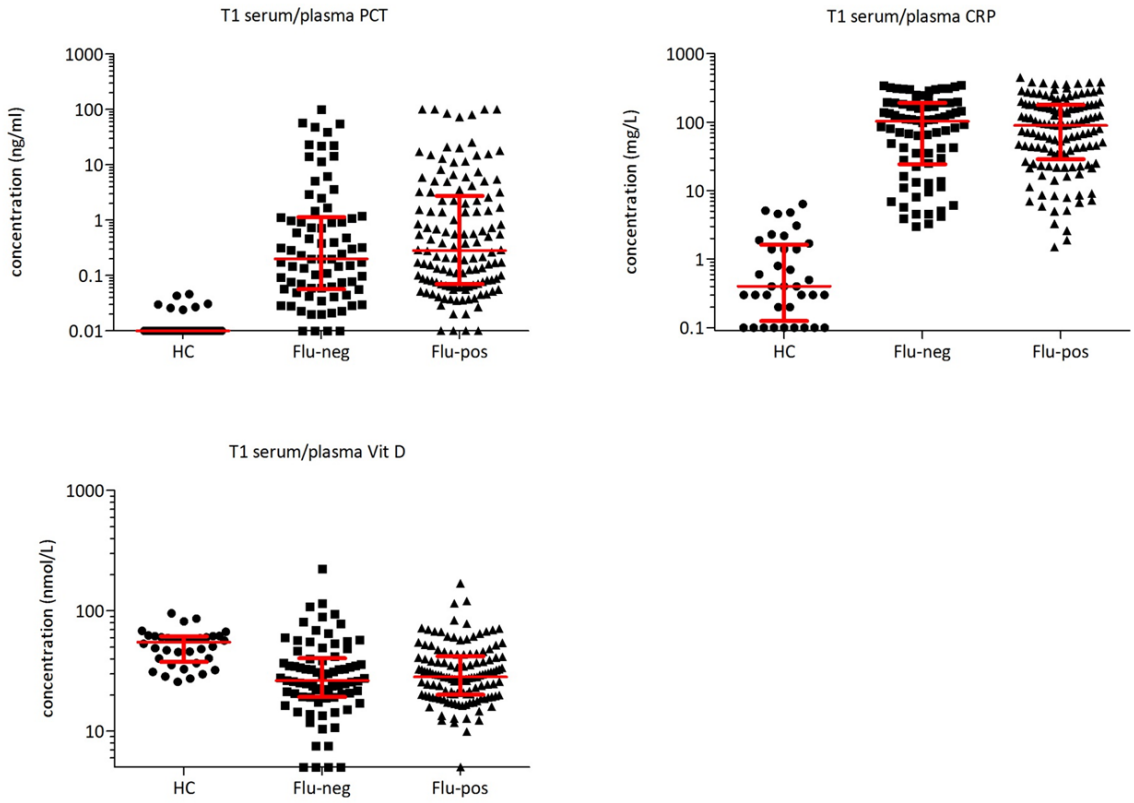


Figure 5.27 Levels of procalcitonin and c-reactive protein are elevated in influenza-positive and influenza-negative patients compared to healthy controls, whereas levels of vitamin D are lower
Procalcitonin (PCT, ng/mL), c-reactive protein (CRP, mg/L) and vitamin D (Vit D, nmol/L) were measured in serum and plasma samples obtained from patients at T1, and in samples from healthy controls. Median levels of each mediator in influenza-negative and influenza-positive patients were significantly different to median levels in healthy controls (Kruskal-Wallis with Dunn's post-test, $p<0.001$). Significant differences were not seen when comparing levels in influenza-negative and influenza-positive patients. Sample groups: HC = adult healthy controls; Flu-neg = influenza-negative adults; Flu-pos = influenza-positive adults.

5.11.2 Comparison of healthy controls and influenza-positive patients according to severity of illness

Median procalcitonin levels did not differ significantly between patients with severity 1 and severity 2 illness, but the median level was significantly higher in severity 3 patients (0.13, 0.21 and 3.2 ng/mL, respectively). Median CRP levels were similar in severity 1 and severity 2 patients (45.3 and 97.35 ng/mL, respectively). The median CRP level in severity 3 patients (167.6 ng/mL) was significantly higher than the median level in severity 1 patients, but not severity 2 patients (45.3 and 97.35 ng/mL, respectively). Relative to healthy controls, median vitamin D levels progressively decreased as severity increased, although significant differences in medians were only seen between severity 1 and severity 3 (32.3 and 20.1 nmol/L, respectively; $p<0.001$) and severity 2 and severity 3 patients (31.1 and 20.1 nmol/L, respectively; $p<0.05$) (figure 5.27).

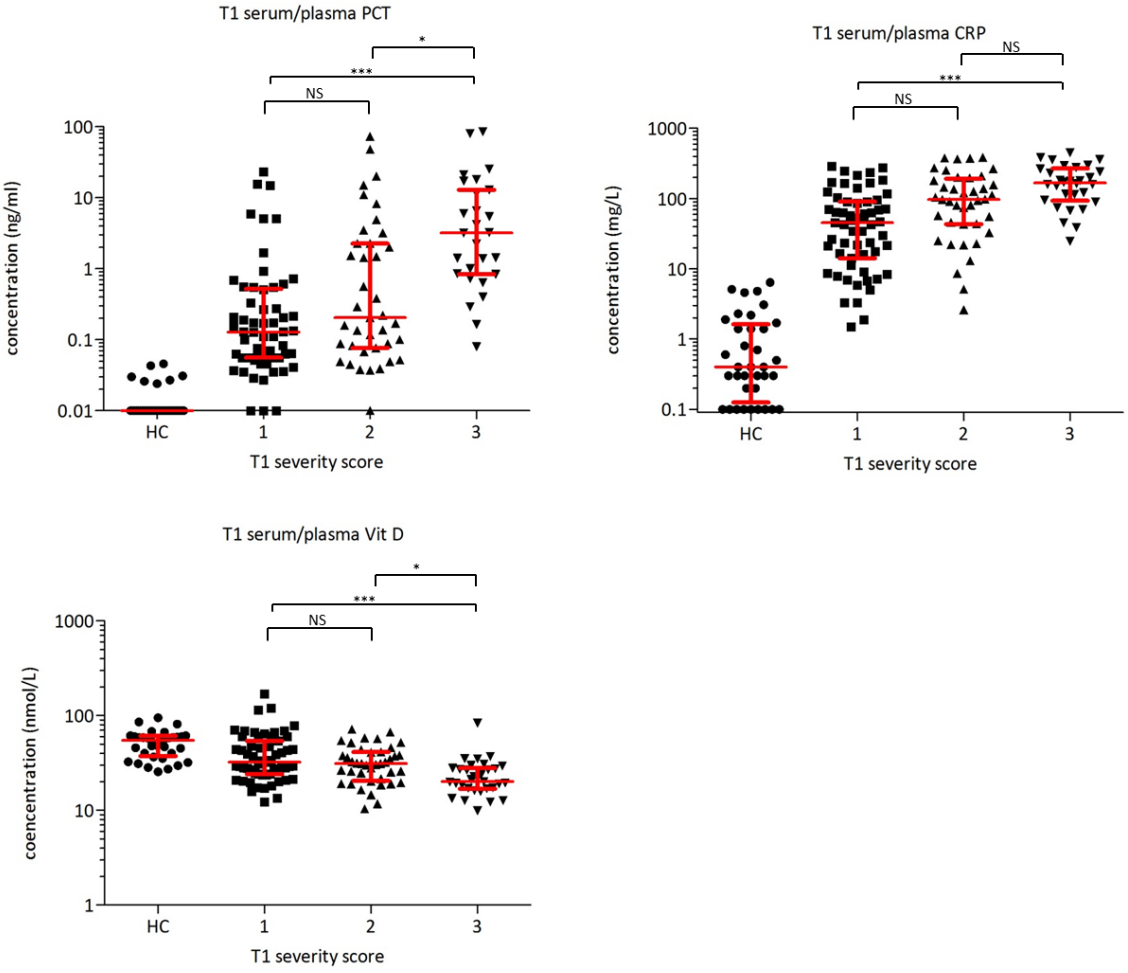


Figure 5.28 Levels of procalcitonin and c-reactive protein are highest in critically-ill influenza patients, whereas levels of vitamin D are lower than in patients with less severe influenza
Procalcitonin (PCT, ng/mL), c-reactive protein (CRP, mg/L) and vitamin D (Vit D, nmol/L) were measured in serum and plasma samples obtained from influenza-positive adult patients at T1, and in samples from healthy controls. Patients were stratified according to severity of illness at T1 (grade 1 severity; grade 2 severity; grade 3 severity). Kruskal-Wallis test with Dunn's post-test was used to assess significance (***p<0.001; *p<0.05; NS = non-significant). Median levels in all severity groups were significantly different from levels in healthy controls.

5.12 Discussion

5.12.1 Successful profiling of soluble mediators and biomarkers in blood and respiratory tract secretions from hospitalised patients with influenza and ILI

To address our hypotheses, we quantified a large number of soluble mediators and biomarkers in serum/plasma and in upper- and lower-respiratory tract secretions obtained at three time points from a large cohort of hospitalised adult patients with differing severities of influenza and ILI, and also samples from healthy controls for comparison. The high-sensitivity multiplex electrochemiluminescence MSD assay performed well for the majority of soluble mediators measured, although levels of some mediators (e.g. IL-8) were often at or above the upper limit of detection. In this initial analysis of T1 mediator and biomarker results, we have identified both differences and similarities between influenza-negative patients, influenza-positive patients, and healthy controls. For each compartment sampled, we have also identified mediator profiles associated with different severities of illness in influenza-positive adults at T1. Significant findings are discussed in the following sections.

5.12.2 IP-10 and IL-8 are significantly elevated in serum and nasal secretions from hospitalized influenza-positive patients

In serum and in NPA, median levels of the Th1/interferon-associated chemokine IP-10 (CXCL-10) and the pro-inflammatory chemokine IL-8 were significantly elevated in influenza-positive and influenza-negative adult patients, relative to levels in adult healthy controls. Median levels of both mediators were significantly higher in influenza-positive patients compared to influenza-negative patients. Many other pro-inflammatory mediators were elevated in serum from both influenza-positive and influenza-negative patients relative to levels in healthy controls, but unlike IL-8 and IP-10, levels of these mediators did not differ significantly between the two groups of patients. IP-10 was also elevated in SAM eluates, but only levels of IP-10 in samples from influenza-positive patients were significantly higher than levels in samples obtained from healthy controls. IL-8 in SAM was elevated in samples from influenza-positive patients, relative to those from influenza-negative patients and healthy controls, but levels of IL-8 in SAM were similar in all three groups.

IP-10 is a member of the CXC chemokine family and is secreted by epithelial, endothelial and stromal cells, as well as keratinocytes, fibroblasts, eosinophils, neutrophils and monocytes (472, 473). Expression of the *IP-10* gene increased in response to IFN-γ produced by Th1 cells, leading to increased secretion of IP-10 protein (472). IP-10 exerts its effects by binding to the CXCR3 receptor, which is also the target receptor for MIG (CXCL9) and I-TAC (CXCL11). The CXCR3 receptor is expressed primarily by activated T cells and NK cells, but is also expressed by macrophages, dendritic

cells and populations of endothelial and epithelial cells. Binding of ligands to CXCR3 increases intracellular $Ca2^{++}$ and activates phosphoinositide 3-kinase and MAP-kinase (474, 475). IP-10 has multiple biological functions, including induction of apoptosis, regulation of cell growth and proliferation, regulation of angiostasis, and regulation of CXCR3+ chemotaxis (476). As a proinflammatory chemokine, IP-10 serves to attract cells involved in the Th1 innate immune response, rather than cells involved in the Th2 response (which express CCR3 and CCR4). Secretion of the protein is stimulated by IFN-γ produced by Th1 cells, IP-10 secretion forms part of a Th1 positive feedback loop (477). Increased secretion of IP-10 has been implicated in the pathogenesis of infections caused by a number of viruses, including hRV (478), RSV (473), enterovirus, hepatitis B virus, hepatitis C virus, HIV (479), Ebola virus (480), Dengue virus (481), SARS coronavirus (482) and influenza A virus (483, 484). A pro-inflammatory role for IP-10 has also been suggested in certain bacterial, protozoan and fungal infections, and also in non-infectious inflammatory conditions (476).

In this study, elevated levels of IP-10 were seen in serum, NPA and SAM, irrespective of whether patients were influenza-positive or influenza-negative. This is not surprising, since we have identified other viral and bacterial infections in the influenza-negative patients with ILI and others have shown that IP-10 is increased (generally measured in serum/plasma) in many different infections. We also detected elevated levels of IP-10 in BAL obtained from 11 critically-ill influenza-positive patients, relative to levels in healthy control BAL. In an animal model of ARDS, mice lacking IP-10 or CXCR3 had decreased severity of lung injury and improved survival, irrespective of whether ARDS was induced by influenza virus infection or acid aspiration. In the same model, neutrophils infiltrating the lungs were shown to be the source of local IP-10 (485). The marked increase in IP-10 in samples from our influenza-positive patients, especially in nasal and nasopharyngeal secretions, suggests that IP-10 is an important soluble mediator in the pathogenesis of severe influenza illness in humans, particularly in patients with A(H1N1)pdm09 virus infection who accounted for the majority of patients recruited to MOSAIC.

IP-10 is markedly increased in BAL fluid obtained from A(H1N1)pdm09 virus-infected mice (484). In IP-10-deficient mice infected with A(H1N1)pdm-09 virus or PR8 (mouse-adapted H1N1) virus, morbidity, mortality and lung histopathology scores are reduced when compared with the same outcome measures in infected mice that are not deficient in IP-10. In this animal model, IP-10 appears to contribute to lung injury through activation of PI3K-Akt-p38-ATF2 and JNK/MAPK signalling pathways. Furthermore, administration of an anti-IP-10 monoclonal antibody significantly improves survival and lung histopathology in the same mouse model (484).

In our influenza-positive adult cohort, the highest median levels of IP-10 were seen in NPA and serum obtained from patients with grade 3 severity of illness. We saw a significant increase when we compared levels in serum from patients with grade 1 and grade 3 severity of illness. By contrast, median levels of IP-10 in SAM eluates were greatest in those with the least severe illness, although differences between the three severity groups were statistically non-significant. It is interesting that the levels in SAM did not follow the same trend that was seen in NPA or serum. This could reflect differences in secretion patterns of IP-10 in different compartments in response to infection, for example the back of the nose (sample by NPA) versus the front of the nose (sampled by SAM). We could not exclude the effect of sampling technique when comparing these two compartments, however, and it should be noted that many patients had IP-10 levels that were at or above the upper limit of detection of the MSD assay. Nevertheless, our findings suggest that IP-10 appears to be an important soluble mediator in influenza virus-infected patients and, when viewed alongside the findings from mouse studies, our findings support the proposal that agents targeting IP-10, such as an anti-IP-10 monoclonal antibody (484), are worthy of consideration as potential immunomodulators for severe influenza virus infection.

IL-8 is an inflammatory chemokine produced by a variety of cells, including monocytes, macrophages, lymphocytes, neutrophils and endothelial and epithelial cells (486). Secretion of IL-8 can be stimulated by IL-1a, IL-1β, IL-17, TNF-α, or by Toll-like receptor-mediated pathways and bacterial lipopolysaccharide. IL-8 binds to two receptors: CXCR2 and, to a lesser extent, CXCR1 (487). A major source of IL-8 is secretion macrophages. The main effector functions of IL-8 are the activation and recruitment of neutrophils to sites of infection or injury (488, 489), but IL-8 also attracts NK cells, T cells, basophils and eosinophils (490). Increased levels of IL-8 can be found in different compartments in a number of infectious (bacterial and viral) and non-infectious inflammatory conditions. As a common mediator of inflammation, it is not surprising that elevated levels (relative to healthy controls) were seen in the influenza-negative and influenza-positive patients in our study, especially in upper respiratory tract secretions. The highest levels were seen in influenza-positive patients, although significant differences were seen only in serum and NPA samples.

5.12.3 Th1 and Th2-associated chemokines are higher in nasal secretions from influenza-positive patients

In addition to IP-10, median levels of the related IFN-associated and Th1-associated chemokines MIG (CXCL9) and I-TAC (CXCL11) were higher in NPA and SAM eluate samples from influenza-positive patients, compared to levels in influenza-negative patients and healthy controls. The difference in

median levels of MIG in SAM eluates between influenza-negative and influenza-positive patients was not significant. However, these results suggest that Th1-mediated inflammation in upper respiratory tract tissues is important in patients with ILI, especially in patients with confirmed influenza. The Th2-associated chemokines MDC (CCL22) and TARC (CCL17) were also increased in NPA from both influenza-negative and influenza-positive patients but in SAM eluates, the levels in influenza-negative patients did not differ significantly from the respective median levels in healthy controls. Furthermore, median levels of eotaxin-1 were significantly elevated in NPA from influenza-positive patients (but not in SAM or serum) relative to healthy controls and influenza-negative patients.

Elevated levels of MDC, TARC, eotaxin-3 (and eotaxin-1) were also detected in BAL fluid obtained from 11 critically-ill patients, relative to levels in healthy control BAL. The detection elevated levels of both Th1- and Th2-associated chemokines in respiratory secretions from influenza-positive patients is interesting. Viewing the results from the patients as a whole, one might speculate that there is counter-regulation of Th1 and Th2 responses. In mice, influenza virus infection induces the simultaneous expression of IFN-γ (a Th1-associated cytokine) and IL-5 (a Th2-associated cytokine) mRNA by CD8+ T cells and multiple cells may be involved in the differential secretion of Th1- and Th2-associated cytokines during the early stages of influenza infection. Although we didn't observe marked elevations in these cytokines, we did observe elevations in Th1 and Th2-associated chemokines, which may be consistent with established Th1 and Th2 responses. If release of these chemokines is simultaneous, then our results suggest similar Th1/Th2 counter-regulation may be occurring, in an attempt to prevent infection-associated immunopathology. However, these results alone do not tell us whether individual influenza virus-infected patients had concomitant increases in Th1 and Th2 chemokines; further analysis is required to look for correlation of Th1 and Th2 in individuals and to investigate whether the presence of Th2-associated conditions, such as asthma, influence the production of Th2-associated cytokines or chemokines following influenza virus infection.

5.12.4 Serum neopterin is higher in influenza-positive patients

Irrespective of the compartment sampled or influenza virus infection status, we did not see evidence of a polarised cytokine response (Th1 or Th2). However, a small but statistically significant increase was seen in the levels of serum neopterin (measured by ELISA) in influenza-negative and influenza-positive patients, relative to the level in healthy control serum and the greatest median level of neopterin was seen in influenza-positive patients. By contrast, the median level of serum RANTES (CCL5) in influenza-positive patients was similar to that of healthy controls, but the median level of RANTES was elevated in influenza-negative patients. When we compared neopterin levels in

influenza-positive patients with different severities of illness, significantly greater levels were observed in critically-ill patients (grade 3 severity). There was no association between severity of illness and median levels of RANTES, which were similar to the median level in healthy controls in influenza-positive patients.

Significant production of neopterin by human macrophages and dendritic cells is induced by interferon-γ, which is produced primarily by activated T cells as part of the Th1 response (491). Our results suggest, based on analysis of all hospitalised influenza patients, that the Th1-mediated inflammation is evident in influenza infection, to a greater extent than in influenza-negative patients. Considering that most patients had established acute illness by the time they presented to hospital, the elevated neopterin levels most likely follow IFN-γ release that had occurred earlier in the course of infection. Elevated serum neopterin levels are seen in a variety of infectious diseases (bacterial, viral, parasitic and fungal), and also in non-infectious inflammatory conditions and malignancies (492, 493). Since urinary neopterin levels are significantly higher in patients with viral pneumonia than in patients with bacterial pneumonia (494), serum neopterin levels, or the ratio of serum CRP to neopterin (495), should be correlated with evidence of bacterial infection in MOSAIC patients, following this initial analysis.

5.12.5 Severity-associated trends for MIG, MDC and TARC differ according to the compartment sampled

When looking at associations between Th1 and Th2-associated chemokines and severity of illness in influenza-positive patients, it was interesting to note that MIG levels increased in serum and NPA as severity increased, but that levels decreased in SAM eluate as severity increased. Possible explanations include differences in Th1 responses occurring in different compartments (e.g. front-of-nose versus back-of-nose), but differences associated with sampling method also need to be considered. MIG was detected in BAL obtained from critically-ill influenza patients, but a healthy control BAL reference range was not available for comparison.

In serum from influenza-positive patients, the Th2 chemokines MDC and TARC decreased as severity increased, whereas in NPA, MDC increased as severity increased (levels of TARC in NPA did not differ with severity). Levels of MDC and TARC did not differ significantly in SAM eluate samples obtained from influenza-positive patients of different severities of illness. MDC and TARC were detected in BAL from critically-ill influenza-positive patients, but at lower levels than the median levels in NPA and serum. The decrease in serum MDC and increase in NPA MDC as severity of illness increases suggests that MDC – and the cells involved in the Th2 response – may be focussed at sites of inflammation, perhaps in an attempt to counter-act Th1 responses.

5.12.6 IL-10 is elevated in serum and nasopharyngeal secretions from critically-ill influenza patients

Levels of the anti-inflammatory cytokine IL-10 were elevated in serum and NPA from critically ill (severity grade 3) patients, who also tended to be at a later stage of illness than those with milder illness. In mice infected with lethal (but not sub-lethal) doses of influenza virus, IL-10 produced by CD8+ T cells in the lung is associated with reduced tissue inflammation (343). Therefore, the elevated IL-10 levels seen in our critically-ill influenza patients may represent a later-stage response (following induction by type I interferons) that is also part of an attempt to counteract a significant and already established pro-inflammatory response. However, in mouse models IL-10 also promotes morbidity and mortality following infection with high doses of influenza viruses (496, 497). IL-10-deficient mice display dramatically increased survival compared to wild-type mice infected with influenza, and the improvement in mortality correlates with increased expression of Th17-associated cytokines in the lung. IL-10 deficiency in mice is also associated with improved viral clearance, independent of increased recruitment of CD4+ or CD8+ T cells into the lung, but others have shown that blockade of IL-10 receptor once infection is established leads to aggravated disease and no alteration of virus clearance or virus titres in the lung (343). Although these divergent effects observed in mice may be influenced by the infecting dose and the type of mouse used, it may be that the effect of IL-10 in humans infected with influenza virus differs according to when peak IL-10 secretion occurs and the immunological and virological context in which it occurs. An IL-10 response was seen in the critically-ill influenza-positive patients in this study, but it may have been inadequate to overcome the marked and on-going pro-inflammatory response, consistent with the failed regulation that is said to underlie “cytokine storm” (498, 499). Furthermore, rather than solely exerting a beneficial, anti-inflammatory effect, IL-10 may have contributed to lung injury in the critically-ill influenza patients, since IL-10 induces collagen production and promotes recruitment of fibrocytes into the lung in mice (500).

5.12.7 Other inflammatory chemokines correlate with severity of influenza illness

The inflammatory chemokines MIP-1α (CCL3), MIP-1β (CCL4) and MCP-1 (CCL2) were markedly elevated in NPA and SAM eluates obtained from critically-ill (severity grade 3) patients. By contrast, only MCP-1 was significantly elevated in serum obtained from critically ill patients. MCP-1 is important in regulating the migration monocytes and macrophages across the vascular endothelium in response to inflammation, as well as a role in routine immunological surveillance of tissues (501). It has been shown that an elevated level of MCP-3 in plasma and nasal lavage is associated with increased severity of illness in influenza-infected patients, irrespective of age group or viral load (502). In the same study, a triad of increased levels of MCP-3, IFN-α2 and IL-10 was shown to be

associated with severity of illness and also correlated with the localisation of conventional, pro-inflammatory monocytes from blood to inflamed tissues (monocytes were measured in nasal lavage). Following our initial analysis, further investigation is required to assess whether levels of these mediators correspond with monocyte and macrophage activity and differences in sub-populations of these cells, through integration of mediator data with whole-blood cellular immunology data from MOSAIC.

The macrophage inflammatory protein (MIP) chemokines are produced by macrophages, classically in response to bacterial endotoxins (503, 504). Their secretion leads to activation of granulocytes (especially neutrophils), leading to acute inflammation, and they also induce the synthesis and release of pro-inflammatory cytokines (e.g. IL-1, IL-6 and TNF-α) (505). Further exploration of MIP-1α and MIP-1β levels and their association with severity of influenza illness is warranted in our cohort, including exploration of association of MIP and other relevant mediators (e.g. IL-8) with the presence of bacteria and with transcriptomic profiles associated with response to bacterial infection.

5.12.8 Specific mediators in different compartments are associated with different levels of influenza severity

In influenza-positive patients, we identified certain mediators that appeared to be increased in a single severity group, relative to levels in healthy controls. These "hallmark" mediators differed according to the compartment sampled. Mediators associated with critical illness (grade 3 severity of illness) were identified most commonly, although mediators associated with the least severe illness (grade 1 severity of illness) were also identified. In serum, critical illness but not milder illness was associated with elevated levels of the type I interferon, IFN-β, the Th1-associated cytokines, TNF-α and IL-2, and the inflammatory chemokine MCP-1 (CCL2). This profile suggests that, in patients with the most severe disease who also tended to be later in the course of illness, there is a sustained Th1-mediated pro-inflammatory response. IFN-β is implicated in response to intracellular bacterial infections and not just anti-viral response (506), although less is known about the role of type I interferons in infections caused by extracellular bacteria, such as *S. pneumoniae* (507, 508). MCP-1 has a pivotal role in inflammatory mononuclear phagocyte recruitment in response to bacterial infection (including *S. pneumoniae* infection) in mice and promotes clearance of bacteria (509). Additionally, very high levels of MCP-1 were seen in BAL fluid obtained from a sub-group of critically-ill influenza-positive patients in our study. Therefore, this combination of mediators may represent tissue inflammation and attempts at resolution or repair in secondary bacterial pneumonia, rather than reflecting an on-going, primary viral infection. Following this initial analysis, correlation with bacteriological and virological data should be performed to test this hypothesis.

IFN-β was also associated with critical illness when measured in NPA, but unlike serum, TNF-α, IL-2 and MCP-1 were not. However, a marked and significant increase in the pro-inflammatory cytokine IL-1β was seen in NPA from critically-ill patients, but not in NPA from patients with less severe illness (who had levels similar to those in healthy controls). Although it has been suggested that there is redundancy in both IL-1β and TNF-α functional responses, receptor knockout models using mice infected with 1918 A(H1N1) virus suggest that signalling through the IL-1 receptor is protective, whereas signalling through the TNF-α receptor leads to increased severity of illness (510). We observed a modest but significant increase in serum TNF-α in critically-ill influenza patients, but serum levels of sTNFR1 and sTNFR2 were markedly elevated in critically ill patients. Since soluble TNF receptor levels reflect the biological activity of TNF-α (511, 512), our results suggest that TNF-α signalling is contributing to the marked Th1 response and inflammation that is observed in critical influenza illness.

IL-1β promotes differentiation of naïve CD4+ T cells into Th17 cells (513). In addition to being a major inflammatory mediator during influenza virus infection, promoting survival of infected mice but also contributing to lung pathology (514), IL-1β also induces the synthesis of IL-8 and IL-17 (486), leading to a neutrophil response that is crucial to clearance of invading bacteria and influenza virus-infected cells, as well as contributing to tissue inflammation. In our study, elevated levels of IL-1β and IL-17 were also seen in BAL fluid obtained from a sub-set of critically-ill patients.

In SAM eluates, a completely different profile was seen. IFN-β was increased in critically-ill patients compared to healthy controls (113.8 pg/mL versus 66.1 pg/mL, respectively), but the difference was non-significant. Critically-ill patients were characterised by small but statistically significant increases in IL-12p70 and GM-CSF. Both of these pro-inflammatory mediators are produced by a variety of activated inflammatory cells, so it is not clear why these mediators should be increased selectively in the anterior nose of critically-ill patients and not in the other compartments sampled. By contrast, markedly elevated levels of MIG (CXCL9) were detected in SAM eluates from patients with the mildest illness (grade 1 severity), but not in SAM eluates from patients with more severe illness, relative to healthy controls. In fact, there was a suggestion of gradient, with MIG levels decreasing in SAM as severity increased, which was in complete contrast to the trends seen in serum and NPA. It may be that patients with mild (and generally earlier) influenza illness secrete MIG from the respiratory epithelium in the anterior nose, in response to virus-induced IFN-γ release, perhaps reflecting more localised disease or an effective upper-respiratory tract anti-viral response that prevents dissemination distally, through the nasopharynx and into the lower respiratory tract.

5.12.9 Hierarchical clustering reveals mediator response patterns in influenza-negative and influenza-positive patients

The clinical and microbiological characterisation of patients in MOSAIC suggests that there is significant heterogeneity in hospitalised patients with influenza and ILI. Whilst useful in obtaining an overall impression of mediator responses in hospitalised patients, comparisons of median levels of a large number of individual mediators across broad groups of patients are unable to reveal mediator patterns in sub-populations of patients. Therefore, we applied the same hierarchical clustering approach that was used for transcriptomic analyses to the MSD mediators. This semi-supervised method, with clustering performed on both conditions (individuals) and mediators has the ability to reveal patients with similar mediator profiles and also changes in immunologically-related mediators.

The initial analysis of influenza-negative patients, influenza-positive patients and healthy controls aimed to look for profiles that were infection-status specific. In serum and NPA, most healthy controls clustered together in a group. Clustering of healthy controls based on SAM profiles was less clear. Regardless of sample type, influenza-positive and influenza-negative patients did not form distinct groups. However, patients with consistently high levels of 25 mediators in NPA clustered together in a sub-group and the majority these patients were influenza positive. The mediators were pro-inflammatory, Th1-associated, and Th2-associated, but high levels of anti-inflammatory IL-10 were also seen.

In serum, a sub population of influenza-negative and influenza-positive patients consistently had high levels of inflammatory mediators (IL-8, MCP-1, MIG, IFN-γ, IP-10, IL-17, IL-15, IL-6) and also anti-inflammatory IL-10. The same group had consistently lower levels of MDC and TARC, reflecting what was seen in the earlier analysis of individual mediators. IFN-α2a was generally low in this group but the other type I interferon, IFN-β was increased in a proportion of patients in the same group. Interestingly, a group of patients with a different signature that lacked such marked increases in inflammatory mediators, including IL-17 and IL-8, also had a relative increase in IFN-β. Although type 1 interferons are classically associated with response to viral infections (515), they are also induced by bacterial infections (often via TLR pathway signalling) (506). Our initial analysis suggests that further clustering analysis of mediator results using other patient characteristics is warranted, including the presence of bacterial infection and/or the presence of a transcriptomic signature associated with bacterial response.

5.12.10 Hierarchical clustering reveals mediator patterns associated with severity of illness in influenza-positive patients.

Having explored hierarchical clustering patterns in all patients (influenza-negative and influenza-positive adults at T1), we then focussed on influenza-positive adult patients. In the transcriptomics analysis, associations were seen between severity of illness and duration of illness at T1. Therefore, we looked for similar associations between severity of illness and duration of illness with soluble mediator expression. Following statistical filtering, 22 serum mediators were included in clustering analysis. The healthy controls clustered together, with the exception of one control subject. Levels of most mediators were low relative to influenza-positive patients, but levels of MIP-1β, MDC, TARC, eotaxin-1, MCP-4, IL-12p70 and IL-4 generally were higher. This is consistent with our earlier analyses of individual mediators. There was relative heterogeneity in mediator profiles, with no distinct clustering seen in association with severity of illness or duration of illness. However, a subpopulation of patients, mainly those with the most severe illness, clustered together and relatively higher levels of specific inflammatory mediators were seen: IL-17, IL-6, TNF-α, IL-8 and MCP-1. This sub-population also had relatively lower levels of the Th2 chemokines MDC and TARC. Within this sub-population, a sub-cluster of patients with high IFN-β could be seen, predominantly patients who had more severe illness (severity grade 3) and who were at a later stage of illness (five days and over). Following this initial analysis, further work is needed to investigate association of high IFN-β levels, and also IL-6, IL-8 and TNF-α, with bacterial infection and the transcriptomic response to bacterial infection, in patients with severe influenza. Other critically-ill influenza-positive patients had markedly different mediator profiles to this group, with less marked elevation and sometimes depressed levels of the same inflammatory mediators. Further exploration of clinical and microbiological characteristics may reveal why their mediator profiles differ.

5.12.11 Critically ill influenza-positive patients have similar NPA mediator profiles

In NPA, 27 mediators were included in clustering analysis following statistical filtering. In contrast to serum, statistically significant differences in IFN-β were not seen in NPA. The heat-map revealed that the majority of critically-ill patients (severity grade 3) clustered together and increased levels of 25 mediators could be seen, including MDC and TARC. IFN-α2 and IFN-λ (IL-29) were not elevated in most patients with critical illness, relative to healthy controls. Patients with less severe illness did not form obvious clusters based on severity grade, nor could cytokine profiles be explained by duration of illness.

5.12.12 A sub-group of non-critically ill patients have a marked increase in interferons in nasal secretions

The highest NPA levels of IFN-α2a, IFN-λ (IL-29) and IFN-γ were not seen in patients with critical illness. Instead, they occurred in patients with grade 1 or grade 2 severity of illness. These patients had variable duration of illness (0-4 days and 5-28 days), suggesting that the higher levels of the interferons seen in this sub-cluster of patients does not simply reflect an early stage of illness. Levels of IL-4 tended to be lower in these patients, although levels of the other Th2 cytokines, IL-5 and Il-13, appeared to be elevated relative to healthy controls and other influenza patients (especially IL-13). Whilst type I interferons induce expression of other mediators that inhibit virus replication and promote T- and B-cell responses following influenza virus infection (515-517), type I interferons produced early in influenza virus infection also decrease neutrophil recruitment and bacterial killing in mouse models of bacterial super-infection (108). IFN-γ produced by T cells also exacerbates secondary bacterial infections but in mouse models, bacterial super-infection occurs prior to an increase in IFN-γ production (109, 518). Following our initial analysis of T1 results, further analysis is needed to look for association of marked IFN-α2a and IFN-γ expression with evidence of bacterial infection at T1. If the same subjects also provided T2 samples, it will be interesting to see how mediator profiles change with time in these patients and whether bacterial infection or a transcriptomic response associated with bacterial infection occurs later in the course of acute illness, and whether this more robust IFN response influences clinical outcomes. However, this may prove difficult since the patients are likely to have received anti-infective agents, which may influence progression of illness caused by influenza virus infection and/or secondary bacterial infection.

The marked elevation in IFN-λ (IL-29) in NPA that was seen in the same, small number of patients is also of interest. Type I and type III interferons share a common JAK/TYK-STAT-mediated signalling pathway, but whereas IFN-α/β receptor is ubiquitously expressed, IFN-λR1 is expressed primarily by epithelial cells (519, 520). In mice, influenza virus infection results in greater levels of IFN-λ than IFN-α and both mediators are produced early on following induction of infection (310). Production of either mediator alone appears to be sufficient in protecting the lung from influenza virus infection in mouse IFN receptor knockout models, but only type I interferons are capable of limiting the systemic spread of influenza virus in fully interferon-competent ($Mx1^{+/+}$) mice (307, 311).

Statistical analysis of SAM mediator results showed that significant differences in levels occurred in a small number of mediators. 18 mediators underwent hierarchical clustering and two major subdivisions occurred, dividing subjects into two groups: one where patients had profiles that were similar (but not identical) to healthy controls, generally with relatively lower levels of mediators, and

another group whether levels of mediators tended to be elevated. In this latter group, a sub-cluster of predominantly critically-ill (severity grade 3) patients was evident, with consistently increased levels of MDC, GM-CSF, eotaxin-1, MIP-1α, MCP-1, IL-10, IL-6, MIP-1β and TNF-α. Some but not all of the patients in this sub-cluster also had elevated levels of IFN-λ (but not IFN-α2a). Elevated IFN-λ was not seen in hierarchical clustering of mediators in NPA samples from critically-ill patients, which could reflect differences in expression of proteins in these different compartments, or perhaps SAM sampling was a more effective sampling technique for the detection of specific mediators. A small proportion of patients in the same sub-cluster of critically-ill patients also had markedly increased levels of IFN-γ but generally, this was not accompanied by an increase in IFN-λ or IFN-α2a. Other critically-ill patients had mediator profiles that were more like the profiles of healthy controls.

A further sub-cluster was identified, consisting of predominantly severity grade 1 patients but also severity grade 2 patients. Some patients were at early stages of illness, whereas others were at a late stage of illness ($\geq$ 5days). This group was defined by consistently elevated levels of IFN-α2a , accompanied by an increase in IFN-λ in most patients. Unlike the sub-cluster of critically-ill patients, this group also had consistent elevation of IGN-γ, I-TAC and IP-10, suggesting a marked Th1 response. However, they lacked such high levels of the inflammatory cytokine GM-CSF and the chemokines MDC and MIP-1α, perhaps reflecting less established and less severe tissue inflammation. Most patients in this sub-cluster also had marked elevation of IL-10, which suggests an anti-inflammatory response to counter-act the marked interferon and Th1 inflammatory response. Although further analysis is required to assess whether these particular patients progressed to more severe illness, it may be that the combination of an early and robust anti-viral response with an effective anti-inflammatory response limits progression of virus-related inflammation and subsequent tissue damage.

5.12.13 Procalcitonin and c-reactive protein increase as the severity of influenza increases

Median levels of serum/plasma procalcitonin (PCT) and c-reactive protein (CRP) were significantly elevated in influenza-negative and influenza-positive adult patients, relative to healthy controls, but no significant difference was seen between the two patient groups. The increase in CRP is not an unexpected finding, since elevations of CRP are common in patients with acute respiratory infections and febrile illnesses, irrespective of the underlying aetiology. Most studies of hospitalised patients have shown that measurement of CRP does not help differentiate between patients with influenza virus infection, bacterial infection and those with mixed viral-bacterial infection (521), although one small study of critically ill patients with A(H1N1)pdm09 virus infection showed that very high levels

(CRP >200 mg/L) could identify mixed viral-bacterial infection (sensitivity 100%, specificity 87.5%) (522). We found that the median levels of PCT were below 0.5 ng/mL in influenza-negative and influenza-positive patients, suggesting that bacterial infection was uncommon in both groups. However, there were individuals in both groups who had PCT levels >2 ng/mL, which suggests that invasive bacterial infection or bacterial pneumonia was highly likely in these patients. When influenza-patients were stratified according to severity of illness, the median level of PCT was <0.5 ng/mL in patients with grade 1 and grade 2 severity of illness, whereas PCT was significantly higher in patients with severity grade 3 illness (median 3.2 ng/mL). In each severity group, patients with low and high PCT values could be seen, however. Median CRP increased as severity of illness increased, but a significant difference was only seen when comparing severity 1 with severity 3 patients. The median level of CRP was 167.6 mg/L in severity 3 patients, but <100 mg/L in those with less severe illness, which supports the observation from the Flu-CIN study that CRP >100 mg/L is associated with severe outcome (defined as requirement for critical care or death in hospital in the Flu-CIN study) (162).

PCT is the prohormone of calcitonin. In animal models of bacterial sepsis, PCT is produced by a large number of organs and parenchymal tissues, including neuroendocrine cells in the lower respiratory tract and intestine (523, 524). In animals, PCT is detectable within hours following induction of sepsis (525, 526), and it is also detectable within hours following challenge with lipopolysaccharide endotoxin in healthy humans (527). Furthermore, the PCT response appears to be longer-lasting than many acute-phase reactants and pro-inflammatory cytokines, with supra-normal levels detectable at seven days following LPS challenge and sometimes lasting as long as 14 days (525, 528, 529). Although PCT release is seen in non-infectious acute severe inflammatory states, such as pancreatitis (530), severe burns (531), and multiple trauma (532), the greatest elevations of PCT are seen in bacterial sepsis (528, 533). Additionally, studies of non-infectious acute severe inflammatory conditions vary in their ability to identify or exclude concurrent or secondary bacterial infection, so elevated PCT levels in these conditions may be wholly explained by the primary condition. Localised bacterial infections and abscesses are not associated with significant elevations in PCT, although small elevations can be seen (534).

In vitro, expression of PCT mRNA in monocytes is increased by stimulation with bacterial endotoxin and also by inflammatory cytokines released during bacterial infections, especially IL-6, IL-1α and TNF-α (526, 529, 535, 536). Since these soluble mediators are often elevated in severe inflammation generally and yet procalcitonin shows relative specificity for bacterial infection, other unidentified mechanisms must be involved in the stimulation of the PCT response. By contrast, the viral infection-

associated mediator IFN-γ inhibits IL-1β-mediated PCT release *in vitro* (537), supporting observations that significant increases of PCT are not seen in patients with 'pure' viral infections.

In addition to being a biomarker for bacterial sepsis, PCT appears to have a role in the pathogenesis of sepsis. When exogenous PCT is administered to hamsters infected with a sub-lethal dose of bacteria, exogenous PCT doubles mortality (525, 538). PCT also increases levels of pro-inflammatory cytokines when added to human lymphocytes, especially levels of TNF-α, which may promote a positive-feedback loop (535, 536, 539). Early and late administration of anti-procalcitonin IgG in animal models of severe sepsis has been shown to decrease mortality significantly and early administration also inhibits LPS-induced release of TNF-α and IL-1β (540, 541).

A meta-analysis of six studies which examined the role of PCT in identifying bacterial infections in patients with influenza virus infection concluded that PCT has high sensitivity for identifying bacterial co-infection, especially in critically-ill patients, but that sensitivity is low (400). The authors recommend that serum PCT should be used as a rule-out test for bacterial co-infection (high negative predictive value). In studies of community acquired pneumonia, levels of PCT were higher in those with pneumonia of proven bacterial aetiology and high levels correlated with more severe disease and worse clinical outcomes (542, 543).

Accepting the paradigm that high levels of PCT (>2 ng/mL) are consistent with bacterial infection (524), our results suggest that bacterial co-infection was present in 14/26 (54%) critically-ill adults with influenza virus infection, at the time T1 samples were obtained. A proportion of patients with severity grade 1 and 2 illness also had high PCT levels. Following this initial analysis, and the associations seen between higher PCT levels and bacterial response pathways in transcriptomics analyses (described in chapter 4), in future work PCT levels should be correlated with microbiological evidence of bacterial infection, antibiotic administration data, and also with levels of the associated mediators TNF-α, IL-6 and IL-1β. The identification of high levels of PCT in many patients with influenza virus infection, especially those with critical illness, suggests that patients with severe influenza illness may be candidates for future clinical trials of anti-PCT antibody therapy. The low levels of serum PCT that were seen in approximately half of the influenza-positive group in our study (predominantly those with non-critical illness) suggests that factors other than bacterial super-infection need to be considered as the cause of complicated influenza in those without critical illness. A small number of critically-ill patients also had low PCT values, but given that some critically ill patients had received care at the time of recruitment, their low levels may reflect a response to successful antibiotic treatment. In future work, the relationship of PCT with receipt and timing of antimicrobials will need to be explored.

5.12.14 Vitamin D levels decrease as influenza severity increases

We observed a gradient in serum/plasma vitamin D levels, whereby the highest levels were seen in healthy controls, and levels were seen to decrease in influenza-positive patients as the severity of illness increased. Findings from numerous studies suggest that low levels of vitamin D are associated with the likelihood of developing symptomatic influenza virus infection. One study of healthy adults found that maintenance of a vitamin D level of ≥38 ng/mL correlated with a statistically significant two-fold reduction in the risk of developing acute respiratory tract infections and also with a reduction in the number of days that individuals were ill (544). A similar reduction in risk of viral upper respiratory tract infections has been demonstrated in children and adolescents (545). Multiple studies have shown that vitamin D supplementation does not reduce the risk of acute upper respiratory tract infections in healthy adults, although subjects generally had normal or near-normal vitamin D levels at baseline (546-549). Additionally, supplementation does not appear to reduce the risk of exacerbations of COPD (550). Although vitamin D levels in people living in temperate regions (including the UK) tend to be lower in seasons when UVB light levels are lower (551), typically seasons when influenza outbreaks occur, other factors that occur at the same time of year, such as changes in environmental temperature and relative humidity, may also contribute to the increase in influenza activity.

The suggestion that low vitamin D levels may be associated with more severe flu goes back to the 1918 H1N1 influenza pandemic, when it was observed that the lowest influenza and pneumonia-related mortality was seen in the US city with the highest UVB light levels, whereas the highest mortality was seen in the city with the lowest UVB light levels (552). There are few data on the association between influenza severity and vitamin D levels, however. For all-cause community acquired pneumonia cases, a study of 300 adult patients showed a negative correlation between 1,25-hydroxy-vitamin D levels and pneumonia severity (measured by the CURB score), but not with 25-hydroxy-vitamin D levels (553). This association was independent of age and season and, when a pathogen was detected, the infecting pathogen (*Streptococcus pneumoniae*, *Listeria monocytogenes*, influenza A virus, and influenza B virus). In the same study, the proportion of patients with vitamin D deficiency (25-OH-vitamin D <20 ng/mL) was greatest in influenza virus-infected patients (n=50), but significantly different transformed 25-OH-vitamin D levels were not detected when comparing patients infected with different respiratory pathogens.

In our analysis of influenza-positive adults from both seasons combined, we found a small and non-significant decrease in the median level of 25-OH-vitamin D in patients with severity 2 illness compared to severity 1 illness. Severity 3 patients had the lowest levels, with significantly lower

levels compared to severity 1 and severity 2 patients. Using accepted UK ranges for 25-OH vitamin D levels in adults (554), the median levels for severity 1 and 2 patients were compatible with insufficiency (25-50 nmol/L), and the median level in severity 3 patients was compatible with deficiency (<25 nmol/L). All three severity groups had significantly lower levels than healthy controls. The median level for healthy controls was compatible with an adequate vitamin D status (50-75 nmol/L). Although healthy controls were recruited during summer months, it is interesting to note that 15/36 (41.7%) had levels suggesting vitamin D insufficiency.

1,25-OH vitamin D_3 has anti-inflammatory properties, including direct and indirect influence on CD4+ T cell differentiation. The overall effect is promotion of Th2 and regulatory T cell responses and attenuation of anti-inflammatory Th1 and Th17 responses (555). However, through the down-regulation of TLR2, TLR4 and TLR9 on monocytes and macrophages, vitamin D also can also suppress production of pro-inflammatory cytokines such as TNF-α and Il-6 (556, 557). 1,25-OH vitamin D3 also induces the production of the peptide cathelicidin, which demonstrates antimicrobial activity against bacteria (Gram-negative and Gram-positive), fungi, and enveloped viruses (558). The human cathelicidin, LL-37, has a significant antiviral effect against influenza virus *in vitro,* acting directly against influenza virions, and LL-37 treatment of influenza virus-infected mice is associated with lower levels of pro-inflammatory cytokines in the lung and decreased mortality (559).

Our results suggest that a lack of vitamin D in critically-ill patients may promote or help to maintain the pro-inflammatory cytokine response that was seen in this group and may also lead to less effective innate anti-viral and anti-bacterial responses. Vitamin D deficiency is common in adult intensive care patients *per* se and deficiency is associated with increased mortality. Although our critically ill patients tended to be recruited later in the course of illness, it is unlikely that vitamin D stores had depleted during acute illness. Rather, we propose that these patients were likely to have been vitamin D deficient at the time they acquired infection and that a lack of vitamin D contributed to their exuberant pro-inflammatory innate response and also to secondary bacterial infections. It is not clear why these patients were deficient in vitamin D, however. Following this initial analysis, further work is required to explore whether very low levels of vitamin D can be explained by underlying comorbidities (including chronic kidney disease and obesity) or differences in ethnicity, and also whether vitamin D levels correlate with specific immunophenotypes in our patients. Little is known about the benefits of correcting vitamin D insufficiency or deficiency in established influenza illness or in other forms of acute severe respiratory infection, but administration of high-dose vitamin D or exogenous cathelicidin may be worth considering in future investigations of adjuvant therapies.

5.12.15 Comparison with other studies of soluble mediators in influenza

The majority of influenza-positive patients in our study had A(H1N1)pdm09 virus infection. Therefore, it is reasonable to compare our findings with other studies of patients infected with the same virus, although comparison with studies of soluble mediators in severe illness caused by other influenza subtypes is also helpful. Unlike MOSAIC, most observational studies have attempted to measure in serum or plasma without measurement of mediators in respiratory tract samples, with the justification that changes in mediators in blood are representative of changes in the lower respiratory tract, since the lungs are highly vascular organs (135, 467). However, as demonstrated in this study, significant differences in levels and trends of mediators can be seen in different compartments, so while the profiling of mediators in blood alone has operational advantages, it does not provide a complete picture of soluble mediator responses in influenza virus infection.

In their study of patients with A(H1N1)pdm09 virus infection in Spain, Bermejo-Martin *et al.* measured 29 soluble mediators in serum using a Bio-Plex 27-plex assay (Bio-Rad) and two individual ELISA assays (334). Twenty hospitalised adult patients (including 10 critically-ill patients), 15 adult outpatients and 15 adult healthy controls were included in their analysis. Patients with bacterial infection or who had been in hospital for more than 5 days were specifically excluded. Day of illness at time of sampling was not stated. The authors found that increased levels of IP-10, MCP-1 and MIP-1β were present in serum from inpatients and also outpatients not requiring hospitalisation, but that hospitalised patients were characterised by high levels of IFN-γ, Th1-associated mediators (TNF-α, IL-15, and IL-12p70) and Th17-associated mediators (IL-8, IL-9, IL-17, and IL-6), relative to levels in outpatients with mild illness. IL-15 and IL-12p70 were increased exclusively in critically-ill patients. Levels of IL-6 were also greatest in critically-ill patients, leading the authors to conclude that increased IL-15, IL-12p70 and IL-6 constituted a mediator hallmark of critical illness. We did not detect a significant increase in serum IFN-γ, IL-12p70 and MIP-1β in serum from our influenza-positive patients, relative to healthy controls, but we did see elevated levels of the other Th1 mediators identified in their study and also the same "Th17-associated" mediators (IL-9 was not measured). Rather than IL-15 and IL-17, we detected a different "hallmark" panel of mediators in critically ill patients: IFN-β, TNF-α, IL-2 and MCP-1. Possible explanations for these discrepant findings include differences in duration in illness and the specific exclusion of patients with bacterial infection in the Bermejo-Martin study. Furthermore, they sampled significantly fewer patients than were included in our study.

Lee *et al*. studied 66 hospitalised adult Chinese patients with A(H1N1)pdm09 virus infection and measured 13 soluble mediators in plasma using cytometric bead arrays and ELISA (333). Patients

were stratified according to severity of pneumonia. Twenty-eight patients (42.4%) had severe pneumonia (hypoxaemia and radiographic pneumonia) and these were compared with 38 patients (57.6%) who had no pneumonia or mild pneumonia (radiographic pneumonia but no hypoxaemia). Median day of illness at presentation was 4 days (range 1-6 days) for severe pneumonia patients and 2 days (range 1-4 days) for no/mild pneumonia patients, although the day of illness at time of sampling was not stated. Patients with community-acquired or nosocomial bacterial infection were not excluded (32.1% severe patients; 7.9% mild patients). Mediators were measured in serum from 24 patients with severe pneumonia and 29 patients with milder illness. Severe illness was associated with marked elevations in IL-6, IL-8 and MCP-1, relative to those with milder illness. High plasma levels of the same three mediators and also sTNFR1 correlated with the extent and progression of radiographic pneumonia. IL-10 was also significantly higher in those with severe illness. The authors comment that the same mediators have been reported to be elevated in HPAI A(H5N1) virus-infected patients, but the increases were less marked in their A(H1N1)pdm09 virus-infected cohort. Allowing for differences in how severity was measured and how patients were grouped according to severity, their findings are consistent with the trends seen in analysis of serum mediators in our much larger hospitalised cohort of predominantly white European patients. However, we found IP-10 to be significantly higher in critically-ill patients, whereas they found no significant difference between IP-10 levels in severe and milder patients. In fact, IP-10 levels were much higher in patients with seasonal A(H1N1) virus-infected patients, which they cited for comparison. We also detected significantly higher levels of MIG, IFN-α2a, IFN-β, IL-15 and IL-17 in critically-ill patients, but these mediators were not measured in their study. Increased TNF-α2a was associated with critical illness in our study but not in their study, which may reflect differences in assay sensitivities. However, we also observed marked elevations of serum sTNFR1 and sTNFR2 in critically-ill patients.

In a separate study, the same group compared the levels of 13 mediators in plasma obtained at presentation to hospital from 34 patients with severe A(H1N1)pdm09 virus infection, 29 patients with mild A(H1N1)pdm09 virus infection, 10 patients with seasonal A(H1N1) virus infection, 35 patients with A(H3N2) virus infection and 8 patients with influenza B virus infection (335). In patients with A(H1N1)pdm09 virus infection, they confirmed their observation that elevated levels of pro-inflammatory mediators (including IL-6, IL-8, MCP-1 and sTNFR1) are associated with increased severity of illness, but also showed decreased levels of IP-10, MIG and IL-17, relative to levels in patients with seasonal influenza. Furthermore, lower IL-17 levels were shown to be associated with respiratory and cardiovascular complications. These observations are at odds with the findings from our study, since we saw small but significant increases of serum IL-17 in influenza-positive patients relative to healthy controls, with the highest levels seen in critically-ill patients. The reason for this

discrepancy isn't clear. Lee *et al.* speculate that Th17-mediated adaptive immune responses are weaker in A(H1N1)pdm09 virus infection compared to seasonal influenza virus infection and that this contributes to delayed clearance of virus and a sustained pro-inflammatory response in severe A(H1N1)pdm09 illness. However, the relative contribution of the adaptive immune response in clearing influenza virus during primary infection is controversial (560).

Few studies have analysed soluble mediator levels in the lower respiratory tract, but mediators have been measured in lung tissue obtained from fatal cases of A(H1N1)pdm09 virus infection. Gao *et al*. used a 27-plex bead-based assay (Bio-Rad) to quantify mediators in homogenised frozen lung tissue obtained at autopsy from 27/50 patients (median age 38; range 4 months – 72 years) (561). Seven of the 27 patients (26%) had bacterial co-infection. Results were compared with those obtained from analysis of 12 normal lung tissue specimens. Marked elevation of 7 mediators were reported in the samples from A(H1N1)pdm09 virus-infected patients: IL-1 receptor antagonist protein (IL-1RA), IL-6, TNF-α, IL-8, MCP-1, MIP-1β and IP-10. With the exception of IL-1RA, which we did not measure, we saw the highest levels of the same mediators in NPA samples from critically-ill patients. All of these mediators were elevated in BAL fluid obtained from a subset of critically-ill patients in our study, relative to levels in healthy control BAL, but many other mediators were also elevated in BAL. Gao et al also reported that cases with bacterial co-infection had markedly higher levels of IL-6 and TNF-α than those who did not have bacterial co-infection. In future work, it will be important to correlate levels of IL-6 and TNF-α with evidence of bacterial co-infection in our subjects.

5.12.16 Conclusions

We have shown that it is possible to accurately quantify many important soluble immune mediators in a large number of hospitalised patients with influenza virus infection and other causes of ILI during an influenza pandemic. In contrast to other studies, we have measured mediators in upper respiratory tract secretions (NPA and SAM eluate) and lower respiratory tract secretions (BAL fluid), and not just in serum or plasma. This broad approach is important in pathogenesis studies because changes of some mediators in blood may not be representative of changes that occur where influenza virus infection actually occurs i.e. the respiratory tract. Indeed, we have shown that certain mediators increase in upper respiratory tract secretions whilst at the same time they are seen to decrease in serum. These compartmental differences may reflect site-specific inflammation, or perhaps the movement of mediators between compartments.

There was enough overlap in symptoms at the time of diagnostic testing for influenza virus infection to have been considered possible in all patients recruited to MOSAIC. In this initial analysis of data from the first acute time-point, the levels of many mediators did not differ significantly between

patients with evidence of active influenza virus infection and with those in whom we could not identify influenza virus infection, reflecting common innate immune responses that can be seen in a wide range of respiratory infections. However, it is clear that hospitalised patients with influenza virus infection have significant elevations of certain mediators that were not seen in influenza-negative patients. Across all compartments, the chemokine IP-10 (CXCL10) stood out as an important pro-inflammatory mediator, consistent with a profound interferon-related/Th1-related innate response to influenza virus. Furthermore, in influenza-positive patients we saw differences in the association between IP-10 levels and severity of influenza illness, increasing in serum and NPA as severity increased, but decreasing in secretions from the anterior nasal compartment (measured in SAM eluates) as severity increased. This suggests that IP-10 release may be focused at the key sites of inflammation. In severe A(H1N1)pdm09 virus infection, the lower respiratory tract is a major site of inflammation. Although we could demonstrate elevated levels of IP-10 in lung secretions from the small number of patients who provided BAL samples, measuring IP-10 in more easily obtained serum and NPA samples may be a useful surrogate. The role of IP-10 in the pathogenesis of influenza-related acute lung injury is unclear, but our identification of IP-10 as a key mediator suggests that IP-10 is a target for novel therapeutic approaches. This could include trials of specific monoclonal antibodies in those with elevated levels of IP-10 or the other related IFN/Th1 chemokines, MIG (CXCL9) and I-TAC (CXCL11), or blockade of their common receptor, CXCR3.

Our results suggest that the term “cytokine storm” is overly simplistic. While marked elevations of pro-inflammatory mediators can be seen in patients with severe influenza, we have shown that even in this initial single acute time-point analysis, there was significant heterogeneity in the magnitude of response of individual mediators, and also in the types of mediators that were increased or decreased. We saw marked elevations of Th1 and IFN-associated mediators, which may be anticipated in a severe viral infection, but we also observed increases in Th2 mediators, which was more surprising. Such a mixed response may represent an attempt at counter-regulation of inflammatory responses, but the contribution of underlying conditions, such as asthma (a condition which is over-represented in patients infected with A(H1N1)pdm09 virus) in determining mediator profiles requires further analysis. Similarly, relatively high levels of the anti-inflammatory mediator IL-10 were observed in critically-ill patients, generally at the same time as high levels of inflammatory mediators were present in blood. While it is tempting to assume that the release of IL-10 is beneficial to a host with such profound inflammation, it is not clear whether this is the case, since mouse models suggest that high levels of IL-10 may be counterproductive to resolution of influenza virus infection and associated severe inflammation. Similarly, although interferons may be beneficial in antiviral response, as suggested by the high levels of IFN-α2a, IFN-λ (IL-29) and IFN-γ in

NPA that were seen in patients with less severe illness, an initial, exuberant IFN/IFN-associated mediator response may leave the host susceptible to later, secondary bacterial infection. Further correlation with influenza viral load data and bacteriological data is required.

To look for "hallmarks" of specific levels of severity of influenza illness, we used the same approach adopted by other studies, whereby we identified mediators that were significantly increased in a single severity group, relative to healthy controls. Without stratification of patients based on duration of illness, and without excluding those with co-infections, we identified high levels of the type I interferon, IFN-β, the Th1-associated cytokines, TNF-α and IL-2, and the inflammatory chemokine MCP-1 (CCL2), in serum from critically ill patients. Additionally, critically-ill patients were unique in having marked and significant increases of IFN-β and IL-1β in NPA, relative to levels in healthy controls. This pro-inflammatory profile may simply represent acute lung injury and systemic inflammatory response syndrome secondary to influenza virus infection and because most of our most severely ill patients were already critically-ill at the time of presentation to hospital, it is impossible to say what immunological (or virological) events preceded – or contributed – to this state. Not knowing what events lead to critical illness is a limitation, but is not unique to our study and it is a reflection of the well-described course of A(H1N1)pdm09 virus infection. Although patients with less severe illness tended not to require critical care later during the period of hospitalisation, some patients did increase from grade 1 to grade 2 severity of illness. Subsequent longitudinal analysis of mediators in T1, T2, and T3 samples, with correlation with clinical and microbiological data, may reveal immunological changes associated with progression or resolution of illness.

Additionally, the mediator responses seen in many of our critically ill patients may have been driven, at least in part, by secondary bacterial infection. Following this initial analysis, further correlation with virological and bacteriological data for all patients is required, including sequential analysis of mediators at different time points. For all mediator trends, correlation with cellular immunology results, transcriptional profiles and host genetic polymorphisms will also be useful.

In an attempt to make sense of changes in large numbers of mediators across different compartments, we also applied a hierarchical clustering method and heat-map analysis of mediators measured by MSD. This has provided a greater granularity and allowed us to identify sub-groups of patients with similar mediator profiles, even within the same severity group. The patterns seen could not simply be explained by duration of illness, but our unbiased analysis revealed that critically-ill influenza patients cluster separately from other patients, especially clustering based on mediators in NPA and in SAM eluate. Although correlation with other clinical and microbiological

variables now needs to be applied, our results suggest that influenza-positive patients with established critical illness are an immunologically distinct group and may require immunomodulators that target a specific group of mediators, if they are to be tried in clinical trials. If performed in real time, such profiling may allow immunomodulation to be tailored to the individual patient and longitudinal profiling could also be used to assess response to treatment.

We measured PCT, CRP and vitamin D in serum/plasma. These biomarkers/mediators can be measured routinely by clinical laboratories. The low PCT levels detected in approximately 50% of the influenza cohort suggests that significant bacterial infection does not contribute to illness in many patients requiring hospitalisation. However, since bacterial rather than viral infections are associated with high PCT levels, our results suggest that a significant proportion of our patients did not have significant bacterial infections, especially those who were critically ill. Therefore, there may be a role for PCT in guiding which influenza virus-infected patients receive or continue to receive antibiotics. This is particularly important since detection of bacteria is difficult, even with extended molecular detection methods. PCT may also be a target for immunomodulatory therapy. Further analyses to correlate PCT with known bacterial infections are warranted and because some patients with less severe illness also had high PCT levels at T1, it will also be interesting to see whether their mediator profiles change between T1 and T2. Finally, a gradient was seen for vitamin D, with levels decreasing as influenza severity increased. Although it is not clear whether vitamin D deficiency predisposes to severe influenza illness or exacerbates it, there may be a role for vitamin D supplementation as part of treatment.

Great care was taken to achieve standardisation of sampling and assays. For technical reasons, we were unable to make dilution adjustments for SAM eluate returns, following the addition of a fixed volume of eluate buffer. However, we do not believe that this has significantly impacted the comparison of SAM eluate samples. Due to limited sample volumes and the large number of assays performed on NPA samples, we were unable to assess sample quality e.g. by quantifying IgA content, nor could we estimate cellularity of NPA samples e.g. by measuring urea content. However, we believe that the standardised approach to NPA sampling will have reduced sampling bias. For certain mediators e.g. IL-8, levels in some patient samples reached or exceeded the manufacturer-defined upper limit of detection for detection. Due to the nature of the multiplex MSD assay, we were unable to dilute samples to prevent this from happening and due to limited sample volumes, we were unable to perform separate ELISA assays of diluted samples for these mediators. After seeking independent statistical advice, we do not believe that this has influenced statistical comparisons of different groups, however.

In conclusion, we have performed a careful and detailed initial analysis of mediators at a single, acute time point in adults. In patients predominantly infected with A(H1N1)pdm09 virus, we have identified patterns of soluble mediator response that differ according to severity of illness and compartment sampled. A marked pro-inflammatory response is frequently seen in severe influenza, irrespective of virus subtype, and our results suggest that the magnitude of the response is greater than that seen in patients with ILI that is not caused by influenza virus infection. Our results suggest that the term “cytokine storm” is too simplistic, however. We have identified sub-groups of patients who share relatively distinct mediator profiles representing activation of different immunological pathways, including Th1, Th2 and anti-inflammatory mediators. This suggests that investigation of immunomodulatory therapies for influenza is worthwhile, but that such treatments need to be tailored to individual patients with established disease. We now plan to explore longitudinal changes in mediators and explore the patterns seen by correlating mediator results with additional clinical and microbiological parameters from the detailed MOSAIC database, as well as integrating these results with outputs from other MOSAIC laboratories.

Chapter Six

6 General Discussion

6.1 Summary of the MOSAIC study

The MOSAIC study was unique in examining the contributions of host, pathogen and co-pathogen factors in a single, integrated study of influenza pathogenesis in humans, following the emergence of a novel pandemic influenza virus. The Consortium responded quickly following the declaration of the 2009-10 pandemic and was successful in recruiting 255 adults and children with severe influenza and influenza-like illness, in two major cities during the second UK pandemic wave and the first post-pandemic winter. Sixty-seven percent of patients had laboratory evidence of active influenza virus infection, predominantly A(H1N1)pdm09 virus infection. To the best of our knowledge, no other group has performed such extensive investigations of a large number of hospitalised patients during an influenza pandemic. Little is known about the pathogenesis of A(H1N1)pdm09 virus infection in humans and we expect that the findings from MOSAIC will make a significant contribution to understanding the mechanisms of severe influenza. MOSAIC's ultimate aims are to integrate all of the different outputs from MOSAIC, create an integrated model of pathogenesis in humans, and stimulate subsequent scientific studies.

6.2 Conclusions and Future Directions

6.2.1 Clinical and microbiological characteristics of hospitalised influenza patients

We have collected extensive and detailed clinical data from a large cohort of hospitalised patients with influenza virus infection (predominantly A(H1N1)pdm09 virus infection) and also from patients with ILI but no evidence of active influenza virus infection. These data were collected using the Flu-CIN data collection tool (retrospective case note analysis), supplemented by an additional CRF to capture specific data in real-time. Initial analysis has shown that our influenza-positive cohort is reasonably representative of populations of hospitalised patients with A(H1N1)pdm09 infection. Adult patients accounted for the majority of cases and patients with known risk factors were represented. These include patients with asthma, immunosuppression, obesity, other chronic disease and women who were pregnant, as well as patients who had no underlying health conditions. A scoring system for severity of illness was developed, which divided patients into three large sub-groups with illness ranging from mild (no respiratory compromise) through to critical illness (requiring invasive mechanical ventilation and/or ECMO) and death. The careful characterisation of patients has facilitated comparison of different patient groups in this work and

will also be extremely useful for future analyses. We have also performed an extensive search for evidence of viral and bacterial co-infections. This included molecular detection methods in addition to standard methods such as bacterial culture. The addition of NHS-obtained microbiological data to our own microbiological investigations means that MOSAIC is well-placed in addressing an important and clinically-relevant question: how common is bacterial and viral co-infection in patients hospitalised with influenza, and how this relates to severity of illness.

In samples obtained at the first acute time point (T1) we detected bacteria associated with respiratory tract infection in upper respiratory samples from 40% of patients with influenza virus infection. The most commonly detected species were *S. pneumoniae*, *S. aureus* and *H. influenzae*. While we cannot identify whether these detections represent infections rather than carriage, or whether these pathogens were also present in the lower respiratory tract, we also detected invasive *S. pneumoniae* infections through analysis of blood culture and urinary antigen testing results. Approximately half of influenza patients had elevated levels of serum procalcitonin, suggesting that significant bacterial infection in a significant proportion of cases. We also detected other respiratory viruses in 35/135 (26%) of influenza virus-infected patients, most commonly rhinovirus and RSV. By very detailed analysis, we have therefore shown that bacterial and viral co-pathogens are much more frequent than previously suspected in cases of influenza.

These findings support the use of antibiotics in addition to antiviral drugs in many of those hospitalised with influenza. However, in some patients antibiotic therapy may be unnecessary and possibly detrimental, leading to side-effects and antibiotic resistance. Since it is known that routine bacteriological detections methods and clinical assessment are poor indicators of bacterial infections, future work should examine how identification of coinfections can be improved. This may include assessment of host response to bacterial infection by transcriptomics and other biomarkers. In this regard, further analysis of MOSAIC data is required, including longitudinal analysis of data from the later time points not described in this thesis (i.e. T2 and T3).

6.2.2 Whole blood transcriptomic profiling of severe influenza virus infection

We have applied whole blood transcriptomic profiling to investigate the immunopathogenesis of severe influenza. To the best of our knowledge, this is the first time that transcriptomic analysis of a large, well-characterised, mixed population of hospitalised patients with predominantly monogenic A(H1N1)pdm09 virus infection has been performed. We have followed an analytical approach that previously provided valuable insights into the pathogenesis of other infectious diseases, including respiratory viral infections, bacterial infections and tuberculosis. Although we performed microarrays of samples obtained at two acute (T1 and T2) and a third, later time point (T3), from

adults and children, our initial analysis focussed on T1 data from adults only. A major strength of our analysis was the inclusion of samples from a large number of carefully matched adult healthy controls, allowing comparison of illness-related changes with the healthy state. We examined results from the 2009/10 and larger 2010/11 separately, in an attempt to validate our initial findings.

Our unbiased analysis has provided important insights into RNA expression changes and how these may contribute to pathogenesis of influenza. Semi-supervised hierarchical clustering, using strict statistical filtering, revealed two major transcriptional profiles in influenza virus-infected adults at T1. The first major transcriptional signature contained a relative over-abundance of interferon-related genes. IPA canonical pathway analysis identified *OAS1, IFIT3, IFIT1* and *Mx1* as important components of the signature in both seasons, and *IFI35* gene was also identified in the 2009/10 analysis. In the second major transcriptional signature, the same interferon-related transcripts were less abundant; instead, we saw an overabundance of signalling and inflammation-related transcripts, many of which are neutrophil-associated genes. Pathway analysis revealed that the first signature was strongly associated with antiviral response, whereas the second signature was associated with bacterial response.

To investigate why there should be two distinct signatures, we examined clinical and microbiological data. Hierarchical clustering analysis revealed that patients with the antiviral response signature were predominantly at an earlier stage (0-4 days) of non-critical illness. By contrast, the 'bacterial'-response signature was seen predominantly in patients with critical illness, who also tended to be a later stage of illness. Analysis of by K-means clustering and pathway analysis confirmed the severity-associated trends. Modular transcriptional analysis showed a decreased expression (relative to healthy controls) in interferon modules in critically-ill influenza patients compared to those with less severe illness, but also showed consistently increased expression in the inflammation modules and particularly the neutrophil modules. Thus, our modular analysis confirmed the severity-associated trends seen with hierarchical and K-means clustering and also highlighted the increased expression of neutrophil-associated genes in those with severe disease.

Although neutrophils are known to ingest influenza virus-infected cells, they are an essential component of response to invasive bacterial infections. Therefore, we applied detailed bacterial detection data to the same hierarchical clustering experiments. Accepting the limitations of bacterial detection (especially in patients already on antibiotics) and that tests for invasive bacterial infection (e.g. blood cultures) were not performed in all patients, clustering analysis suggested that known bacterial infections were common in influenza-positive patients who had the bacterial-response signature i.e. those who tended to be critically ill and who were at a later stage of illness (5 days and

over). The same trend was also observed when we applied data for detections of bacterial species identified most commonly in A(H1N1)pdm09 virus-infected patients, *S. pneumoniae*. Further, those patients with the bacterial response signature generally had high serum procalcitonin, whereas those with the antiviral response signature (i.e. patients with less severe illness and at an earlier stage) generally had low procalcitonin levels.

In future work we will focus on analysing those patients who had appropriate testing to confirm or exclude invasive and non-invasive *S. pneumoniae* infection. We expect that this will strengthen the signals seen in this initial analysis. We will also perform longitudinal analysis, comparing transcriptional changes across the three time points to identify whether some patients with the antiviral response signature go on to develop the bacterial response signature, and whether this is associated with a change in severity of influenza illness or bacterial detection. We will take into account receipt of antibacterial and antiviral therapies and RNA expression data will also be correlated with the results of cellular immunology studies, host genetics (e.g. SNPs identified through exome sequencing) and virology (e.g. viral load).

In hierarchical clustering analysis, patients with confirmed influenza virus infection did not cluster separately from patients in whom influenza virus was not detected. This may be explained by the fact that the influenza-negative patients were a heterogeneous group with a mixture of viral and bacterial infections, and that there may be considerable overlap in transcriptional responses in the analytical approach we used. For this reason, we did not go on to identify or test the specificity of disease-specific signatures. However, this may be attempted in future work, once final clinical and microbiological diagnoses have been applied to the patients in the non-influenza group, with comparisons of influenza-positive patients against subgroups of patients in whom we are confident of the underlying infectious aetiology. Furthermore, we could not exclude the possibility of recent influenza virus infection (preceding hospitalisation) in the influenza negative patients. We plan to analyse serological data (and vaccination history data) for the influenza-negative patients, hoping to identify such patients and then assess whether their transcriptomic profiles and bacterial detection data suggest secondary bacterial infection.

6.2.3 Soluble immune mediator and biomarker responses in severe influenza

We measured a large number of soluble immune mediators in blood and respiratory secretions across multiple time points. As with our RNA analysis, my initial analysis is focussed on T1 results from adults. In future, results for T2 and T3 samples will allow longitudinal analysis of mediator changes over time; we will also be able to integrate mediator results with other MOSAIC outputs to

obtain a holistic view of immunopathogenesis of severe influenza and thereby discover new possible treatment modalities.

We have identified a large number of mediators - predominantly pro-inflammatory - that were elevated in patients with ILI compared to healthy controls. Increased levels of certain mediators, such as IP-10 (CXCL10), were much more marked in patients with influenza virus infection irrespective of the compartment sampled. IP-10 levels also increased as severity of illness increased, suggesting that IP-10 an important and specific marker of severity. A priority for future work will be to correlate IP-10 protein levels with data on IP-10 genetic polymorphisms and IP-10 RNA expression, and to investigate the possibility of blocking IP-10 to ameliorate disease.

Through the initial analysis of severity and duration of illness, we have shown that although exuberant mediator responses occur in severe influenza, profiles are diverse and that trends seen in blood do not always reflect trends in respiratory tract secretions. We have seen increases in Th1, Th2 and anti-inflammatory IL-10 mediators occurring at the same time, indicating that inflammatory processes observed in vivo may not follow rules derived from mice or in vitro cultured cells. Our results highlight the importance of quantifying mediators in different compartments when investigating pathogenesis, and suggest that the term “cytokine storm” is overly simplistic. This has important implications for the development of immunomodulatory agents, suggesting that therapies will need to be tailored to individuals and that mediator profiling will be useful in identifying patients that may benefit from specific personalised treatments.

To make sense of the vast body of data, we applied hierarchical clustering techniques with visualisation of trends using heat maps. This provided many insights into the complexity of mediator responses. Although we observed severity-associated patterns, clustering of patients according to mediator profiles was not fully explained by severity or duration of illness. For example, a subgroup of patients (predominantly the critically-ill) had marked elevation of IFN-β, IL-17, IL-6, TNF-α and MCP-1 (CCL-2) in serum. It may be that these patients had secondary bacterial infections but further correlation with bacteriological and transcriptomic data is required. Correlation with other data, including virological and co-morbidity data, is also essential in an attempt to explain the variability of mediator profiles.

By identifying mediators that were elevated in a single severity groups, we identified mediators that appear to be characteristic of critical influenza illness. In serum, these mediators were IFN-β, TNF-α, IL-2 and MCP-1 (CCL2). This contrasts with the findings of another much smaller study, which suggested that increased IL-15, IL-12p70 and IL-6 in serum was a hall-mark of critical illness. That

study specifically excluded patients with bacterial coinfection, whereas ours did not. We believe that including patients with bacterial complications is important since it is more representative of clinical experience of severe A(H1N1)pdm09 virus infection.

We have also shown that serum procalcitonin levels increase as influenza severity increases. This supports the conclusion from RNA expression work and the bacteriological analysis that bacterial coinfection is an important component of critical influenza illness. By contrast, we observed that vitamin D decreases as severity increases. While it is not clear whether vitamin D deficiency predisposes to or reflects more severe illness, the results are intriguing and should stimulate further investigation, including correction of deficiency in those with critical illness.

Soluble immune mediator profiling is an important component of pathogenesis studies. It provides an insight into more downstream processes and focuses on relevant immune markers. However, for a novel pathogen, even a large assay panel may exclude important mediators. Consideration of dynamic range is also important, since assays may lack necessary sensitivity or may be saturated when there are high levels of protein, as we saw with certain mediators in this study. These and other potential advantages and disadvantages are summarised in table 6.1.

	Whole blood transcriptomic profiling	**Soluble immune mediator profiling**
Advantages	• Thousands of transcripts/genes can be studied • Includes unknown factors • Results from data mining can be hypothesis generating • Biological snapshot in time	• Targets relevant immune markers • Closer to effector action • Local as well as systemic measures • Wide dynamic range • Biological snapshot in time
Disadvantages	• Full interpretation is difficult (perhaps impossible) • Influenced by cellular composition • Removed far upstream from effector functions • Biological snapshot in time	• Best-guess assay panel for a novel pathogen • Important mediators may be missed • Assay may lack sensitivity or may saturate • Limited value if receptor expression levels are not known • Not functional measures of biological effects • Biological snapshot in time

Table 6.1 Comparison of whole blood transcriptomic profiling and soluble immune mediator profiling

6.2.4 MOSAIC as a model for future outbreak pathogenesis studies

The MOSAIC study was an ambitious undertaking. It is unique in its approach of studying pathogen, host and co-pathogen factors in a single, integrated study. Within a remarkably short period of time, an effective consortium of experienced UK researchers was developed and protocols were developed. Our sampling protocol proved to be practical and effective, producing standardised samples for evaluation by multiple laboratories. The clinical database is very detailed and will serve as an important resource for further work by MOSAIC and other investigators. Although such detailed analyses will take time, we are now at the stage of being able to integrate the different outputs from MOSAIC to develop models of influenza pathogenesis. We expect that our results will generate many new hypotheses to be tested in animal and *in vitro* models, in addition to clinical and human challenge studies.

Many of the MOSAIC cases were recruited in the post-pandemic winter of 2010/11. Despite the extraordinary efforts of the investigators and funders to launch the study, the first pandemic wave and the peak of the second wave had already passed by the time recruitment could begin. This was due in part to administrative delays, something that has been highlighted a report on the regulation and governance of health research (562)MOSAIC was fully operational by the end of the second wave allowing on-going refinements in the light of initial experience. This explains why 2010/11 recruitment was so successful and demonstrates that for an outbreak study be effective, an established core of investigators (e.g. the Centre for Respiratory Infection, or CRI) is essential, with plans and protocols in place ahead of an outbreak. Ideally, the research plans should be tested in advance. Considering the unpredictable nature of pandemics, using a similar model to study seasonal outbreaks of respiratory viruses may be a way to achieve this.

An important legacy of MOSAIC is the ability to inform the development of future outbreak studies, both in the UK and elsewhere. Part of this process is assessing what worked and what didn't work. In MOSAIC, we recruited in two geographically distinct cities. Whilst this helped to avoid bias related to specific geographical populations, it also introduced logistical difficulties. It is highly likely that future global outbreaks will involve London, so there is an argument for focussing recruitment across London's hospitals. Protocols that depend on batched frozen samples have distinct advantages, but if analysis of fresh samples is essential, local laboratory resources are badly needed. Sampling should be easy to perform and require minimal training and should be simple enough to be performed by clinical staff.

The Flu-CIN data form was extensive and took a long time to process. Retrospective case note analysis missed data that are not commonly recorded in hospital notes. Collection of standardised,

core clinical data, ideally electronically and in real-time, should provide a more focussed clinical data set that is easier to analyse.

MOSAIC has been remarkable in contributing to the development of well tested templates, sampling protocols and clinical data collection tools through its involvement in initiatives led by the International Severe Acute Respiratory and emerging Infection Consortium (ISARIC)(563) and the World Health Organisation (WHO). The open-access standardised flexible tiered protocols will allow international researchers to conduct research rapidly and according to their local resources, following the emergence of a novel pathogen or an infectious disease outbreak. Although information from MOSAIC about the immunopathogenesis of influenza is undoubtedly important, the legacy of this unique collaboration in ISARIC and the Platform for European Preparedness Against (Re-)emerging Epidemics (PREPARE) network (564) is also a landmark achievement that could not have been realised without the collaborative model that we established in this study.

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Appendices

Appendix 1: Information sheet and consent form for adults able to consent

Mechanisms of Severe Acute Influenza Consortium (MOSAIC) Study

INFORMATION SHEET FOR ADULTS

Principal investigator: Peter Openshaw

We would like to invite you to take part in a research study. Before you decide you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully. Talk to others about the study if you wish and ask us if there is anything that is not clear or if you would like more information.

What is the study about?

Influenza ('flu') affects millions of people around the world every year. Most cases are mild, but some people with flu become very unwell. We do not understand why this happens. We want to investigate how our body fights the flu infection (including 'swine flu') and what affect other viruses and bacteria have on these responses. We also want to look at how our genes (DNA) and those of the flu virus, influence infection. This information will hopefully help us understand how and why flu causes more severe disease in some people. It will also help us come up with new treatments and plan for future pandemics.

Why have I been chosen?

You have been invited to take part in the study because you have recently been admitted to hospital and a doctor looking after you suspects that you may have influenza (flu) or you may have had a confirmed diagnosis of flu that your doctor will have told you about.

What will happen if I agree to take part?

- A member of the research team will review your medical records and ask some questions about you and any medicines you might be taking. These data will be entered into a central study database and be given a code so that you cannot be identified from the information in the database.
- We would also like to take the following samples from you:

A) Swabs and secretions from your nose and throat

B) Blood sample – up to 60mls (4 tablespoons worth)

C) Sputum (spit)

D) Urine

E) Stool (faeces)

- These samples will be collected throughout your hospital stay but specifically at three different time points; within 24 hours of recruitment into the study, 2-3 days after recruitment and

one month after going home from hospital (only samples A) to C) will be collected at this latter point). 20mls (4 teaspoons worth) of blood will be taken at the first time point (within 24 hours of recruitment), 60mls (4 tablespoons worth) of blood at the second time point (2-3 days after recruitment) and 60mls (4 tablespoons worth) at the outpatients follow up visit. Where possible we will collect these samples at the same time as you have routine samples taken.

- We would like to suck out some of the fluid/mucous in your nose by placing a thin flexible tube into the nose and applying gentle suction for 5 seconds.

- For a small group of patients we would like to take a nasal scrape (a sample of surface cells from the inside lining of your nose) and you will be informed by the researcher if we would like to take this sample from you

- If you have suspected flu but after laboratory testing it is confirmed that you do not have flu we would like, with your permission, to retain any initial samples we have taken for research and we would still like to take further samples from you as described in this leaflet so that we can compare your samples to those from patients who have been diagnosed with flu

- In addition, if you are having, or already have had, respiratory sample collection as part of your routine care, for example tracheal aspirates (sample from the windpipe), bronchial lavage (washings from the lungs) and other samples we would like to take an additional amount of these samples for our research.

What will happen to the samples?

- These samples will be used to find out how our immune systems respond to the flu virus (how our body fights the infection) and whether there are any genes (DNA) which determine who gets more severe disease (DNA analysis).

- All the samples will be labelled with a code with no identifying information about you. The samples will then be tested in a number of laboratories in different parts of the UK.

- If you agree, we would also like to keep any of your samples that are left over. These would be stored and used for future ethically approved research. At any time you wanted, you could ask for these samples to be destroyed.

- In the unlikely event that you are not able to make decisions for yourself about this study during the course of your illness we would like, with your permission, to continue to include you in the study by continuing to take samples and collect data as described in this information sheet.

Are there any disadvantages to taking part in this study?

The only disadvantages from taking part in this study are those associated with collecting the samples. Both taking blood and collecting respiratory swabs/secretions can be uncomfortable. However, we shall, where possible, collect these samples at the same time that you are having them taken as part of your routine care. The brief nasal suctioning may tickle, make your eyes water slightly or feel slightly uncomfortable, but it should not be painful.

Are there any benefits to taking part in this study?

No, there are none. While this study will not benefit you directly, it may help us to understand more about swine flu and hopefully help us to find new treatments in the future.

What if something goes wrong?

Imperial College London holds insurance policies which apply to this study. If you experience harm or injury as a result of taking part in this study, you may be eligible to claim compensation without having to prove that Imperial College is at fault. This does not affect your legal rights to seek compensation.

If you are harmed due to someone's negligence, then you may have grounds for a legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the Investigator using the below contact details. The normal National Health Service complaints mechanisms are also available to you. If you are still not satisfied with the response, you may contact the Imperial College Joint Research Office.

Who is organising and funding the research?

The research is co-ordinated by Imperial College, London and has been funded by the Wellcome Trust and the Medical Research Council (MRC). It has also been reviewed and given a favourable opinion by the Outer West London and Scotland A Research Ethics Committee. It is being carried out by experts in 'flu from around the country as a collaboration between NHS Trusts and universities (a list of these collaborators is available to you should you wish). None of the investigators performing the research or participants taking part will benefit financially from the study.

Will the information on me be kept confidential?

Yes, all personal information will be kept confidential and secure. Only people involved in the study will have access to the information. This study will be published in medical journals but it won't be possible to identify you from what is written.

Can I withdraw from participation in this study?

Your participation in this study is voluntary and you have the right to decline to participate. You are free to withdraw at any time and do not have to give a reason for this, even after you have agreed to take part. Being part of this study will not affect your normal medical care, either now or in the future.

What if I have any problems or would like further information about the study?

You can either contact the Principal Investigator on xxxxxxxxxxx or the hospital Patient Advice and Liaison Service (PALS) is available for independent advice.

This information can be made available in other languages and formats if requested.

CONSENT FORM

Mechanisms of severe acute influenza consortium (MOSAIC)

Principal investigator: Peter Openshaw

Please tick

1. I confirm that I have read and understand the participant information sheet dated Version 5, 24th September 2010 for the above study and been given a copy to keep. I have had the opportunity to ask questions and have had these answered satisfactorily. ☐

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

3. I understand that sections of any of my medical notes and data collected during the study may be looked at by responsible individuals from the above named hospital or associated university or from regulatory authorities, where it is relevant to my taking part in this research. I give permission for these individuals to access my records. ☐

4. I agree to the use of my samples in this research project, as described on the patient information sheet. ☐

5. I agree to the collection and use of an additional amount of my respiratory samples for research during routine clinical procedures. ☐

6. In the unlikely event that I am not able to make decisions for myself about this study, I agree to continue to participate as described in the information sheet ☐

7. I agree to the use of my samples in any future ethically-approved studies ☐

8. I agree to take part in this research project. ☐

_____	_____	_____
Name of Subject	Signature	Date (DD/MM/YYYY)
_____	_____	_____
Name of Person taking consent	Signature	Date (DD/MM/YYYY)
_____	_____	_____
Principal Investigator	Signature	Date (DD/MM/YYYY)

1 copy for participant; 1 copy for Principal Investigator; 1 copy to be kept with hospital notes.

Appendix 2: Information sheet and declaration form for personal consultee of adult unable to consent

Mechanisms of Severe Acute Influenza Consortium (MOSAIC) Study

INFORMATION FOR PERSONAL CONSULTEES (ENGLAND AND WALES)

Principal investigator: Professor Peter Openshaw

We would like to invite your relative/friend to take part in a research study. We feel your relative/friend is unable to decide for himself/herself whether to participate in this research. To help decide if he/she should join the study, we would like to ask your opinion whether or not they would want to be involved. We'd ask you to consider what you know of their wishes and feelings, and to consider their interests. Please let us know of any advance decisions they may have made about participating in research. These should take precedence.

Before you decide it is important for you to understand why the research is being done and what it would involve for your relative/friend. Please take time to read the following information carefully. Talk to others about the study if you wish. Ask us if there is anything that is not clear or if you would like more information and take time to decide. If you decide that your friend/relative would not wish to take part it will not affect the standard of care they receive in any way.

The following information is the same as would have been provided to your relative/friend.

What is the study about?

Influenza ('flu') affects millions of people around the world every year. Most cases are mild, but some people with flu become very unwell. We do not understand why this happens. We want to investigate how our body fights the flu infection (including 'swine flu') and what affect other viruses and bacteria have on these responses. We also want to look at how our genes (DNA) and those of the flu virus, influence infection. This information will hopefully help us understand how and why flu causes more severe disease in some people. It will also help us come up with new treatments and plan for future pandemics.

Why has my friend/relative been chosen?

You are being asked about your friend/relatives participation in this study because they have recently been admitted to hospital and a doctor looking after them suspects that they may have influenza (flu) or they may have had a confirmed diagnosis of (flu) that their doctor will have told you about.

What will happen if my friend/relative takes part in this study?

- A member of the research team will review their medical records to obtain clinical data. These data will be entered into a central study database and be given a code so that they cannot be identified from the information in the database.

- We would also like to take the following samples from your friend/relative:

A) Swabs and secretions from their nose and throat

B) Blood sample – up to 60mls (4 tablespoons worth)

C) Sputum (spit)

D) Urine

E) Stool (faeces)

- These samples will be collected throughout their hospital stay but specifically at three different time points; within 24 hours of recruitment into the study, 2-3 days after recruitment and one month after going home from hospital (only samples A) to C) will be collected at this latter point). 20mls (4 teaspoons worth) of blood will be taken at the first time point (within 24 hours of recruitment), 60mls (4 tablespoons worth) of blood at the second time point (2-3 days after recruitment) and 60mls (4 tablespoons worth) at the outpatients follow up visit. Where possible we will collect these samples from existing drip or monitoring lines and samples will be taken by trained and experienced staff.

- We would like to suck out some of the fluid/mucous in their nose by placing a thin flexible tube into the nose and applying gentle suction for 5 seconds.

- For a small group of patients we would like to take a nasal scrape (a sample of surface cells from the inside lining of the nose) and you will be informed by the researcher if we would like to take this sample from your friend/relative

- If they have suspected flu but after laboratory testing it is confirmed that they do not have flu we would like, with your assent, to retain any initial samples we have taken for research and we would still like to take further samples from them as described in this leaflet so that we can compare their samples to those from patients who have been diagnosed with flu.

- In addition, if they are having, or already have had, respiratory sample collection as part of their routine care, for example tracheal aspirates (sample from the windpipe), bronchial lavage (washings from the lungs) and other samples then we would like to take an additional amount of these samples for our research.

What will happen to the samples?

- These samples will be used to find out how our immune systems respond to the flu virus (how our body fights the infection) and whether there are any genes (DNA) which determine who gets more severe disease (DNA analysis).

- All the samples will be labelled with a code with no identifying information about your friend/relative. The samples will then be tested in a number of laboratories in different parts of the UK.

- If you agree, we would also like to keep any of their samples that are left over. These would be stored and used for future ethically approved research. At any time you wanted, you could ask for these samples to be destroyed.

Are there any disadvantages to taking part in this study?

We anticipate that the side effects from the above described research procedures will be minimal. Where possible we will collect these samples from existing drip or monitoring lines and samples will be taken by trained and experienced staff. The brief nasal suctioning may tickle, make their eyes water slightly or feel slightly uncomfortable, but it should not be painful.

Are there any benefits to taking part in this study?

No, there are none. While this study will not benefit your friend/relative directly, it may help us to understand more about swine flu and hopefully help us to find new treatments in the future.

What if something goes wrong?

Imperial College London holds insurance policies which apply to this study. If your friend/relative experiences harm or injury as a result of taking part in this study, they may be eligible to claim compensation without having to prove that Imperial College is at fault. This does not affect their legal rights to seek compensation.

If they are harmed due to someone's negligence, then they may have grounds for a legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way your friend/relative has been treated during the course of this study then you should immediately inform the Investigator using the below contact details. The normal National Health Service complaints mechanisms are also available to you. If you are still not satisfied with the response, you may contact the Imperial College Joint Research Office.

Who is organising and funding the research?

The research is co-ordinated by Imperial College, London and has been funded by the Wellcome Trust and the Medical Research Council (MRC). It has also been reviewed and given a favourable opinion by the Outer West London Research Ethics Committee. It is being carried out by experts in 'flu from around the country as a collaboration between NHS Trusts and universities (a list of these collaborators is available to you should you wish). None of the investigators performing the research or participants taking part will benefit financially from the study.

Will the information be kept confidential?

Yes, all personal information will be kept confidential and secure. Only people involved in the study will have access to the information. This study will be published in medical journals but it won't be possible to identify your friend/relative from what is written.

Can I request that my friend/relative is withdrawn from the study at any point?

Yes. Your agreement is entirely voluntary and you have the right to decline to act as a personal consultee. You are free to withdraw your friend/relative at any time and do not have to give a

reason for this, even after you have agreed for them to take part. Being part of this study will not affect their normal medical care, either now or in the future.

What if I have any problems or would like further information about the study?

You can either contact the Principal Investigator on 0207 594 0944 or the hospital Patient Advice and Liaison Service (PALS) is available for independent advice.

This information can be made available in other languages and formats if requested.

Mechanisms of Severe Acute Influenza Consortium (MOSAIC) Study

PERSONAL CONSULTEE DECLARATION FORM

Principal investigator: Professor Peter Openshaw

Please initial box

1. I have been consulted about [ ]'s participation in this research project. I have had the opportunity to ask questions about the study and understand what is involved. ☐

2. In my opinion he/she would have no objection to taking part in the above study. ☐

3. I understand that I can request he/she is withdrawn from the study at any time, without giving any reason and without his/her care or legal rights being affected. ☐

4 I understand that relevant sections of his/her care record and data collected during the study may be looked at by responsible individuals from the above named hospital, our collaborators working at Imperial College, London or from regulatory authorities, where it is relevant to their taking part in this research. ☐

5. In my opinion he/she would have no objection to the use of her/his tissue samples in any future ethically-approved studies. ☐

_____	_____	_____
Name of Personal Consultee	Signature	Date (DD/MM/YYYY)

_____

Relationship to participant

_____	_____	_____
Person undertaking consultation (if different from researcher)	Signature	Date (DD/MM/YYYY)

_____	_____	_____
Researcher	Signature	Date (DD/MM/YYYY)

When completed: 1 (original) to be kept in care record, 1 for personal consultee; 1 for researcher site file

Appendix 3: Flu-CIN data collection form

NHS

Network ID Number

Department of Health – FLU-CIN

FLU-CIN Data Collection Form

(See guidance notes for completion)

1. Patient Demographics

1.1 (Complete all fields)

D.O.B.: ☐☐ / ☐☐ / ☐☐☐☐ or age (yrs) ☐☐ or age if < 12 months ☐☐

Gender: M ☐ F ☐

Hospital Number: ..... Postcode .....

1.2 Admitting Trust

Nottingham Hospitals ☐ Leicester Hospitals ☐ Sheffield Hospitals ☐

Imperial College Hospitals ☐ Liverpool Hospitals ☐

Hospital name: ..... Non-sentinel trust name: .....

1.3 Patient details

episode ☐

Patient defined ethnicity: (Tick one box)

White ☐ Mixed ☐ Asian/Asian British ☐

Black/Black British ☐ Chinese ☐ Other Ethnic Group ☐

1.4 Admission route

GP ☐ 999 ☐ A&E/ED ☐ OPD ☐

Other Hospital ☐ Community Midwife ☐ NHS 24 ☐ NHS Flu Line ☐

1.5 Social situation

Lives alone ☐ Residential/Nursing Home ☐ 'Looked after' child ☐

Military barracks ☐ Hall of residence ☐ Prison/Police custody ☐

Lives with others ☐ Number of persons in household (inc patient) ☐☐

1.6 GP Name & Practice Address

Name: .....

Address: .....

.....

1 Version 2

1.7 Transfer from other hospital

Hospital name	Admission date (to that hospital)	Transfer date (from that hospital)

1.8 Admission

Date and Time first seen (This admission, this hospital) | | | | | 09 /10* | : | (24 hour clock)

Previously seen in AMU/ED with the same problem but not admitted?

Yes ☐ No ☐ Don't know ☐ Date seen: | | | | | 09 /10*

Previous admission with the same problem?

Yes ☐ No ☐ Don't know ☐ Date seen: | | | | | 09 /10*

*Delete as appropriate

1.9 FLU-CIN category

(please tick one box)

1. Case of confirmed influenza A/H1N1v ☐
2. Case of suspected influenza A/H1N1v ☐
3. Influenza-like illness during pregnancy ☐
4. Clinician-suspected influenza complications ☐
5. Other acute respiratory illness considered to be consistent with influenza (eg exacerbation of COPD, asthma, pneumonia, LRTI, etc.) ☐

This section relates to Triage Criteria on opposite page.

☐ Discharge criteria met

(Tick if discharge criteria met even if subsequently admitted for other reasons)

Children: Discharge criteria met at 4 hours

- No evidence of respiratory distress
- Respiratory rate < 40 bpm
- SpO_2 – >92% in air
- No evidence of dehydration
- Infants tolerating ¾ oral feeds

Adults: Discharge criteria met at 4 hours

- No evidence of respiratory distress
- Respiratory rate < 30 bpm
- SpO_2 – >92% in air
- No evidence of dehydration
- Tolerating oral fluids

Emergency Department or AMU ONLY

1.10 TRIAGE CRITERIA FOR HOSPITAL TREATMENT

(to be completed on presentation: tick **all** boxes that apply)

Criteria Met (✔)	Presenting symptoms	
	Children under 16 years	Adults
☐ A	**Severe respiratory distress** Lower chest wall indrawing, sternal recession, grunting or noisy breathing when calm.	**Severe respiratory distress** Severe breathlessness: eg unable to complete sentences in one breath, use of accessory muscles, supra-clavical recession, tracheal tug or feeling of suffocation.
☐ B	**Increased respiratory rate** Measured over at least 30 seconds ≥ 50 breaths per minute if under 1 year or ≥ 40 breaths per minute if over 1 year.	**Increased respiratory rate** Measured over at least 30 seconds. Over 30 breaths per minute.
☐ C	**Oxygen saturation ≤ 92% in air *or* on oxygen** Absence of cyanosis is a poor discriminator for severe illness.	**Oxygen saturation ≤ 92% on pulse oximetry, breathing air *or* on oxygen** Absence of cyanosis is a poor discriminator for severe illness.
☐ D	**Respiratory exhaustion or apnoeic episode** Apnoea defined as a ≥ 20 second pause in breathing.	**Respiratory exhaustion** New abnormal breathing pattern e.g. alternating fast and slow rate or long pauses between regular breaths.
☐ E	**Evidence of severe clinical dehydration or clinical shock** Sternal capillary refill time > 2 seconds, reduced skin turgor, sunken eyes or fontanelle.	**Evidence of severe clinical dehydration or clinical shock** Systolic blood pressure <90mmHg and/or diastolic blood pressure <60mmHg. Sternal capillary refill time > 2 seconds, reduced skin turgor.
☐ F	**Altered conscious level** Strikingly agitated or irritable, seizures or floppy infant.	**Altered conscious level** New confusion, striking agitation or seizures.
☐ G	**Causing other clinical concern to the clinical team or specialist doctor** e.g. rapidly progressive or unusually prolonged illness.	**Causing other clinical concern to their specialist doctor or clinical team** e.g. rapidly progressive or unusually prolonged illness.
☐ **Other** (specify):		
☐ **None identified**		

1.11 AVPU on presentation:

A ☐ V ☐ P ☐ U ☐ (tick one box: highest level patient meets, A to U)

or Glasgow Coma Scale score ☐

- **Alert** – a fully awake (although not necessarily orientated) patient. This patient will have spontaneously open eyes, will respond to voice (although may be confused) and will have bodily motor function.
- **Voice** – the patient makes some kind of response when you talk to them, which could be in any of the three component measures of Eyes, Voice or Motor – e.g. patient's eyes open on being asked "are you okay?!". The response could be as little as a grunt, moan, or slight move of a limb when prompted by the voice of the attendant.
- **Pain** – the patient makes a response on any of the three component measures when pain stimulus is used on them. Recognized methods for causing the pain stimulus include a sternal rub, pinching the patient's ear or pressing a pen barrel across the bed of the patient's fingernail. A fully conscious patient would normally locate the pain and push it away, however a patient who is not alert and who has not responded to voice (hence having the test performed on them) is likely to exhibit only withdrawal from pain, or even involuntary flexion or extension of the limbs from the pain stimulus.
- **Unresponsive** – Sometimes seen noted as 'Unconscious', this outcome is recorded if the patient does not give any Eye, Voice or Motor response to voice or pain.

2. Emergency Department Assessment

2.1 Presenting symptoms (tick all that apply)

Fever or feverish	☐	Malaise	☐	Dry cough	☐	Sore throat	☐	Headache	☐
Myalgia	☐	Chills	☐	Coryza	☐	Anorexia	☐	Sneezing	☐
Arthralgia	☐	Productive cough	☐	Nausea	☐	Diarrhoea	☐	Vomiting	☐
Dyspnoea	☐	Conjunctivitis	☐	Epistaxis	☐	Rash	☐	Altered consciousness	☐
Seizures	☐	Persistent crying (children)	☐	Preganant: Y ☐ N ☐ Number of weeks ☐ Number of fetuses ☐					

Other: .....

.....

.....

2.2 Date of onset

☐☐☐☐☐☐ Onset: Abrupt ☐ Gradual ☐ Not known ☐

2.3 Any recent admission / attendance for febrile illness

None ☐ GP / Primary Care Centre ☐ Emergency Department ☐

Date of previous attendance: ☐☐☐☐☐☐

Examination

2.4 Temperature

Enter on ED observations table

2.5 CVS

Enter heart rate & BP on ED observations table

Oedema ☐ Hepatomegaly ☐ Heart sounds / murmurs:

2.6 Respiratory

Enter respiratory rate on Emergency Department observations table

Breathlessness ☐ Cough ☐

2.7 Abdomen/Gastro-intestinal

Vomiting ☐ Diarrhoea ☐ Clinically obese (physician defined) ☐

2.8 ENT:

.....

.....

2.9 Skin:

.....

.....

2.10 Eyes

2.11 CNS

AVPU/GCS – enter on ED observations table below:

Change in behaviour during this illness ☐

New seizures ☐

Meningism ☐

New focal neurology ☐

New confusion ☐

2.12 Musculoskeletal system

Muscle tenderness ☐ Muscle weakness ☐

2.13 Medications (long-term or routine medications only)

Name	Dose and route	BNF code

2.14 ED Observations

Date								
Time								
AVPU								
or GCS								
Temperature								
Heart rate								
Respiratory rate								
Pulse oximetry								
BP								
O_2 administered	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
% O_2 given or								
litres/min								
	mask or prongs	mask or prongs	mask or prongs	mask or prongs	mask or prongs	mask or prongs	mask or prongs	mask or prongs

2.15 Initial investigations ordered

(Chest x-ray, throat swabs, other microbiology/virology etc.)

Date : time	Investigation	Results found and entered in DB

2.16 Admitted to hospital or discharged from ED

Admit ☐	Decision at: time and date	☐☐:☐☐ ☐☐/☐☐/☐☐
	Admitted at: time and date	☐☐:☐☐ ☐☐/☐☐/☐☐
Admitted to:	Ward ☐ HDU ☐ Critical Care ☐	
Discharge ☐	Decision at: time and date	☐☐:☐☐ ☐☐/☐☐/☐☐
	Admitted at: time and date	☐☐:☐☐ ☐☐/☐☐/☐☐

Interventions at time of decision:

Supplemental oxygen ☐	IV fluids ☐	IV antibiotics ☐
Oral antibiotics ☐	Oseltamivir ☐	Zanamivir ☐

2.17 Children only (< 6 years)

Birth history (for children <6yrs)

Mode of delivery:

SCBU: Yes ☐ No ☐ Neonatal problems: Yes ☐ No ☐

Details if Yes:

Immunisations up to date (children <6yrs) Yes ☐ No ☐

Gestation at birth: ☐ weeks ☐ days e.g. 39 weeks 5 days

Birth weight: ☐ lb ☐ oz ☐ kilograms

Childhood immunisations (Circle as appropriate)

DTP/HiB/IPV	1 2 3 B	**Men C**	1 2 B	**PCV**	1 2	**MMR**	1 B	**BCG**	1

3. Initial Assessment and History

3.1 Where assessed?

HDU/ICU ☐ Acute Medical Unit ☐ Ward ☐ Maternity department ☐

Other (please specify) .....

.....

3.2 History of Influenza-like illness

Date of onset of first symptoms [ ][ ] / [ ][ ] 2009 / 10* *Delete as appropriate

Time of onset: Overnight ☐ Morning ☐ Afternoon ☐ Evening ☐	Yes	No	Not known
Was the onset abrupt? (see guidance notes)			
Is patient a routine recipient of annual influenza vaccine?			
Did patient receive influenza vaccination in 08/09 season?			
Did patient receive influenza vaccination in 09/10 season?			
Did patient receive pandemic influenza vaccination (swine flu) in 09/10 season?			
If "Yes" has patient received 1 dose of pandemic vaccine?			
If "Yes" has patient received 2 doses of pandemic vaccine?			
Please specify number of weeks since last dose of pandemic vaccine:			
Has patient ever received pneumococcal (or pneumonia) vaccine?			
Has patient visited or consulted with GP about influenza during this illness?			

Medications

Pre-admission antiviral treatment this illness?		Yes ☐ No ☐	
	Date started (if known)	How many days completed?	Still taking it on admission?
Oseltamivir (Tamiflu)			Yes ☐ No ☐
Zanamavir (Relenza)			Yes ☐ No ☐
Other (state):			Yes ☐ No ☐

Pre-admission antibiotic treatment this illness?			Yes ☐ No ☐	
Name	Dose	Date started (if known)	How many days completed?	Still taking it on admission?
				Yes ☐ No ☐
				Yes ☐ No ☐
				Yes ☐ No ☐
				Yes ☐ No ☐
				Yes ☐ No ☐
				Yes ☐ No ☐

Self-bought medicines this illness?		Yes ☐ No ☐	
Name	Date started (if known)	How many days completed?	Still taking it on admission?
			Yes ☐ No ☐
			Yes ☐ No ☐
			Yes ☐ No ☐
			Yes ☐ No ☐

3.3 Influenza initial symptoms at onset of illness (tick all that apply)

Fever or feverish		Malaise		Dry cough		Sore throat		Headache	
Myalgia		Chills		Coryza		Anorexia		Sneezing	
Arthralgia		Productive cough		Nausea		Diarrhoea		Vomiting	
Dyspnoea		Conjunctivitis		Epistaxis		Rash		Altered consciousness	
Seizures		Persistent crying (children)		Other:					

3.4 Current presenting symptoms on admission (tick all that apply)

Fever or feverish		Malaise		Dry cough		Sore throat		Headache	
Myalgia		Chills		Coryza		Anorexia		Sneezing	
Arthralgia		Productive cough		Nausea		Diarrhoea		Vomiting	
Dyspnoea		Conjunctivitis		Epistaxis		Rash		Altered consciousness	
Seizures		Persistent crying (children)		Other:					

3.5 Weight and height

Weight on admission:	☐ kg	self-reported ☐	measured ☐
Height: feet/inches	☐ ft ☐	self-reported ☐	measured ☐
metres	☐		

ACUTE MEDICAL UNIT/WARD ASSESSMENT

Past medical history

3.6 Co-morbidities

(tick all that apply)

Asthmatic		COPD		Cystic fibrosis		Other chronic lung disease		Diabetes	
Other metabolic disease		Neurological disease		Sickle cell disease		Chronic renal disease		Chronic liver disease	
Hypertension		Heart disease		Atrial fibrillation		Cerebrovascular disease		Pregnant: Y / N	
								no. of weeks	
								no. of foetuses	
Clinically obese (physician defined)				Other (please specify):					

3.7 Immune compromised

On treatment for immune deficiency		On immunosuppressant drugs		Recently completed course of chemotherapy	
Transplant recipient		HIV positive			

3.8 Cancer

No		Yes: Active*		If Yes:	Solid tumour	
		Yes: Recent (within last 12 months)*			Haematological	
* excluding non-melanoma skin cancer					On maintenance medication	

3.9 Risk factors

Smoking	Current daily		Occasional		Ex-smoker		Never
Does any other household member smoke? ☐ Yes ☐ No							
IVDU ☐							
Alcohol – units per week:							

3.10 Allergies

No		Yes: (please specify)

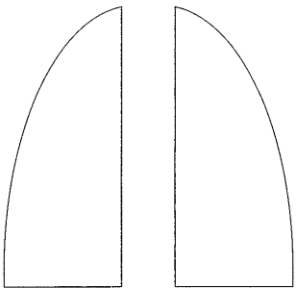
3.11 Other past medical history

3.12 Medications

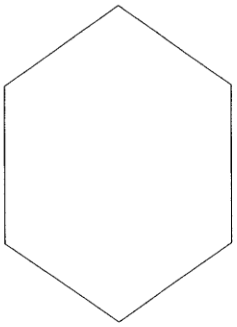
(long term or routine medications – those being taken prior to admission)

NAME	DOSE/FREQ	ROUTE	BNF CODE

Clinical Findings Upon Examination

3.13 Cardiology	Clinical evidence of cardiac failure ☐
3.14 Respiratory system 	Breathlessness ☐ Cough ☐ Enter chest X-ray findings in 3.21
3.15 Central nervous system	Any of following **new to this illness** episode? New seizures ☐ New focal neurology ☐ Meningism ☐ New confusion ☐ Change in behaviour ☐
3.16 Skin/Cutaneous	Rash: Yes ☐ No ☐ (please describe in free text): Lymphadenopathy: Yes ☐ No ☐ If "Yes": Local ☐ Generalised ☐
3.17 Musculoskeletal system	Muscle tenderness ☐ Muscle weakness ☐
3.18 Eyes	
3.19 ENT	

3.20 Abdominal



Clincally obese (physician defined) ☐
Vomiting ☐
Diarrhoea ☐

Investigations (If undertaken)

3.21 Chest X-ray

CXR not performed ☐
CXR normal ☐
Findings consistent with pneumonia ☐
CXR abnormal, DESCRIBE:

If possible attach copy of the report or transcribe main findings.

Interstitial shadowing consistent with infection	Left	Right	Bilateral
Alveolar shadowing/ consolidation	Left	Right	Bilateral
Effusion	Left	Right	Bilateral
Pneumothorax	Left	Right	Bilateral
Cardiomegaly	Yes		No
Other (specify below)	Left	Right	Bilateral

3.22 ECG findings

Not performed ☐ Normal ☐ Abnormal ☐

If abnormal describe rhythm from entry in notes.

3.23 Laboratory results

Hb		INR		ALT	
WCC		Na		γGT	
Platelets		K		Bilirubin	
Haematocrit		Urea		Alk Phos	
Neutrophils		Creat		Albumin	
Lymphocytes		GFR		Tot. prot	
		CPK		CRP	

3.24 Arterial blood gases

On air	Y/N	**On O_2**	If yes, give $\%O_2$ given or l/min
PaO_2		$PaCO_2$	
HCO_3		BE	
SaO_2		lactate	

3.25 Urinalysis

SG		bilirubin	
pH		ketones	
glucose		nitrites	
protein		leukocytes	
blood			

3.26

Pneumococcal antigen (if done) +ve ☐ -ve ☐

Haemoglobinuria (if done) +ve ☐ -ve ☐

Proteinuria (if done) +ve ☐ -ve ☐

3.27 Microbiology/Virology

(give results in as much detail as possible e.g. H1N1 +ve etc., detail organisms for microbiology)

	Date of specimen	M/V	Negative	Positive culture detail	Drug resistance
Nose/throat swab:					
Blood:					
Urine:					
Stool:					
Sputum:					
Other:					

M = microbiology, V = virology

3.28 Respiratory virus laboratory diagnostic status at time of initial assessment (if known, tick all that apply)

Status	**Tick**	**Date of specimen**
Influenza +ve		
Influenza A +ve		
Influenza A/H1N1 (swine flu) +ve		
RSV +ve		
Other +ve (state):		

3.29 Observations

Date							
Time							
AVPU							
or GCS							
Temperature							
Heart rate							
BP							
Respiratory rate							
Pulse oximetry							
O_2 administered	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N	Y / N
FiO_2							

3.30 Other clinical observations upon initial assessment:

Date / Time	Comment / clinical notes

3.31 Diagnosis & risk assessment

Reason for hospital admission

Initially admitted for other reasons and acquired influenza in hospital ☐

If yes, please complete section below at time of onset of influenza

For all other patients complete below at time of initial assessment

Uncomplicated Influenza		**Complicated Influenza**	
Additional or alternative diagnoses: (detail below):		**Complicated by:** (tick as applicable; these are not co morbidites but acute problems e.g. heart failure, myocarditis, fetal distress, meningitis etc.)	Respiratory
			Exacerbation asthma
			Exacerbation COPD
			Pneumonia
			LRTI
			Other respiratory comp
			GI
			CNS
			CVS
			Obstetric
			Underlying disease
			Other (specify below)
		Complication as stated in notes:	

Experienced clinician's opinion on risk of mortality (circle as appropriate)

Low (<3%)	**Moderate** (3-30%)	**High** (31-80%)	**Very high** (>80%)
Current level of care required:			
0	1	2	3
Level of care admitted to:			
0	1	2	3

Level 0 – *Patients whose needs can be met through normal ward care.*

Level 1 – *Patients at risk of deterioration or recently relocated from higher levels of care, whose needs can be met on an acute ward with additional advice and support from the critical care team.*

Level 2 – *Patients requiring more detailed observation or intervention including support for a single failing organ system or post-operative care, and those 'stepping down' from higher levels of care. (HDU).*

Level 3 – *Patients requiring advanced respiratory support alone or basic respiratory support together with support of at least two organ systems. This includes all complex patients requiring support for multi-organ failure. (ICU).*

Other observations on risk assessment and care level required:

3.32 Children Only (< 6 years)

Birth history

Mode of delivery:

SCBU: Yes ☐ No ☐ Neonatal problems: Yes ☐ No ☐

Details if Yes:

.....

.....

Immunisations up to date (children <6yrs) Yes ☐ No ☐

Gestation at birth: ☐ weeks ☐ days e.g. 39 weeks 5 days

Birth weight: ☐ lb ☐ oz ☐ kilograms

Childhood immunisations – circle as appropriate

DTP/HiB/IPV	1 2 3 B	**Men C**	1 2 B	**PCV**	1 2	**MMR**	1 B	**BCG**	1

4. Daily Care Observations

4.1 Day ☐

(day 1= the day or part of a day before the first night as an inpatient; day 2 =following day etc)

Level of Care

Ward ☐ HDU ☐ ITU ☐ Date: ☐☐/☐☐/☐☐

Complete this page for all care levels:

4.2 Record first documented observation in period indicated

Observations	00:00–05:59	06:00–11:59	12:00–17:59	18:00–23:59
Time				
Temperature				
Heart rate				
Blood pressure				
Respiratory rate				
WCC				
Supplemental O_2 required	Y/N	Y/N	Y/N	Y/N
Litres/min or				
$\%O_2$ given				
SpO_2 (pulse oximetry or arterial line)				
Conscious level – AVPU or GCS				
Confusion – (tick if confused)				
Temperature spike >38°C within last 24 hours	Y/N			

(A – alert; V – responds to voice; P – responds to pain; U – unresponsive)

4.3 Progress

Decision made to intensify care?		Being cared for at optimal level?		If no: what is optimal level?	Has patient been diagnosed with pneumonia (with radiological changes on x-ray) in last 24 hours? Y / N
Condition change from previous day:	Same ☐ Better ☐ Worse ☐			Specialist advice sought?	Y / N

4.4 Laboratory results

Hb		INR		ALT	
WCC		Na		γGT	
Platelets		K		Bilirubin	
Haematocrit		Urea		Alk Phos	
Neutrophils		Creat		Albumin	
Lymphocytes		GFR		Tot. prot	
		CPK		CRP	

4.5 Arterial blood gases

On air	Y/N	On O_2	If yes, give $\%O_2$ given or l/min
PaO_2		$PaCO_2$	
HCO_3		BE	
SaO_2		lactate	

4.6 Microbiology/Virology

	Date of specimen	M/V	Negative	Positive culture detail	Drug resistance
Nose/throat swab					
Blood					
Urine					
Stool					
Sputum					
Other					

M = microbiology, V = virology

4.7 Urinalysis

SG		bilirubin	
pH		ketones	
glucose		nitrites	
protein		leukocytes	
blood			

4.8

Pneumococcal antigen (if done) +ve ☐ -ve ☐

Haemoglobinuria (if done) +ve ☐ -ve ☐

Proteinuria (if done) +ve ☐ -ve ☐

4.9 Therapy

	Antiviral 1: Y / N	**Antiviral 2: Y / N**	**Antibiotic 1: Y / N**	**Antibiotic 2: Y / N**	**Steroid: Y / N**
Name:					
Route					
Dosage					
Frequency					
Commence Tick as appropriate	Commence	Commence	Commence	Commence	Commence
Continue Tick as appropriate	Continue	Continue	Continue	Continue	Continue
Recommence Tick as appropriate	Recommence	Recommence	Recommence	Recommence	Recommence
Stop Tick as appropriate	Stop	Stop	Stop	Stop	Stop

4.10 Escalation of care

Treatment decisions	Yes	No
Was consideration given to escalating treatment?		
If considered but decided against, give reasons (circle all that apply):		
Lack of response to treatments / deteriorating condition / not required		
Resource restrictions:		
Nursing Staff		
Medical Staff		
Lack of essential equipment / drugs / disposables (list):		

4.11 Decisions around admitting to HDU/ITU

Critical care admissions / referrals	Yes	No
Was patient admission declined?		
If declined, give reasons:		
Inappropriate referral		
Appropriate referral but insufficient resources		
Resource restrictions		
Level 2 beds		
Level 3 beds		
Nursing staff		
Medical staff		
Essential equipment / drugs / disposables (list)		

4.12 Further clinical observations & notes:

Time	Comment / clinical notes

Level 2/3 Only (HDU/Critical Care)

4.13 For readings for respiratory assistance and ventilator settings (below), document here the first reading of the day (from 06:00)

Respiratory assistance		CPAP / BiPAP (NIV)		FiO_2		Pressure settings		Respiratory rate	

Ventilator settings		Mode		FiO_2		PEEP		Mean airway pressure		Rate	

4.14 From the HIGHEST SOFA score during the day – circle the appropriate level for each measure (Adults only)

(Circle appropriate level and database will calculate total SOFA score)

SOFA Score	0	1	2	3	4
Respiration (PaO_2/FiO_2)	>53.3 Kpa	40.1-53.3 Kpa	26.7-40 KPa	"13.4-26.6 Kpa with respiratory support"	≤ 13.3 Kpa with respiratory support
Cardiovascular MAP or inotrope support	MAP ≥70mmHg	MAP < 70 mmHg	Dopamine ≤ 5 mics/kg/min or Dobutamine (any dose) For at least I hour	Dopamine > 5 mics/kg/min or epinephrine ≤ 0.1mics/ kg/min or norepineparine≤ 0.1mics/kg/min	Dopamine >15 mics/kg/min or epinepherine > 0.1mics/ kg/min or norepinepherine > 0.1mics/kg/min
Renal Creatinine (umol/L) or urine output	<110 micromols/L	110-170 micromols/L	171-299 micromols/L	300 -440 micromols/L or 201-500ml/day	> 440 micromols/L or <200ml/day
CNS Glasgow Coma Scale	15	13-14	10 to 12	6 to 9	< 6
Liver Bilirubin (umol/L)	<20 micromols/L	20-32 micromols/L	33-101 micromols/L	102-204 micromols/L	>204 micromols/L
Coagulation Platelets (10^3/ mm^3)	>150	101-150	51-100	21-50	≤20

5. Pregnancy

5.1 Pregnancy/fetal wellbeing assessment

Date of pregnancy/fetal wellbeing assessment		Date of investigation			
5.2 Pregnancy/Fetal Wellbeing *Comments:*	**CTG (if done)**		Normal	Abnormal	Fetal Heart Rate:
	Ultrasound		Normal	Fetal Growth Restriction	Oligo-Hydramnios
	Doppler scan		Normal	Abnormal (describe):	

5.3 Pregnancy outcome

Single or multiple pregnancy?	Single/ Multiple		**Gestation at onset of influenza:**	weeks	
Medical or obstetric complications: (comments below)	Gestational Diabetes		**Gestation when antiviral commenced:**		
	Pre-eclampsia		**Antiviral agent:**	oseltamivir:	
	Respiratory			zanamivir:	
	Cardiac				
	Renal		**Evidence of fetal compromise:**	IUGR	
	Other: (specify)			Unexplained oligohydramnios/ polyhydramnios	
				Abnormal Doppler findings	
				Abnormal CTG findings	
	Ante-partum haemorrhage			Biophysical profile	
				Abnormal fetal USS	
Gestation at delivery:	weeks		**Mode of delivery:** (tick as appropriate)		
PPROM (preterm, pre-labour rupture of membrane)			Normal vaginal	Instrumental	
Onset of labour:			C-section emergency	C-section elective	
Spontaneous			C-section pre-labour	C-section in labour	
Induced			**Indication for C-section/instrumental delivery:**		
N/A					

5.4 Neonate 1

Live birth		Still birth		Neonatal death	
Necrotising enterocolitis		Birthweight (kg):		Male / Female	
Abnormal intracranial USS		APGAR score at 5 minutes		Respiratory Distress Syndrome	
Cord Blood pH (arterial)		Cord blood pH (venous)		Other complication?	
Resuscitation required		Facial oxygen			
If neonatal death:		Age at death (days):		Cause of death:	

5.5 Neonate 2

Live birth		Still birth		Neonatal death	
Necrotising enterocolitis		Birthweight (kg):		Male / Female	
Abnormal intracranial USS		APGAR score at 5 minutes		Respiratory Distress Syndrome	
Cord Blood pH (arterial)		Cord blood pH (venous)		Other complication?	
Resuscitation required		Facial oxygen			
If neonatal death:		Age at death (days):		Cause of death:	

6. Discharge/Death in hospital

6.1 Date of discharge from hospital ☐☐/☐☐/☐☐

6.2 Patient ready for discharge on this date? * please circle as appropriate	Ready	Y / N
	Not yet ready – but capacity issues on ward / unit / hospital	Y / N
	Ready before this date, but appropriate discharge destination not available	Y / N
6.3 Discharge destination: (tick as appropriate)	Original domicile	
or step-down to:	Warden aided accommodation	
	Residential home	
	Nursing home	
	Rehabilitation / Cottage hospital	
	Home of relative	
	Other – specify (including transfers)	

6.4 Death in hospital ☐	**Time:** :	**Date:** / /	**Place: ED / AMU / Ward / HDU / ICU**

Cause of death – list all causes as per death certificate:

Referred for post mortem? ☐

Coroner's case? ☐

Appendix 4: Adult subject folder and supplementary CRF

MOSAIC Primary Record Sheet (Adult)

MOSAIC number_____

Subject's name_____

Hospital Number_____

Site code_____

DOB_____

Date admitted_____

Date recruited_____

Recruited by_____

T0 details

Date taken_____

Time taken_____

Sample Taken by_____

Sample Type: Flocked NP swab ☐ Combined nose & throat ☐ NP aspirate ☐

Other ☐ _____

Nostril (where applicable) Left ☐ Right ☐

Date delivered to Clinical Virology_____

Time delivered to Clinical Virology_____

Result_____ Method_____

Date reported_____ Time reported_____

MOSAIC Clinical Record Form (Adult)

MOSAIC Number:_____ Date record completed:_____

Day of acute illness at recruitment:_____ Destination: Ward ☐ HDU ☐ ICU ☐

Comorbidities: None ☐ Asthma ☐ Other chronic respiratory disease ☐ Cardiovascular disease ☐
Chronic renal disease ☐ Neurological disease ☐ Pregnancy ☐ Immunosuppressed ☐
Other (please state):_____

Steroids: None ☐ Long-term ☐ Newly prescribed ☐ Inhaled ☐ Oral ☐ IV ☐

CXR: Not done ☐ Normal ☐ Unilateral infiltrate ☐ Bilateral infiltrates ☐ Pl. effusion ☐

O_2 saturations on admission (%)_____On room air ☐ On O_2 ☐ (% or L/min)_____

PRE-HOSPITAL ANTIVIRAL use **None** ☐

ANTIVIRAL	DOSE	FREQ	ROUTE	DATE STARTED	DATE FINISHED*
Oseltamivir ☐					
Zanamivir ☐					

Number of missed doses_____

INPATIENT ANTIVIRALS received at time of recruitment **None** ☐

ANTIVIRAL	DOSE	FREQ	ROUTE	DATE STARTED	DATE FINISHED*
Oseltamivir ☐					
Zanamivir ☐					

PRE-HOSPITAL ANTIBIOTIC use **None** ☐

ANTIBOTIC†	DOSE	FREQ	ROUTE	DATE STARTED	DATE FINISHED*
1)					
2)					

INPATIENT ANTIBIOTICS received by the time of recruitment **None** ☐

ANTIBOTIC†	DOSE	FREQ	ROUTE	DATE STARTED	DATE FINISHED*
1)					
2)					

† enter the name of each antibiotic, including those given as stat doses.
* state the date when the course was finished, where applicable, including a change to therapy.

Pre-hospital ANTIPYRETIC/ANALGESIC use (including stat doses) **None** ☐

ANTIPYRETIC/ANALGESIC†	DOSE	FREQ	ROUTE	DATE STARTED	DATE FINISHED*
1)					
2)					

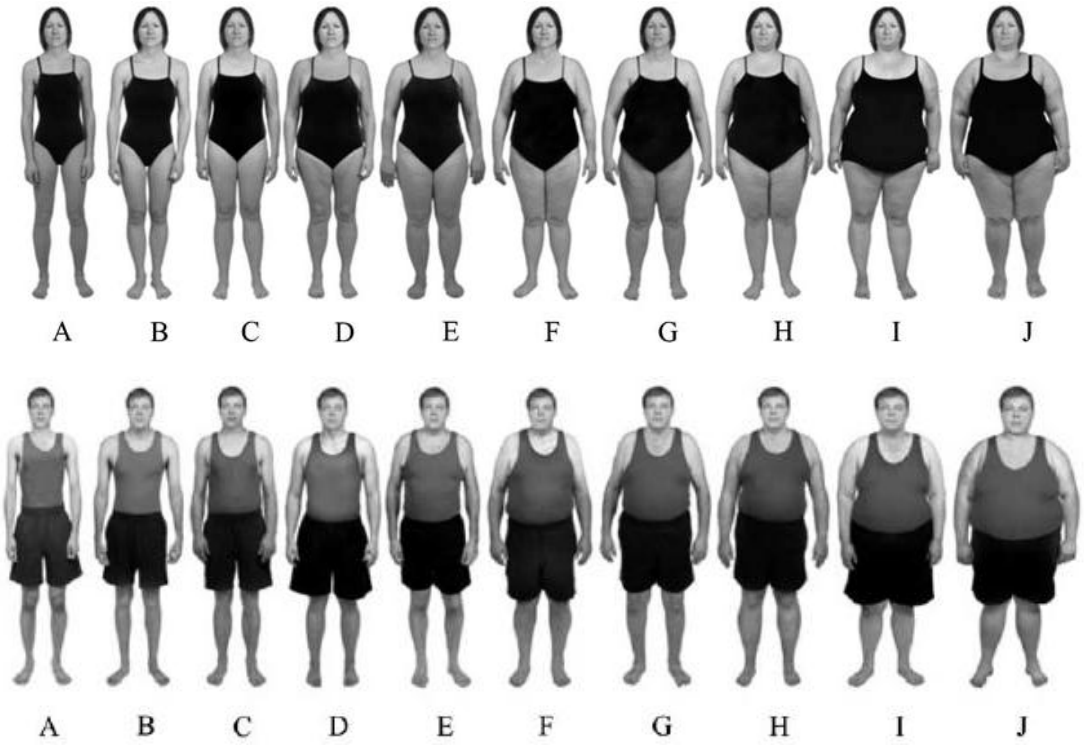
* state the date when the course was finished, where applicable, including a change to therapy.
† enter the name of each antipyretic/analgesic, including those taken as stat doses.

Body Mass Assessment **** Always endeavour to obtain recordings of weight and height ****

Height (state units)_____ **Body weight (state units)**_____

Data provided by: Clinical team ☐ Patient ☐ MOSAIC staff ☐

Visual estimate of body mass (circle the letter under the image that best represents the subject):



Smoking History **Current smoker** ☐ **Ex-smoker** ☐ **Never smoked** ☐ **Lives with a smoker** ☐

Number of cigarettes/ounces of tobacco smoked per day_____

Age subject started smoking_____ Age stopped smoking (if applicable)_____

Vaccination History Subject has never received influenza or pneumococcal vaccinations ☐

Seasonal influenza vaccine ☐ Date(s) given_____ Dose(s) given_____

Standalone H1N1 "Swine flu" vaccine e.g. *Pandemrix* or *Celvapan* ☐ Date(s) given_____ Dose(s) given_____

Pneumococcal (Pneumovax/Prevenar) ☐ Date(s) given_____ Dose(s) given_____

MOSAIC T1 Sample Record Sheet (Adult)

MOSAIC number_____

Date of sampling_____

Time of sampling_____

Samples taken by_____

Blood sample	**Taken**	**Not Taken**
6 ml clotted serum tube (WP3, WP6)	☐	☐
3 ml K2 EDTA (WP3)	☐	☐
3 ml K2 EDTA (WP6)	☐	☐
3 ml K2 EDTA (WP7)	☐	☐
2 x 3 ml Tempus (WP4)	☐	☐

Time of primary centrifugation of serum (red top) tube_____

Time of delivery of specimens to MOSAIC lab_____

Deviations from T1 blood sampling protocol/comments

Respiratory tract sample	**Taken**	**Not Taken**
Bacterial (m/c/s) throat swab	☐ side_____	☐
Plain (16S) throat swab	☐ side_____	☐
Viral throat swab in UTM (*only if NPA not obtained*)	☐ side_____	☐
SAM strip in Cryotube	☐ side_____	☐
Nasopharyngeal aspirate	☐ side_____	☐

Time of delivery of specimens to MOSAIC lab_____

Deviations from T1 respiratory sampling protocol/comments

MOSAIC T2 Sample Record Sheet (Adult)

MOSAIC number_____

Date of sampling_____

Time of sampling_____

Samples taken by_____

Blood sample	**Taken**	**Not Taken**
6 ml clotted serum tube (WP6)	☐	☐
3 ml K2 EDTA (WP3)	☐	☐
3 ml K2 EDTA (WP6)	☐	☐
2 x 3 ml Tempus (WP4)	☐	☐
4 x 10 ml Sodium heparin (WP5)	☐	☐

Time of primary centrifugation of serum (red top) tube_____

Time of delivery of specimens to MOSAIC lab_____

Deviations from T2 blood sampling protocol/comments

Respiratory tract sample	**Taken**	**Not Taken**
Bacterial (m/c/s) throat swab	☐ side_____	☐
Plain (16S) throat swab	☐ side_____	☐
SAM strip	☐ side_____	☐
Nasopharyngeal aspirate	☐ side_____	☐
Viral throat swab in UTM (*only if NPA not obtained*)	☐ side_____	☐

Time of delivery of specimens to MOSAIC lab_____

Deviations from T2 respiratory sampling protocol/comments

Clinical Impression

Status quo ☐ Improving ☐ Deteriorating ☐

Expected continuing care

Ward ☐ Critical Care ☐ Discharge ☐ RIP ☐

MOSAIC Additional Sample Record Sheet (Adult)

MOSAIC number_____

Urine

Sample type: MSU ☐ CSU ☐ Other ☐ _____

Date and time produced_____

Date and time delivered to MOSAIC specimen reception_____

Stool

Date produced_____

Date delivered to MOSAIC specimen reception_____

Sputum

Date produced_____

Date delivered to MOSAIC specimen reception_____

Deviations from additional sample collection protocol

MOSAIC Critical Care Additional Sample Record Sheet (Adults)

MOSAIC number_____

Lower Respiratory Tract Specimen 1 NBL ☐ BAL ☐ BW ☐

Volume for MOSAIC_____

Collected by_____

Date sample collected_____

Liquid type (e.g. 0.9% saline)_____Volume instilled_____Volume returned _____

Date delivered to MOSAIC specimen reception_____

Lower Respiratory Tract Specimen 2 NBL ☐ BAL ☐ BW ☐

Volume for MOSAIC_____

Collected by_____

Date sample collected_____

Liquid type (e.g. 0.9% saline)_____Volume instilled_____Volume returned _____

Date delivered to MOSAIC specimen reception_____

Deviations from additional sample collection protocol

MOSAIC Hospital Discharge Sheet (Adult)

MOSAIC number_____

Date of Discharge_____

Subject's telephone number:_____

Date of T3 follow up appointment_____

Time of T3 follow-up appointment_____

T3 follow-up location_____

Appointment made by_____

MOSAIC T3 Sample Record Sheet (Adult)

MOSAIC number_____

Date of sampling_____

Time of sampling_____

Samples taken by_____

Blood sample	**Taken**	**Not Taken**
6 ml clotted serum tube (WP3 & WP6)	☐	☐
3 ml K2 EDTA (WP3)	☐	☐
3 ml K2 EDTA (WP6)	☐	☐
2 x 3 ml Tempus (WP4)	☐	☐
2 x 10 ml Sodium heparin (WP5)	☐	☐
2 x 10 ml sodium heparin (WP6)	☐	☐

Time of primary centrifugation of serum (red top) tube_____

Time of delivery of specimens to MOSAIC lab_____

Deviations from T3 blood sampling protocol/comments

Respiratory tract sample	**Taken**	**Not Taken**
Bacterial (m/c/s) throat swab	☐	☐
Plain (16S) throat swab	☐	☐
Viral throat swab in UTM (*only if NPA not obtained*)	☐	☐
SAM strip	☐	☐
Nasopharyngeal aspirate	☐	☐

Time of delivery of specimens to MOSAIC lab_____

Deviations from T3 respiratory sampling protocol/comments

MOSAIC T3 Adult Clinical Evaluation Form

MOSAIC number_____

Date of Assessment_____

Assessor_____

Assessment Location_____

Discharged from Hospital to_____

Overall impression:

Recovered ☐ Residual symptoms ☐ New Symptoms ☐

Details:

Clinical Examination Key Findings

Further follow-up required? No ☐ MOSAIC ☐ NHS ☐

Details including appointment information:

MOSAIC Clinically Significant Result Reporting: Adult Record Sheet

MOSAIC Number_____

MOSAIC Site_____

Notifying Laboratory_____

Laboratory Contact_____

Date MOSAIC clinical researcher notified_____

Sample type_____

Sample ID_____

Date sample taken_____

Date sample received by lab_____

Date sample processed_____

Result_____

Action

MOSAIC clinical researcher_____

Clinically significant result? Yes ☐ No ☐

Trust clinician notified? Yes ☐ No ☐

Name of Trust clinician_____

Contact details_____

Date Trust Clinician notified_____

Comments

Appendix 5: Adult T1 sampling standard operating procedure

MOSAIC Time Point 1 (T1) Adult Sampling SOP

Important

- The subject must have signed the appropriate MOSAIC consent form prior to T1 sampling
- The MOSAIC researcher should wear their identity badge at all times when in a clinical area
- The subject reserves the right to withdraw from MOSAIC at any time during the study period
- T1 sampling can take place before the results of the clinical T0 sampling are known; it is expected that this will be the case for the majority of subjects
- Inform the attending doctor or nurse that the patient has consented to participate in MOSAIC and that you will be taking blood and respiratory samples for research purposes
- All T1 sampling equipment and consumables are contained in the T1 sampling pack
- Appropriate personal protective equipment should be used when taking samples, in line with local Trust recommendations
- Sharps should be placed in the appropriate container and clinical waste disposed of in appropriately marked waste bins within the clinical environment
- It is recommended that T1 blood sampling is performed prior to obtaining T1 respiratory samples; please follow the order of sampling as set out in this document

Blood samples

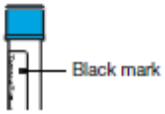
Equipment

- Green (21 G) Vacutainer Safety-Lok butterfly needle with attached Vacutainer tube holder
- Disposable tourniquet
- Skin cleaning swab
- Cotton wool ball
- Elastoplast

1. Inform the patient that you are taking blood samples as part of the MOSAIC study
2. Remove the bottles from the sampling pack and place in a cardboard tray
3. Put on disposable gloves
4. Remove the Safety-Lok butterfly Vacutainer collection device from its packaging

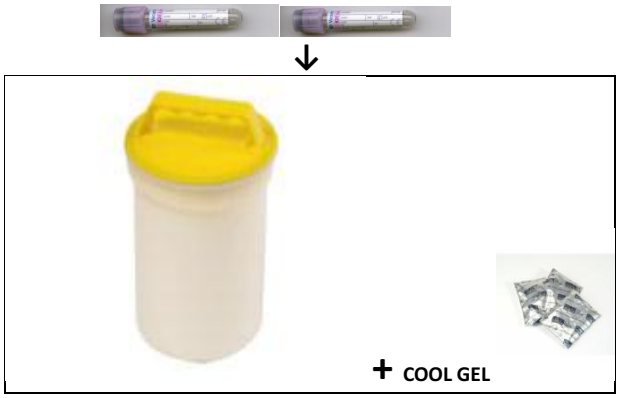


5. Identify an appropriate peripheral vein
6. Apply the disposable tourniquet at least 10cm above the chosen vein
7. Clean the vein with the disposable cleaning swab, allowing 30 seconds for the cleaning fluid to evaporate
8. Achieve venepuncture and immediately withdraw blood samples in the following order, ensuring all bottles are filled to maximum volume
    i. **6 ml clotted serum tube (red top)** 
    ii. **3 x 3ml K2 EDTA tubes (lavender top)** 
    
    iii. **2 x Tempus tubes (blue top)** 
    - **NB: Fill each Tempus tube exactly to the black mark on the side of the tube**



9. Release the tourniquet
10. Remove the butterfly needle from the vein, apply the Safety-Lok and place in the tray
11. Apply cotton wool ball to venepuncture site and ask the patient to apply pressure
12. **Immediately shake each Tempus tube vigorously for 20 seconds**
13. **Invert the red-top serum tube 5 times.**
14. **Invert each EDTA tube 10 times.**
15. Apply the specific T1 sample labels from the subject's folder to the appropriate bottles
16. Remove cotton wool ball and apply Elastoplast (unless patient is allergic to plasters)
17. Dispose of sharps in the sharps bin
18. Place the bottles in the following Biojars:


tube upright  keep





COOL (4°C) Biojar **ROOM TEMP Biojar**

- **Complete the T1 blood sampling record in the subject's folder**

Now obtain the respiratory samples

Throat swabs

Equipment

- 1 x Sterilin transport throat swab



- 1 x Sterilin F155CA plain throat swab

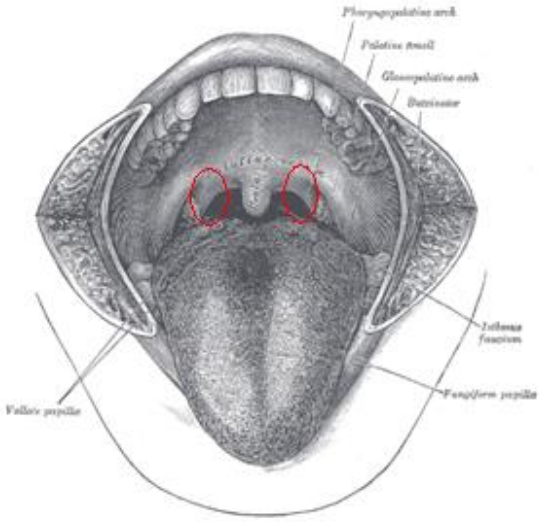


- 1 x Sterilin/Copan (502CS01) flocked throat swab with 1 ml UTM ***ONLY REQUIRED IF NPA UNOBTAINABLE***



- Tongue depressor & pen torch  
-

1. Gently depress the subject's tongue using the tongue depressor
2. Identify the palatopharyngeal arches (red circles in diagram below) using the pen torch for illumination



3. Open up the **bacterial (blue top) transport swab** pack and taking care not to touch the tip, remove the swab from the container.



Swab the palatopharyngeal arch by making 5 small circular motions, ensuring that the tip is in contact with the mucosa. Avoid touching the patient's tongue. Return the swab to its dedicated container.

4. Open up the **plain (white top) throat swab** pack and taking care not to touch the tip, remove the swab from the container



Swab the SAME palatopharyngeal arch by making 5 small circular motions, ensuring that both tips are in contact with the mucosa. Avoid touching the patient's tongue. Return the swab to its dedicated container.

5. ONLY OBTAIN FLOCKED VIRAL THROAT SWAB IF NPA IS NOT OBTAINED

Open up the **flocked viral throat swab** and taking care not to touch the tip, remove the swab from it from its packaging. Remove the cap from the bottle of UTM ensuring it is kept upright to avoid spillage of contents.



Swab the OPPOSITE palatopharyngeal arch by making 5 circular small motions, ensuring that the tip is in contact with the mucosa. Place the tip of the swab in the pot of UTM, and stir the medium with the tip five times before snapping the swab shaft at the breakpoint and screwing on the lid tightly. Invert the UTM bottle five times.



→



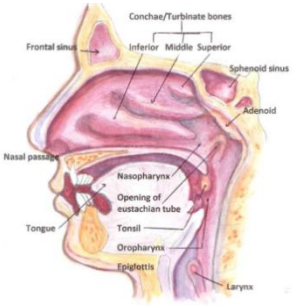
6. Dispose of the tongue depressor in the clinical waste bin. Dispose of any packaging in the appropriate waste bin.
7. Apply the specific T1 sample labels from the subject's folder to the appropriate containers.
8. Place all three swab samples in the **COOL Biojar** and replace the lid.

SAM sample

Equipment

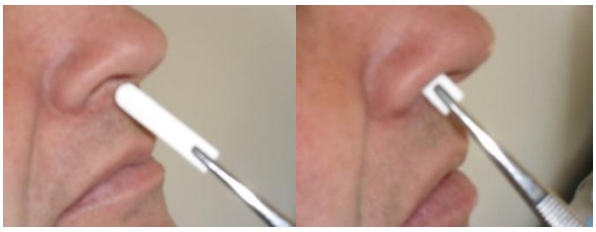
- Sterile disposable plastic forceps (note metal forceps shown in diagrams)
- Small grip bag containing one SAM Accuwick strip and one Cryotube

1. With gloved hands, open the pack containing the sterile disposable plastic forceps.
2. Remove the Cryotube tube from the bag and loosen the screw cap.
3. Remove the Accuwick SAM strip from the bag using the forceps
4. With the patient sitting comfortably upright, and the head extended backwards, identify the **RIGHT inferior turbinate**.





5. A single Accuwick SAM strip is placed on the lateral wall of the **RIGHT nasal cavity** (against the inferior turbinate) using blunt-ended forceps. The strip should be placed almost completely into the nostril, leaving a small external portion visible to enable retrieval.



6. The SAM strip makes contact with the nasal lining fluid through light pressure with a finger applied to the outside of the nose (**nasal pinch**); strips are left to absorb nasal secretions for **exactly 2 min.**
7. Remove the strip using the forceps and place it in the Cryotube.



8. Screw on the cap so it is tightly sealed and write on the MOSAIC subject number using the freeze-proof pen. Place the Cryotube in a grip-seal bag and apply the appropriate T1 sample label from the subject's folder and place the bag in the **COOL Biojar.** Replace the Biojar lid.

Nasopharyngeal Aspirate (NPA)

Equipment





- 10 CH black-tip Argyle suction catheter
- Suction trap pack with container lid
- Connector tubing to connect suction trap to wall suction
- 5 ml plastic vial of sterile 0.9% saline

PPE and infection control requirements

Check and follow your current local NHS Trust guidelines. NPA is ideally performed in a side room, but it may be performed in an open area as long all those in attendance observe PPE requirements. Irrespective of vaccination history, the operator should wear:

- disposable gloves
- plastic gown
- FFP3 respirator (ideally fit-tested)
- eye protection

1. Inform the patient that NPA is not a painful sampling procedure but that it can cause some mild nasal irritation (e.g. tickling sensation, feeling of wanting to sneeze and eye watering).
2. Using gloved hands, remove the suction trap from its packaging and attach the suction trap to suction canister of the wall suction using an appropriate length of plastic connector tubing connected to the suction trap port. Keep the suction trap screw-top lid in the packaging

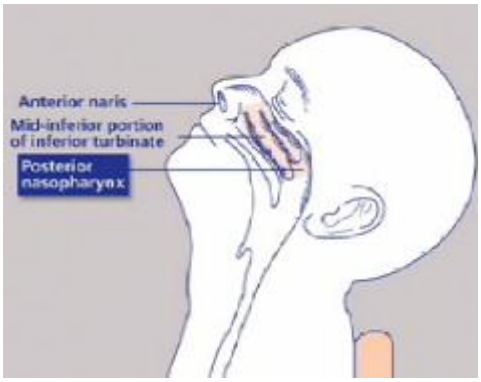


3. Open the end of the suction catheter tubing nearest to the black suction control on the catheter, keeping the catheter within its packaging until ready for use. Attach the black end of the catheter to the remaining port on the suction trap.
4. Remove the top of the vial of the sterile saline and place upright on a surface within easy reach
5. With the patient either sitting upright or with on a trolley with the back positioned at around 45°, tilt the patient's head back slightly



6. Switch on wall suction to **13 kPa** (100 mmHg) and maintaining an aseptic technique, remove the catheter from its packaging

7. Without applying suction, insert the catheter into the patient's **LEFT nostril** to reach the posterior nasopharynx, approximately half way along an imaginary line drawn between the left nostril and the left ear lobe





8. Apply suction by occluding the hole in the black end of the suction catheter and then withdraw the catheter over 5 seconds whilst rotating your wrist. **Keep the suction trap upright during this procedure**. Release suction when the catheter has been removed from the patient's nose.
9. Examine the catheter and trap to look for secretions coating the inside of the catheter and trap. If no secretions are seen, repeat the procedure (document repeat attempts in the appropriate section of the subject folder)
10. Place the tip of the catheter in the vial of saline, apply suction and aspirate the entire contents into the trap. Release suction and turn off the wall suction.
11. Unscrew the top (port lid) of the suction trap, remove the screw-cap from the packaging and screw onto the sputum trap container.
12. Dispose of the catheter, suction tubing and sputum trap port lid in the clinical waste bin.
13. Apply the appropriate T1 specimen label to the specimen container and place it in the **cool Biojar** and replace the lid.
    - **Complete the T1 respiratory sampling record in the subject's folder**

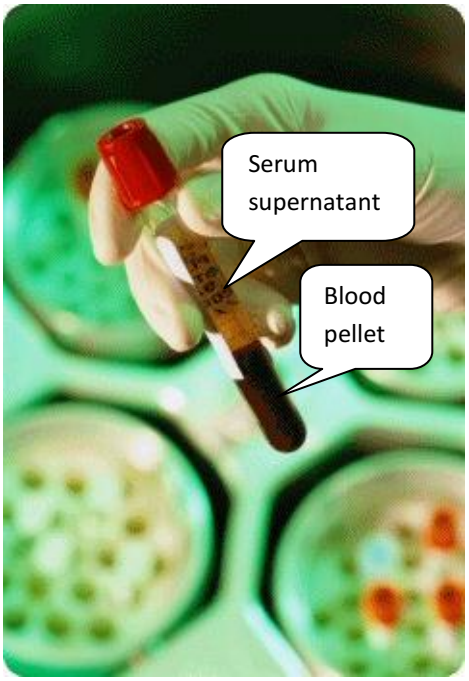
Place the Biojars in the MOSAIC specimen transport bag



<u>Serum sample processing</u>

The sample should be left for 60 minutes after being drawn to allow the blood to clot. The clotted sample needs to be processed quickly, <u>60-90 minutes after</u> the clotted serum sample has been drawn. If a laboratory technician is unavailable, then the recruiter will need to perform the centrifugation and aliquoting, and then deposit the processed samples in the designated -80C freezer. If the recruiter cannot deliver the samples to MOSAIC specimen reception within 1 hour, the recruiter will need to perform centrifugation and aliquoting in the field (if recruiting off-site).

1. Put on a pair of NITRILE (non-latex) gloves.
2. Place the red top clotted serum tube in the cooled (4°C) centrifuge.
3. Ensure the centrifuge is balanced, using counterbalance tubes where necessary.
4. Centrifuge at 700 x g for 5-10 minutes (to produce a layer of serum supernatant)

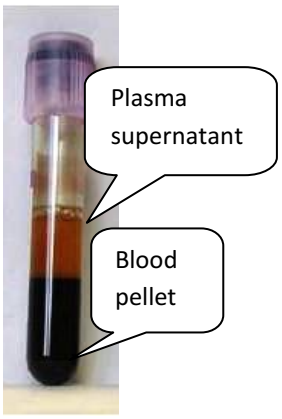


5. Remove the lid of serum tube and using a disposable sterile graduated pipette, aliquot 0.5 ml of supernatant into each of 4 Cryotube. Seal the Cryotubes and dispose of the pipette in a clinical waste bin.
6. Apply the appropriate T1 MOSAIC labels to each Cryotube. If labels are not available, write on the MOSAIC number, "serum" and "T1" on each tube in freezer-proof ink.
7. Replace the lid of the serum tube, ensuring the tube is adequately sealed. Apply the appropriate T1 sample label to the serum if not already labelled.
8. Put the serum tube containing the blood clot pellet and any remaining supernatant, along with the Cryotubes in the **cool Biojar** and return it to the MOSAIC specimen transport bag.

WP6 EDTA (Plasma) sample processing

Centrifuge the WP6 EDTA sample as soon as possible, 60-90 minutes after the EDTA blood sample has been drawn. If a laboratory technician is unavailable, then the recruiter will need to perform centrifugation and aliquoting. If the recruiter cannot deliver the samples to MOSAIC specimen reception within 1 hour, the recruiter will need to perform centrifugation and aliquoting in the field (if recruiting off-site). The EDTA tube can be centrifuged at the same time as the red-top serum tube, as long as the centrifuge remains balanced.

1. Put on a pair of NITRILE (non-latex) gloves.
2. Place the lavender top EDTA tube in the cooled (4°C) centrifuge.
3. Ensure the centrifuged is balanced, using counterbalance tubes where appropriate.
4. Centrifuge at 700 x g for 5-10 minutes (to produce a layer of plasma supernatant



5. Keeping the tube upright, remove the lid of the centrifuged EDTA tube and using a disposable sterile graduated pipette, aliquot 0.5 ml of plasma supernatant into each of two Cryotube (avoiding contact with the blood pellet at the base of the tube).
6. Apply the appropriate MOSAIC label to each aliquot Cryotube. If labels are unavailable, write the MOSAIC number on each tube (using freezer-proof ink), along with "WP6 T1 plasma". The blood pellet and pipette should be disposed of in the appropriate clinical waster bin.
7. Put the Cryotubes in the **cool Biojar** and return it to the MOSAIC specimen transport bag.
   - **Deliver the MOSAIC specimen transport bag to the MOSAIC specimen reception**
   - **Document any deviations from protocol on the appropriate record sheets**
   - **REMEMBER TO TAKE THE MOSAIC SUBJECT FOLDER WITH YOU**

<u>Instructions for MOSAIC Specimen Reception Processing</u>

These instructions are intended to provide guidance to the recruiter-sampler on how to process/handle the specimens once they reach the designated MOSAIC specimen reception, should a laboratory technician be unavailable (e.g. out of hours). Recruiter-samplers who are asked to process samples should have first received appropriate local training in the required techniques and laboratory health and safety.

All samples in the cool Biojar should be kept cool in the Specimen Reception (4°C) while processing.

All pipetting, decanting and aliquoting should be performed in a class II safety cabinet.

Sample	Container(s)	Work Package(s)	Immediate processing in MOSAIC Specimen Reception	MOSAIC Specimen Reception Storage/handling
WP3 EDTA (plasma)	1 x 3ml Lavender-top EDTA tube in the cool Biojar	WP3	Centrifugation of the sample should take place at 60-90 minutes after being drawn (at the same time as the clotted blood serum tube). If plasma aliquots were not already made in the field, centrifuge the EDTA tube at 700g for 5-10 minutes in a balanced, cooled (4°C) centrifuge. Pipette 2 x 0.5 ml aliquots of plasma supernatant into 2 Cryotubes. Apply the appropriate MOSAIC sample labels or write on each Cryotube the MOSAIC number and Tube 1: WP3 HPA T1 EDTA Tube 2: WP3 GLA T1 EDTA	2 x 0.5 ml WP3 plasma ("EDTA") aliquots into designated -80°C freezer immediately.
WP3 flocked viral throat swab in UTM (only if no NPA obtained)	UTM pot (red or green lid) in cool Biojar	WP3 (Glasgow)	Ensure correct label attached	Place in designated -80°C freezer immediately.

Sample	Container(s)	Work Package(s)	Immediate processing in MOSAIC Specimen Reception	MOSAIC Specimen Reception Storage/handling
Clotted blood (serum)	1 x 6ml red top tube in room temp Biojar	WP6 (Imperial St Mary's)	1. The red top tube should have been allowed to clot for 60 mins at room temperature after being drawn. Centrifugation of the sample should take place at 60-90 mins after being drawn. If serum aliquots were not already made in the field, centrifuge the tube at 700g for 5-10 minutes in a balanced, cooled (4°C) centrifuge. Pipette 2 x 0.5 ml aliquots of serum supernatant in two Cryotubes. Apply the correct WP6 MOSAIC labels to each Cryotube, or write on the MOSAIC number, "WP6", "T1" and "serum". Return the labelled red top tube to the fridge (4°C) for further processing	Place 2 x WP6 serum aliquots in designated -80°C freezer immediately.
		WP3 (HPA)	2. The following day, remove the red top tube from the fridge. Centrifuge at 700g for 5-10 mins in a cooled (4°C), balanced centrifuge. Pipette any remaining serum into a Cryotube and apply the appropriate MOSAIC label, or write on the MOSAIC number "serum", "T1" and "WP3 HPA".	Place 1 x WP3 HPA serum aliquot in designated -80°C freezer immediately.

Sample	Container(s)	Work Package(s)	Immediate processing in MOSAIC Specimen Reception	MOSAIC Specimen Reception Storage/handling
WP4 Tempus (whole blood)	2 x blue-top Tempus tubes in cool Biojar	WP4 (Imperial St Mary's)	Ensure samples are correctly labelled.	Place both tubes in designated -80°C freezer immediately.
WP6 EDTA (plasma)	1 x 3 ml lavender-top EDTA tube in cool Biojar	WP6 (Imperial St Mary's)	Centrifugation of the sample should take place at 60-90 minutes after being drawn (at the same time as the clotted blood serum tube). If plasma aliquots were not already made in the field, centrifuge the EDTA tube at 700g for 5-10 minutes in a balanced, cooled (4°C) centrifuge. Pipette 2 x 0.5 ml aliquots of plasma supernatant into 2 Cryotubes. Apply the appropriate MOSAIC sample labels, or write on the MOSAIC number, "plasma", "T1" and "WP6".	2 x 0.5 ml WP6 plasma aliquots into designated -80°C freezer immediately.
WP7 EDTA blood	1 x 3 ml lavender-top EDTA tube in cool Biojar	WP7 (Edinburgh)	Ensure sample is correctly labelled with the MOSAIC label or write on the MOSAIC number, "WP7" and "T1".	Place in designated -80°C freezer immediately.
NPA	Universal container (approx. 5 ml "NPA stock") in the cool Biojar	WP3 (HPA and Glasgow), WP6 (Imperial St Mary's) and WP8 (Imperial Royal Brompton)	1. Aliquots should only be made using a safety cabinet. Gently shake the universal container. Using a sterile pipette, make 4 x 1ml aliquots using 4 x Cryotubes. Apply appropriate MOSAIC labels or write on the following, in addition to the subject's MOSAIC number: Tube 1: WP3 GLA NPA T1 Tube 2: WP3 HPA NPA T1 Tube 3: WP6 NPA T1 Tube 4: WP8 16S NPA T1	Place 2 x WP3 NPA aliquots, WP6 NPA aliquot and WP8 16S NPA aliquot in designated -80°C freezer immediately. **SEE NEXT TABLE**

Sample	Container(s)	Work Package(s)	Immediate processing in MOSAIC Specimen Reception	MOSAIC Specimen Reception Storage/handling
NPA cont'd			2. Leave the remaining volume in the universal container, ensuring that the lid is screwed on tight. This is the sample for fresh bacterial culture. Ensure it is labelled with the MOSAIC number and "T1". Keep cool and if immediate delivery to the local microbiology lab is not possible, keep the sample in the fridge (4°C).	Deliver remaining volume in its original universal container, with appropriate request form, ASAP to local microbiology lab. If kept in local fridge, it should be delivered within 24 hours of the sample having been collected. DO NOT FREEZE.
WP6 SAM strip	1 x Cryotube containing SAM strip, in cool Biojar	WP6 (Imperial St Mary's)	Ensure sample is correctly labelled with MOSAIC label, or write on MOSAIC number and "T1 WP6 SAM"	Place in designated -80°C freezer immediately.
WP8 16S throat swab	1 x white-top throat swab in dedicated container, in cool Biojar.	WP8 (Imperial Royal Brompton)	Ensure sample is correctly labelled, with MOSAIC label or write on MOSAIC number and "T1 WP8 16S"	Place in designated -80°C freezer immediately.
WP8 m/c/s throat swab	1 x blue-top throat swab in dedicated container, in cool Biojar.	WP8 (local microbiology lab)	Ensure sample is labelled with the MOSAIC number and "T1". Keep cool and if immediate delivery to the local microbiology lab is not possible, keep the sample in the fridge (4°C).	Deliver, with appropriate request form, ASAP to local microbiology lab. If kept in local fridge, it should be delivered within 24 hours of the sample having been collected. DO NOT FREEZE.

Sample	Container(s)	Work Package(s)	Immediate processing in MOSAIC Specimen Reception	MOSAIC Specimen Reception Storage/handling
Sputum (not time-point dependent)	Sputum in universal container, in cool Biojar.	WP8 (local microbiology lab and Imperial Royal Brompton)	Gently shake the sample. In a safety cabinet, pipette half the contents into a Cryotube. Write the MOSAIC number on this tube, along with "sputum WP8 16S" and the date of sampling. Replace the lid on the universal container, ensuring it is tightly sealed and labelled with the MOSAIC number.	1. Place the aliquot for WP8 16S in the designated -80°C freezer immediately. 2. The remaining volume in the original universal container should be sent, with appropriate request form, ASAP to local microbiology lab. If kept in local fridge, it should be delivered within 24 hours of the sample having been collected. DO NOT FREEZE.
Urine (not time-point dependent)	Urine in a universal container	WP6 (Imperial St Mary's)	Ensure sample is correctly labelled with MOSAIC number and date of sampling.	Place in designated -80°C freezer immediately.
Stool (not time-point dependent)	Faeces in a stool pot (blue-top universal container)	WP3	Ensure sample is correctly labelled with MOSAIC number and date of sampling.	Place in designated -80°C freezer immediately.
BAL/NBL (not time-point dependent)	BAL/NBL in universal container, in cool Biojar.	As per NPA	Process as per NPA instructions (except replace "NPA" with "BAL" and write date rather than time point.	As per NPA instructions. DO NOT FREEZE ALIQUOT FOR FRESH BACTERIAL CULTURE.

Appendix 6: Paediatric sampling hierarchy

Hierarchy of blood volumes for research in addition to clinical samples

	Total Amount	1. EDTA sample	2. Clotted sample	3. Heparinised sample	4. Tempus sample
< 1 year or 10kg	2ml	1ml	1ml	X	X
1-5 years or 10-20kg	6ml	1ml	2ml	2ml	1ml
5-10 years or 20-30 kg	13ml	3ml	5ml	4ml	1ml
10-16 years or > 30kg	20ml	3ml	6ml	10ml	1ml

- Hierarchy based on WPs prioritised by study funders
- Every effort will be made to collect research samples at the same time as clinical samples; given vagaries of collecting blood from children, it may not be possible to get complete sample sets from all children
- Blood amounts based on total blood volume being approximately 80 ml/kg (MCRN guidelines less clinical draw [~2ml]). Sampling regime equivalent to ≤ 3 % of the total blood volume over 4 weeks, ≤ 1% at any single time

Appendix 7: Canonical pathway lists for influenza-positive patients

Major cluster name	Canonical pathway	-log(p-value)	Ratio	Molecules (symbol)
T- and B-cell pathways	Primary Immunodeficiency Signalling	5.65	10/62 (0.161)	CD4 CD19 CD3D CD40LG CD79A CD8A IL7R LCK ZAP70
	Role of NFAT in regulation of the immune response	5.55	18/199 (0.09)	CD4 CD247 CD3D CD79A CD79B CSNK1G3 FCER1A GNA12 GNG7 HLA-DQA1 HLA-DQB1 LCK MEF2C NFATC3 RCAN3 RRAS2 ZAP70
	T cell receptor signalling	5.31	13/109 (0.13)	CD4 CD247 CD3D CD3E CD8A CD8B CTLA4 LCK NFATC3 RASGRP1 RRAS2 TXK ZAP70
	iCOS-iCOSL signalling in T helper cells	4.84	13/123 (0.11)	CD4 CD247 CD3D CD3E CD40LG HLA-DQA1 HLA-DQB1 LCK NFATC3 PLEKHA1 PLEKHA3 TRAT1 ZAP70
	CTLA4 Signalling in Cytotoxic T lymphocytes	4.12	11/98 (0.11)	CD4 CD247 CD3D CD3E CTLA4 HLA-DQA1 HLA-DQB1 LCK PPP2R2B TRAT1 ZAP70

Supplementary table 1: Top five IPA canonical pathways and their constituent genes identified in the T- and B-cell-associated pathways major transcriptional cluster, influenza-positive adult patients from the 2009/10 season.

Major cluster name	Canonical pathway	-log(p-value)	Ratio	Molecules (symbol)
Interferon and immune pathways	Interferon signalling	10.03	9/36 (0.25)	BAK1 IFI35 IFIT1 IFIT3 MX1 OAS1 SOCS1 STAT1 STAT2
	Role of pattern recognition receptors in recognition of bacteria and viruses	9.12	12/106 (0.11)	C5 C1QB C1QC DDX58 EIF2AK2 IFIH1 IRF7 OAS1 OAS2 OAS3 TLR2 TLR5
	Activation of IRF by cytosolic pattern recognition receptors	7.49	9/72 (0.13)	DDX58 DHX58 IFIH1 IFIT2 IRF7 ISG15 STAT1 STAT2 ZBP1
	Role of JAK family kinases in IL-6-type cytokine signalling	3.80	4/27 (0.15)	MAPK14 SOCS1 SOCS3 STAT1
	Complement system	4.12	11/98 (0.11)	C5 C1QB C1QC SERPING1

Supplementary table 2: Top five IPA canonical pathways and their constituent genes identified in the interferon and immune pathways major transcriptional cluster, influenza-positive adult patients from the 2009/10 season.

Major cluster name	Canonical pathway	-log(p-value)	Ratio	Molecules (symbol)
Inflammation/ signalling and cell-cycle pathways	Cell cycle control of chromosomal replication	3.10	3/32 (0.09)	MCM2 MCM4 RPA4
	Airway pathology in COPD	2.94	2/9 (0.22)	MMP8 MMP9
	Cell cycle: G2/M DNA damage checkpoint regulation	2.53	3/49 (0.06)	CCNB2 GADD45 TOP2A
	p53 signalling	2.49	4/96 (0.04)	BIRC5 GADD45 GADD45 TP53I3
	Melatonin degradation III	2.19	1/6 (0.17)	MPO

Supplementary table 3: Top five IPA canonical pathways and their constituent genes identified in the inflammation and cell-cycle pathways major transcriptional cluster, influenza-positive adult patients from the 2009/10 season.

Major cluster name	Canonical pathway	-log(p-value)	Ratio	Molecules (symbol)
T- and B-cell pathways	Primary Immunodeficiency Signalling	5.72	11/62 (0.18)	CD4 CD3D CD3E CD40LG CD79A CD8A CIITA IL7R LCK TNFRSF13C ZAP70
	CTLA4 signalling in cytotoxic T lymphocytes	5.30	14/98 (0.14)	CD4 CD28 CD3D CD3E CTLA4 HLA-DOA HLA-DQA1 HLA-DQB1 LCK PLCG1 PPP2R2B PPP2R5C TRAT1 ZAP70
	iCOS-iCOSL signalling in T helper cells	4.53	14/123 (0.11)	CD4 CD28 CD3D CD3E CD40LG HLA-DOA HLA-DQA1 HLA-DQB1 IL2RB LCK PLCG1 PLEKHA1 TRAT1 SAP70
	Natural killer cell signalling	4.39	14/117 (0.12)	CD244 KIR3DL3 KLRB1 KLRC1 KLRC2 KLRC3 KLRD1 LCK NCR3 PLCG1 RRAS2 SH2D1A SH2D1B ZAP70
	T cell receptor signalling	4.36	13/109 (0.12)	CD4 CD28 CD3D CD3E CD8A CD8B CTLA4 LCK PLCG1 RASGRP1 RRAS2 TXK ZAP70

Supplementary table 4: Top five IPA canonical pathways and their constituent genes identified in the T- and B-cell-associated pathways major transcriptional cluster, influenza-positive adult patients from 2010/11 season.

Major cluster name	Canonical pathway	-log(p-value)	Ratio	Molecules (symbol)
Cell cycle pathways	Mitotic roles of polo-like kinase	6.05	8/70 (0.11)	CCNB1 CCNB2 CDC20 CDC25A CDK1 KIF11 KIF23 PRC1
	DNA damage-induced 14-3-3σ signalling	5.73	5/21 (0.24)	CCNB1 CCNB2 CCNE1 CCNE2 CDK1
	Eisotrgen-mediated S-phase entry	5.19	5/28 (0.18)	CCNA2 CCNE1 CCNE2 CDC25A CDK1
	Cyclins and cell cycle regulation	4.46	7/90 (0.08)	CCNA2 CCNB1 CCNB2 CCNE1 CCNE2 CDC25A CDK1
	GADD45 signalling	4.17	4/23 (0.17)	CCNB1 CCNE1 CCNE2 CDK1

Supplementary table 5: Top five IPA canonical pathways and their constituent genes identified in the cell-cycle associated pathways major transcriptional cluster, influenza-positive adult patients from the 2010/11 season.

Major cluster name	Canonical pathway	-log(p-value)	Ratio	Molecules (symbol)
Interferon and immune pathways	Interferon signalling	8.91	7/36 (0.19)	IFI35 IFIT1 IFIT3 MX1 OAS1 SOCS1 STAT1
	Activation of IRF by cytosolic pattern recognition receptors	8.35	8/72 (0.11)	DDX58 DHX58 IFIH1 IFIT2 IRF7 ISG15 STAT1 ZBP1
	Role of pattern recognition receptors in recognition of bacteria and viruses	8.15	9/106 (0.09)	C1QB C1QC DDX58 IFIH1 IRF7 OAS1 OAS2 OAS3 PRKCE
	Complement system	5.79	5/35 (0.14)	C2 C1QB C1QC CFB SERPING1
	Role of RIGI-like receptors in antiviral innate immunity	3.83	4/49 (0.08)	DDX58 DHX58 IFIH1 IRF7

Supplementary table 6: Top five IPA canonical pathways and their constituent genes identified in the interferon and immune pathways major transcriptional cluster, influenza-positive adult patients from the 2010/11 season.

Major cluster name	Canonical pathway	-log(p-value)	Ratio	Molecules (symbol)
Signalling/ inflammation pathways	IL-10 signalling	5.54	10/78 (0.13)	CCR1 IL18RAP IL1R1 IL1R2 IL1RN IL4R MAP2K6 MAPK14 NFKBIA SOCS3
	IL-6 signalling	4.18	11/124 (0.09)	IL18RAP IL1R1 IL1R2 IL1RN IL6ST MAP2K6 MAPK14 NFKBIA SOCS3 TNFAIP6 VEGFA
	Role of macrophages, fibroblasts and endothelial cells in rheumatoid arthritis	4.13	19/336 (0.06)	CREBBP FCGR1A IL18R1 IL18RAP IL1R1 IL1R2 IL1RN IL6ST IRAK3 MAP2K6 MAPK14 NFKBIA OSM PDGFA SOCS3 TLR2 TLR5 TNFSF13B VEGFA
	Toll-like receptor signalling	3.58	7/62 (0.11)	EIF2AK2 IRAK3 MAP2K6 MAPK14 NFKBIA TLR2 TLR5
	p38 MAPK signalling	3.45	10/118 (0.9)	CREBBP IL18RAP IL1R1 IL1R2 IL1RN IRAK3 MAP2K6 MAPK14 MEF2A MKNK1

Supplementary table 7: Top five IPA canonical pathways and their constituent genes identified in the signalling and inflammation (bacterial response) pathways major transcriptional cluster, influenza-positive adult patients from the 2010/11 season.

Major cluster name	Canonical pathway	-log(p-value)	Ratio	Molecules (symbol)
Metabolic pathways	Oestrogen receptor signalling	2.53	4/136 (0.03)	GTF2A1 MED13 MED15 TAF13
	Oxidised GTP and dGTP detoxification	1.86	1/11 (0.09)	DDX6
	Assembly of RNA polymerase II complex	1.64	2/56 (0.04)	GTF2A1 TAF13
	Phosphatidylcholine biosynthesis I	1.49	1/17 (0.06)	CEPT1
	phosphatidylethanolamine biosynthesis II	1.44	1/18 (0.06)	CEPT1

Supplementary table 8: Top five IPA canonical pathways and their constituent genes identified in the metabolic pathway major transcriptional cluster, influenza-positive adult patients from the 2010/11 season.

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Figure 1.1 Schematic diagram of the influenza virus life cycle

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Figure 1.8: The origins of influenza A(H1N1)pdm09 virus

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Figure 1.9: Sequential chest radiographs from a hospitalised patient with A(H1N1)pdm09 virus infection

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