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*Published in:*
The European respiratory journal

*DOI:*
10.1183/13993003.00787-2023

*Publication date:*
2024

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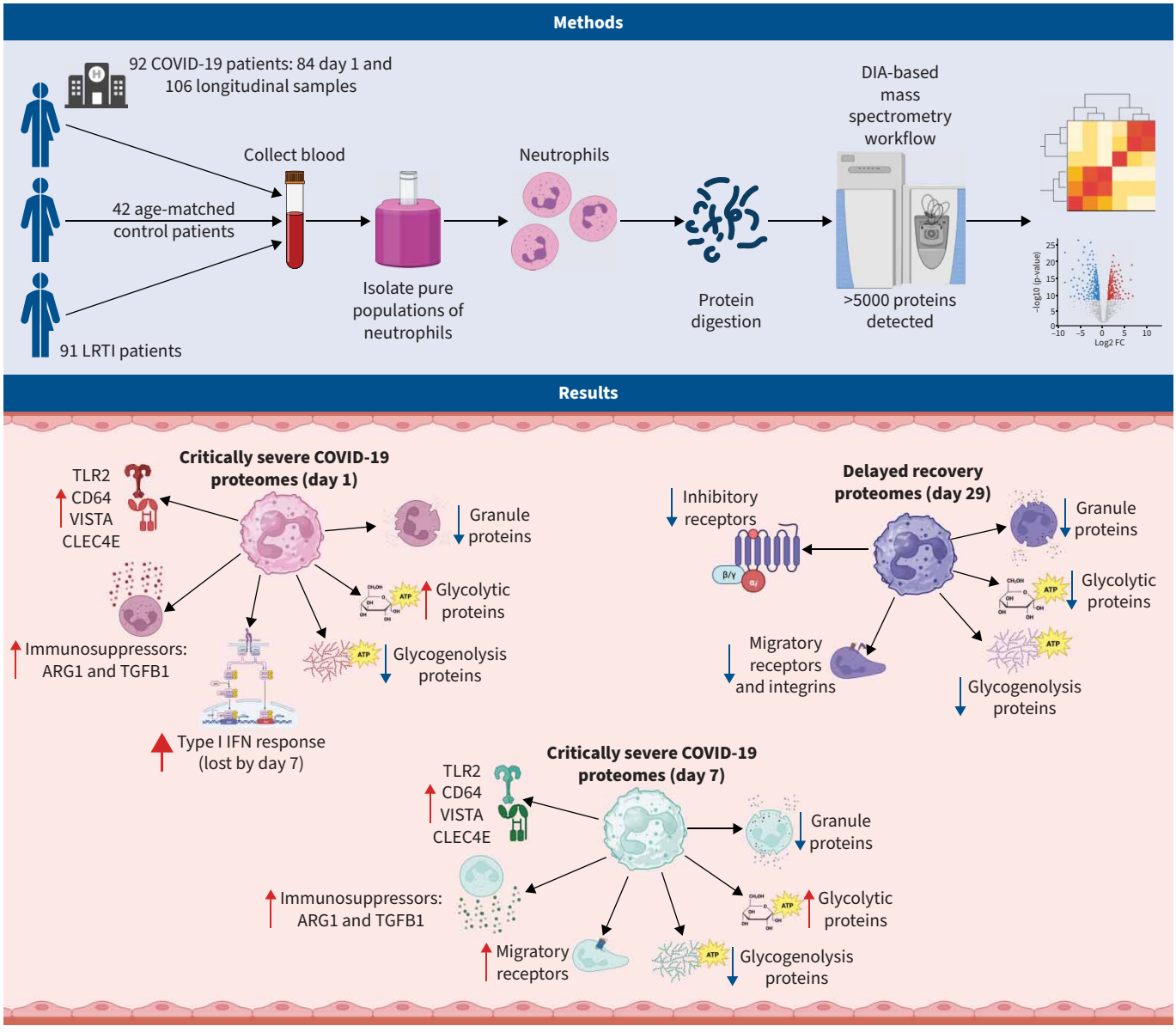


**GRAPHICAL ABSTRACT** Summary of the study. Peripheral blood neutrophils from >200 hospitalised patients across three patient groups (coronavirus disease 2019 (COVID-19), non-COVID-19 lower respiratory tract infection (LRTI) and matched controls) were comprehensively profiled using mass spectrometry, revealing novel proteomic changes in acute and convalescent COVID-19. DIA: data-independent acquisition; TLR: Toll-like receptor; ARG: arginase; TGF: transforming growth factor; IFN: interferon.

# Extensive acute and sustained changes to neutrophil proteomes post-SARS-CoV-2 infection

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**Cite this article as:** Long MB, Howden AJM, Keir HR, *et al*. Extensive acute and sustained changes to neutrophil proteomes post-SARS-CoV-2 infection. *Eur Respir J* 2024; 63: 2300787 [DOI: 10.1183/13993003.00787-2023].



This article has an editorial commentary: https://doi.org/10.1183/13993003.00137-2024

Received: 14 May 2023
Accepted: 23 Nov 2023

## Abstract

***Background*** Neutrophils are important in the pathophysiology of coronavirus disease 2019 (COVID-19), but the molecular changes contributing to altered neutrophil phenotypes following severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection are not fully understood. We used quantitative mass spectrometry-based proteomics to explore neutrophil phenotypes immediately following acute SARS-CoV-2 infection and during recovery.

***Methods*** Prospective observational study of hospitalised patients with PCR-confirmed SARS-CoV-2 infection (May to December 2020). Patients were enrolled within 96 h of admission, with longitudinal sampling up to 29 days. Control groups comprised non-COVID-19 acute lower respiratory tract infection (LRTI) and age-matched noninfected controls. Neutrophils were isolated from peripheral blood and analysed using mass spectrometry. COVID-19 severity and recovery were defined using the World Health Organization ordinal scale.

***Results*** Neutrophil proteomes from 84 COVID-19 patients were compared to those from 91 LRTI and 42 control participants. 5800 neutrophil proteins were identified, with >1700 proteins significantly changed in neutrophils from COVID-19 patients compared to noninfected controls. Neutrophils from COVID-19 patients initially all demonstrated a strong interferon signature, but this signature rapidly declined in patients with severe disease. Severe disease was associated with increased abundance of proteins involved in metabolism, immunosuppression and pattern recognition, while delayed recovery from COVID-19 was associated with decreased granule components and reduced abundance of metabolic proteins, chemokine and leukotriene receptors, integrins and inhibitory receptors.

***Conclusions*** SARS-CoV-2 infection results in the sustained presence of circulating neutrophils with distinct proteomes suggesting altered metabolic and immunosuppressive profiles and altered capacities to respond to migratory signals and cues from other immune cells, pathogens or cytokines.

## Introduction

Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection has a diverse spectrum of presentations, from asymptomatic or pre-symptomatic, to critical illness involving development of pneumonia and acute respiratory distress syndrome (ARDS) [1–3].

The immune response plays a key role in determining the outcome of SARS-CoV-2 infection with either immunodeficiency or an excessive inflammatory response causing morbidity and mortality. Major immune events following COVID-19 include a type I interferon response (IFN-I) in many cells [4, 5], T-cell lymphopenia [6–8], cytokine storm [6, 7, 9, 10], myeloid compartment dysregulation [4, 8, 11, 12] and procoagulant pathway activation [10, 13]. Neutrophils are key effector cells of the innate response to pathogens [14, 15] and in severe COVID-19 there are increased blood neutrophil counts [2, 16–18], along with evidence of emergency myelopoiesis and the appearance of circulating immature neutrophils in peripheral blood [11, 19–22]. Moreover, neutrophil activation signatures and increased neutrophil extracellular trap production (NETosis) predict disease trajectory [23–25]. Neutrophil populations following SARS-CoV-2 infection have been characterised using mass cytometry [22, 26, 27], flow cytometry [21, 28] and by single-cell RNA sequencing [4, 29–31], and profound changes in circulating neutrophils associated with SARS-CoV-2 infection have been identified in these studies. However, it is recognised that in-depth quantitative analysis of cellular proteomes can provide insights that are not obtained from transcriptomes, particularly relating to neutrophil biology [32]. Proteomic analysis of neutrophils has identified an IFN-I and prothrombotic hyperinflammatory signature in neutrophils isolated from SARS-CoV-2-infected individuals with ARDS [33]. However, there has been no systematic analysis of neutrophil proteomes in patients with COVID-19 of differing severity, and exploration of the persistence of changes in neutrophil phenotypes following SARS-CoV-2 infection has been limited. Interestingly, recent evidence indicated persistent neutrophil activation several months after acute COVID-19 in patient subsets demonstrating impaired lung function [34]. Accordingly, the current study used mass spectrometry to provide a global overview of changes in COVID-19 neutrophils compared to control populations. This study subsequently tested hypotheses that changes in neutrophil proteome are related to disease severity, and that the proteome changes over time in relation to clinical status. This extensive patient cohort and longitudinal sampling strategy up to 29 days post-enrolment has provided an in-depth analysis of the neutrophil proteome overall and provided novel insights into changes in neutrophils during acute SARS-CoV-2 infection and during the disease recovery phase.

## Methods

The PREDICT-COVID-19 study was a prospective observational case–control study conducted at Ninewells Hospital (Dundee, UK). Patients with suspected or confirmed COVID-19 were enrolled within 96 h of hospital admission, with SARS-CoV-2 infection confirmed by reverse transcriptase (RT)-PCR performed on combined oropharyngeal and nasopharyngeal swabs. The study was approved by the East of Scotland Research Ethics Committee (identifier number: 20/ES/0055). Written informed consent was provided by all participants. Two control populations were included: 1) hospitalised patients presenting with community-acquired lower respiratory tract infections (LRTI) not due to SARS-CoV-2 infection and 2) age matched, noninfected controls in hospital for other reasons.

Blood sampling was performed at enrolment (study day 1), and on day 7, 15 and 29 for the COVID-19 cohort while hospitalised. Additionally, at day 29, all participants who had been discharged were invited to return for follow-up sampling. Full inclusion and exclusion criteria are shown in the supplementary material. Inclusion criteria for controls were age $\geqslant$16 years, absence of an infection-related diagnosis, judged as clinically stable by the investigator and able to give informed consent. Exclusion criteria were known or past SARS-CoV-2 infection in the past 3 months, known contact with a COVID-19 positive case in the preceding 14 days, any current infection and any contraindication to venepuncture or participation in the study as judged by the investigator.

### *Clinical variables*

Baseline severity was classified according to the World Health Organization (WHO) scale as WHO3 (hospitalised, not requiring supplementary oxygen), WHO4 (hospitalised, requiring oxygen through facemask or nasal prongs) and WHO5/6 (requiring high-flow nasal oxygen, continuous positive airway pressure or invasive mechanical ventilation). Patients were categorised as either recovered, being discharged with no ongoing symptoms reported (WHO1), or nonrecovered (patients classified as WHO2, who were discharged but still reporting ongoing symptoms, and WHO3, who were still hospitalised) at day 29 follow-up.

### *Neutrophil and peripheral blood mononuclear cell isolation and sample preparation for liquid-chromatography mass spectrometry*

Neutrophils were isolated from blood using the EasySep Direct Human Neutrophil Isolation Kit (Stemcell Technologies #19666) within 2 h of venepuncture. Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient separation using Lymphoprep and SepMate columns. Isolated cells were pelleted and stored at −80°C until analysis.

### *Proteomic sample preparation and mass spectrometry analysis*

Neutrophil and PBMC pellets were processed for mass spectrometry as described [33]. For each sample, 1.5 mg of peptide was analysed on a Q Exactive HF-X (Thermo Scientific) mass spectrometer coupled with a Dionex Ultimate 3000 RS (Thermo Scientific). The mass spectrometer was operated in data-independent acquisition (DIA) mode, and the raw mass spectrometry data were processed using Spectronaut [35]. Full details of sample preparation for LC-MS and LC-MS analysis are described in the supplementary methods. All the processed proteomic data is openly available at the Immunological Proteome Resource [36]. All the mass spectrometry files, as well as the processed search result files are available at the PRoteomics IDEntifications database [37] under the identifiers PXD036082 and PXD036089.

### *Statistical methods*

The differential expression analyses were performed in R using Limma [38]. q-values were calculated with the Bioconductor package qvalue. Differences were considered significant when the q-value was $\leqslant 0.05$. p-values for protein families and the PBMC proteins were calculated in R using Welch's t-test; p-values $\leqslant 0.05$ were considered significant. Differences in study group demographics used Chi-squared or Fisher's exact test for qualitative variables and Mann–Whitney test for continuous variables after Shapiro–Wilk normality test; $p \leqslant 0.05$ was considered significant. Full details including overrepresentation analyses are described in the supplementary methods.

## Results

217 patients were enrolled between May 2020 and December 2020 including 84 individuals with confirmed COVID-19, 91 with acute LRTI and 42 noninfected controls (table 1). COVID-19 and control populations were generally well matched at baseline (day 1). Details on age, gender and clinical data are shown in table 1. Pathogens identified in the LRTI group are listed in supplementary table S11.

### *The protein landscape of neutrophils from hospitalised COVID-19 patients*

Peripheral blood neutrophil proteomes from patients with COVID-19, non-COVID-19 LRTI and control groups were analysed using mass spectrometry (figure 1a). More than 5800 unique proteins were identified with a median of 4923 proteins per sample (figure 1b). There were no significant differences in the total protein content of neutrophils from control, LRTI or COVID-19 patients (figure 1c), but there were differences in protein landscapes. 300 proteins were significantly increased and 123 proteins significantly decreased in abundance in neutrophils from LRTI patients compared to controls (figure 1d; supplementary table S1), including azurophilic granules containing elastase and myeloperoxidase. A stratified analysis of LRTI patients based on severity revealed similar results (supplementary figure S1). Neutrophil proteomes from COVID-19 patients showed 1748 proteins significantly changed in abundance (figure 1e; supplementary table S2); with 1008 proteins significantly increased compared to controls. This included IFN-induced proteins and metabolic proteins involved in glycolysis and fatty acid oxidation (FAO). Proteins decreased in expression in neutrophils from COVID-19 patients included CD10, components of endosomal sorting complexes and key enzymes controlling glycogenolysis.

COVID-19 patients were stratified based on the WHO severity scale and proteomic changes were found to be proportional to disease severity (supplementary table S3). 221 proteins were significantly changed in neutrophils from WHO3 (moderate), 779 in WHO4 (severe) and 1483 in WHO5–6 (critically severe) patients compared to noninfected controls (figure 1f). There was a core signature of 171 neutrophil proteins changed across all COVID-19 groups (figure 1g; supplementary table S4). This included 101 IFN-I signature proteins that were increased in abundance in neutrophils from WHO3, WHO4 and WHO5–6 patients, including MX1 and MX2 (figure 1h; supplementary figure S2). MX1 in particular was highly abundant with a pronounced and robust increase in COVID-19. The core signature also included 70 proteins that were decreased in abundance with functions linked to nuclear rigidity, *e.g.* SYNE1, SYNE2 and SUN2 (figure 1i) and the laminin B receptor (figure 1j).

Neutrophils from WHO3 patients displayed very few significant changes in metabolic proteins, whereas neutrophils from WHO5–6 patients had increased expression of proteins controlling FAO, the electron transport chain, the tricarboxylic acid cycle and key glycolytic proteins (figure 2a). In addition, neutrophils from WHO5–6 patients had reductions in proteins controlling glycogen breakdown such as glycogen phosphorylase (PYG)L (figure 2b) and PYGB (figure 2c), and inhibitors of glycogen synthesis glycogen synthase kinase (GSK)3A (figure 2d) and GSK3B (figure 2e). They also displayed changes in cell surface receptors (figure 2f), with significant reductions of C5AR1 and CXCR2 in WHO4 and WHO5–6 patient neutrophils and LTB4R and S1PR4 only in WHO5–6 patients. Additionally, neutrophils from WHO5–6 patients displayed higher abundance of IL1R2 (figure 2g), a decoy interleukin-1 receptor, the inhibitory receptor VISTA (figure 2h) and the pattern recognition receptor Toll-like receptor (TLR)2 (figure 2i),

**TABLE 1** Patient characteristics at enrolment

| | SARS-CoV-2-positive | Non-COVID-19 LRTI | Noninfected controls |
|---|---|---|---|
| **Patients** | 84 | 91 | 42 |
| **Participant demographics** | | | |
| Age years | 66.2±14.7<br>69.5 (55.8–78.5) | 65.7±16.7<br>71.0 (58.0–78.0) | 57.4±18.6*<br>61.0 (35.8–74.2) |
| Sex at birth (male) | 41 (48.8) | 46 (50.5) | 16 (38.1) |
| **Comorbidities** | | | |
| Chronic cardiac disease | 36 (42.9) | 37 (40.7) | 14 (35.0) |
| Chronic respiratory disease | 24 (28.6) | 42 (46.2)* | 11 (27.5) |
| Diabetes | 10 (11.9) | 13 (14.3) | 7 (17.5) |
| BMI >30 kg·m$^{-2}$ | 24 (28.6) | 29 (31.9) | 11 (27.5) |
| **Severity at enrolment, clinical measurements and dexamethasone treatment** | | | |
| Hospitalisation | | | |
| Hospitalised, not requiring oxygen | 32 (38.1) | 52 (57.1)* | NA |
| Hospitalised, requiring oxygen | 33 (39.3) | 35 (38.5) | NA |
| Requiring ventilatory support | 19 (22.6) | 4 (4.4)*** | NA |
| Radiography findings | | | |
| No changes | 20 (23.8) | 36 (39.6)* | NA |
| Unilateral pneumonia | 14 (16.7) | 41 (45.1)**** | |
| Bilateral pneumonia | 49 (58.3) | 0**** | |
| Not done | 1 (1.2) | 14 (15.4)*** | |
| Time from symptom onset to hospitalisation (days) | 9.7±12.4<br>7.0 (4.0–11.0) | Not recorded | NA |
| Length of hospital stay (days) | 11.6±10.3<br>7.0 (4.0–15.5) | 7.1 ±6.1**<br>6.0 (1.0–8.0) | NA |
| 28-day mortality | 13 (15.5) | Not recorded | Not recorded |
| Dexamethasone treatment while hospitalised | 46 (53.6) | Not recorded | NA |
| Blood neutrophil count ×10$^9$ cells·L$^{-1}$ | 5.6±2.8 | 8.2±3.8**** | NA |
| Blood eosinophil count cells·µL$^{-1}$ | 40.1±71.9 | 121.3±173.3**** | NA |

Data are presented as n, mean±SD, median (interquartile range) or n (%), unless otherwise stated, and include all participants with baseline (day 1) samples available. SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; COVID-19: coronavirus disease 2019; LRTI: lower respiratory tract infection; BMI: body mass index; NA: not applicable. *: p⩽0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001 *versus* COVID-19 group.

along with proteins capable of lymphocyte immunosuppression, such as arginase (ARG)1 (figure 2j) and transforming growth factor (TGF)B1 (figure 2k). We also explored changes associated with dexamethasone (DEX) treatment across the patient groups, but found that >99.5% of proteins were unchanged between patients with or without DEX (supplementary table S5), although sample numbers available for this comparison were limited.

A stratified analysis of patients in the WHO3 group at baseline was performed based on subsequent deterioration to require supplemental oxygen. Proteins that were significantly increased in abundance in WHO3 patients who worsened showed enrichment for the translation initiation complex, the proteasome and terms related to vesicle trafficking proteins (supplementary figure S3a). They also recapitulated patterns linked to severity, including increased abundance of glycolytic proteins such as lactate dehydrogenase (LDH) A (supplementary figure S3b) and α-enolase (ENO1) (supplementary figure S3c), increased abundance of ARG1 (supplementary figure S3d), IL17RA (supplementary figure S3e), TLR2 (supplementary figure S3f) and CLEC4D (supplementary figure S3g). Furthermore, WHO3 patients who deteriorated also showed significantly higher abundance of the cap methyl transferase CMTR1 (supplementary figure S3h) reported to regulate a subset of IFN-induced proteins, BABAM2 (supplementary figure S3i) reported to deubiquitinate the IFNα receptor, UBE2L6 (supplementary figure S3j) the E2 enzyme responsible for ISG15nylation and increased abundance of a subset of IFN-induced proteins (supplementary figure S3k–m). In WHO3 patients, a larger IFN signature was associated with subsequent deterioration.

### *Longitudinal analysis of neutrophil proteomes following SARS-CoV-2 infection*

To gain insight into changes in neutrophil proteomes during disease progression, we also examined neutrophils from COVID-19 patients 4, 7, 15 and 29 days post-recruitment into the study. A principal

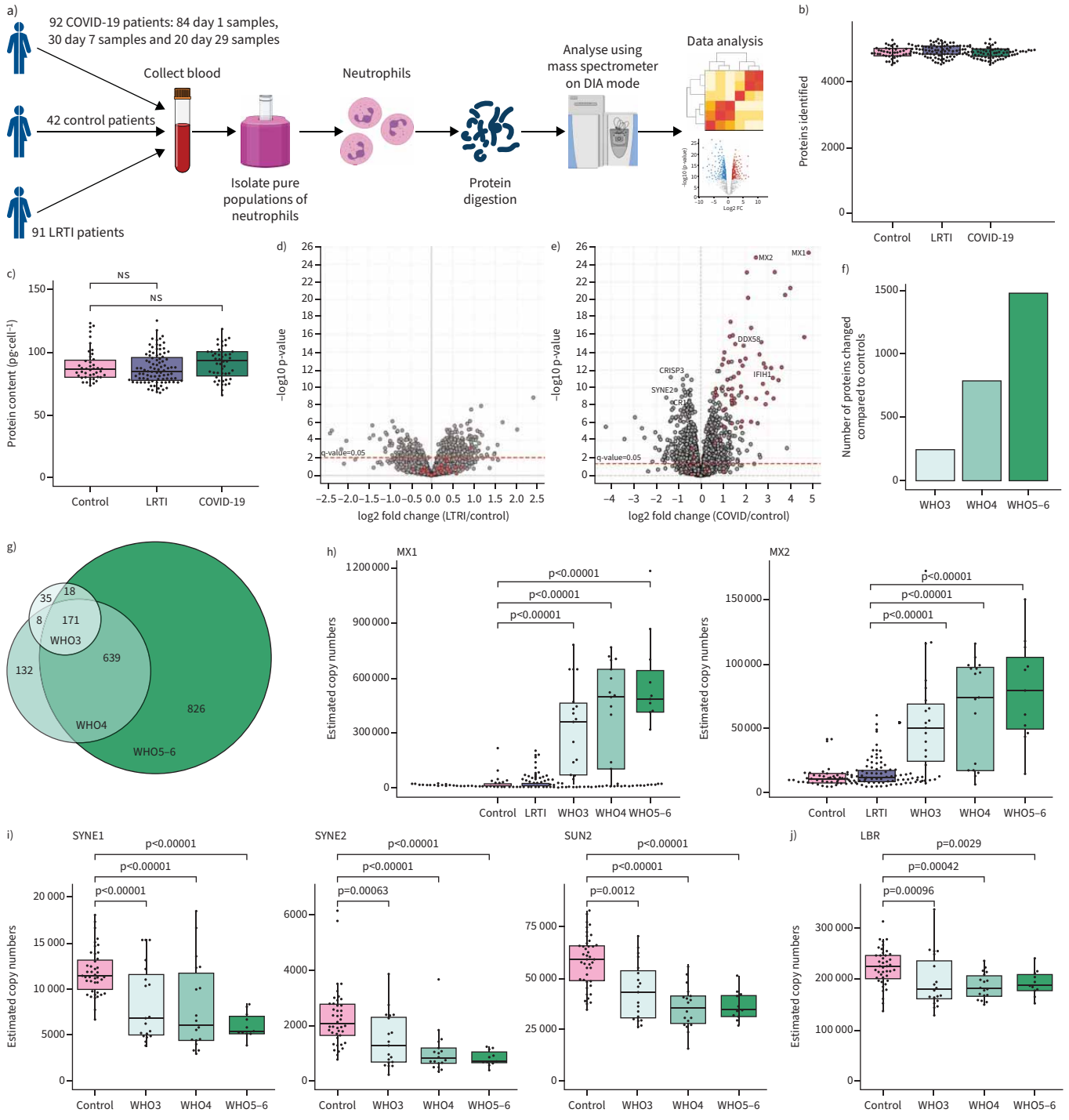


**FIGURE 1** Core coronavirus disease 2019 (COVID-19) neutrophil proteomic signature. a) Sample collection and processing workflow. 84 COVID-19 samples were included at day 1, and samples from an additional eight participants from whom day 1 samples were unavailable were also included in later longitudinal analyses. b) Number of proteins identified across all samples for control (n=42), lower respiratory tract infection (LRTI) (n=91) and COVID-19 (n=84). c) Estimated protein content for all samples for control, LRTI and COVID-19. Group numbers were the same as in b). d) Volcano plot showing the fold change and p-value comparing the neutrophil proteomes of LRTI patients to the controls. Interferon (IFN)-induced proteins are shown in red. The red dotted line represents q=0.05. e) Neutrophil proteomes of COVID-19 patients compared to the controls. IFN-induced proteins are shown in red. The red dotted line represents q=0.05. f) Number of statistically significant differentially abundant proteins in the neutrophil proteomes of World Health Organization (WHO)3 (moderate), WHO4 (severe) and WHO5-6 (critically severe) COVID-19 patients compared to the controls. g) Overlap of significantly altered proteins across the stratified COVID-19 patient cohorts *versus* controls. h) Estimated protein copy numbers for MX1 and MX2 across control (n=42), LRTI (n=91), WHO3 (n=18), WHO4 (n=19) and WHO5–6 (n=11) COVID-19 patients. Estimated protein copy numbers for i) SYNE1, SYNE2 and SUN2 and j) laminin B receptor (LBR) across control (n=42), LRTI (n=91), WHO3

(n=18), WHO4 (n=19) and WHO5–6 (n=11) patients. All p-values were calculated using empirical Bayes methods with moderated t-tests. For all boxplots, the whiskers extend from the hinge to the largest and smaller values no further than 1.5×interquartile range. DIA: data-independent acquisition; NS: nonsignificant.

component analysis of all COVID-19 neutrophil samples showed a clear separation between the neutrophil proteomes of day 1 WHO4 and WHO5–6 and all other samples, with progressive differences from day 7 to 29 (supplementary figure S4a). To explore this further, we compared the proteomes of COVID-19 patients at day 7 to those at day 1 (supplementary table S6) and found only 23 proteins were changed in patients who were WHO3 at baseline, while >1000 were changed in WHO4 and WHO5–6 patients (supplementary figure S4b).

Increases in abundance of metabolic proteins were conserved from day 1 to day 7 in WHO5–6 patients, as were the increases in abundance of the immunosuppressive proteins ARG1, TGFB1 and VISTA (supplementary figure S5). However, there were 538 proteins that were increased in abundance in neutrophils from day 7 when compared to day 1 neutrophils from WHO5–6 patients. These included GSK3α and GSK3β (supplementary figure S4c and d), migratory receptors, such as C5AR1 (supplementary figure S4e), S1PR4 (supplementary figure S4f), FPR1 (supplementary figure S4g), FPR2 (supplementary figure S4h) and CD177 (supplementary figure S4i), chemokines such as CXCL8 (supplementary figure S4j), tumour necrosis factor (TNF) signalling proteins such as TNFRSF1A (supplementary figure S4k), TRAF2 (supplementary figure S4l) and IKBKG (Nemo; supplementary figure S4m), the immunosuppressive ligand LGALS3 (supplementary figure S4n), the IL1R2 (supplementary figure S4o) and CSF2RA (supplementary figure S4p).

Analysis of proteins that were decreased in expression in neutrophils from COVID-19 patients at day 7 compared to day 1 found enrichment in proteins that mediate antiviral responses, necroptosis and chromatin silencing (supplementary figure S6a and b). One protein with the biggest reduction in abundance in day 7 *versus* day 1 neutrophils was cap methyltransferases (CMTR)1, the translational regulator of IFN-I proteins (supplementary figure S6c). Similarly, the neutrophil proteomes of WHO5–6 and also WHO4 patients displayed dramatic reductions in abundance of major IFN-I response proteins at day 7 compared to day 1 (supplementary figure S6d–f), suggesting progressive suppression of IFN signalling. The neutrophils from day 7 WHO5–6 patients also showed marked reductions in abundance of proteins associated with chromatin structure like H1 histones (supplementary figure S6g) and high mobility group proteins (supplementary figure S6h) compared to neutrophils analysed on day 1, suggesting changes in nucleosome structure by day 7. Reduced abundance of signalling molecules and receptors important for inflammatory responses, including DAP12 (TYROBP; supplementary figure S6i), FcRy (FCER1G; supplementary figure S6j), NFKBIB (supplementary figure S6k) and NFKBIE (supplementary figure S6l) was also evidenced in this group at day 7.

### *Neutrophil proteomic signatures of recovered* versus *nonrecovered COVID-19 patients*

To explore longer term effects of SARS-CoV-2 infection on proteins associated with neutrophil phenotypes we analysed neutrophils derived from COVID-19 patients 29 days after enrolment. For these analyses, patients were stratified into those who were recovered (WHO1) and those with persistent symptoms and limitations or still hospitalised (nonrecovered; WHO2–3). Participant characteristics for those within this stratified analysis are detailed in supplementary table S12. 404 proteins demonstrated significantly different expression at day 29 in recovered participant neutrophils compared to control participants (figure 3a, supplementary table S7), in nonrecovered participants there were 1111, with some of the changes being small in magnitude (only 257 proteins with fold change >1.5 in recovered patients and 540 in nonrecovered patients). The acute changes seen in IFN-I, signalling receptors and immunosuppressive proteins were no longer present at day 29. We calculated the estimated protein content per cell and found the neutrophils from recovered patients were not significantly different to controls (figure 3c), unlike nonrecovered patients, thus the neutrophil proteomes of recovered patients were increasingly similar to controls. However, there were exceptions such as increased expression of the colony-stimulating factor (CSF) receptor CSF2RA (figure 3d), and reduced abundance of the Fc receptor common γ chain FCER1G (figure 3e), glycogen phosphorylase PYGL (figure 3f) and the migratory receptors C3AR1 and LTB4R (figure 3g).

All of these proteins (figure 3d–g) were also significantly altered in the neutrophils from nonrecovered patients. However, nonrecovered patients had a more profoundly different proteome that also included markers of immature neutrophils (figure 3h and i). Levels of the activation marker CD64 were also

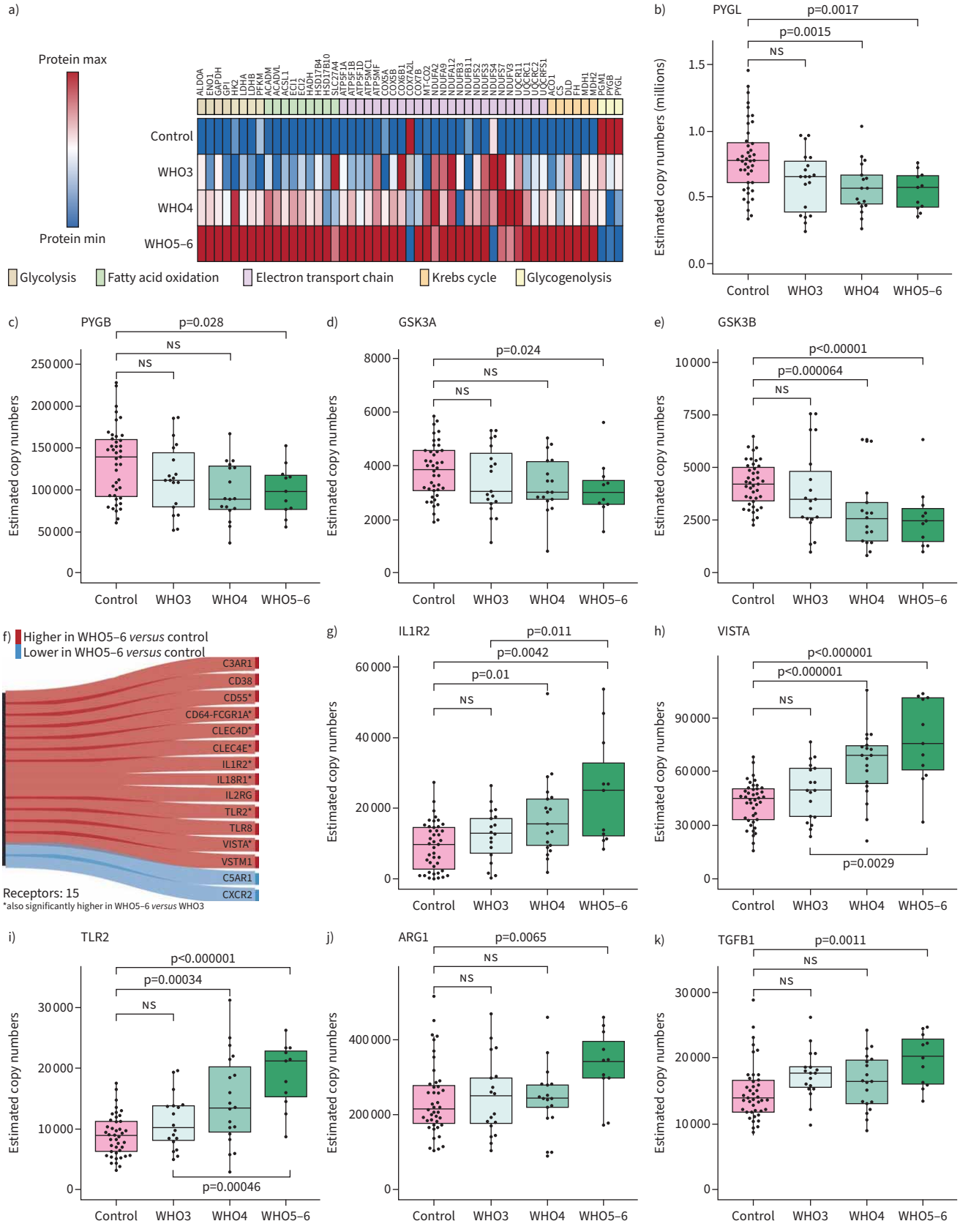


**FIGURE 2** Metabolic, signalling and immunosuppressive changes in coronavirus disease 2019 (COVID-19). a) Metabolic proteins that were significantly changed in abundance in World Health Organization (WHO)5–6 COVID-19 patients. The data are normalised to the maximum value of

each protein. Estimated protein copy numbers for b) glycogen phosphorylase (PYG)L, c) PYGB, d) glycogen synthase kinase (GSK)3A and e) GSK3B in the neutrophil proteomes across control (n=42), WHO3 (n=18), WHO4 (n=19) and WHO5–6 (n=11) patients. f) Immunomodulatory receptors significantly altered in the neutrophil proteomes of WHO5–6 compared to controls. Proteins in red are significantly increased in abundance in the neutrophil proteomes of WHO5–6 patients; proteins in blue were significantly decreased in abundance. Estimated protein copy numbers for g) interleukin-1 receptor type 2 (IL1R2), h) VISTA, i) Toll-like receptor (TLR)2, j) arginase (ARG)1 and k) transforming growth factor (TGF)B1 in the neutrophil proteomes across control (n=42), WHO3 (n=18), WHO4 (n=19) and WHO5–6 (n=11) patients. All p-values were calculated with limma using empirical Bayes methods with moderated t-tests. For all boxplots the whiskers extend from the hinge to the largest and smaller values no further than 1.5×interquartile range. NS: nonsignificant.

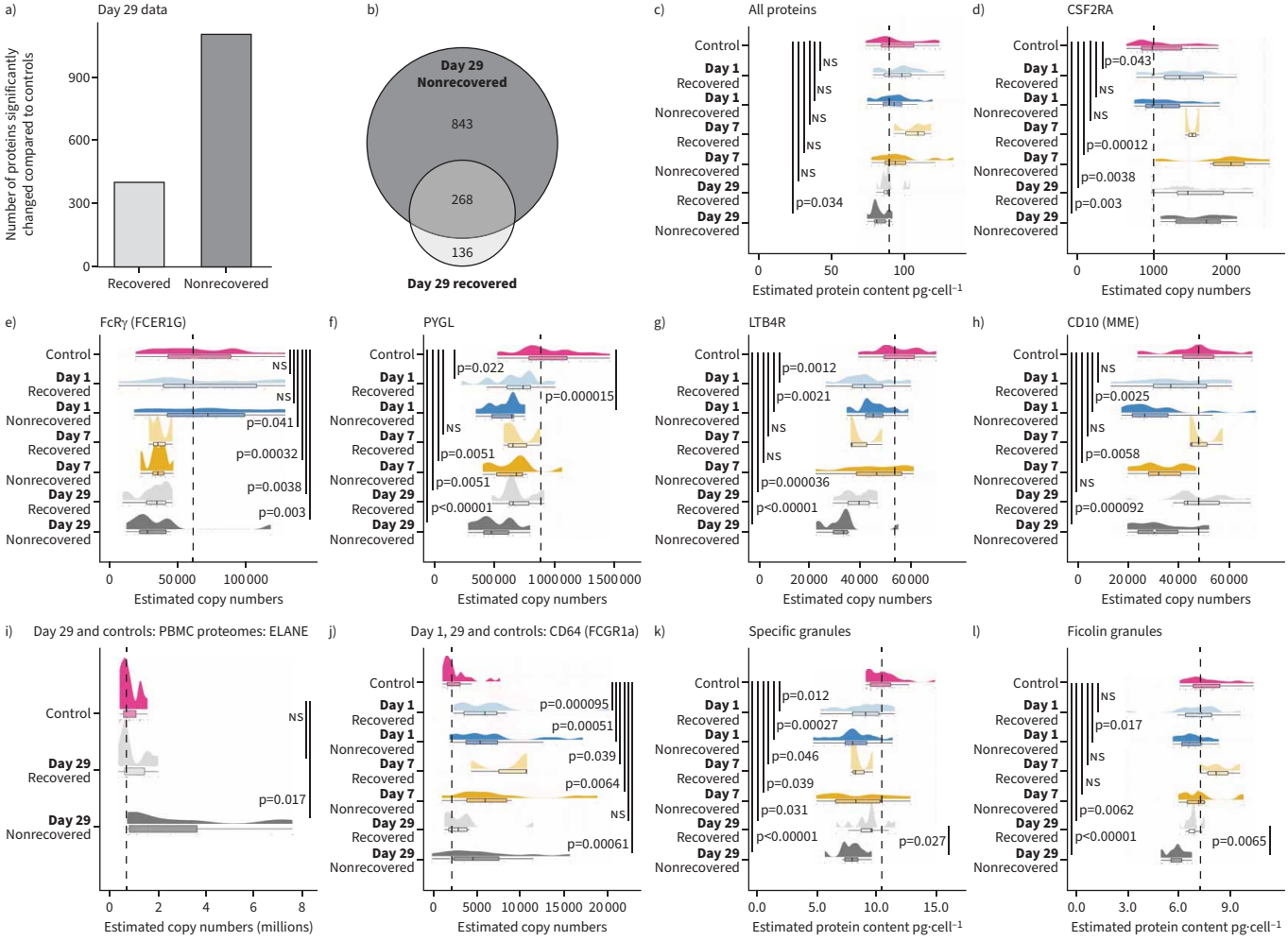


**FIGURE 3** Changes in recovered and nonrecovered patients at day 29. a) Number of statistically significant differentially abundant proteins overall in the neutrophil proteomes of recovered or nonrecovered patients at day 29 compared to controls. b) Overlap of significantly changed proteins in the recovered and nonrecovered patients at day 29 compared with controls. c) Raincloud plot showing the estimated protein content in picograms per cell. Coronavirus disease 2019 (COVID-19) patients were stratified by day 29 status (recovered or nonrecovered). Raincloud plot showing the estimated protein copy numbers for d) CSF2RA, e) FcRy (FCER1G), f) glycogen phosphorylase (PYG)L, g) LTB4R and h) CD10 across controls, day 1, day 7 and day 29 COVID-19 patients stratified into recovered and nonrecovered (*i.e.* by day 29 World Health Organization (WHO) score). Each data point represents protein levels in a unique sample provided at the specified time point. i) Raincloud plot showing the estimated protein copy numbers in the peripheral blood mononuclear cell (PBMC) proteomes for ELANE across controls and COVID-19 patients at day 29 stratified into WHO1 (*i.e.* recovered) and WHO2–3 (*i.e.* nonrecovered). Raincloud plot showing j) the estimated protein copy numbers for CD64, the estimated protein content for k) specific (secondary) granules and l) ficolin granules across controls, day 1, day 7 and day 29 COVID-19 patients stratified into recovered and nonrecovered. Controls (n=22), day 1 recovered (n=14), day 1 nonrecovered (n=13), day 7 recovered (n=3), day 7 nonrecovered (n=10), day 29 recovered (n=10), day 29 nonrecovered (n=10). c), i), k) and l) have p-values calculated using Welch's t-test. All other p-values were calculated using empirical Bayes methods with moderated t-tests. All raincloud plots include a density blot and a boxplot. For all boxplots the whiskers extend from the hinge to the largest and smaller values no further than 1.5×interquartile range. NS: nonsignificant.

significantly higher in abundance in the proteomes of neutrophils from nonrecovered patients compared to controls (figure 3j). Neutrophils from nonrecovered patients had reductions in a select set of granule proteins (figure 3k), including significant reductions in proteins from specific and ficolin granules (figure 3l). These changes did not appear linked to neutrophil maturity markers; gelatinase granules and secretory vesicles, the last set of granules to be produced during development, were not significantly reduced in the neutrophils from nonrecovered patients compared to control neutrophils (supplementary figure S7).

A distinctive feature of the neutrophil proteome in nonrecovered patients was the systematic reduction in proteins involved in vital metabolic pathways. These changes included reduced abundance of rate-limiting regulators of glycolysis like the glucose transporter SLC2A3, hexokinase 3 (HK3) and the lactate transporter SLC16A3 (figure 4a). Interestingly reduced expression of SLC2A3 and HK3 (figure 4b and c) was only found in the neutrophils isolated at day 29 from nonrecovered patients and was not seen earlier in the course of disease. This was also the case for glycogen synthesis regulators such as the GBE1, a glycogen branching enzyme, and glycogenin 1 (GYG1) (figure 4d and e).

Neutrophils derived from nonrecovered patients showed consistent reductions in the abundance of receptors that control neutrophil migration (figure 4f), including C3AR1 and LTB4R, which were surprisingly also decreased in neutrophils from recovered patients. However, in nonrecovered individuals this also included other vital receptors like the sphingosine-1-phosphate receptor S1PR4 (figure 4g) and the chemokine receptor CXCR2 (figure 4h). Additionally, reduced abundance of integrins CD18, CD11b, CD11a (figure 4i and j, supplementary figure S8) and reduced expression of SYK (figure 4k), a kinase that mediates integrin and Fc receptor signalling, was observed; all of these changes were only present at day 29. Day 29 nonrecovered patients also showed modest reductions of signalling molecules that mediate the functions of inhibitory receptors such as SH-2-containing inositol 5′ phosphatase SHIP-1 (INPP5D; figure 4m), and the SH-2-containing tyrosine phosphatase SHP-1 (PTPN6; figure 4n). Furthermore, reductions in abundance of inhibitory receptors of the C-type-lectin family and leukocyte immunoglobulin-like family were identified (figure 4l).

## Discussion

This study provides a valuable resource centred around the large-scale proteomic characterisation of hundreds of neutrophil proteomes in a control cohort, in acute disease and in recovery, providing in-depth mapping of longitudinal changes related to COVID-19. Our study is one of many demonstrating the potential for large-scale proteomics and multi-omics to profile immune responses in respiratory disease [39–42]. We identified proteomic signatures associated with severe acute disease such as transient IFN-I signalling and upregulation of key neutrophil receptors and metabolic pathway components. Importantly, both persistent and emerging changes associated with nonrecovery at day 29 post-enrolment were found, including a reduction in certain granule protein groups and reduced inhibitory and migratory machinery and metabolic protein abundance.

Prior studies of small cohorts with COVID-19-associated ARDS identified an IFN-I proteomic signature in neutrophils [33, 43]. The present data revealed that this signature is present in most neutrophil proteomes derived from COVID-19 patients at day 1, regardless of disease severity. However, some differences were present, where an increased IFN-I signature in WHO3 patients associated with subsequent worsening condition, conversely an increase in WHO5–6 patients correlated with better outcomes (supplementary table S8). As IFN-I levels can reflect viral load, the link with viral titres and outcomes in mild and severe COVID-19 would be of interest for future investigations [44, 45]. Furthermore, the IFN-I signature was transient and its kinetics associated with disease severity. Neutrophils from WHO3 patients sustained an IFN-I signature for >1 week post-admission, whereas WHO4 and WHO5–6 patients lost this signature more rapidly. This divergence suggests the potential for stratified therapeutic interventions, using MX1 as a marker for IFN-I activity, as described previously [46], whereby levels of MX1 could be measured in blood using a lateral flow test [47]. This could theoretically be used to stratify critically ill patients lacking an IFN-I response who could potentially benefit from IFN-β treatment during acute severe viral infection.

In addition, the data identified neutrophil markers of disease severity including molecules known to have immunosuppressive functions, such LGALS3, ARG1 and TGFB1, all of which were still elevated in WHO5–6 COVID-19 patients for ⩾7 days post-admission. At day 7, increased abundance of important migratory receptors such as S1PR4, FPR1 and FPR2, along with higher abundance of CXCL8, a potent chemoattractant, was revealed in WHO5–6 patients; this suggests that their neutrophils may have increased capacity to migrate into the tissues while having an augmented immunosuppressive capacity. Severity analyses also highlighted important receptors like the inhibitory receptor VISTA and the pattern recognition receptors TLR2 and CLEC4D, all of which remained significantly increased in abundance

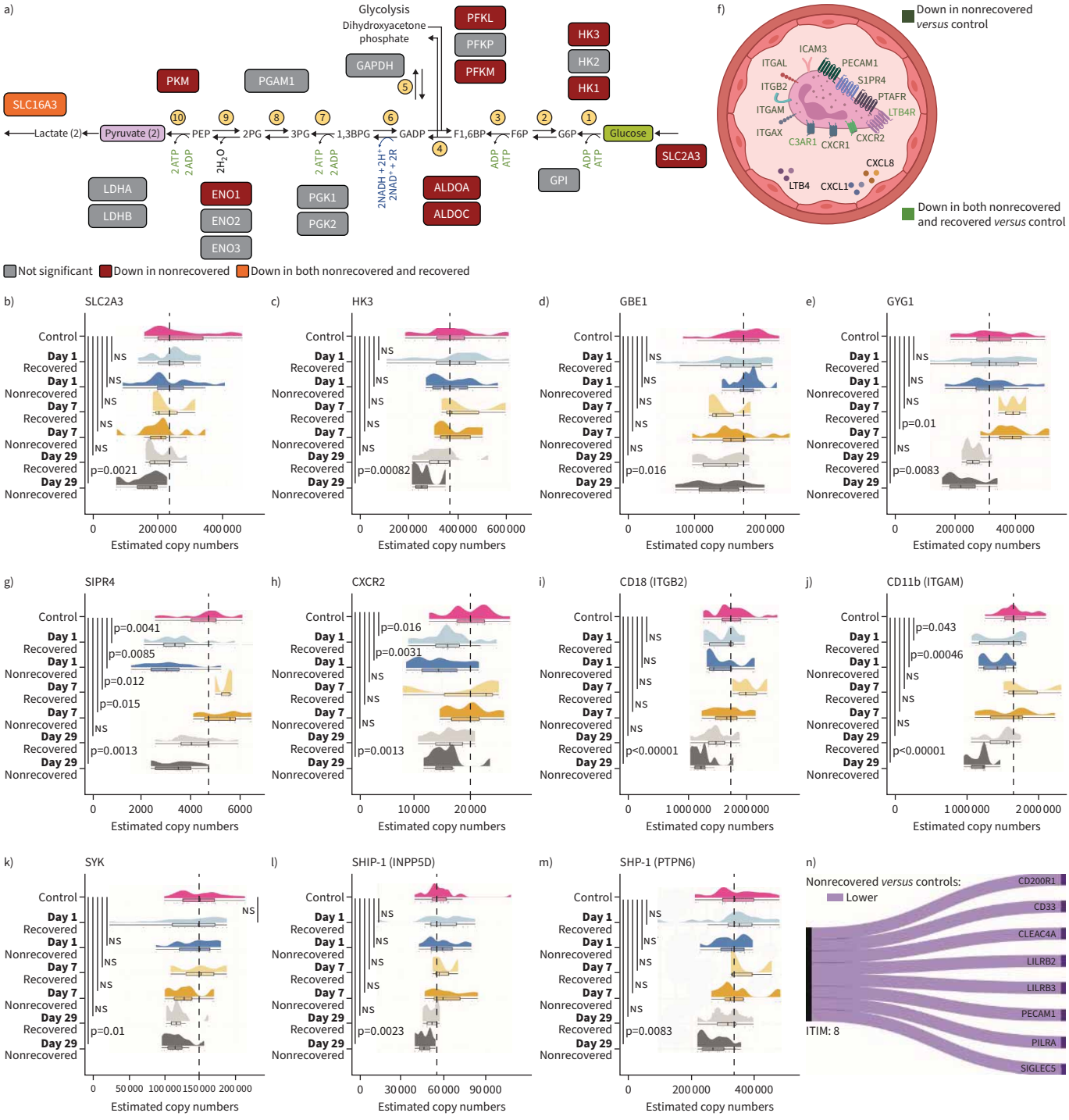


**FIGURE 4** Neutrophil migratory, metabolic and inhibitory machinery at day 29. a) Glycolytic pathway highlighting proteins that were significantly reduced in abundance in the nonrecovered patients. Raincloud plot showing the estimated protein copy numbers for b) SLC2A3, c) hexokinase 3 (HK3), d) GBE1 and e) glycogenin 1 (GYG1) across controls, and day 1, day 7 and day 29 coronavirus disease 2019 (COVID-19) patients stratified into recovered and nonrecovered. Each data point represents protein levels in a unique sample provided at the specified time point. f) Migratory receptors and integrins that are exclusively reduced in abundance in the nonrecovered patients compared to controls. Raincloud plot showing the estimated protein copy numbers for g) sphingosine-1-phosphate receptor 4 (S1PR4), h) CXCR2, i) CD18 (ITGB2), j) CD11b (ITGAM), k) SYK, l) SH-2-containing inositol 5′ phosphatase (SHIP)-1 (INPP5D), m) SH-2-containing tyrosine phosphatase (SHP)-1 (PTPN6) across controls, day 1, day 7 and day 29 COVID-19 patients stratified into recovered or nonrecovered (*i.e.* not recovered at day 29). n) Sankey diagram showing the inhibitory receptors that are significantly decreased in abundance in the neutrophil proteomes of nonrecovered patients. COVID-19 patients providing samples at the respective time points were stratified by their day 29 status (recovered or nonrecovered). Controls (n=22), day 1 recovered (n=14),

day 1 nonrecovered (n=13), day 7 recovered (n=3), day 7 nonrecovered (n=10), day 29 recovered (n=10), day 29 nonrecovered (n=10). All p-values were calculated using empirical Bayes methods with moderated t-tests. All raincloud plots include a density blot and a boxplot. For all boxplots the whiskers extend from the hinge to the largest and smaller values no further than 1.5×interquartile range.

⩾7 days post-admission. The association of high VISTA and TLR2 abundance in neutrophils with COVID-19 disease severity is intriguing and offers potential targets to limit inflammation. TLR2 has been reported to bind to SARS-CoV-2 spike protein as well as other viral pathogens such as respiratory syncytial virus and trigger inflammatory cascades [48, 49], suggesting antagonistic antibodies could limit inflammation. Similarly, VISTA is both an inhibitory ligand and receptor, and has been suggested as a target to manage excessive innate immune activation [50]. The TNF-receptor TNFR1 (TNFRSF1A) was also significantly increased from baseline to day 7 in the most severe patients; TNFR1 has been associated with ageing and risk of mortality in COVID-19 [51], with a role in neutrophil hyperactivation and mobilisation [52]; and TNFR1 antibody treatment has shown promising anti-inflammatory effects in healthy volunteers [53].

We also noted metabolic changes in neutrophils associated with severity and recovery status. Critically ill patients displayed changes in abundance of proteins across a wide array of metabolic pathways, some of which could be explained by an increased proportion of immature neutrophils in patients with severe disease, and others (*e.g.* LDHA) have been linked to hypoxia [54, 55] and NET production [56]. These changes were present at earlier time points, but resolved by day 29. The only consistent metabolic change across all time points was a reduction in the abundance of the rate-limiting enzymes of glycogenolysis, PYGL and PYGB.

COVID-19 causes prolonged illness in a subset of patients and a novel aspect of this study was the analysis of neutrophil proteomes during recovery. At day 29 post-enrolment, recovered patients displayed neutrophil proteomes increasingly similar to controls, while nonrecovered patients had abnormal metabolic profiles distinct from the changes seen at day 1 and 7. Neutrophils have been shown to depend on glycolysis for energy production when the environment is nutrient rich [14, 57–59] and to default to glycogen breakdown when it is not [49]. These two metabolic pathways are vital to neutrophil functions, and our data show significantly lower abundance of key rate-limiting proteins of both glycolysis and glycogenolysis in the proteomes of nonrecovered patients. Reductions in both pathways impair the bioenergetic capacity of neutrophils and have been shown to lead to impaired killing and impaired survival capacities [60].

The potentially dysfunctional phenotype of post-COVID-19 neutrophils was not limited to metabolism. Peripheral blood neutrophils depend on receptors and integrins to recognise migratory signals and perform the extravasation process. The neutrophil proteomes of nonrecovered patients displayed a consistent reduction in migratory receptor levels, from the chemokine and complement receptors to spingosine-1-phosphate receptors. They also showed significantly reduced abundance of subunits of the Mac-1 and LFA-1 complexes that mediate leucocyte extravasation [61]. These changes may suggest reduced capacity of neutrophils to migrate from the blood into the sites of inflammation, potentially increasing vulnerability to secondary infections; mutations that affect function or abundance of CD18 in human neutrophils cause leukocyte adhesion deficiency and result in increased susceptibility to bacterial infections [62]. Nonrecovered COVID-19 patients also displayed a systematic reduction in the abundance of inhibitory receptors and phosphatases which are required to limit neutrophil activation [63–67]. A recent study following-up patients 3 and 6 months after severe acute COVID-19 showed persistent and differential neutrophil-associated signatures in plasma samples in those demonstrating interstitial lung changes [34]. The reduction in inhibitory machinery proteins evidenced in the present study might explain the loss of granule proteins; potentially representing a persistent degranulating neutrophil phenotype at day 29. Whether this hypothesised phenotype could have deleterious consequences remains to be determined; it has previously been identified as a part of a tissue protective mechanism [68] and also as a feature of an activated phenotype in the bloodstream with potentially lethal consequences [14].

A key limitation of the present study is its descriptive nature, and therefore mechanistic conclusions cannot be made. Evaluation of relevant neutrophil functions alongside proteomic analysis in a further cohort with acute respiratory infection would be pertinent further work in addition to characterisation of airway cells. This study was conducted predominantly during the first and second waves of the UK COVID-19 pandemic and includes only hospitalised patients. Therefore, the impact of subsequent circulating variants on systemic neutrophil functions in a predominantly vaccinated population and in outpatients, remains to

be confirmed. In addition, while we studied symptoms up to day 29, some patients with COVID-19 experience much longer-term symptoms; whether the persistent neutrophil dysfunction that we have observed continues beyond day 29 requires further study.

In conclusion, COVID-19 and other respiratory viral infections remain a risk to global health. Utilising data from patients enrolled during the first and second waves of the COVID-19 pandemic, these data highlight important areas to direct further research, including key follow-up studies with neutrophil functional testing post-infection. We performed one of the most in-depth proteomic profiling investigations of peripheral blood neutrophils to date, and identified a core neutrophil proteomic signature associated with acute disease and identified key neutrophil receptors linked to disease severity which could be potential therapeutic targets. Furthermore, this study characterised a molecular phenotype linked to delayed recovery which may further understanding of later, long COVID as well as avenues for investigation of delayed recovery in other viral infections such as influenza, and should be investigated further in these patients.

Acknowledgements: We would like to thank Sarah Walmsley (Centre for Inflammation Research, Institute for Regeneration and Repair, University of Edinburgh, Edinburgh, UK) for insightful discussions relating to the data. We thank the team members of the proteomics facility at the University of Dundee (Dundee, UK), in particular Kenneth Beatie, Douglas Lamont and Abdelmadjid Atrih, for support in processing the mass spectrometry samples.

Data availability: All the mass spectrometry files, as well as the processed search result files are available at PRIDE under the identifiers PXD036082 and PXD036089. All the processed data are available to be explored on the Immunological Proteome Resource.

Ethics statement: This study was approved by the East of Scotland Research Ethics Committee (REC number: 20/ES/0055).

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Conflict of interest: H.R. Keir has received speaker fees from Insmed. A. Shoemark has received grant funding from AstraZeneca. A.J. Brenes has received support for attending meetings from the British Society for Proteome research. J.D. Chalmers has received research grants from AstraZeneca, Boehringer Ingelheim, GlaxoSmithKline, Gilead Sciences, Grifols, Novartis, Insmed and Trudell, and received consultancy or speaker fees from Antabio, AstraZeneca, Boehringer Ingelheim, Chiesi, GlaxoSmithKline, Insmed, Janssen, Novartis, Pfizer, Trudell and Zambon. All other authors declare no conflicts of interest.

Support statement: This work was funded by the Chief Scientist Office Rapid Response COVID-19 Research Grant (COV/DUN/20/01 and COV/DUN/20/04), the UK Coronavirus Immunology Consortium (MR/V028448) and the Wellcome Trust (205023/Z/16/Z). D.A. Cantrell is supported by a Wellcome Trust Principal Research fellowship (097418/Z/11/Z). J.D. Chalmers is supported by the GSK/Asthma and Lung UK Chair of Respiratory Research (C17-2) and a Scottish Senior Fellowship from the Chief Scientist Office (SCAF17/03). Funding information for this article has been deposited with the Crossref Funder Registry.

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