Progression of whole-blood transcriptional signatures from interferon-induced to neutrophil-associated patterns in severe influenza

Jake Dunning1,8, Simon Blankley2, Long T. Hoang1, Mike Cox3, Christine M. Graham2, Philip L. James3, Chloe I. Bloom2, Damien Chaussabel4, Jacques Banchereau5, Stephen J. Brett6, MOSAIC Investigators7, Miriam F. Moffatt3, Anne O’Garra2* and Peter J. M. Openshaw1*

Transcriptional profiles and host-response biomarkers are used increasingly to investigate the severity, subtype and pathogenesis of disease. We now describe whole-blood mRNA signatures and concentrations of local and systemic immunological mediators in 131 adults hospitalized with influenza, from whom extensive clinical and investigational data were obtained by MOSAIC investigators. Signatures reflective of interferon-related antiviral pathways were common up to day 4 of symptoms in patients who did not require mechanical ventilator support; in those who needed mechanical ventilation, an inflammatory, activated-neutrophil and cell-stress or death (‘bacterial’) pattern was seen, even early in disease. Identifiable bacterial co-infection was not necessary for this ‘bacterial’ signature but was able to enhance its development while attenuating the early ‘viral’ signature. Our findings emphasize the importance of timing and severity in the interpretation of host responses to acute viral infection and identify specific patterns of immune-system activation that might enable the development of novel diagnostic and therapeutic tools for severe influenza.
superimposed on these patterns that were, however, related mainly to the duration and severity of influenza.

**Results**

**Clinical cohorts.** Adult patients with laboratory-confirmed influenza were recruited in 2009–2010 \( (n = 22\) patients) and 2010–2011 \( (n = 22\) 109). The majority were infected with pH1N1 influenza virus (95.5% in 2009–2010, and 86.2% in 2010–2011). In each cohort, the majority of patients had at least one comorbidity (81.8% in 2009–2010, and 74.3% in 2010–2011). 13.6% participants in 2009–2010 and 25.7% patients in 2010–2011 had illness categorized as severity 3 (described below) at the first sampling time point (T1) (Table 1).

**Whole-blood transcriptomics.** Principal-component analysis of the 18,974 most abundant transcripts among whole-blood RNA at enrollment (time 1 (T1): 2010–2011 season; 109 cases) showed clustering distinct from that of age-, ethnicity- and sex-matched healthy control subjects \( (n = 130)\). There was no discernible difference between patients infected with influenza A virus and those infected with influenza B virus (Fig. 1a). Samples at the final time point (time 3 (T3): > 4 weeks after T1) obtained from patients who achieved clinical resolution were similar to those from healthy control subjects, but they remained abnormal in patients who remained unwell (data not shown).

Modular analysis\(^{22}\) of the samples from the 2010–2011 cohort showed a greater abundance of transcripts from the genes in the interferon-inducible module (M3.1) and neutrophil module (M2.2) in patients with influenza than in healthy control subjects (Fig. 1b). Transcripts representing plasma cells (module M1.1), a subset of myeloid-lineage genes (module M2.6) and two inflammation modules (M3.2 and M3.3) were also increased, while expression of genes in a T cell module (M2.8) and B cell module (M1.3) was decreased (Fig. 1b). The calculated index ‘molecular distance to health’ (MDTH) (derived from analysis of 4,526 transcripts significantly above background, filtered for low expression\(^{23}\)) was higher in most patients with influenza than in healthy control subjects (Fig. 1c), although this was affected by disease stage and severity (discussed below). A combination of expression-level and statistical filtering identified 1,255 transcripts expressed differentially in patients relative to their expression in healthy control subjects. Supervised hierarchical clustering revealed transcripts that were over- or under-expressed in patients with influenza relative to their expression in healthy control subjects (Fig. 1d); applying this same 1,255-transcript set to the 2009–2010 cohort (22 patients with influenza and 25 matched healthy control subjects) replicated these profiles (Supplementary Fig. 2), which indicated that viral variation between the two seasons\(^{22}\) did not appreciably affect transcriptomic patterns.

Ingenuity pathway analysis identified the top five canonical pathways associated with upregulated and downregulated transcripts (Fig. 1d). Transcripts upregulated in patients with influenza were associated with the categories ‘interferon signalling genes’ (including IFITM1, IFI35, IFI17, OAS1, IFIT3 and IFI35; Supplementary Fig. 1), ‘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 downregulated were those associated with the categories ‘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).

Hierarchical clustering of the top 25 most significant transcripts in the 2010–2011 group of patients with influenza showed two major clusters (Fig. 1c). Transcripts of the interferon-stimulated gene IFI27 were overexpressed in almost all cases, while transcription of FCER1A was usually decreased. Independent analysis of the 25 transcripts from the 2009–2010 dataset showed similar clustering (Supplementary Fig. 2b). Patients with activation of type I interferon–induced genes (for example, RSAD2, IFI6 and IFI44L) typically did not express transcripts encoding neutrophil-associated or bacterial response–associated molecules (for example, DEFA4, ELANE and MMP8) and vice versa (Fig. 1c and Supplementary Fig. 2b). Together these results showed that patients with acute influenza had activation of whole-blood transcriptomic pathways indicative of responses to type 1 and type 2 interferons and of inflammatory markers and possibly combined with the effects of depletion of some cell types from the blood.

**Transcriptomics and disease severity.** Patients in the 2010–2011 cohort were grouped according to their severity of illness at T1 by the following three-point scale: 1, no supplemental oxygen required; 2, oxygen by mask required; 3, mechanical ventilation required. Transcriptomic abnormality (mean MDTH; 4,526 transcripts) was greater in patients with illness categorized as severity 1–2 than in healthy control subjects and was further increased in patients with illness categorized as severity 3 (Fig. 2a). By modular analysis\(^{22}\), there was an over-abundance of transcripts in the plasma-cell module (M1.1), neutrophil module (M2.2) and myeloid lineage

### Table 1 | Characteristics of patients and healthy control subjects

|                      | 2010–2011 influenza \( (n = 109)\) | 2010–2011 healthy \( (n = 130)\) | 2009–2010 influenza \( (n = 22)\) | 2009–2010 healthy \( (n = 25)\) |
|----------------------|-------------------------------------|----------------------------------|----------------------------------|----------------------------------|
| 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                               |
| Comorbidity          |                                     |                                  |                                  |                                 |
| 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                               |
| Pregnant women       | 10 of 43 (23.3%)                    | 1 of 75 (1.3%)                  | 2 of 8 (25%)                    | 0                               |
| (15–49 years of age) |                                     |                                  |                                  |                                 |
| 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                              |

Percentages may not add up to 100% for all variable due to rounding. NA, not applicable.
module (M2.6) in influenza virus–infected patients, a result that was most evident in those with illness of the greatest severity. Patients with illness categorized as severity 3 also showed an abundance of transcripts in inflammation modules M3.2 and M3.3. In contrast, interferon-related transcripts (module M3.1) were most evident in patients with illness categorized as severity 1 or 2 (Fig. 2b). Therefore, patients with the most-severe illness had transcriptional patterns that differed from those with less-severe illness, with an increased abundance of inflammation-related transcripts and a decrease in interferon-related transcripts.

Semi-supervised hierarchical clustering of 231 transcripts with a change in expression of more than twofold between patients with illness categorized as severity 1–2 and those with illness categorized as severity 3 showed that the expression of transcripts associated with the gene-ontology (GO) term ‘response to virus’ was typical of that in patients with milder disease (Fig. 3a; Supplementary Table 1), whereas patients who needed mechanical ventilation (severity 3) showed a marked abundance of transcripts associated with the GO term ‘response to bacterium’ (Supplementary Table 2). Patients with severe illness typically showed a relative under-abundance of transcripts associated with the GO term ‘cellular defense response’ (Fig. 3a).

The same 231-transcript list noted above was verified by hierarchical clustering analysis of the 2009–2010 cohort. Patients with influenza of severity 1 or 2 were again characterized by transcripts associated with the GO term ‘response to virus’, whereas three patients with influenza of severity 3 instead showed transcripts associated with the GO term ‘response to bacterium’ (Supplementary Fig. 2c). We determined the relationship between total ‘molecular score’ for the 51 transcripts associated with the GO term ‘response to virus’ (Supplementary Table 1) and the 112 transcripts associated with the GO term ‘response to bacterium’ (Supplementary Table 2) from patients in the 2010–2011 cohort, at T1 (n = 109) (Fig. 3b). Patients with influenza who had high ‘viral responses’ (> 500) were exclusively from the groups with illness of severity 1 or 2, whereas most patients with high ‘bacterial scores’ (> 500) had illness of severity 3 and had low ‘viral scores’ (reflective of the modular analysis). However, a few patients with illness of severity 1 or 2 had low ‘viral molecular scores’ and moderately high ‘bacterial molecular scores’. The removal of six patients with known bacterial co-infection did not eliminate this subgroup. Similar findings were obtained for the 2009–2010 cohort (Supplementary Fig. 2d).

Reciprocal expression was observed for activated and repressed biofunctions of the 231 differentially expressed genes in patients with illness of severity 3, compared with their expression in patients with illness of severity 1 or 2 (Fig. 3c,d). Nine genes encoding molecules associated with neutrophil activation were upregulated (for example, MPO, DEFA1 and ELANE), along with three genes encoding molecules associated with leukocyte influx (MPO, MMP9 and LCN2) in a similar comparison (Fig. 3c). The repressed biofunctions in patients with illness of severity 3 were in the categories ‘activation of cytotoxic T cells’, ‘adhesion of immune cells’ and ‘quantity of leukocytes’ (Fig. 3d). These results showed that patients with the most-severe disease show upregulation of genes encoding products associated with neutrophil activation and leukocyte influx that was not seen in those with less-severe influenza.

**Effect of illness duration, severity and viral load on transcriptional patterns.** Patients with 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).
Patients with illness of severity 3 showed higher ‘bacterial molecular scores’ than those of patients with less-severe disease, even at first presentation, whereas ‘bacterial molecular scores’ were low in patients with illness of severity 1 or 2 regardless of the time of sampling (Fig. 4b).

In patients in the 2010–2011 cohort with repeat samples (T1 and T2, separated by 2–5 d; n = 59), the ‘viral molecular score’ usually (but not always) decreased between T1 and T2 (Supplementary Fig. 3a). In patients for whom samples at T2 were obtained 2 d after T1 (n = 41), the reduction in ‘viral score’ was significant (P = 0.0002 (two-tailed Mann-Whitney test); Supplementary Fig. 3b). Changes between T1 and T2 in ‘bacterial molecular scores’ were more variable (Supplementary Fig. 3c,d). A decrease in viral load (measured in mucus obtained by nasopharyngeal suction) was observed between T1 and T2 (Supplementary Fig. 3e), but there was no clear relationship between viral load and ‘viral transcriptomic score’ (Supplementary Fig. 3f). Together these results showed that the relative dominance of ‘viral’ or ‘bacterial’ transcriptomic responses was influenced by both the severity of illness and the duration of illness.

Effect of bacterial infection and carriage on transcriptomic patterns. To investigate the role of bacterial infection in driving ‘bacterial’ GO terms, we identified a subgroup of influenza virus–infected patients who had been thoroughly investigated for bacterial infection by analysis of the nasopharyngeal aspirate (NPA) and throat swabs at T1 by culture and analysis of the NPA at T1 by detection of bacterial pathogens via PCR (in addition to testing for pneumococcal antigen in blood cultures and urine for most patients). Incomplete bacteriological sampling excluded 36 of 109 patients (33%); 34 patients (47%) provided at least four of five sample types. Of the 73 cases with adequate samples, 39 (53%) were deemed to have potentially pathogenic bacteria detected in at least one sample type and were classified by an expert clinical review panel to have potentially pathogenic bacteria detected in at least one sample type.

Comparison of those patients with influenza in whom clinically relevant bacterial co-infection was identified with those in whom no clinical relevant bacterial co-infection was identified showed that the average ‘viral molecular score’ was lower in those with bacterial infection at all times up to day 12 after the onset of illness (Fig. 4c), and the average ‘bacterial score’ was greater in those with bacterial co-infection between day 3 and day 14 (Fig. 4d). However, the transcriptomic scores showed similar time trends regardless of the presence or absence of bacterial co-infection. Similar findings were obtained when stricter exclusion criteria were applied to the subgroup analysis, with the exclusion of 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 small sample size (only 13 patients provided all five sample types and did not have bacteria detected).

To assess the influence of treatment of bacterial infection on the observed ‘viral’ and ‘bacterial’ responses, we stratified ‘bacterial’ scores and ‘viral scores’ at T1 and T2 in patients with influenza in the 2010–2011 cohort according to antibiotics prescription. In the MOSAIC study, 92% of the patients (234 of 253) were treated with antibiotics at some time. Antibiotics before T1 had no demonstrable effect on transcriptomic patterns (Supplementary Fig. 4a). Comparison of patients with influenza who were not given antibiotics (n = 7) with those given sustained antibiotic treatment after T1 (n = 24) or throughout illness (including T1 and T2; n = 27) showed that there was no discernable or statistically significant effect of antibiotic administration on the ‘bacterial molecular scores’ (Supplementary Fig. 4b).

We next assessed the abundance of 16S rRNA transcripts (indicative of bacterial load) in the throat-swab and NPA samples of patients classified as having ‘bacterial co-infection’ or ‘viral infection without bacterial infection’. The abundance of the 16S rRNA in throat swabs was not different in these groups, but the bacterial load in NPA samples was greater in those patients with confirmed bacterial co-infection (Supplementary Fig. 4c).

Finally, we investigated the utility of procalcitonin (PCT) as a possible guide to the presence of substantial bacterial infection26–27. The concentration of PCT showed no relationship with the ‘viral molecular score’ (Fig. 4e), and there was no correlation between ‘viral molecular scores’ at T1 and T2 and the concentration of PCT measured at the corresponding time point (data not shown). However, ‘bacterial molecular scores’ tended to be higher in those patients with the most-severe disease and the highest concentration of PCT regardless of the presence or absence of significantly detectable bacteria (Fig. 4f).

In summary, ‘viral molecular scores’ were seen in disease of up to 5 days’ duration. Even during this early phase, patients who needed mechanical ventilation had low ‘viral scores’, and this was especially
true in those with clinically determined bacterial co-infection. On the other hand, expression of ‘bacterial response genes’ was seen only in patients with the most-severe influenza; significant bacterial infection enhanced this signal, but the ‘bacterial score’ was evident in those with severe influenza regardless of the presence of bacterial co-infection (especially if the disease had lasted a week or more).

Effect of illness duration, severity and bacterial co-infection on soluble mediators. An advantage of the MOSAIC study is that it provides linked data of whole-blood transcriptomic signatures and, for example, levels of 35 soluble mediators in the blood, NPA and anterior nasal fluid (‘nasadsorption’ samples using synthetic adsorptive matrices) at up to three time points.

The changes observed depended on the mediator and compartment. For example, the concentration of the cytokine IL-1β in serum showed no trend when plotted against disease severity (Fig. 5a), but the concentration of IL-1β in NPA or nasadsorption samples was higher in those with severe disease (Fig. 5b,c). In contrast, the concentration of IL-6 in serum increased with disease severity (Fig. 5d); in NPA samples, IL-6 was undetectable in most of the healthy control subjects but was detected in most of the patients with influenza and increased with disease severity (Fig. 5e). The concentration of IL-6 was more consistent in nasadsorption samples than in NPA samples, was measurable in healthy control subjects, and was highest in most patients with influenza but did not reflect disease severity (Fig. 5f).

The concentration of the chemokine CXCL8 in serum tended to be higher in patients with influenza than in healthy control subjects and again increased with disease severity (Fig. 5g); in NPA samples, the concentration of CXCL8 was variable but generally increased with influenza disease severity and tended to saturate the assay (Fig. 5h). The concentration of CXCL8 in in nasadsorption samples was...
even higher, often saturating the assay even for samples from some healthy control subjects (Fig. 5i). Interferon-α2a (IFN-α2a) was measurable in only a portion of subjects, but its concentration was increased in serum in patients with milder illness (severity 1 or 2) but not in those with severe disease (Fig. 5j). In NPA or nas adsorption samples, the concentration of IFN-α2a was increased in some patients with milder illness (a result that was not statistically significant; Fig. 5k,l).

The concentration of IL-17 in serum increased with severity at T1 (Supplementary Fig. 5a) and was higher in the bronchoalveolar lavage fluid of eight patients (from whom samples were obtained for clinical indications) than in that of healthy control subjects (Supplementary Fig. 5b). In addition, we found a significant positive correlation of the serum concentration of IL-17 (Pearson; r = 0.44; P < 0.001) and TNF (Pearson; r = 0.40 (Spearman); Supplementary Fig. 5d) with MDTH.

As for the effects of timing, the concentrations of the chemokine CXCL10, IL-6 and chemokine CCL2 were elevated in serum from patients with severe influenza, especially between day 5 and day 10 (Fig. 6a,b and data not shown). Proven bacterial co-infection...
had no evident additional effect on CXCL10 (Fig. 6c), but IL-6 was abundant in serum not only in patients with severe influenza (even early in disease; Fig. 6b) but especially in patients with bacterial co-infection (especially between day 5 and day 10; Fig. 6d).

In the NPA samples, the concentration of most mediators (for example, CXCL10, IL-6, CCL2 and CXCL8) was markedly increased in severe disease and especially after day 4 (Fig. 6e,f and data not shown). The concentration of CXCL10 in NPA samples was again

Fig. 5 | Concentration of selected mediators in various compartments according to severity of illness. Concentration (log scale) of IL-1β (a–c), IL-6 (d–f), CXCL8 (g–i) and IFN-α2a (j–l) in serum (a,d,g,j), NPA samples (b,e,h,k) and nasabsorption eluates (c,f,i,l) (above columns) obtained at T1 from patients with influenza (severity of illness (horizontal axis) at T1) and healthy control subjects (serum: n = 36 (HC), 58 (severity 1), 43 (severity 2) and 31 (severity 3); NPA: n = 35 (HC), 50 (severity 1), 32 (severity 2) and 27 (severity 3); nasabsorption eluate: n = 36 (HC), 60 (severity 1), 43 (severity 2) and 30 (severity 3), presented as box plots (center line, median; box margins, interquartile range; extended lines, range); zero values and values below the lower limit of detection were assigned half the geometric mean lower limit of detection for presentation here (upper limit of detection, 2,500 pg/ml). NS, not significant (P > 0.05); *P < 0.05, **P < 0.01 and ***P < 0.001 (Kruskal-Wallis test with Dunn’s post-test).
unaffected by confirmed bacterial disease (Fig. 6g), whereas the concentration of IL-6 (and of CCL2 and CXCL8; data not shown) was particularly increased in patients with bacterial co-infection (Fig. 6h).

In the nas adsorption samples, the concentration of mediators decreased slowly with time even in less-severe disease; the concentration of CXCL10 was decreased by known bacterial co-infection.

Fig. 6 | Relationships among severity of illness, duration, bacterial infection, and selected mediators. Concentration of CXCL10 (a,c,e,g) and IL-6 (b,d,f,h) in serum (a-d) and NPA samples (e-h) obtained at T1 and T2 from patients with influenza, stratified by severity of illness (key) (a,b,e,f) or by the presence (n = 39 subjects; 63 samples) or absence (n = 34 subjects; 52 samples) of proven bacterial infection (key) (c,d,g,h), plotted against time of illness (presented as in Fig. 4). P values (top right corners), deviance of generalized linear models (χ² test).
but the concentration of IL-6, CCL2 and CXCL8 was unaffected by disease severity or bacterial status (data not shown).

Since the ‘bacterial load’ (assessed as copy number of 16S rRNA in NPA samples) was greater in patients with clinically relevant bacterial infection than in those without such infection (Supplementary Fig. 4c), we regressed this parameter against viral or bacterial MDTH. There were high values for viral MDTH only in those with lower bacterial loads in the NPA samples (Supplementary Fig. 5e), and high values for bacterial MDTH were seen only in a subset of those with a higher bacterial load (16S rRNA) in the NPA samples (Supplementary Fig. 5f).

These data were consistent with a role for bacterial load in the inflamed respiratory tract in driving levels of soluble mediators in mucosal fluids and serum as well as transcriptional signatures in the blood; however, the effects of influenza severity and time after disease onset remained the dominant determinants of host responses.

**Discussion**

The MOSAIC study is exceptional in including a large number of well-characterized patients hospitalized with influenza, studied prospectively and sampled intensively. We found that whole-blood RNA-expression profiles of patients hospitalized with influenza evolved over time and that the pattern reflected severity. Patients with mild (or early) disease typically showed responses dominated by interferon-inducible genes and type I interferons, but that ‘viral’ signature was replaced during severe (or late) disease by a pattern reflective of inflammation and neutrophil activation, more typically associated with the GO term ‘response to bacteria’, including genes encoding regulators of apoptosis and anaerobic metabolism.

The ‘viral’ response was rarely seen in patients beyond day 4, whereas the inflammation–neutrophil activation signal peaked during the second week.

In severe disease, the early ‘viral’ response was typically absent, whereas the ‘bacterial’ signature was present, at enrollment in the study; this was especially so in patients with proven bacterial co-infection whereas the ‘bacterial’ signature was present, at enrollment in the ‘viral’ response was rarely seen in patients beyond day 4, whereas the inflammation–neutrophil activation pattern was seen.

Soluble protein mediators were generally abundant in the serum and nasopharyngeal samples of patients with severe disease, even early after onset. Inflammatory mediators (for example, IL-17, IL-1β and IL-6) were augmented in those with clinically relevant bacterial co-infection, whereas IFN-α levels tended to be low or undetectable in most compartments in those with very severe influenza; however, interferon-related secondary mediators (for example, CXCL10 in serum) were generally more abundant in patients with severe disease. These findings suggest complex interactions between mechanisms for sensing and responding to viruses and those for sensing and responding to bacteria that have evolved over time.

To investigate the issue of bacterial co-infection specifically, we identified patients in whom pathogenic bacteria was found in mucosal samples or blood culture as a subgroup with clinically confirmed bacterial sepsis. Three of these six patients needed mechanical ventilation and had a markedly elevated ‘bacterial signature’ without any increase in ‘viral score’; one patient had elevated ‘bacterial scores’ and ‘viral scores’. The remaining two patients with bacteremia did not have marked elevations in their ‘bacterial scores’; both had mild disease (severity 1). We next used stringent criteria to identify patients with influenza whom we investigated extensively for bacteria and found to be not co-infected, and compared those with patients in whom pathogenic bacteria were identified with certainty. Patients with confirmed bacterial co-infection had higher ‘bacterial molecular scores’ overall, but progression of the transcriptional signatures was similar over time. Therefore, severe infection with influenza virus alone seemed able to drive the ‘bacterial’ signature, but this response was enhanced by bacterial co-infection. We conclude that transcriptomic data from the blood are an unreliable guide to the presence or absence of bacterial co-infection but need careful interpretation in the context of the timing and severity of disease. We cannot determine the extent to which these changes might be driven by injury caused by influenza virus or by innate sensitivity to resident microbiota that leads to activation of pathways of the T<sub>β</sub>17 subset of helper T cells triggered by endotoxins from mucosal surfaces.

In animal models of lung disease, dysregulated host immune responses and interferon production can lead to complex inflammatory responses that contribute to pathogenesis. In macaques, administration of recombinant IFN-α2a initially upregulates the expression of genes encoding antiviral molecules and prevents viral infection, but continued treatment causes desensitization and a paradoxical decrease in the expression of genes encoding antiviral molecules. These paradoxical immunosuppressive effects can impede viral control or trigger inflammation and tissue damage. In mice, infection with influenza virus causes an early local influx of neutrophils, followed by a virus specific CD8<sup>+</sup> T cell response. Neutrophils might facilitate the development of this antigen-specific response by guiding influenza virus–specific CD8<sup>+</sup> T cells into sites of infection by laying chemokine trails. Our findings for human influenza are generally compatible with these animal studies.

We have presented here only selected results of an extended study of data on soluble immunological mediators from the MOSAIC cohort. Our main findings were of decreased concentrations of IFN-α2a and increased concentrations of IL-1β, IL-6 and CXCL8 in the nasal and/or serum compartments in patients with severe disease. This apparent reciprocity might relate to the known cross-regulatory functions of IL-1 and type I interferons in experimental models. Our results generally fit with the proposal that levels of mediators such IL-1β, IL-6 and IL-17 are influenced by bacterial co-infection in severe influenza but are not driven by it. However, many additional possible analyses remain to be performed. We chose to illustrate only those most relevant to the transcriptomic analysis and the question of bacterial superinfection. Additional correlations can be explored with our online data as a resource, and we welcome discussions about additional interpretations.

Our study has important limitations. Despite its ambition, scope and intensity, we had limited numbers of repeat samples from individual patients. Our description of trends over time depends largely on summative data and on subjective reporting of the time of disease onset. Ideally, our findings need validation in other time-series studies of simple and complicated acute viral disease with frequent sampling at multiple sites. We were unable to study the early or preclinical phases but were limited to investigation of symptomatic patients presenting with disease of sufficient severity to require hospitalization. Ongoing studies of experimental infection with pH1N1 in volunteers should allow us to overcome some of these limitations.

In summary, virus-induced type I interferon–related pathways were activated during the first 4 d of symptomatic influenza in hospitalized patients. These ‘viral’ pathways were then downregulated and were replaced by inflammatory, activated-neutrophil and apoptosis-related pathways associated with IL-17 abundance, host-mediated tissue damage and expression of gene clusters in the category ‘response to bacteria’, particularly in patients with a high bacterial load (16S rRNA) in their nasopharyngeal secretions. In patients with severe illness, the ‘viral’ response was diminished even early in disease, accompanied by an increase in IL-1β and IL-17. These findings emphasize that the stage and severity of disease need to be taken into account in the interpretation of host responses to infection and in the development of potential diagnostic tests to distinguish between possible causes and appropriate therapies.
Methods
Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41590-018-0111-5.

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Author contributions
J.D., A.O.G. and P.J.M.O. conceived of and designed the study, with input from D.C. and J.B.; J.D., S.B., L.T.H. and C.I.B. analyzed the microarray data, with supervision by A.O.G. and P.J.M.O. and input from J.B. and D.C.; M.C., P.L.J. and M.F.M. performed and analyzed the microbiome experiments; C.M.G. performed the microarray experiments; J.D., S.J.B. and P.J.M.O. developed clinical protocols, recruited subjects and collated clinical data; and J.D., S.B., L.T.H., A.O.G. and P.J.M.O. wrote and revised the manuscript.

Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to A.O. or P.J.M.O.
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MOSAIC Investigators
Maximillian S. Habibi1, Sebastian L. Johnston1, Trevor T. Hansel1, Mike Levin1, Ryan S. Thwaites1, John O. Warner1, William O. Cookson3, Brian G. Gazzard9, Alan Hay10, John McCauley10, Paul Aylin11, Deborah Ashby11, Wendy S. Barclay12, Ruth A. Elderfield12, Simon Nadel13,14, Jethro A. Herberg13, Lydia N. Drumright15, Laura Garcia-Alvarez15, Alison H. Holmes16, Onn M. Kon1,14, Stephen J. Aston16, Stephen B. Gordon16, Tracy Hussell17, Catherine Thompson18, Maria C. Zambon18, Kenneth J. Baillie19, David A. Hume19, Peter Simmonds19, Andrew Hayward20, Rosalind L. Smyth21, Paul S. McNamara22, Malcolm G. Semple22, Jonathan S. Nguyen-Van-Tam23, Ling-Pei Ho24, Andrew J. McMichael24, Paul Kellam25, Walt E Adamson26, William F Carman26 and Mark J. Griffiths27

1Chelsea and Westminster NHS Foundation Trust, London, UK. 20Francis Crick Institute, World Influenza Centre, London, UK. 11School of Public Health, Imperial College London, London, UK. 15Department of Paediatrics, Imperial College London, London, UK. 16Department of Respiratory Medicine, Imperial College Healthcare Trust, London, UK. 17Centre for Infection Prevention, Imperial College London, London, UK. 18Respiratory Infection Group, Liverpool School of Tropical Medicine, Liverpool, UK. 19Manchester Collaborative Centre for Inflammation Research (MCCIR), Manchester, UK. 110Public Health England (formerly Health Protection Agency), London, UK. 21The Roslin Institute, University of Edinburgh, Edinburgh, UK. 22University College London, London, UK. 23UCL Institute of Child Health, London, UK. 24University of Liverpool, Liverpool, UK. 25University of Nottingham, Nottingham, UK. 26University of Oxford, Oxford, UK. 27Wellcome Trust Sanger Institute, London, UK. 28West of Scotland Specialist Virology Centre, Glasgow, UK. 29Royal Brompton and Harefield NHS Foundation Trust, London, UK.
Methods

Study population and inclusion criteria. Patients \( \geq 16 \) years of age were recruited during two successive winters (1 December 2009 to 3 March 2011). Patients with suspected influenza were identified by medical or nursing staff or investigators and were notified by hospital diagnostic laboratories. Patients in London were recruited from four Imperial College Healthcare NHS Trust hospitals, the Chelsea and Westminster Hospital, and the intensive care unit at the Royal Brompton Hospital (a national referral center for severe respiratory failure). In Liverpool, patients were recruited from the Royal Liverpool, Liverpool Women’s and Arrow Park Hospitals. Patients were included irrespective of prior or concurrent comorbidity (most commonly asthma, pregnancy, immunocompromising conditions or co-infection with other respiratory pathogens), to reflect the populations known to be at greatest risk of severe influenza. Adult healthy control subjects were recruited and matched to the patient cohorts for age, sex and ethnicity and were screened to exclude known illnesses or current use of medications (Registered Clinical Trial NCT00965354).

Research ethics committee approval. The study was approved by the NHS National Research Ethics Service, Outer West London REC (09/H079/52, 09/MRE00/67). Patients or their legally authorized representatives provided informed consent. Additional adult healthy control subjects were recruited as part of a separate study and consented to their samples’ being used in additional studies (Central London 3 Research Ethics Committee, 09/H076/141). Informed consent was obtained from all participants, and we complied with all relevant ethical regulations.

Biological sampling. Research samples were obtained at three time points: T1 (recruitment), T2 (approximately 48 h after T1) and T3 (at least 4 weeks after T1). Only samples at T1 and T2 were included in this report. Whole-blood samples for transcriptomics were collected during the two recruitment periods: 2009–2010 and 2010–2011. Of 85 MOSAIC participants presenting with influenza-like illness in 2009–2010, 23 (27%) were adults with confirmed influenza, and T1 transcriptomic samples were available from 22 adults. Of 171 MOSAIC participants presenting with influenza-like illness in 2010–2011, 111 (65%) were adults with confirmed influenza, and T1 transcriptomic samples were available from 109 of 111 (98%). RNA extraction and microarray were successful for all available patient samples from both cohorts. Microarrays were also performed on samples from adult healthy control subjects of age, sex and ethnicity similar to that of the study patients (Table 1). One sample from a healthy control subject in the 2009–2010 cohort was not included in final analysis because it failed quality-control assessments.

Of the 199 adult patients recruited in 2010–2011 and included in this analysis, 94 (86%) were infected with A(H1N1)pdm09 influenza virus, and the remainder were infected with influenza A(H3N2) virus, non-subtyped influenza A virus or influenza B virus. 1 of 22 adult patients recruited during 2009–2010 was infected with A(H1N1)pdm09 virus; remaining patients were infected with A(H1N1)pdm09 virus. Due to the natural evolution of influenza activity during the 2009–2010 pandemic in the UK, the 2009–2010 cohort was smaller than originally anticipated. Therefore, to assess the host response in the blood transcriptional signature as thoroughly as possible, we focused our analysis on the larger 2010–2011 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.

Influenza virus quantification. Nasopharyngeal secretions were collected into sterile universal sputum traps by suction catheterization. After 5 s of suctioning, any contents remaining within the catheter were flushed through with 5 ml normal saline. Samples were stored at \(-80^\circ C\) until analysis. Viral nucleic acids were extracted using the QiaGen MDx Biorebot automated extractor with the QIAamp Virus MDx Kit according to the manufacturer’s instructions. qRT-PCR reactions were set up to a total volume of 15 μl using the QiaGen One-Step RT-PCR kit, using primers (influenza A matrix (M) or pH1N1 neuraminidase (NA)) as described previously5 on an ABI Prism 7500 SDS real time platform (Applied Biosystems). For viral-load quantification, we first derived the crossing threshold (Cv) value (at the inflexion slope of the sigmoid amplification curve to capture the point at which DNA amplification is exponential) performed in a batched assay as a relative expression of viral burden against each sample. Subsequently, this was measured against a standard curve of Cv, value to plaque-forming units per ml, generated by measurement of plaque-forming units when MDCK canine kidney cells were inoculated with a known amount of pH1N1.

Clinical data collection and assignment of scores for severity of illness. Clinical data were extracted from hospital case notes and recorded in the Flu-CIN data-collection tool5 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 substantial respiratory compromise, with blood oxygen saturation of \( > 93\% \) while the patient was breathing room air; 2, oxygen saturation of \( \leq 93\% \) while the patient was 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 (extracorporeal membrane oxygenation). 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 pathogens from nasopharyngeal aspirates: 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 obtained at T1 also underwent culture and microscopy. Where available, urine samples collected between T1 and T2 underwent pneumococcal antigen testing (BinaxNow, Alere). Clinical microbiology data were obtained from hospital laboratory databases, including results of blood cultures (when obtained 48 h before and after T1) and urinary pneumococcal antigen results (for patients who did not have a researcher-requested urinary antigen sample). An independent blinded assessment of the significance and validity of positive blood-culture results, in an attempt to exclude cases of pseudobacteremia caused by commensal contamination.

Soluble immunological mediators. Serum, nasopharyngeal aspirate (NPA) and nasal-aspiration fluid were collected at recruitment (T1) from participants with confirmed influenza and from adult healthy control subjects. Clotted blood was centrifuged at 1,000 \( \times g \) at 4°C, and aliquots of serum supernatant were stored at \(-80^\circ C\). Each NPA was collected using a 10 F Argyle suction catheter, inserted to reach the posterior nasopharyngeal wall, moderate suction was applied while the catheter was withdrawn over 5 s. The catheter was flushed with through with 5 ml of sterile normal saline, and the total contents were collected in a universal container. Aliquots of NPA were stored at \(-80^\circ C\). Nasal-aspiration fluid was collected from the lateral wall of the nasal cavity using a synthetic absorptive matrix (SAM) strips (Leukosorb, Pall) and was stored at \(-80^\circ C\) until analysis. On the day of analysis, 500 μl Milliplex assay buffer (Millipore) 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 \( \times g \) for 5 min at 4°C, and eluates were kept on ice.

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

Blood procalcitonin assay. Procalcitonin (PCT) in plasma or serum (collected at T1) was quantified using a commercial Brahms HemosIL PCT test (Brahms) from the Cobas e602 platform. Samples with a PCT value at the upper limit of detection (ULOD) were arbitrarily assigned a 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 gene encoding 16S rRNA was targeted with 0.3 μl each of 10 μM universal primers 520 F 5′-AYT GGG YDT AAA GNC and 802 R 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 1.5 μl of TriPure Clean water (Mobio). Reactions were 30 cycles, with hot-start thermal cycling carried out on a VIAA 7 Real-Time PCR System. Thermal-cycling conditions were 90°C for 3 min, 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 for a cloned (TOPO TA, Invitrogen) gene encoding full-length Vibrio natriegens DSMZ 749 16S rRNA was included in order to be able to calculate an absolute abundance from Cv values together with no template controls. The resulting copy number of 16S rRNA (bacterial load) was log-transformed before being used analytically.

Microarray gene-expression profiling. At each time point, 3 ml of whole blood was collected into each of two Tempus tube (Applied Biosystems/Ambion) by trained research staff following a standard phlebitomy protocol. Blood was vigorously mixed immediately following collection and was stored at \(-80^\circ C\) 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. 230 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, 750 ng of labeled cRNA was hybridized 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 was 0.99. Signal values were then set to a threshold level of 10, were log2-transformed and were per-chip normalized using a 75th percentile-shift algorithm. Each gene was normalized 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 hybridization were filtered for low expression in GeneSpring GX 12.5, whereby the only transcripts retained were those with a change of at least twofold from the median normalized intensity value in at least 10% of all samples. Principal-component analysis of all transcripts significantly above background in at least 10% of samples (18,974 transcripts) was performed using R 3.3.2 (R Development Core Team). To derive the 1,255 transcript list, non-parametric statistical filters were applied (P < 0.01 (Mann-Whitney unpaired test with Bonferroni family-wise error rate (FWER) multiple-testing correction)), followed by filtering by change (fold values) between groups (transcripts were retained with a change greater than twofold between any two groups). For severity analysis, 231 normalized intensity value transcripts were obtained by filtering for low expression and then applying statistic filters (P < 0.01 (Kruskal-Wallis test with Bonferroni FWER)), followed by filtering by change (fold values) between groups (transcripts were retained with a change of greater than twofold between patients with illness of severity 3 and those with illness of severity 1 and 2). All heat maps were generated in GeneSpring GX 12.5 (semi-supervised analysis, clustered by Pearson’s un-centered method with average linkage rule).

Comparison Ingenuity Pathway Analysis (IPA) (Ingenuity Systems) was used to determine the most significant canonical pathways for upregulated and downregulated transcripts (P < 0.05 (Fisher's exact test)). Additionally, IPA was used to generate the graphed presentation of selected canonical pathways and network diagrams. For the 231 transcript list, significantly activated biofunctions (z-score > 2) and significantly repressed biofunctions (z score < 2) were identified in IPA and are presented in gene-network diagrams. GO Term analysis (Gene Ontology Consortium) integrated with GeneSpring GX12.5 was used to identify biological processes, according to GO annotations.

The molecular distance to health (MDTH) and molecular scores were calculated using methods described previously and were applied to different signatures. Transcriptional modular analysis was applied as described previously. In brief, raw expression levels of all transcripts significantly above or below background were compared between each sample and all the controls present in a given dataset. The percentage of differentially expressed genes in each module is represented by the color 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 significant genes and the mean change in expression of these genes (fold values) compared to the controls in specific modules are presented in graphical form (P < 0.00001 (unpaired t-test)). MDTH and modular analysis were calculated in Microsoft Excel 2010 (Microsoft). GraphPad Prism V5 for Windows (GraphPad Software) and R 3.3.2 (R Development Core Team) were used to generate graphs and perform additional statistical analyses.

Reporting summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability statement. The raw and normalized microarray data that support the findings of this study have been deposited in GEO with the accession code GSE111368.

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1. **Sample size**
   - Describe how sample size was determined.
     Although an estimated sample size calculation was performed for the MOSAIC study at the time the study was designed, the nature of the 2009-10 pandemic and the associated challenges of recruiting participants necessitated opportunistic (proximity) sampling.

2. **Data exclusions**
   - Describe any data exclusions.
     Data were not excluded from the analyses. Although samples were collected from adult and pediatric participants in the MOSAIC study, the intention was to analyse results from adult participants only in the present study.

3. **Replication**
   - Describe the measures taken to verify the reproducibility of the experimental findings.
     For the primary focus of study - transcriptional responses to influenza - results from two distinct patient cohorts were compared: patients recruited in winter of 2009/10 and patients recruited in the winter of 2010/11. Although the cohorts differed in size, the findings were sufficiently similar to demonstrate consistency of findings. Both cohorts were dominated by influenza A(H1N1)pdm09 infections.

4. **Randomization**
   - Describe how samples/organisms/participants were allocated into experimental groups.
     Participants with influenza were recruited sequentially as they presented to healthcare services and according to the study protocol's inclusion criteria. Healthy controls were recruited independently and according to agreed inclusion criteria (including successful completion of a qualifying questionnaire). Samples were randomised before assignment to the arrays, to avoid experimental bias, and were analysed en masse with their matched healthy control samples. Influenza virus infection status of participants was revealed following completion of analysis of their samples.

5. **Blinding**
   - Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
     Influenza virus infection status was known at the time of clinical data collection, consistent with the study protocol. For transcriptomic and soluble immune mediator profiling, the influenza virus infection status of participants was revealed only following completion of laboratory analysis of their samples. Supervised and semi-supervised data analyses have been indicated where applicable.

Note: all in vivo studies must report how sample size was determined and whether blinding and randomization were used.
6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

- [ ] The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
- [ ] A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- [ ] A statement indicating how many times each experiment was replicated
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  - *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
- [ ] A description of any assumptions or corrections, such as an adjustment for multiple comparisons
- [ ] Test values indicating whether an effect is present
  - *Provide confidence intervals or give results of significance tests (e.g. P values) as exact values whenever appropriate and with effect sizes noted.*
- [ ] A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
- [ ] Clearly defined error bars in all relevant figure captions (with explicit mention of central tendency and variation)

See the web collection on statistics for biologists for further resources and guidance.

7. Software

Describe the software used to analyze the data in this study.

GenomeStudio (Illumina); GeneSpring GX version 12.5 (Agilent Technologies); R 3.3.2 (R Development Core Team); Ingenuity Pathway Analysis (IPA) (Ingenuity Systems Inc., Redwood, CA); Microsoft Excel 2010 (Microsoft Corp.); GraphPad Prism V5 for Windows (GraphPad Software Inc., La Jolla, CA, USA)

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a third party.

- No unique materials were used.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

- No antibodies were used.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

b. Describe the method of cell line authentication used.

c. Report whether the cell lines were tested for mycoplasma contamination.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

- Eukaryotic cell lines were not used.
- Eukaryotic cell lines were not used.
- Eukaryotic cell lines were not used.
- No commonly misidentified cell lines were used.

11. Animals and human research participants

Provide all relevant details on animals and/or animal-derived materials used in the study.

- No animals were used.
12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

Study population characteristics have been described in Table 1 of the manuscript.