

1 **Progression of whole blood transcriptional signatures from**
2 **interferon-induced to neutrophil-associated patterns in patients**
3 **with severe influenza**

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33 **Abstract**

34 Transcriptional profiles are increasingly used to investigate the severity, subtype and pathogenesis
35 of disease. We now describe whole blood RNA signatures and local and systemic immune mediator
36 levels in a large cohort of adults hospitalised with influenza from which extensive clinical and
37 investigational data was obtained. Signatures reflecting interferon-related antiviral pathways were
38 common up to day 4 of symptoms in cases not requiring mechanical ventilatory support; in those
39 needing mechanical ventilation, an inflammatory, activated neutrophil and cell stress/death
40 ('bacterial') pattern was seen, even early after disease onset. Identifiable bacterial co-infection was
41 not necessary for this 'bacterial' signature but could enhance its development while attenuating the
42 early 'viral' signature. Our findings emphasise the importance of timing and severity in the
43 interpretation of transcriptomic profiles and soluble mediator levels, and identify specific patterns of
44 immune activation that may enable the development of novel diagnostics and therapeutics.

45

46 Influenza viruses present a continuous threat to global health, mutating and spreading within and
47 between species. It is estimated that one billion cases of human influenza occur worldwide each
48 year, causing 3-5 million cases of severe illness and 300,000 to 500,000 deaths¹. While most deaths
49 and illnesses are attributable to seasonal influenza, pandemics caused by novel viruses regularly
50 pose an unpredictable challenge to public health.

51 Infection with pandemic 2009 H1N1 influenza A virus (pH1N1) resulted in generally mild disease²,
52 but still caused an estimated 250,000 – 500,000 additional deaths during the first 12 months of
53 global circulation³. Whereas seasonal influenza commonly causes severe disease in the old and
54 infirm, serious pH1N1 disease mostly occurred in infants and younger adults, presenting as viral
55 pneumonia and sometimes complicated by multi-organ failure^{4, 5}. It has been suggested that severe
56 influenza may in part result from an over-exuberant host reaction to infection (sometimes termed
57 “cytokine storm”), but is also driven by a high viral load in affected persons^{6, 7, 8}. Although analysis of
58 transcriptional signatures and mediator levels has helped to clarify the pathogenesis of severe
59 influenza, the relationship between severity, timing and complications of infection remains unclear.
60 Previous studies of gene expression patterns in influenza have typically involved small numbers of
61 individuals, healthy subjects undergoing experimental challenge or patients suffering from mild
62 disease^{9, 10, 11, 12, 13, 14, 15}. Transcriptomic analysis has also been used to study a variety of acute and
63 chronic infections, including bacterial sepsis, dengue virus infection and tuberculosis¹⁶ and to
64 examine differences and similarities between infectious and non-infectious inflammatory disorders,
65 such as systemic lupus erythematosus¹⁷.

66 To further elucidate influenza pathogenesis, the Mechanisms of Severe Acute Influenza Consortium
67 (MOSAIC) recruited 255 hospitalised patients with suspected influenza in England over two
68 consecutive seasons (2009/10 and 2010/11). By analysing biological samples taken at multiple time-
69 points and correlating this with extensive clinical data, MOSAIC aimed to define the contributions
70 made by influenza virus sequence variation, co-pathogens (non-influenza viruses and bacteria) and
71 host factors (genetic and transcriptional differences, soluble mediator responses and cellular
72 immune responses) to disease pathogenesis. Sample analysis resulted in a cumulative total of 2.1 x
73 10⁷ data items on this population, a dataset that we now describe in outline and provide as a
74 resource. To date, MOSAIC has reported enrichment for a host genetic variant, the interferon-
75 inducible transmembrane protein 3 (*IFITM3*) allele SNP rs12252-C in some hospitalised patients with
76 influenza¹⁸, and that viral sequence changes that accumulate over time may contribute to the
77 variation in disease severity^{19, 20, 21, 22}. The exceptional size and depth of the MOSAIC study provides a
78 unique database to allow these complex issues to be resolved.

79 In the present study, we used whole blood transcriptional RNA analysis and data from soluble
80 immune mediator (cytokines and chemokine) measurements from mucosal fluids and serum to
81 define associations between individual responses to infection and clinical and laboratory findings in
82 hospitalised adults with influenza. The richness of clinical and ancillary data in the MOSAIC study
83 allowed us to examine the extent to which specific patterns of inflammation arise from progression
84 of antiviral and inflammatory responses induced simply by viral infection, or whether they reflect a
85 response to pathogenic or commensal bacteria. We found that transcriptomic signatures and
86 mediator levels are strongly associated with both severity and duration of illness, indicating a phased
87 and graded activation of interferon-related and inflammatory genes. Clinically-evident bacterial co-
88 infection influenced the pattern of gene expression, but effects were superimposed on patterns
89 governed by the duration and severity of influenza.

90

91 **Results**

92 **Influenza is characterized by an overabundance of interferon and inflammation-related transcripts**

93 Principal Component Analysis (PCA) of the 18,974 most abundant transcripts from whole blood RNA
94 at enrolment (T1) showed that influenza patients from the 2010/11 season (n=109) formed a distinct
95 cluster that was clearly differentiated from matched healthy controls (n=130). PCA indicated no
96 discernible difference between patients with influenza caused by diverse influenza A or influenza B
97 viruses (Fig. 1a).

98 Microarray profiles of whole blood RNA from the first and second acute illness sampling time-points
99 (T1 vs. T2) were indistinguishable except in that they reflected different times after symptom onset
100 (see below). Results from samples from the final time-point (T3, at least 4 weeks after T1) were
101 similar to those from healthy controls in cases that were clinically resolved, but were highly
102 abnormal in patients who remained unwell and in hospital (data not shown). Since T3 samples were
103 highly diverse, they are not described further in the present report.

104 Modular analysis²³ of the 2010/11 samples revealed a marked overabundance of transcripts within
105 the interferon-inducible (M3.1) and neutrophil (M2.2) genes relative to healthy controls (Fig. 1b).
106 Transcripts representing plasma cells (M1.1), a subset of myeloid lineage genes (M2.6) and two
107 inflammation modules (M3.2 and M3.3) were also increased. There was a decrease in expression of
108 T- (M2.8) and B-cell (M1.3) modules (Fig. 1b). The calculated index termed 'molecular distance to
109 health' (MDTH, derived from analysis of 4526 transcripts significantly detected from background
110 filtered for low expression²⁴) was increased in most cases of influenza compared to healthy controls

($P < 0.0001$; Fig. 1c), although this varied according to the disease stage and severity (see below). In the 2010/11 cohort, a combination of expression-level and statistical filtering identified 1255 differentially-expressed transcripts compared to healthy controls. Supervised hierarchical clustering of these transcripts (expressed as a heat-map) revealed transcripts that were relatively over- or under-expressed in influenza patients, relative to healthy controls (Fig. 1d). When the 1255 transcript signature from the 2010/11 cohort was applied to the 2009/10 cohort (22 influenza patients and 25 matched healthy controls), the 2009/10 profiles appeared to be the same as the 2010/11 profiles (Supplementary Fig. 1a), indicating that viral variation between the two seasons²² did not appreciably affect transcriptomic patterns.

Ingenuity Pathway Analysis (IPA) identified the top five canonical pathways associated with up-regulated and down-regulated transcripts ($P < 0.05$, Fisher's Exact Test; Fig. 1d). Transcripts that were up-regulated in influenza patients were associated with 'interferon signalling genes' (including *IFITM1*, *IFI35*, *IFIT1*, *OAS1*, *IFIT3* and *IFI35*; Fig. 1e), 'activation of pattern recognition receptors by bacteria and/or viruses', 'activation of IRF by cytosolic pattern recognition receptors', 'hepatic fibrosis/hepatic stellate cell activation', and 'IL-6 signalling'. Transcripts that were down-regulated in influenza patients were those associated with 'ICOS-ICOSL signalling in T helper cells', 'primary immunodeficiency signalling', 'role of NFAT in regulation of the immune response', 'OX40 signalling pathway', and 'T cell receptor signalling' (Fig. 1d).

Heterogeneity in gene expression of the 25 most significant transcripts in hospitalised adults with influenza

The heat-map generated by hierarchical clustering of the top 25 most significant transcripts in the 2010/11 influenza patients (identified by mean fold-change over healthy controls) showed that the influenza cases clustered into two major groups, albeit with two further sub-clusters (Fig. 1f). The transcripts for the IFN-stimulated gene *IFI27* were over-expressed in almost all influenza patients, but most also showed decreased *FCER1A* transcription. Independent analysis of the same 25 transcripts applied to data from the 2009/10 comparison cohort showed similar clustering effects (Supplementary Fig. 1b). Patients with type I interferon-induced gene activation typically did not express neutrophil-associated and bacterial response-associated transcripts, and those samples with an overabundance of neutrophil-associated and bacterial response-associated transcripts (e.g. *DEFA4*, *ELANE*, *MMP-8*) did not simultaneously show consistent overabundance of antiviral response-associated transcripts (e.g. *RSAD2*, *IFI6*, *IFI44L*); these patterns were generally mutually exclusive (Fig. 1f and Supplementary Fig. 1b).

Modular transcriptional analysis and disease severity

To investigate whether severity of illness might explain the sub-clustering of the transcriptional responses seen in influenza patients and the heterogeneity of influenza transcriptional responses suggested by data presented in Figure 1f, 2010/11 cases were grouped according to their severity of illness at the first sampling time point (T1) using a three-point severity score based on treatment decisions in relation to the presence and severity of respiratory failure (severity 1: no supplemental oxygen requirement; 2: oxygen by mask; 3: mechanical ventilation). Relative to healthy controls, a similar increase in mean MDTH was seen in patients with severity 1 and 2 illness, but a greater increase was seen in severity 3 patients (4526 transcripts; Fig. 2a). Using modular analysis²³, we noted an over-abundance of plasma cell (M1.1), neutrophil activation (M2.2), and myeloid lineage (M2.6) transcripts in influenza-infected patients that was most marked in those with the greatest severity. Severity 3 cases also showed the greatest abundance of transcripts in the inflammation modules M3.2 and M3.3. By contrast, increased abundance of interferon-related transcripts (M3.1) was most clearly evident in cases with severity 1 or 2, but was less evident in patients with severity 3 disease (Fig. 2b).

Relationship between GO Term clusters and severity of illness

Semi-supervised hierarchical clustering of 231 differentially expressed transcripts was performed and results were expressed as a heat-map (with transcripts retained if there was greater than two-fold change between severity 3 and severity 1 and 2; Fig. 3a). This heat-map suggested that severity 1 and 2 patients had similar over- and under-abundance patterns, and there was marked over-abundance of transcripts associated with 'response to virus' identified by GO Terms analysis (Supplementary Table 1). By contrast, severity 3 patients had less marked abundance of 'response to virus' transcripts, but much more marked over-abundance of 'response to bacterium' transcripts that are often associated with (but not exclusive to) bacterial infection (Supplementary Table 2), as compared to patients with severity 1 or severity 2 illness. Additionally, patients with severity 3 illness demonstrated greater under-abundance of transcripts associated with 'cellular defence response', relative to patients with severity 1 or severity 2 illness.

The same 231 transcript list was tested by hierarchical clustering analysis of the 2009/10 comparison cohort (Supplementary Fig. 1c). Influenza patients within the cluster were characterised again by an over-abundance of transcripts associated with 'response to virus' by GO terms analysis, and these patients had either severity 1 or severity 2 illness. By contrast, the cluster of patients with over-abundance of transcripts associated with 'response to bacterium' by GO terms analysis, but much

less marked abundance of transcripts associated with 'response to virus', included all three patients with severity 3 illness (Supplementary Fig. 1c).

A molecular score was calculated for each individual for the 51 'response to virus' and the 112 'response to bacterium' transcripts identified by GO Terms analysis across the 2010/11 influenza patients of different severities at the first time of sampling (T1 only; n=109; Fig. 3b; Supplementary Tables 1 and 2 respectively). Influenza patients with high 'viral' molecular scores (> 500) were exclusively from the severity 1 and severity 2 groups. The majority of patients with high 'bacterial' scores (> 500) had severity 3 illness, and these patients had low 'viral' scores, in keeping with the modular analysis. A small minority of severity 1 and 2 patients had relatively low 'viral' molecular scores with moderately high 'bacterial' molecular scores. Six patients with known bacteraemia were included in the analysis but removal of cases with known bacterial co-infection did not influence the appearance of the 'bacterial' molecular signature (data not shown). A similar effect was observed in the influenza patients from 2009/10 (Supplementary Fig. 1d).

Reciprocal expression of activated and repressed biofunctions of identified genes was observed in severity 3 patients, compared to severity 1 and 2 patients combined (Fig. 3c, 3d). Nine genes associated with neutrophil activation were shown to be upregulated, including the genes *MPO*, *DEFA1*, and *ELANE*. Additionally, three genes associated with activation of leukocyte influx were upregulated in severity 3 patients: *MPO*, *MMP9* and *LCN2* (Fig. 3c). The associated repressed biofunctions in severity 3 patients were 'activation of cytotoxic T cells', 'adhesion of immune cells' and 'quantity of leukocytes' (Fig. 3d).

Effect of illness duration on molecular signatures

Patients with influenza symptoms of up to 4 days' duration at the time of sampling typically had elevated 'viral' molecular scores, but not if they required mechanical ventilation (Severity 3); in such cases, the 'viral' score was low, even early in the disease (Fig. 4a). 'Bacterial' molecular scores were low in patients with severity 1 and 2 illness regardless of the time of sampling, whereas patients with severity 3 illness showed higher 'bacterial' molecular scores than patients with less severe disease, irrespective of illness duration (Fig. 4b).

Focussing on influenza patients (2010/11 cohort) with repeat samples (T1 and T2; n=59) separated by 2-5 days, the 'viral' molecular score usually (but not always) decreased between T1 and T2 (Supplementary Fig. 2a). In those cases where T2 samples were obtained 48 hours after T1 (n=41, 2010/11 cohort), the reduction in 'viral' molecular score was statistically significant ($P = 0.0002$;

Supplementary Fig. 2b). Changes in 'bacterial' molecular scores between T1 and T2 were much more heterogeneous than changes in 'viral' scores, irrespective of the actual timing of T2 sample collection in relation to T1 (Supplementary Fig. 2c, 2d).

Effect of confirmed infection and treatment on transcriptomic signatures

We next sought correlations between influenza viral load (estimated from the samples of mucus obtained from the nasopharyngeal space by suction catheter at T1 and T2, Supplementary Fig 2e) and the transcriptional 'viral' and 'bacterial' scores at T1 and T2. No association was found between viral load and the 'viral' transcriptomic score (Supplementary Fig. 2f, and additional data not depicted).

To investigate whether bacterial infection is necessary for the observed activation of neutrophils and 'response to bacterium' by GO terms analysis, we analysed a subgroup of influenza-infected patients that had been thoroughly investigated for bacterial infection by analysis of five sample types: T1 nasopharyngeal aspirate (NPA) for PCR detection of bacterial pathogens; T1 NPA for culture; T1 throat swab for culture; blood for culture; urine for pneumococcal antigen testing. To account for incomplete bacteriological sampling in some patients, we excluded 36/109 (33%) patients for whom two or more sample-types were not available for analysis. Of the remaining 73 patients, 39 (53%) had potentially pathogenic bacteria detected in at least one sample type, and 34 (47%) patients provided at least four out of five sample-types and did not have significant bacteria detected by review of all the data available to the clinical panel. All patients thus categorised as 'bacteria not detected' had provided NPA and throat swab samples; blood cultures were not obtained from 10 patients and urine for pneumococcal antigen testing was not obtained from 11 patients.

Comparing those cases of influenza in which significant bacterial infection was confirmed (Bac+) with those in whom no bacterial infection was found despite extensive investigation (Bac-), the average 'viral' molecular score was lower in those with bacterial infection at all times up to day 12 after illness onset (Fig. 4c). The average 'bacterial' score was greater in those with bacterial co-infection between day 3 and 14 (Fig. 4d), but the time-trends in either case showed a similar pattern regardless of the presence or absence of significant bacteria. Similar trends were observed when stricter exclusion criteria were applied to the subgroup analysis, excluding patients from the 'bacteria not detected' group if they had not provided all five sample-types (data not shown); in this case, statistical analysis could not be performed due to the low sample size (only 13 patients provided all five sample types and did not have bacteria detected).

To investigate further whether bacterial infection drives the transcriptional response, serum (or plasma) levels of procalcitonin (PCT) were used as a possible discriminant of invasive bacterial infection^{25, 26, 27}. Serum procalcitonin levels showed no relationship to 'viral' molecular score (Fig. 4e), and there was no correlation between 'viral' molecular scores at T1 and T2 and PCT levels measured at respective time-points (data not shown). However, 'bacterial' molecular scores tended to be raised in those cases with the highest PCT levels (Pearson $r = 0.44$, $P < 0.001$) regardless of the presence or absence of significant detectable bacteria (Fig. 4f).

Therefore, the 'viral' molecular score was greatest early in disease, being lost after about 5 days. Even early in disease, cases needing mechanical ventilation had low 'viral' scores; this was especially true in those with bacterial co-infection. On the other hand, expression of 'bacterial' response genes is seen only in the most severe cases of influenza; bacterial infection enhances this signal, but the 'bacterial' score was increased in those with influenza regardless of measurable bacterial co-infection, especially if the disease had lasted over one week. Taken together, our data show that viral infection alone can induce the up-regulation of neutrophil-related genes, but induction of these genes is enhanced in severe disease or by detectable bacterial co-infection.

We further examined the possible influence of treatment of bacterial infection on the observed 'viral' and 'bacterial' responses, by stratifying T1 and T2 'bacterial' and 'viral' scores in 2010/11 influenza patients according to receipt of antibiotics. Almost all patients recruited to the MOSAIC study (92%; 234/255) were treated with antibiotics on clinical grounds at some time. Antibiotics prior to first sampling had no demonstrable effect on transcriptomic patterns (Supplementary Fig. 3a). Comparing patients who were not given antibiotics ($n=7$) with those given sustained antibiotic treatment following T1 ($n=24$) or throughout illness (including when T1 and T2; $n=27$), there was no statistically significant effect of antibiotic administration on the 'bacterial' molecular scores (Supplementary Fig. 3b). We next examined the levels of the 16S rRNA gene (bacterial load) in the throat swab and NPA samples from cases that were classified as 'viral and bacterial co-infection' or 'viral infection, no bacterial infection'. The levels of the 16S rRNA gene were no different between these groups on throat swabs, but the NPA bacterial load was greater in those cases with confirmed bacterial co-infection (Supplementary Fig. 3c).

Effect of illness duration, severity and bacterial co-infection on soluble mediators

We next sought to validate the 'bacterial' and 'viral' transcriptional signatures observed in blood with and protein-level data of mediators in the blood, NPA and anterior nasal fluid (SAM) at three time points (35 mediators, data available online).

Serum levels of the pro-inflammatory cytokine IL-1 β , which has limited anti-viral activity, showed no trend against severity (Fig. 5a), but was significantly increased in the NPA in those with the most severe influenza disease (Fig. 5b); nasal-absorption samples similarly showed an increase in IL-1 β only in those with greatest severity (Fig. 5c). Serum IL-6 levels were raised in those with influenza and especially in those with most severe disease (Fig. 5d); in the NPA, IL-6 was undetectable in most of the healthy controls but increased in most of the cases of influenza and especially in those with severe disease (Fig. 5e). The levels of IL-6 in nasal-absorption samples paralleled those in serum and NPA (Fig. 5f). CXCL8 serum levels tended to be higher in cases of flu than in controls, again increasing with disease severity (Fig. 5g). CXCL8 levels in NPA samples were highly variable but increased alongside influenza severity (Fig. 5h); however many NPA and nasabsorption CXCL8 measurements were so high as to be unquantifiable without dilution, even in a proportion of healthy controls (Fig. 5i). By contrast to these predominantly inflammatory/bacterially-driven mediators, IFN α 2a, which was measurable only in a proportion of individuals, was raised in serum in milder (severity 1 or 2) rather than severe (severity 3) disease (Fig. 5j). IFN α 2a levels in NPA and nasal-absorption samples were similarly higher in some milder influenza cases, relative to severe cases, though such differences were not significant. (Fig. 5k, 5l). The analysis of inflammatory mediators generally supports the association in the transcriptomics data between severe disease at T1 and increased inflammatory/'bacterial' markers, along with decreased 'viral' markers, in keeping with their known role in infection and disease pathogenesis⁷.

Serum levels of IL-17 were increased with severity at T1 (Supplementary Fig. 4a) and were elevated in the BAL of eight patients in whom samples were available, relative to healthy controls (Supplementary Fig. 4b). By inter-relating transcriptomic findings with mediator levels, we found a significant positive correlation between serum IL-17 (a marker of bacterial inflammation, acting on stromal cells to drive production of antimicrobial peptides and neutrophil chemoattractants), and the bacterial MDTH (Supplementary Fig. 4c). A similar trend was seen between TNF α and MDTH (Supplementary Fig. 4d). Since 'bacterial load' (as measured as NPA 16S rRNA copy number) was raised in cases of significant bacterial infection (Supplementary Fig. 3c), we regressed this parameter against viral or bacterial MDTH. High levels of viral MDTH only occurred in those with low bacterial load in the NPA (Supplementary Fig. 4e). By contrast, high bacterial MDTH were seen only in those with raised 16S bacterial load in the NPA (Supplementary Fig. 4f). These data supported a strong 'bacterial' transcriptomic signature to be associated with a neutrophilic/anti-bacterial inflammatory response and higher respiratory tract bacterial load. This signature was in turn associated with severe influenza, or later disease time points.

305 Considering the importance of time in the transcriptomics data, we next accounted for symptom
306 duration in this protein mediator data. CXCL10, IL-6 and CCL2 were elevated in serum from severe
307 cases of influenza (especially between days 5 and 10: Fig. 6a, 6b and not depicted). Proven bacterial
308 co-infection had no evident additional effect on CXCL10 (Fig. 6c), but serum IL-6 was more abundant
309 not only in severe cases (even early in disease, Fig 6b) but especially in cases of bacterial co-infection
310 (especially between days 5 and 10, Fig. 6d). In the NPA, most mediators (e.g. CXCL10, IL-6, CCL2 and
311 CXCL8) were markedly increased in severe disease, especially after day 4 (e.g. Figs. 6e and 6f, and
312 not depicted). NPA CXCL10 was again unaffected by confirmed bacterial disease (Fig. 6g) whereas
313 levels of IL-6 (and CCL2 and CXCL8, data not shown) were particularly increased in patients with
314 bacterial co-infection (Fig. 6h). In the anterior nasal fluid (SAM), mediator levels declined slowly with
315 time even in less severe disease; CXCL10 was depressed by bacterial co-infection but IL-6, CCL2 and
316 CXCL8 levels at this site were unaffected by severity or bacterial status (not depicted; data online).

Discussion

The MOSAIC study is exceptional in presenting data from a large number of exceptionally well-characterised hospitalised patients with influenza, studied prospectively. We found that whole-blood RNA expression profiles of patients hospitalised with influenza evolve over time and that the speed and scale of this evolution reflects severity. Patients with mild (or early) disease typically showed a pattern dominated by interferon-inducible genes and type 1 interferon, but this 'viral response' signature was replaced during severe (or late) disease by a pattern reflecting inflammation and neutrophil activation, more typically associated with the GO term 'response to bacteria'. The 'viral' response was rarely seen in patients beyond day 4 of symptoms; the inflammation/neutrophil activation signal dominated during the second week, but resolved during clinical recovery. In patients providing multiple samples during the first 3 days of illness, the 'viral' molecular score decreased rapidly between the first and subsequent sample, whereas the 'bacterial' score showed less consistent change.

The decline in viral response and the dominance of inflammation and neutrophil activation seen in severe disease was enhanced by proven bacterial co-infection, but did not depend on it. However, the bacterial load in the nasopharynx (quantified by 16S copy number) needed to be low for a 'viral' signature to be high, and high for an inflammatory/cell activation response to be evident. This finding suggests an interaction between viral and bacterial sensing and response mechanisms. In terms of soluble protein mediators, serum and nasopharyngeal levels were generally highest in cases of severe disease, even during the early stages. Levels of inflammatory mediators (e.g. IL-1 β and IL-6) were augmented in those with clinically significant bacterial co-infections. Interferon α levels tended to be low or undetectable in most compartments in those with very severe influenza, but interferon-related mediators (e.g. CXCL10 in serum) were generally most abundant in severe cases.

We were unable to show any qualitative difference in the response to different infecting viruses (e.g. influenza virus A vs. B). From previous studies, it seems that viral load does, in part, drive disease severity in patients infected with highly pathogenic strains of influenza^{6,7,8}, but we were unable to show a correlation between the viral load in the nasopharyngeal aspirate and the degree of abnormality of whole-blood RNA signatures. Measurement of viral load in influenza is technically difficult and depends on variations in sampling of nasopharyngeal mucus. For ethical and practical reasons we were unable to obtain routine samples from the lower respiratory tract; our findings should not be cited as evidence that viral load is irrelevant to disease severity but only that we were unable to demonstrate a relationship using the methods available to us.

The linked nature of MOSAIC cohort data allowed us to adopt an integrated approach to data exploration. Cases with early or mild influenza showed a transcriptional signature typical of viral infection (up-regulation of Type I interferon related genes *IFIT1*, *IFIT3*, *OAS1*, *IFITM1* and *IFI35*, and type II interferon-stimulated genes *IFITM1* and *IFI35*) in the JAK-STAT activation pathways. This was independently evident in samples collected in 2009/10, but in the larger 2010/11 cohort there was a distinct subgroup of cases expressing both IFN α -inducible protein 27 (*IFI27*) transcripts and antiviral response genes (e.g. *RSAD2*, *IFI6* and *IFI44L*). *IFI27* encodes an IFN-regulated mitochondrial protein that has antiviral effects *via* sensitization of cells to pro-apoptotic stimuli²⁸. Although *IFI27* expression has been proposed as a potential biomarker for influenza infection¹¹, overexpression of this transcript is not unique to influenza; it is also strongly upregulated in human airway epithelial cells and peripheral blood after infection with respiratory syncytial virus⁹. In our study, its translation was not associated with the stage, severity, or complications of influenza but persisted in the absence of up-regulation of other ISGs.

Following the initial interferon-dominated phase, patients with severe or prolonged symptoms activate a broad range of genes in addition to those classically associated with viral responses (reviewed elsewhere¹⁶). After the first four days of illness, these genes include those that encode inflammatory cytokines and chemokines, classical ‘antibacterial’ effector molecules (especially from neutrophils), and regulators of apoptosis and anaerobic metabolism²⁹. We find that this occurs irrespective of identifiable bacterial co-infection, mirroring studies in macaques in which administration of recombinant IFN α 2a initially up-regulates the expression of antiviral genes and prevents viral infection, but continued IFN α 2a treatment subsequently causes desensitization and a decrease in antiviral gene expression³⁰. In animal studies, it has been shown that IFN α / β is not only antiviral but can also promote inflammation and disease. This occurs *via* immunosuppressive effects that impede viral control³¹ or by triggering inflammation and tissue damage³². In mice, influenza infection also causes an early influx of neutrophils into the lung followed by a virus specific CD8⁺ T-cell response^{33, 34, 35}. Neutrophils might facilitate the development of this antigen-specific response as they may serve as antigen-presenting cells in influenza infection in mice^{35, 36} and guide influenza-specific CD8⁺ T cells into sites of infection by laying chemokine trails containing CXCL12³⁷. In animal models of viral lung disease, dysregulated host immune responses³⁸ and interferon production³² can lead to complex inflammatory responses which contribute to pathogenesis^{39, 40}. We recognise that transcriptomic data do not always reflect protein data, although genes down-stream of cytokine signalling may remain over-expressed⁴¹. This is expected, since it is well established that cytokines are under tight regulatory mechanisms and their expression at the level of mRNA and protein is short-lived⁴², limiting such correlations.

Given our observed 'bacterial' transcriptomic signature, we sought to define the incidence of clinically significant bacterial infections in our patients. To optimise the characterisation, respiratory tract sampling was supplemented with blood cultures for significant bacterial species and urinary pneumococcal antigen, where available. This sampling protocol and analysis went well beyond that normally used in clinical practice, possibly leading to detection of inconsequential bacterial carriage, rather than just those contributing to disease. Additionally, clinicians did not request blood cultures or urinary pneumococcal antigen tests for all patients, restricting the numbers of cases in which we could definitively determine presence or absence of significant bacterial infection. Clearly, we cannot exclude the possibility of bacterial co-infection in patients who were categorised as 'no significant bacteria detected'; we base our conclusions on the best evidence available to us and exhaustive case-by-case analysis, selecting only those cases which our expert panel felt could confidently be classified as 'bacterially infected' or 'uninfected'. The difficulties that we encountered in confirming or excluding the presence of bacterial infection in patients with respiratory tract infections have been highlighted by others^{43, 44}.

When measuring total bacterial loads in nasopharyngeal samples, we were surprised to find them of some predictive value: only in cases with low bacterial loads did we see high levels of 'viral' response gene activation, while low bacterial load was almost never associated with 'bacterial' response gene expression patterns. Indeed this 'bacterial' MDTH signature was also associated with serum IL-17 levels; along with higher IL-6 and IL-1 β levels (but lower IFN- α 2a) at T1, these data were indicative of an immune response biased toward anti-bacterial immunity early in severe influenza. We also examined the possible use of serum or plasma procalcitonin as an indicator of invasive bacterial disease. The strength of the transcriptomic 'bacterial' signature showed a significant positive correlation with procalcitonin levels. If procalcitonin were a true marker of bacterial invasive disease this would support the contention that the 'bacterial' transcriptional pattern indicates bacterial infection. However, procalcitonin release is suppressed by high levels of type II interferon (which can result from viral infection) and elevated levels of procalcitonin are seen in some non-bacterial inflammatory conditions^{26, 45}. In our study, elevated levels of procalcitonin were indicative of severe lung inflammation and did not help in deciding the presence or absence of significant bacterial infection.

To narrow the focus still further, we identified cases with pathogenic bacteria found in blood culture as a subgroup with definite and unequivocal bacterial sepsis. Three of these six cases had markedly elevated 'bacterial' molecular score without any increase in 'viral' molecular score; one patient had elevated 'bacterial' and 'viral' scores. All of these patients needed invasive mechanical ventilation.

The remaining two cases with bacteraemia did not have marked elevations in their ‘bacterial’ scores, despite detection of relevant bacteria in blood (*Streptococcus pneumoniae* and Group B *Streptococcus*); both had mild (Severity 1) disease. Interestingly, one of these patients had a high ‘viral’ score but the other did not. Early intervention with antibiotics was also considered as a potential explanation of transcriptomic changes, but in the wider cohort, prior administration of antibiotics had no effect on transcript abundance or the presence or absence of ‘viral’ or ‘bacterial’ signatures. From careful analysis of these cases, we conclude that transcriptomic scores are not invariably a reflection of the presence or absence of bacterial co-infection, as far as it has been possible for us to determine.

We next used stringent criteria to identify influenza cases that were extensively investigated for bacterial co-infection and yet found not to be infected, and cases in which pathogenic bacteria were identified with certainty. Progression of the transcriptomic signatures observed over time was similar in these two groups, but patients with confirmed bacterial infection had higher ‘bacterial’ molecular scores overall, compared to those in whom bacteria were not detected, reaching statistical significance at some day-of-illness time-points. We therefore conclude that influenza virus infection alone can drive what has been referred to in the literature and by GO terms as the ‘bacterial’ signature relating to neutrophil and inflammation-associated genes in patients with severe influenza, but that this response is enhanced by bacterial co-infection. The mechanism by which this occurs is open to speculation but might include alterations of innate sensitivity to resident microbiota in the gut, activation of Th17 pathways and leakage of endotoxins from the intestinal lumen⁴⁶.

Our study has important limitations. Despite its ambition, scope and intensity we had limited numbers of repeat samples in individual patients. Our description of trends over time since onset of illness depends in large part on summative data and on subjective reporting of disease onset. Ideally, the findings need validation in time-series studies simple and complicated acute viral disease with frequent sampling at multiple sites. For ethical and practical reasons, we were unable to plan more than 3 sampling points and not all samples were available from all patients. In addition, we were unable to study the early or preclinical phases but were limited to investigation of symptomatic cases presenting with disease of sufficient severity to reach hospital. For logistical and practical reasons we could not recruit mild cases seen in the community. The prodromal early stage of infection can only be studied with ease in infection challenge of volunteers (in whom severe disease is not expected), but our ongoing studies of experimental infections with pH1N1 in volunteers will allow us to resolve some of these limitations in the future.

We here present only selected results of an extended study of soluble immune mediator data from the MOSAIC cohort. Our main findings were of decreased IFN α 2a and increased IL-1 β , IL-6 and CXCL8 levels in the nasal and/or serum compartments in patients with severe disease. This apparent reciprocity may relate to the known cross-regulatory functions of IL-1 and type I IFNs in experimental models^{29, 47}. Our results generally fit with the proposal that responses seen in severe influenza are strongly influenced by bacterial co-infection, which contributes to driving high levels of mediators such IL-1 β , IL-6 and IL-17. However, there are many additional possible analyses to be performed. We chose only to illustrate those most relevant to the transcriptomic analysis and the question of bacterial superinfection, and we invite readers to explore additional correlations using our online data as a resource. We will welcome discussion with respect to additional analyses.

In summary, virus-induced type I interferon-related pathways are activated during the first four days of symptomatic influenza in hospitalised patients. These 'viral' pathways are then down-regulated, to be replaced by inflammatory, activated neutrophil and apoptosis-related pathways associated with IL-17 abundance, host-mediated tissue damage and 'response to bacteria', particularly in cases with a high 16S bacterial load in the nasopharyngeal secretions. In severe cases of influenza, this 'viral' response may be depressed early in disease and is accompanied by an increase in IL-1 β and IL-17. These findings emphasise that the stage and severity of disease need to be taken into account in interpreting host responses to infection and in the development of potential diagnostic tests to differentiate between treatable causes.

468 Online Methods

469 **Study population and inclusion criteria**

470 Patients ≥ 16 years of age were recruited during two successive winters (01 December 2009 to 03
471 March 2011). Patients with suspected influenza were identified by medical or nursing staff, or
472 notified to investigators by hospital diagnostic laboratories. Patients in London were recruited from
473 four Imperial College Healthcare NHS Trust hospitals, the Chelsea and Westminster Hospital, and the
474 intensive care unit at the Royal Brompton Hospital (a national referral centre for severe respiratory
475 failure). In Liverpool, patients were recruited from the Royal Liverpool, Liverpool Women's and
476 Arrowe Park Hospitals. Patients were included irrespective of prior or concurrent comorbidity (most
477 commonly asthma, pregnancy, immunocompromising conditions, or co-infection with other
478 respiratory pathogens), to reflect the populations known to be at greatest risk of severe influenza.
479 Adult healthy controls were recruited and matched to the patient cohorts for age, sex and ethnicity
480 and were screened to exclude known illnesses or current use of medications.

481 **Research Ethics Committees' Approvals**

482 The study was approved by the NHS National Research Ethics Service, Outer West London REC
483 (09/H0709/52, 09/MRE00/67). Patients or their legally authorised representatives provided
484 informed consent. Additional adult healthy controls were recruited as part of a separate study and
485 consented to their samples being used in additional studies (Central London 3 Research Ethics
486 Committee, 09/H0716/41).

487 **Biological sampling**

488 Research samples were obtained at three time points: T1 (recruitment); T2 (approximately 48h after
489 T1); T3 (at least 4 weeks after T1). Only T1 and T2 samples were included in this report. Whole blood
490 samples for transcriptomics were collected during the two recruitment periods, 2009/10 and
491 2010/11. Of 85 MOSAIC participants presenting with influenza-like illness in 2009/10, 23 (27%) were
492 adults with confirmed influenza, and T1 transcriptomic samples were available from 22 adults. Of
493 171 MOSAIC participants presenting with influenza-like illness in 2010/11, 111 (65%) were adults
494 with confirmed influenza, and T1 transcriptomics samples were available from 109/111 (98%). RNA
495 extraction and microarray were successful for all available patient samples from both cohorts.
496 Microarrays were also performed on samples from adult healthy controls of similar age, sex and
497 ethnicity to the study patients (Table 1). One healthy control sample for the 2009/10 cohort was not
498 included in final analysis because it failed quality control assessments.

Of the 109 adult patients recruited in 2010/11 and included in this analysis, 94 (86%) were infected with A(H1N1)pdm09 influenza virus, the remainder being infected with influenza A(H3N2) virus, non-subtyped influenza A virus, or influenza B virus. One of 22 adult patients recruited during 2009/10 was infected with A(H3N2) virus; remaining patients were infected with A(H1N1)pdm09 virus. Due to the natural evolution of influenza activity during the 2009-10 pandemic in the UK, the 2009/10 cohort was smaller than originally anticipated. Therefore, to assess the host response in the blood transcriptional signature as thoroughly as possible, we focussed our analysis on the larger 2010/11 cohort and then compared findings with the smaller 2009/10 cohort.

Influenza virus infection status

For each participant, influenza virus infection status was determined by reverse transcription polymerase chain-reaction (RT-PCR) testing of an appropriate respiratory tract sample by local clinical virology laboratories, as part of routine clinical care. Clinical laboratories followed nationally agreed and validated PCR protocols, and a panel of experts reviewed all results.

Clinical data collection and severity of illness scoring

Clinical data were extracted from hospital case notes and recorded in the Flu-CIN data collection tool⁴⁸ by trained researchers. Prescription charts were examined to determine whether antibiotics were being administered before, during or after sampling time points.

Severity of illness was graded at T1 and T2 according to the following criteria: (1) no significant respiratory compromise, with blood oxygen saturation >93% whilst breathing room air; (2) oxygen saturation ≤ 93% whilst breathing room air, justifying or requiring supplemental oxygen by face mask or nasal cannulae (with or without continuous positive airway pressure support or non-invasive mechanical ventilation); (3) respiratory compromise requiring invasive mechanical ventilation with or without ECMO. All clinical data underwent extensive validation and quality checking by independent data collection staff.

Detection of bacteria

Nasopharyngeal aspirates and swabs collected at T1 underwent microscopy and culture for bacteria. Additionally, multiplex PCR was performed to detect the following common respiratory bacteria in these samples: *Staphylococcus aureus*, *Chlamydia pneumoniae*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Pneumocystis pneumoniae*, *Legionella* species, *Klebsiella pneumoniae*, *Salmonella* species, *Moraxella catarrhalis*, *Mycoplasma pneumoniae*, and *Bordetella pertussis*. Throat swab samples taken at T1 also underwent culture and microscopy. When available, urine samples collected between T1 and T2 underwent pneumococcal antigen testing (BinaxNow, Allere,

Stockport, UK). Clinical microbiology data were obtained from hospital laboratory databases, including results of blood cultures (when taken 48 hours either side of T1) and urinary pneumococcal antigen results (for patients who did not have a researcher-requested urinary antigen sample). An independent microbiologist assessed the significance and validity of positive blood culture results, in an attempt to exclude cases of pseudobacteraemia caused by commensal contamination.

Soluble immune mediators

Serum, nasopharyngeal aspirate (NPA) and nasal-absorption fluid were collected at recruitment (T1) from participants with confirmed influenza and from adult healthy controls. Clotted blood was centrifuged at 1000 x g at 4°C and aliquots of serum supernatant were stored at -80°C. Each NPA was collected using a 10F Argyle suction catheter, inserted to reach the posterior nasopharyngeal wall; moderate suction was applied while the catheter was withdrawn over 5 seconds. The catheter was flushed through with 5 mL of sterile normal saline and the total contents were collected in a universal container. Aliquots of NPA were stored at -80°C. Nasal-absorption fluid was collected from the lateral wall of the nasal cavity using a synthetic absorptive matrix (SAM) strips (Leukosorb, Pall, UK) and stored at -80°C until analysis. On the day of analysis, 500 µl Milliplex assay buffer (Millipore, UK) was added to each thawed SAM strip before being placed in a Costar Spin-X centrifuge filter of pore size 0.22 µm held within an Eppendorf tube. Samples were centrifuged at 16,000 x g for 5 minutes at 4°C and eluates were kept on ice.

IL-1β, IL-6 and CXCL8 were quantified in each sample type using a 10-plex inflammatory soluble immune mediator electrochemiluminescence assay analysed on an MSD SECTOR instrument (Meso Scale Discovery, USA). For each mediator, a percentage coefficient variation cut-off of 10% was used to set the lower limit of detection. Sample results below the GM-LLOD were assigned half the value of the respective GM-LLOD.

Blood procalcitonin assay

Procalcitonin (PCT) in plasma or serum (collected at T1 and T2) was quantified using the Elecsys BRAHMS PCT assay on a calibrated Cobas e602 platform. Samples with a PCT value at the upper limit of detection (ULOD) were arbitrarily assigned the value of 100 ng/mL (the ULOD). Results may be interpreted as follows: <0.5 ng/mL, low probability of significant bacterial infection; 0.5-2.0 ng/mL, medium probability of significant bacterial infection; >2.0 ng/mL, high probability of significant bacterial infection.

16S rRNA gene bacterial load measurement

The 16S rRNA gene was targeted with 0.3 µl each of 10 µM universal primers 520F 5'-AYT GGG YDT AAA GNG and 802R 5'-TAC NVG GGT ATC TAA TCC added to 7.5 µl of SYBR Fast qPCR Kit Master Mix (KapaBio) and 5 µl of a 1:5 dilution of sample DNA extract and 1.9 µl of PCR Clean water (Mobio). Reactions were prepared in triplicate and thermal cycling carried out on a VIIA-7 Real-Time PCR System. Thermal-cycling conditions were 90°C for 3 mins, then 40 cycles of 95 °C for 20 s, 50 °C for 30 s, 72 °C for 30 s with default melt conditions. A standard curve of cloned (TOPO TA, Invitrogen) full length *Vibrio natriegens* DSMZ 749 16S rRNA gene was included in order to be able to calculate an absolute abundance from C_T values together with no template controls. The resulting 16S rRNA gene copy number (bacterial load) was log transformed prior to using analytically.

Microarray Gene Expression Profiling

At each time point, 3 ml of whole blood were collected into each of two Tempus tubes (Applied Biosystems/Ambion) by trained research staff following a standard phlebotomy protocol. Blood was vigorously mixed immediately following collection and stored at -80°C before RNA extraction. For each patient, the contents of one tube were used for analysis and the other tube was retained in case of assay failure. RNA was isolated using 1.5 ml whole blood and the MagMAX-96 Blood RNA Isolation Kit (Applied Biosystems/Ambion), as per 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). RNA yield was assessed using a NanoDrop8000 spectrophotometer (NanoDrop Products, Thermo Fisher Scientific). High-quality (>6.5 RIN) whole blood RNA was successfully obtained and processed by microarray in all cases. Biotinylated, amplified antisense complementary RNA (cRNA) targets were prepared from 200-250 ng of globin-reduced RNA using the Illumina CustomPrep RNA amplification kit (Applied Biosystems/Ambion). For each sample, seven hundred and fifty nanograms of labelled cRNA were hybridised overnight to Illumina Human HT12 V4 BeadChip arrays (Illumina), which contained greater than 47,000 probes. The arrays were washed, blocked, stained and scanned on an Illumina iScan, as per the manufacturer's instructions. GenomeStudio (Illumina) was used to perform quality control and generate signal intensity values.

Microarray Data Processing

Raw microarray data were processed using GeneSpring GX version 12.5 (Agilent Technologies). Following background subtraction, each probe was attributed a flag to denote its signal intensity detection *P* value. Filtering on flags removed probe sets that did not result in a 'present' call in at

least 10% of the samples, where the 'present' lower cut-off = 0.99. Signal values were then set to a threshold level of 10, $\log^2$ transformed, and per-chip normalised using a 75th percentile-shift algorithm. Each gene was normalised by dividing each mRNA transcript by the median intensity of all samples. Statistical analysis was performed after these steps had been performed.

Microarray Data Analysis

Transcripts significantly detected from background hybridisation were filtered for low expression in GeneSpring GX 12.5, whereby the only transcripts retained were those with at least two-fold change from the median normalised intensity value in at least 10% of all samples. Principal component analysis of all transcripts significantly above background in at least 10% of samples (18974 transcripts) was performed using R 3.3.2 (R Development Core Team). To derive the 1255 transcript list, non-parametric statistical filters (Mann-Whitney unpaired test with Bonferroni family-wise error rate multiple testing correction, $p < 0.01$) were applied, followed by fold-change filtering between groups (transcripts were retained if greater than two-fold change between any two groups). For severity analysis, 231 normalised intensity value transcripts were obtained by filtering for low expression and then applying statistic filters (Kruskal-Wallis test with Bonferroni FWER, $P < 0.01$), followed by fold change filtering between groups (transcripts were retained if greater than two-fold change between those from severity 3 patients and severity 1 and 2 patients). All heat-maps were generated in GeneSpring GX 12.5 (semi-supervised analysis, clustered by Pearson's un-centred method with average linkage rule).

Comparison Ingenuity Pathway Analysis (IPA) (Ingenuity Systems Inc., Redwood, CA) was used to determine the most significant canonical pathways for up-regulated and down-regulated transcripts ($P < 0.05$ Fishers Exact test). Additionally, IPA was used to generate graphical representations of selected canonical pathways, and network diagrams. For the 231 transcript list, significantly activated (z score > 2) and significantly repressed (z score < 2) biofunctions were identified in IPA and expressed in gene network diagrams. GO Term (Gene Ontology Consortium) analysis integrated with GeneSpring GX12.5 was used to identify biological processes, according to GO annotations⁴⁹.

The molecular distance to health (MDTH) and molecular scored were calculated using methods described previously²⁴ and applied to different signatures. Transcriptional modular analysis was applied as described previously²³. Briefly, raw expression levels of all transcripts significantly detected from background were compared between each sample and all the controls present in a given data set. The percentage of significantly expressed genes in each module was represented by the colour intensity, with red indicating over-expression and blue indicating under-expression. Statistical testing was performed using Student's t-test ($P < 0.05$). The mean percentage of

627 significant genes and the mean fold change of these genes compared to the controls in specific
628 modules were shown in graphical form (unpaired t-test, $P < 0.00001$). MDTM and modular analysis
629 were calculated in Microsoft Excel 2010 (Microsoft Corp.). GraphPad Prism V5 for Windows
630 (GraphPad Software Inc., La Jolla, CA, USA) and R 3.3.2 (R Development Core Team) were used to
631 generate graphs and perform additional statistical analyses.

Figure 1. Transcriptional signature of influenza compared to healthy controls. (a) Principal component analysis of all transcripts significantly above background in at least 10% of samples (130 healthy controls (green squares), 97 influenza A (red circles: H1N1; green triangles, H3N2), and 12 influenza B (purple squares); all from 2010/11). (b) Modular analysis of influenza patients relative to healthy controls. The expression of the modules is shown on the left according to the colour intensity display; the corresponding modules are identified in the key to the right. (c) Weighted 'molecular distance to health' (MDTH²⁴) of Influenza patients compared to healthy controls, undertaken on 4526 transcripts that were significantly detected above background, filtered for low expression (transcripts retained if >2 fold-change (FC) from median normalised intensity value in more than 10% of all samples). Box whisker plot with min/max lines; statistical test: Mann-Whitney $P < 0.0001$. (d) Heat-map of 1255 normalised intensity value transcripts, filtered for low expression then statistically filtered (Mann-Whitney with Bonferroni multiple testing correction $P < 0.01$) followed by fold change filter between groups (transcripts retained if >2FC between any 2 groups). Listed next to the heat-map are the top five IPA canonical pathways (by significance $P < 0.05$, Fisher's Exact test) for upregulated and downregulated transcripts. (e) IPA canonical pathway for Interferon Signalling. Red shading represents up-regulated genes, blue represents down-regulated genes. (f) Heat-map of normalised intensity values of the top 25 significant transcripts by mean fold-change between healthy controls and influenza groups clustered on entities and by individuals (Pearson's uncentered (cosine) with averaged linkage).

Figure 2. Severity of disease is associated with diminished expression of interferon-related modules and over-expression of inflammation modules. (a) Weighted molecular distance to health (MDTH) of Influenza patients grouped by severity of illness score (1: normoxic; 2: hypoxia requiring correction by mask oxygen; 3: mechanical ventilation), compared to healthy controls (HC), based on 4526 transcripts that were significantly expressed above background and filtered for low expression (transcripts retained if >2FC from median normalised intensity value in more than 10% of all samples). Box whisker plots are shown with min/max lines. (b) Modular analysis of influenza patients grouped by severity, relative to healthy controls. The colour intensity correlates with the percentage of genes in that module that are significantly differentially expressed.

Figure 3. Severe disease is associated with lower expression of "viral" response genes, compared to early and less severe influenza. (a) Heat-map of 231 normalised intensity value transcripts, obtained by filtering for low expression followed by statistical filtering (Kruskal-Wallis with Bonferroni multiple testing correction $P < 0.01$) followed by fold change filter between groups (restricted to initial T1 samples, transcripts retained if >2FC between severity 3 and severity 1&2).

Listed next to the heat-map are the top GO terms for the 3 major subdivisions of the dendrogram (clustered by Pearson's uncentered (cosine) with average linkage rule). **(b)** Weighted molecular score (relative to healthy controls) of the 112 'bacterial response' transcripts plotted against molecular score of the 51 'viral response' transcripts for the 109 influenza individuals at the T1 time point. Severity of illness is indicated by different colours of dots: severity 1, black dots; severity 2, blue dots; severity 3, red dots. Circled dots identify patients with confirmed bacteraemia. **(c)** IPA significantly activated (z score >2) or **(d)** repressed (z score <2) biofunctions, identified by analysis of 231 transcript list; selected networks of biofunctional genes are shown.

Figure 4. Relationship between severity of illness, bacterial infection, procalcitonin and molecular scores. 'Viral' and 'Bacterial' response scores (according to GO terms, as described in Fig. 3) calculated for individual cases of confirmed influenza according to clinical categories at both the first and second sampling time-points (T1 and T2). Loess fitting was used to interpolate and estimate mean values non-parametrically from the data (solid lines); dashed lines show the estimated 95% confidence interval values of the mean; statistical significance of differences were calculated using Chi-squared tests to compare the deviance of generalized linear models. **(a)** Separating cases into grades of clinical severity, cases without need for mechanical ventilatory support (i.e. severity 1 and 2), showing high 'viral' molecular score at the early stage of the disease. **(b)** Those requiring mechanical ventilatory support (severity 3) had higher 'bacterial' molecular scores regardless of time since onset. **(c)** When classified according to the presence of absence of clinically significant bacterial co-infections, those with proven bacterial co-infection (39 subjects, 63 samples) had lower 'viral' molecular scores than those without identifiable co-infection (34 subjects, 52 samples) regardless of time since disease onset. **(d)** Those with proven bacterial co-infection (39 subjects, 63 samples) had higher 'bacterial' molecular scores than those without identifiable co-infection (34 subjects, 52 samples) regardless of time since disease onset. **(e)** The 'viral' molecular score was unrelated to serum procalcitonin; the 'bacterial' molecular score **(f)** tended to be high in those with raised procalcitonin, but was unaffected by proven bacterial co-infection.

Figure 5. Levels of selected mediators in different compartments according to severity of illness and clinical designation of probable bacterial co-infection status. Serum, nasopharyngeal aspirate (NPA) and nasabsorption eluates from participants with confirmed influenza were obtained at recruitment (T1) and compared with samples from adult healthy controls. Individual values (pg/mL, log scale) are shown with median and interquartile ranges. Zero values and values below the lower limit of detection were assigned half the geometric mean lower limit of detection for display purposes. The upper limit of detection for all assays shown was 2500 pg/mL. Kruskal-Wallis test with

Dunn's post test was used to assess significance (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; NS = not significant). Severity of illness at T1 is shown. HC = healthy controls. Serum samples for HCs and participants with severity 1, 2, and 3 illness (**a, d, g, and j**): $n = 36, 58, 43$, and 31 , respectively. NPA samples for healthy controls and participants with severity 1, 2, and 3 illness (**b, e, h and k**): $n = 35, 50, 32$, and 27 , respectively. Nasabsorbion eluate samples for healthy controls and participants with severity 1, 2, and 3 illness (**c, f, i and l**): $n = 36, 60, 43$, and 30 , respectively.

Figure 6: Relationships between severity of illness, bacterial infection, and selected mediators.

Levels of CXCL10 and IL-6 in serum (**a-d**) and NPA (**e-h**) according to day of illness at both the first and second sampling time-points (T1 and T2). Loess fitting was used to demonstrate time trends of mean values interpolated non-parametrically from the data (solid lines); dashed lines show the estimated 95% confidence interval values of the mean. Statistical significance of differences was calculated using Chi-squared tests to compare the deviance of generalized linear models. Patients requiring mechanical ventilation (severity 3 illness) had significantly higher mean levels of CXCL10 and IL-6 in both serum (**a, b**) and NPA (**e, f**) than patients with less severe diseases (severity 1 and 2). Classifying cases according to the presence or absence of clinically significant bacterial co-infections, those with proven bacterial infection (39 subjects, 63 samples) had higher mean IL-6 levels in both serum (**d**) and NPA (**h**) compared to patients who did not (34 subjects, 52 samples), regardless of time since disease onset. Bacterial infection had no discernible effect on serum (**c**) or NPA (**g**) levels of CXCL10.

Supplementary Figure 1. Validation of transcriptional signatures in an independent cohort. (a)

2009/2010 cohort clustered on individuals and transcripts (Pearson's uncentered with averaged linkage) using 1255 transcript list (from Figure 1). **(b)** 2009/2010 cohort clustered on individuals and transcripts (Pearson's uncentered with averaged linkage) using 25 transcript list (from Figure 1). **(c)** 2009/2010 cohort clustered on individuals and transcripts (Pearson's uncentered with averaged linkage) using 231 transcript list of severity (from Figure 2, transcripts retained if $>2FC$ between severity 3 and severity 1&2). GO Terms analysis of 3 major branches of the transcripts dendrogram was undertaken and listed next to the heat-map. **(d)** Using 51 and 112 transcripts lists (from Figure 3) 'viral response' and 'bacterial response' molecular scores were calculated and plotted for each influenza patient (relative to healthy controls). Cases were coded according to severity of illness, indicated by the colour of the respective dots (severity 1, black; severity 2, blue; severity 3, red).

Supplementary Figure 2. Change of 'viral' and 'bacterial' molecular scores over time and association with viral load. (a) 'Viral' molecular scores plotted for 59 influenza patients (2010/11

cohort) who provided T1 and T2 samples, plotted against respective day of illness at time of sampling. **(b)** Change in 'viral' molecular score between first (T1) and precise second time point (48 hours after T1) in 41 patients with appropriate samples available ($P = 0.0002$, Mann-Whitney test). **(c)** 'Bacterial' molecular score plotted for 59 influenza patients who had both a T1 and a T2 sample, shown plotted against respective day of illness. **(d)** Change in 'bacterial' molecular score between T1 and precise T2 (48h post T1), in 41 patients with appropriate samples available (*NS*, Mann-Whitney test). **(e)** Influenza viral load estimation (Pfu/ml) in nasopharyngeal samples obtained at T1 and T2. **(f)** Relationship between influenza viral load (Pfu/ml) at T1 or T2 and the simultaneous 'viral' molecular score on whole blood.

Supplementary Figure 3. Administration of antibiotics does not affect 'bacterial' or 'viral' molecular scores. **(a)** 109 Influenza patients (2010/11 cohort) presenting within the first 14 days of illness grouped by receipt of any antibiotic in the 24 hours prior to T1 sample. There was no difference (*NS*, Mann-Whitney test) in either bacterial or viral molecular scores between the two groups. **(b)** Prescription of antibiotics after T1 did not significantly influence 'bacterial' molecular score. Fifty-nine influenza patients who had both T1 and T2 samples were grouped by those who did not receive antibiotics ($n=7$), those whose antibiotics were stopped at T1 ($n=1$), those who had antibiotics prescribed after T1 but before T2 ($n=24$), and those who were receiving antibiotics at both T1 and T2 ($N=27$). **(c)** Total 16S copies at T1 in throat swabs and NP aspirate in patients adjudicated to be with or without bacterial co-infection. Those with confirmed bacterial infections had greater levels of total 16S copies in NP aspirate than those deemed to be without co-infection ($P = 0.036$).

Supplementary figure 4. Correlation of serum cytokines and bacterial load in nasopharynx with 'viral' and 'bacterial' molecular distance to health. Levels of IL-17 in the serum of healthy controls (HC) and influenza infected patients (severity 1-3) **(4a)**. Concentration of IL-17 in bronchoalveolar lavage (BAL) of HC and from BAL, NPA, SAM and Serum of patients **(4b)**. Serum levels of IL-17 **(4c)** and TNF α **(4d)** correlate with the bacterial MDTH (IL-17, Spearman $R = 0.39$, P value < 0.001 ; TNF α $R = 0.4$, $P < 0.01$). The total 16S rRNA gene copies in NP aspirate samples were inversely correlated with the viral MDTH (Spearman $R = -0.28$, P value < 0.05) **(4e)** but positively correlated with bacterial MDTH (Spearman $R = 0.37$, P value < 0.05) **(4f)**.

Author Contributions

Conceived and designed the study: PJMO, AOG, CMG, DC, JB, SB, and JD. Performed the microarray experiments: CMG. Developed clinical protocols, recruited subjects and collated clinical data: JD. Analysed the microarray data: JD, CIB, LH and SB with supervision by AOG and PJMO. Wrote and revised the manuscript: JD, SB, LH, AOG, PJMO.

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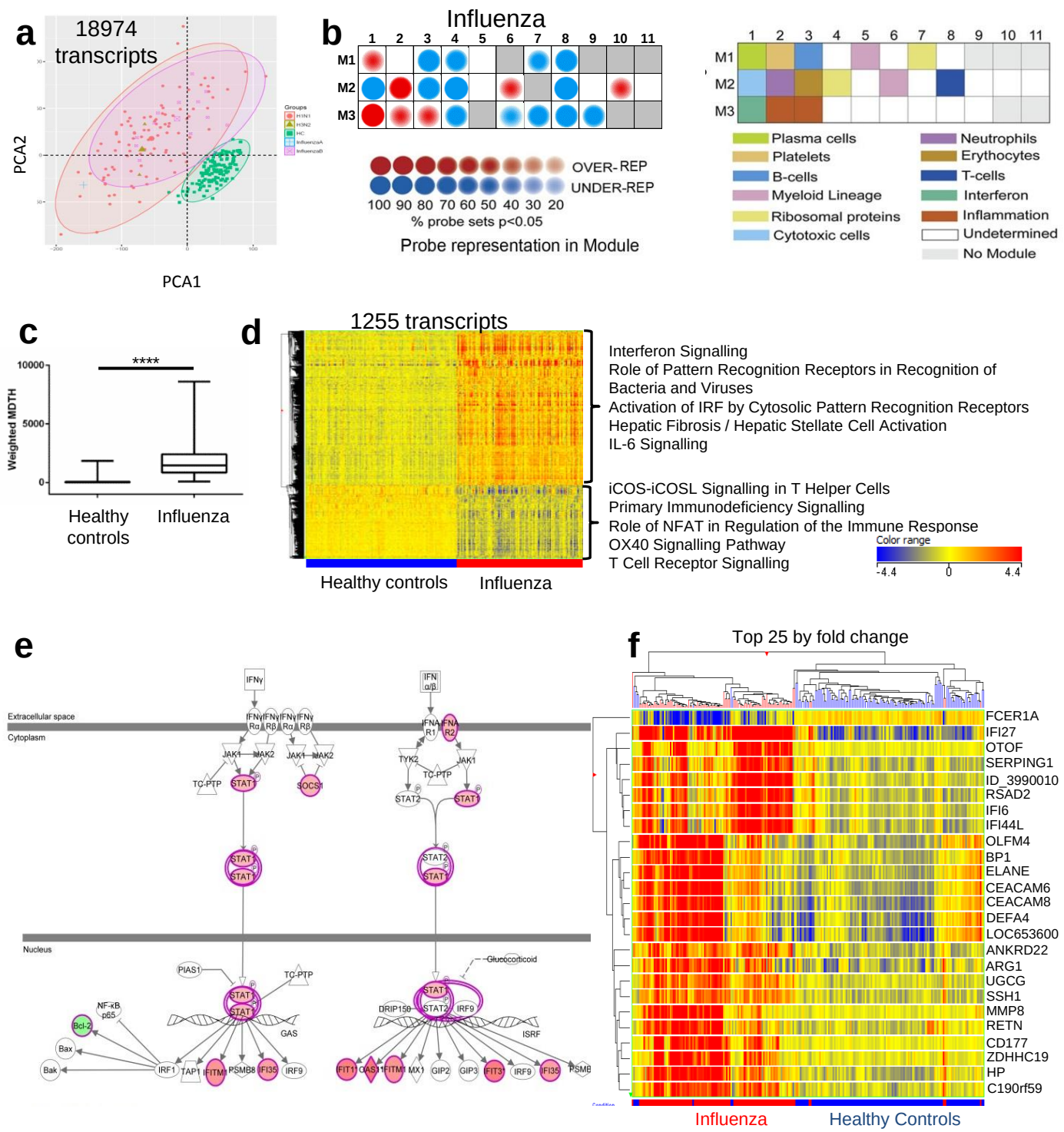
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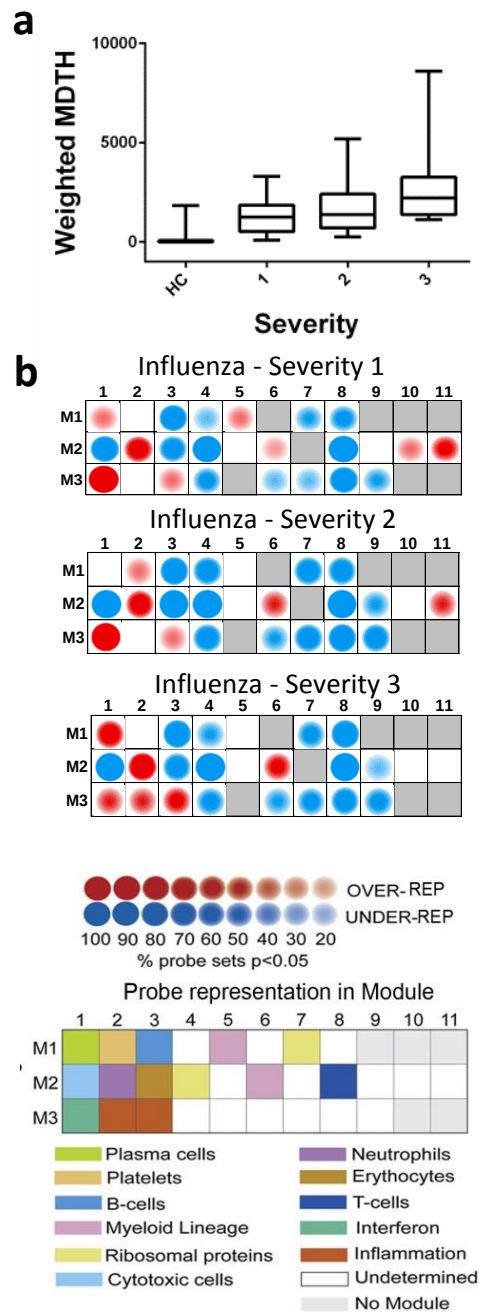
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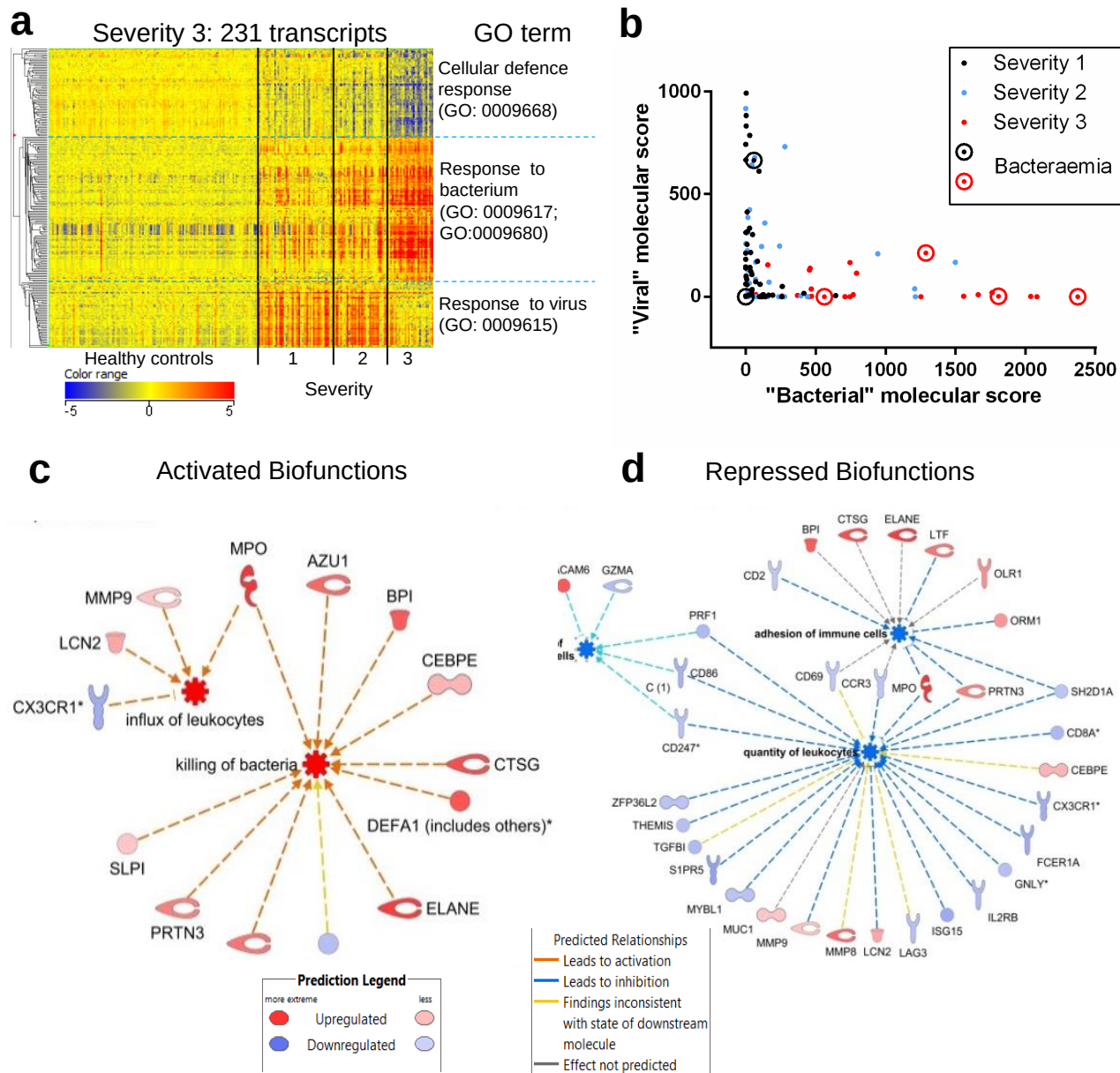
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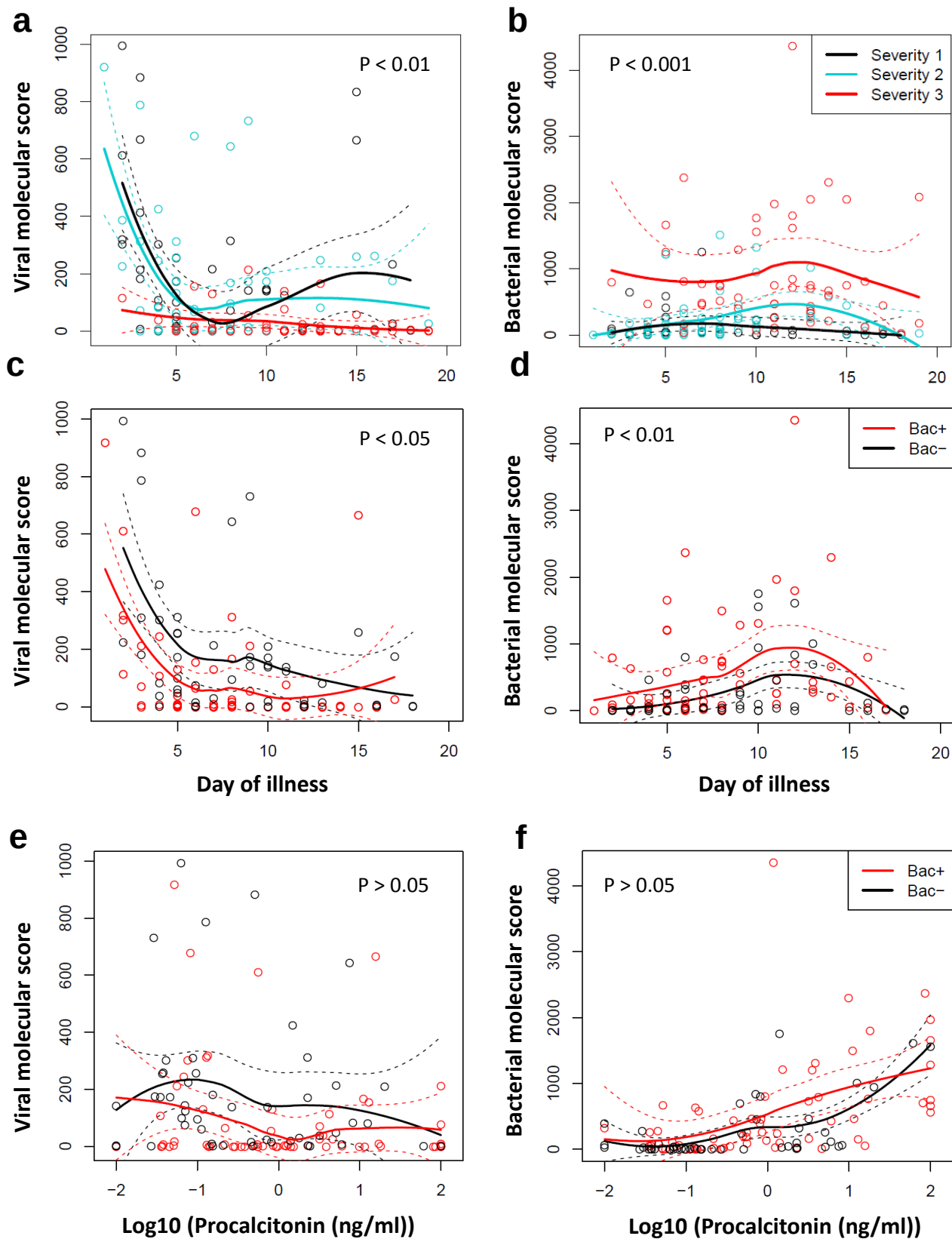
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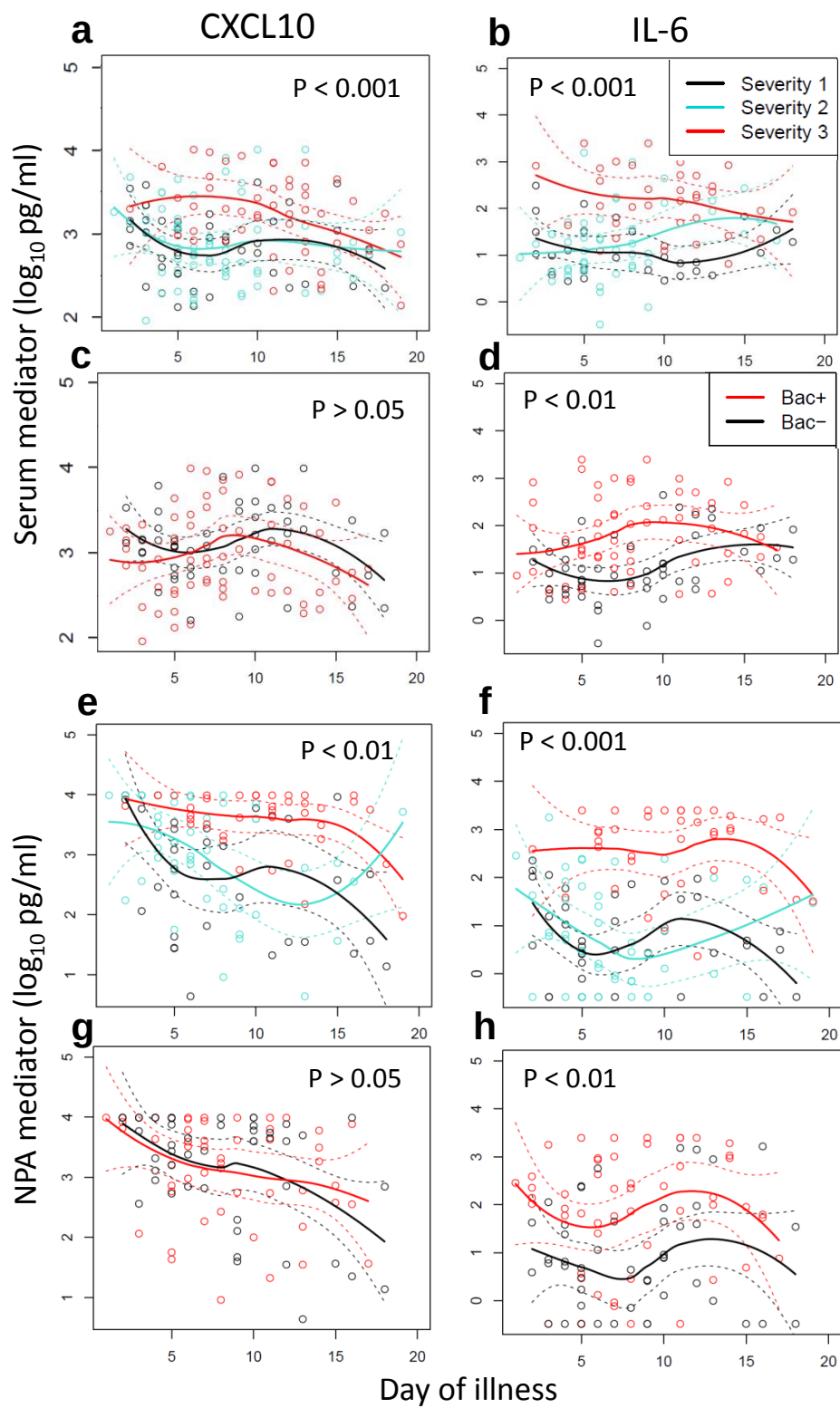


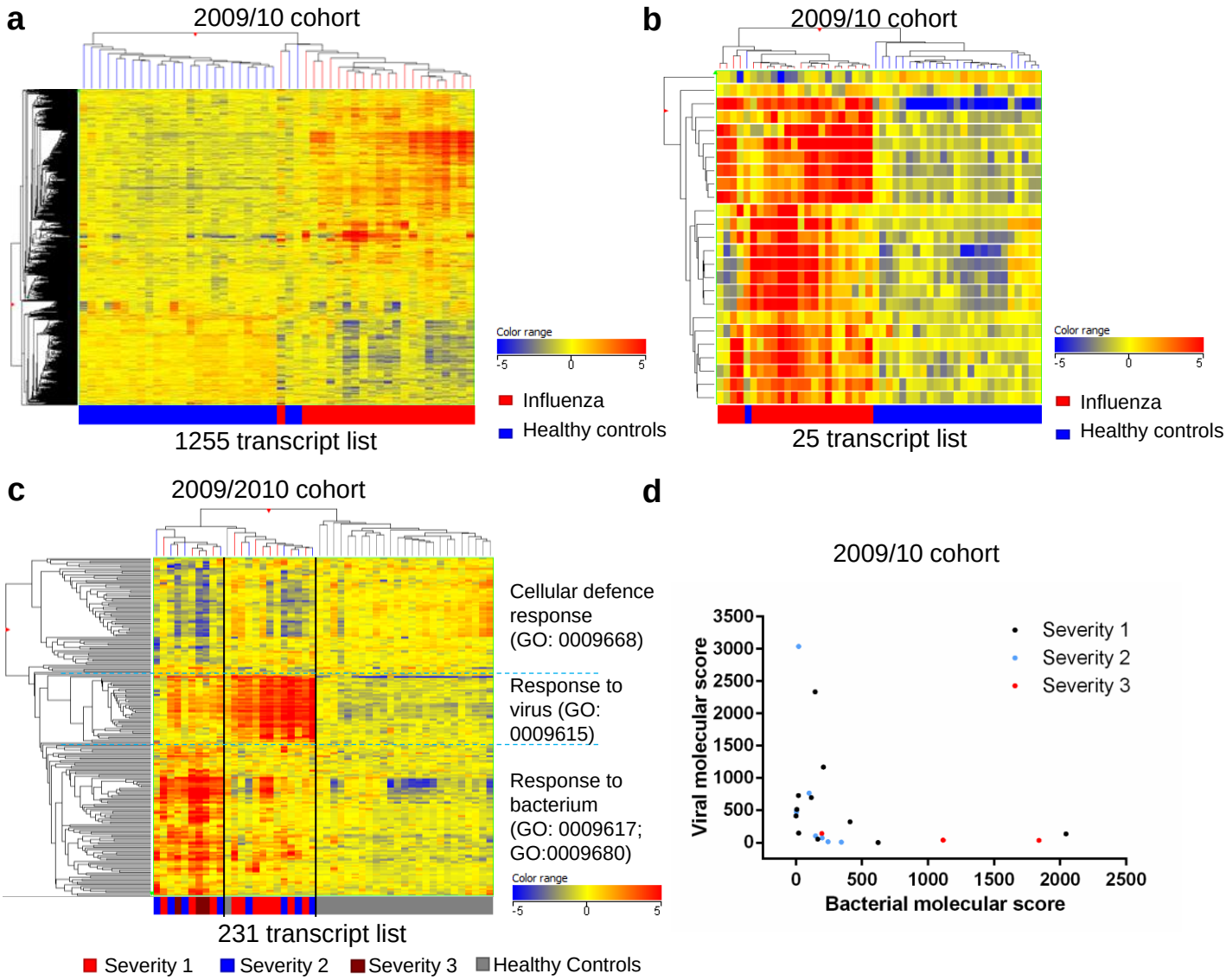
Dunning *et al* Figure 1

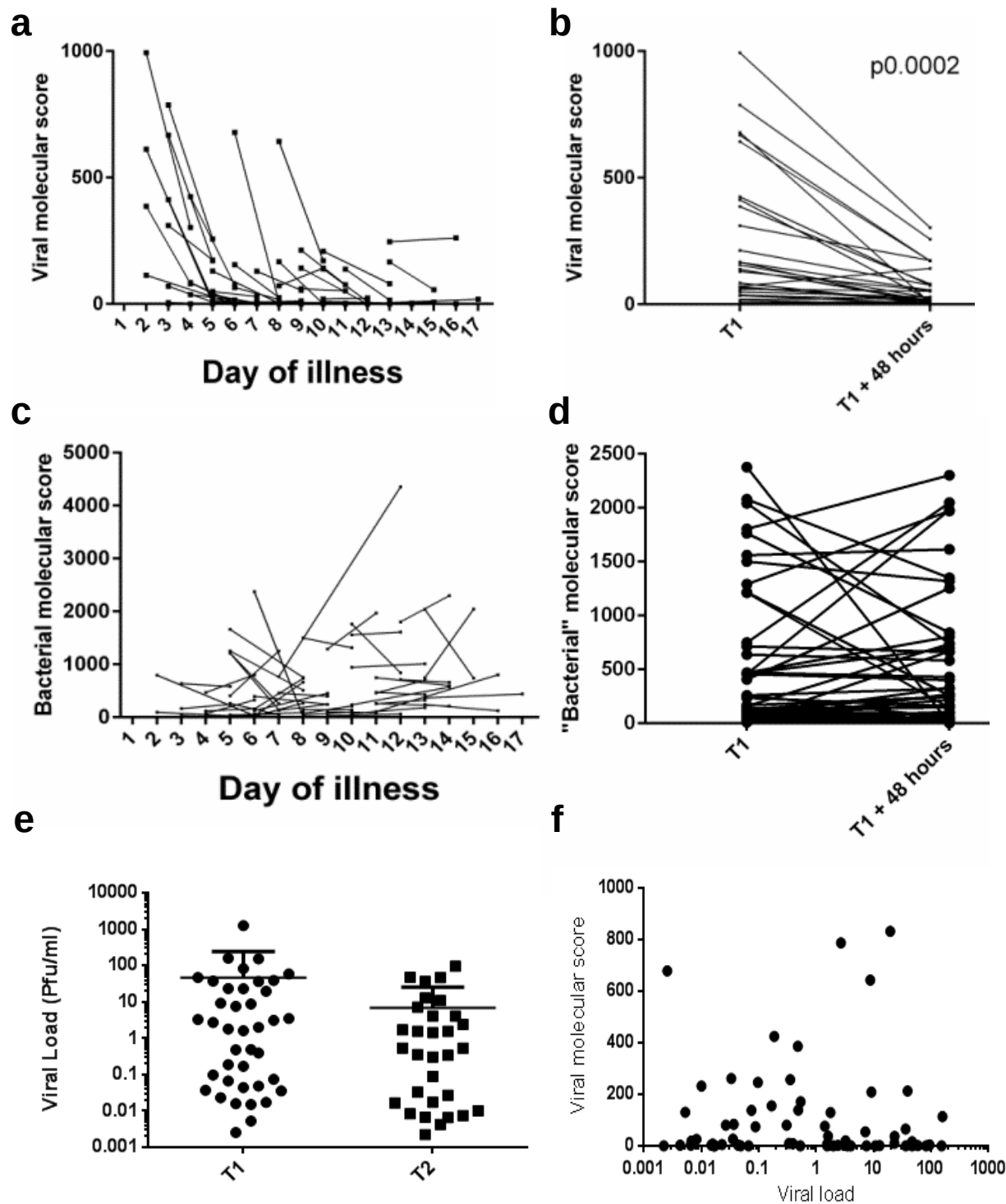


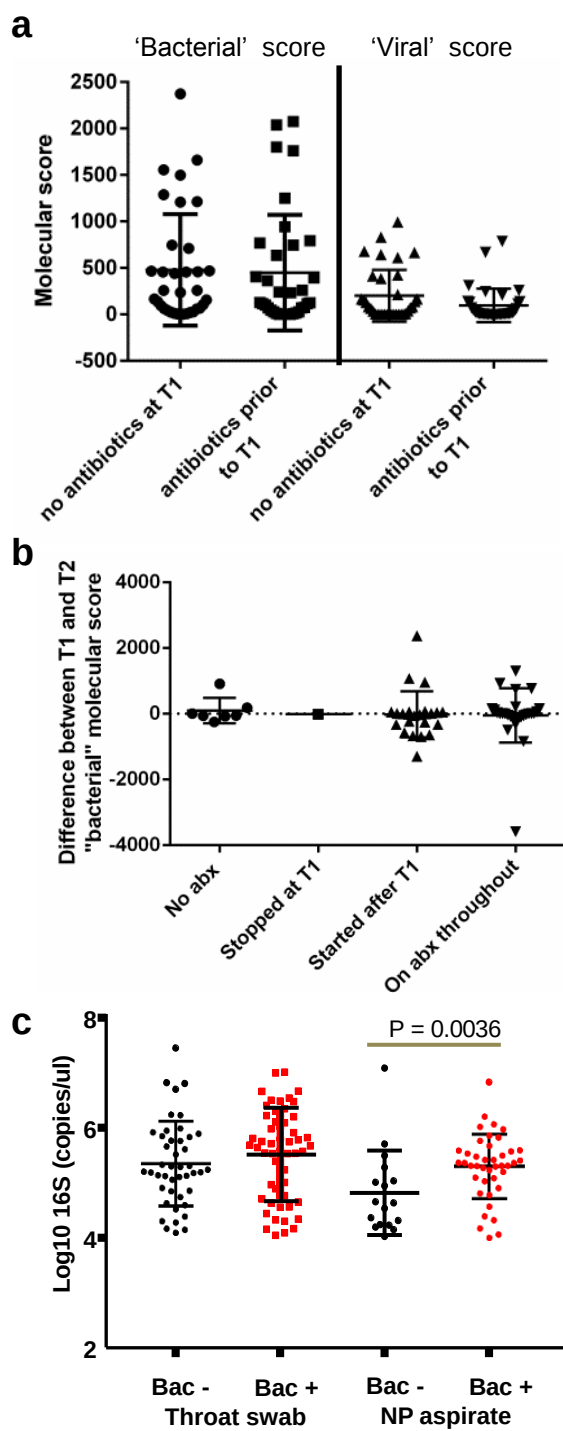












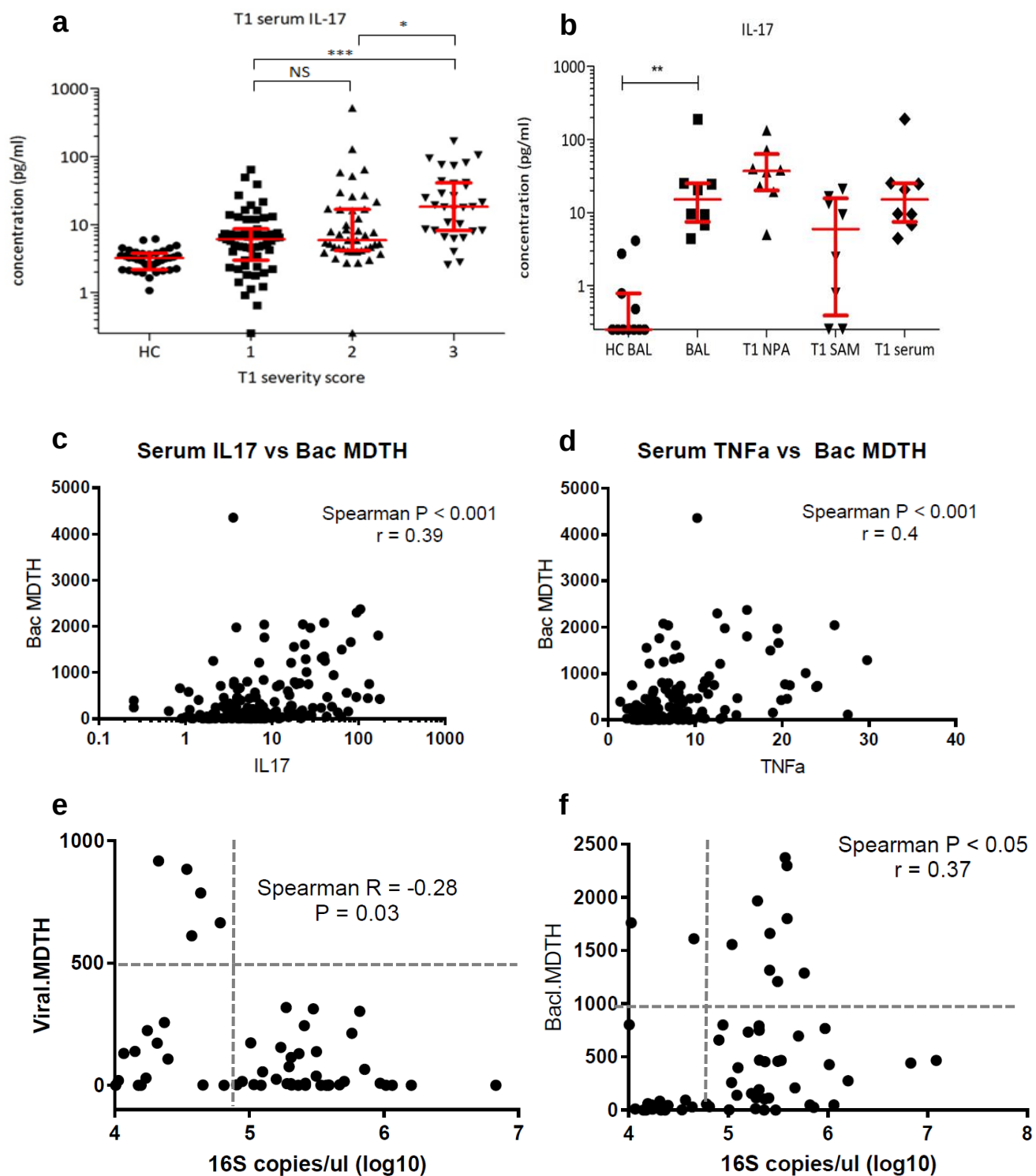


Table 1. Characteristics of recruited patients and healthy controls

	2010/11 Cohort (n=109)	2010/11 Healthy Controls (n=130)	2009/10 Cohort (n=22)	2009/10 Healthy Controls (n=25)
Mean age in years (range)	41 (17-71)	35 (20-68)	44 (23-74)	37 (21-54)
Female (%)	53 (48.6)	75 (57.7)	10 (45.5)	14 (56)
Ethnicity (%)				
White	78 (71.6)	90 (69.2)	10 (45.5)	14 (56)
Black	17 (15.6)	23 (17.7)	5 (22.7)	5 (20)
Asian	9 (8.3)	15 (11.5)	0	6 (24)
Other	5 (4.6)	2 (1.5)	7 (31.8)	0
Comorbidities				
None	28 (25.7)	130 (100)	4 (18.2)	25 (100)
1	31 (28.4)	0	12 (54.5)	0
2	28 (25.7)	0	3 (13.6)	0
≥ 3	22 (20.2)	0	3 (13.6)	0
Women age 15-49y who were pregnant	10/43 (23.3)	1/75 (1.3)	2/8 (25)	0
Influenza type				
pH1N1	94 (86.2)	NA	21 (95.5)	NA
A (H3N2)	2 (1.8)	NA	1 (4.5)	NA
A (unknown)	1 (0.9)	NA	0	NA
B	12 (11)	NA	0	NA
Severity of illness at T1 (%)				
Severity 1	47 (43.1)	NA	11 (50)	NA
Severity 2	34 (31.2)	NA	8 (36.4)	NA
Severity 3	28 (25.7)	NA	3 (13.6)	NA
Peak severity for illness episode (%)				
Severity 1	35 (32.1)	NA	6 (27.3)	NA
Severity 2	44 (40.4)	NA	12 (54.5)	NA
Severity 3	30 (27.5)	NA	4 (18.2)	NA

Note that percentages may not add up to 100 for all variables due to rounding. NA: not applicable.