Characterization of the Inflammatory Response to Severe COVID-19 Illness

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At a Glance

Scientific Knowledge: The characteristics of the cytokinemia associated with COVID-19 are incompletely understood. Data on immunometabolic alterations in patients with severe illness are scarce.

Add to the Field: Here we demonstrate that the COVID-19 cytokinemia is distinct from that observed in other critical care presentations, with marked differences in the balance between pro-inflammatory and anti-inflammatory cytokines, and a blunted alpha-1 antitrypsin acute-phase response. Neutrophils display altered immunometabolism in severe COVID-19.

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Abstract

RATIONALE: Coronavirus disease 2019 (COVID-19) is a global threat to health. Its inflammatory characteristics are incompletely understood.

OBJECTIVES: To define the cytokine profile of COVID-19, and to identify evidence of immunometabolic alterations in those with severe illness.

METHODS: Levels of interleukin (IL)-1β, IL-6, IL-8, IL-10 and soluble TNF receptor 1 (sTNFR1) were assessed in plasma from healthy volunteers, hospitalized-but-stable COVID-19 patients ($COVID_{stable}$), COVID-19 patients requiring intensive care unit (ICU) admission ($COVID_{ICU}$) and individuals with severe community-acquired pneumonia requiring ICU support (CAP_{ICU}). Immunometabolic markers were measured in circulating neutrophils from patients with severe COVID-19. The acute phase response of alpha-1 antitrypsin (AAT) to COVID-19 was also evaluated.

MAIN RESULTS: IL-1β, IL-6, IL-8 and sTNFR1 were all increased in patients with COVID-19. $COVID_{ICU}$ patients could be clearly differentiated from $COVID_{stable}$, and demonstrated higher levels of IL-1β, IL-6 and sTNFR1 – but lower IL-10 – than CAP_{ICU}. COVID-19 neutrophils displayed altered immunometabolism, with increased cytosolic PKM2, phosphorylated PKM2, HIF-1α and lactate. The production and sialylation of AAT increased in COVID-19, but this anti-inflammatory response was overwhelmed in severe illness, with the IL-6:AAT ratio markedly higher in patients requiring ICU admission ($P<0.0001$). In critically unwell COVID-19 patients, increases in IL-6:AAT predicted prolonged ICU stay and mortality, while improvement in IL-6:AAT was associated with clinical resolution ($P<0.0001$).

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CONCLUSIONS: The COVID-19 cytokinemia is distinct from that of other types of pneumonia leading to organ failure and ICU need. Neutrophils undergo immunometabolic reprogramming in severe COVID-19 illness. Cytokine ratios may predict outcomes in this population.

Abstract word count: 250 words

Key words: Coronavirus; COVID-19; Interleukin-1β; Interleukin-6; Interleukin-10; Neutrophils, Alpha-1 antitrypsin; Immunometabolism.

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Introduction

In late 2019, multiple pneumonia cases of unknown origin were identified in Wuhan, China (1). The inciting pathogen was subsequently identified as a novel enveloped RNA betacoronavirus, now known as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), giving rise to coronavirus disease 2019 (COVID-19). The World Health Organization (WHO) has since declared COVID-19 a public health emergency of international concern. As of May 2020, more than 8 million laboratory-confirmed cases have been documented globally, with over 450,000 deaths (2).

Little is known about the pre-hospital course of the disease. However, in-hospital studies have described a pro-inflammatory syndrome with a disproportionately high rate of progression to acute respiratory distress syndrome (ARDS), acute renal failure, shock and arrhythmia (3, 4). Profound hypoxemia at initial presentation is common. Current management remains supportive, focusing on supplemental oxygen, vasopressors to maintain perfusion pressure, and mechanical support in the event of end-organ failure. Despite the implications for global health, the inflammatory characteristics of patients with COVID-19 are incompletely understood, as are the inflammatory mediators and attendant molecular mechanisms underlying them. Indeed, whether a distinct COVID-19 inflammatory profile exists – as opposed to the inflammation seen in these patients being merely the product of a generic response to severe illness – remains unclear, as does the question of whether subphenotypes exist within the COVID-19 cohort. Similarly, the degree of the endogenous anti-inflammatory response to SARS-CoV-2 infection, and the role of this response in delineating those who recover from those who experience severe morbidity and mortality, has not been fully elucidated.

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Critical illness is notable for markedly increased energy demands. Adenosine triphosphate (ATP) serves as the building block of this energy, and is produced via two linked metabolic pathways, glycolysis and the tricarboxylic acid (TCA) cycle, also known as Krebs' cycle. While quiescent human neutrophils demonstrate TCA cycle activity, their metabolism is predominantly glycolytic. Certain circumstances, such as hypoxemia, infection and inflammation, stand to shift the metabolism of circulating neutrophils further towards glycolysis.

The key step in glycolysis is the conversion of phosphoenolpyruvate (PEP) to pyruvate, a nonequilibrium reaction catalyzed by pyruvate kinase (PK). Two major PK isoenzymes exist. PKM1 is considered to be the more enzymatically active form, and promotes entry of pyruvate into Krebs' cycle via pyruvate dehydrogenase. PKM2, on the other hand, displays lower enzymatic activity than PKM1, and instead promotes increased glycolysis with resultant cytosolic accumulation of lactate and other metabolic intermediates (5). PKM2 can assume different conformations, dimers existing in equilibrium with tetramers. Critically, PKM2 dimers are capable of nuclear translocation, where they directly interact with the transcription factor hypoxia-inducible factor-1α (HIF-1α) (6, 7). At the nucleus, in addition to driving the adaptor response to hypoxia (8), HIF-1α transcribes glycolytic machinery and pro-inflammatory cytokines (9), in particular the master pro-inflammatory cytokines interleukin (IL)-1β, a pivotal cause of chronic and acute inflammation (10), and generation of the febrile response in humans (11), and IL-6. This pro-inflammatory shift is compounded by concomitant downregulation of anti-inflammatory and pro-resolution cytokines such as IL-10 (6). In addition to their well-defined role in systemic inflammation, IL-1β, IL-6 and IL-10 have been shown to drive airway inflammation in ARDS, a syndrome characterized by acute hypoxemic respiratory failure,

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disruption of the alveolar-capillary barrier, and excessive neutrophil-predominant inflammation (12, 13).

In this study we evaluated the in-hospital cytokine profile of the COVID-19 patient, to determine whether the clinical status of these individuals could be identified using such a profile, and whether the inflammation seen in COVID-19 could be distinguished from that seen in patients with severe non-COVID pneumonia requiring ICU support. Given the role of immunometabolism in inflammatory cytokine regulation, we also searched for evidence of metabolic reprogramming of circulating immune cells during severe COVID-19 illness. Finally, we assessed the acute phase anti-inflammatory response of alpha-1 antitrypsin (AAT) to COVID-19, and whether shortcomings in this response were associated with worse outcome in those with severe illness.

Methodology

Ethical approval was received from the Beaumont Hospital Ethics Committee (REC #18/52, #17/06, with both projects amended specifically for inclusion of COVID-19 subjects). Healthy controls (HC, n=15) and patients with a laboratory-confirmed diagnosis of COVID-19 infection who were hospitalized and symptomatic but clinically stable 7 days after onset of symptoms ($COVID_{stable}$, n=20), were compared to patients positive for COVID-19 and symptomatic for 7 days who required admission to the ICU at that time for intubation and mechanical ventilation in the context of hypoxemic respiratory failure ($COVID_{ICU}$, n=20). Patients with severe community-acquired pneumonia (CAP) receiving intubation and mechanical ventilation were also studied for the purpose of further comparison (CAP_{ICU}, n=15). Initial blood samples for cytokine measurements and hospital-based blood testing in the ICU cohorts were obtained from

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the same blood draw at the time of intubation, with follow-up sampling at 2-day intervals while in ICU. A confirmed case of COVID-19 was defined by a positive result on a reverse-transcriptase–polymerase-chain-reaction (RT-PCR) assay of a specimen collected on a nasopharyngeal swab. Prolonged ICU stay was defined as an ICU length of stay of greater than 12 consecutive days. Patients were excluded if they were immunosuppressed, receiving long-term oral corticosteroids, anti-IL-1, anti-IL-6 or anti-TNF therapy, known to be pregnant, had active neoplasia, or had a history of vasculitis or connective tissue disease.

Sample preparation

Human blood neutrophils were isolated as previously described (14) using dextran sedimentation followed by lymphoprep (Axis-Shield Poc AS, Oslo, Norway) centrifugation. Neutrophil cytosolic and nuclear fractions were obtained at 4^{o}C in the presence of protease and phosphatase inhibitors (Table S1) as previously described (15). For each neutrophil cytosol preparation, $1x10^7$ neutrophils were used. For nuclear extracts, $8.8x10^6$ neutrophils were required. Plasma was isolated by centrifugation of whole blood in lithium heparin tubes at 240 x *g* for 5 min at room temperature (RT). AAT protein phenotypes in plasma were determined by immunofixation of serum glycoforms via isoelectric focusing gel electrophoresis prior to analysis (16).

Plasma cytokines

Cytokine levels were measured in plasma from the whole blood of HC (n=15), $COVID_{stable}$ (n=20), $COVID_{ICU}$ (n=20), and CAP_{ICU} (n=15). IL-1β, IL-6, IL-8, IL-10 and soluble TNF receptor 1 (sTNFR1), were measured by ELISA (all R&D systems, Minneapolis MN, USA) in accordance with the manufacturer's instructions. The lower limits of detection for each assay are provided in Table S2. Where cytokine levels were undetectable, a value of 0 was assigned.

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Immunometabolic markers

PKM2, phosphorylated PKM2 and HIF-1α were detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blotting. Cytosolic lactate and pyruvate were measured by colorimetric assay (both Abcam, Cambridge, UK). For experiments involving immunometabolic markers, samples from $COVID_{ICU}$ patients (n=8) were compared to samples from HC (n=8) matched for age, sex and body mass index (BMI). A complete list of product details for key resources used is available in Table S2.

Statistical analysis

Results are reported as absolute numbers, medians, or means and standard deviations, as appropriate. Categorical variables are summarized as counts and percentages. No imputation was made for missing data. Changes in the levels of biomarkers between groups were analyzed using GraphPad Prism 8.0 software for Windows, with ~~a paired~~ an unpaired t-test used for comparisons between two groups in the event of normally distributed data, and nonparametric Mann-Whitney testing used in the event of data failing the test for normality. Comparisons across 3 or more groups were by ANOVA or Kruskal-Wallis test. Changes in biomarkers over time between patient groups were analysed using patient group and direction of change in biomarker (slope) as factors. P-values were derived by Tukeys' post-hoc multiple comparison test to control for familywise error rate. Where significant interaction between factors was observed, groupwise P-values are used to refer to simple effects within factors. A value of $P<0.05$ was considered statistically significant.

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Results

Characteristics of the COVID-19 cohorts

The demographic and clinical characteristics of the COVID-19 patients are shown in Table 1, with further details regarding the $COVID_{ICU}$ group, as well as the HC and CAP_{ICU} groups, provided in Tables S3-S5. The mean (+/-SD) age of the overall COVID-19 cohort was 55.5 +/- 17.7 years; 62.5% were men. The most common symptoms on admission to the hospital were fever, dyspnea and cough. The mean duration of symptoms prior to blood sampling was 7 days. 15% had a history of travel to a country where COVID-19 was declared endemic within the previous month (China, South Korea, Italy, Spain or Iran). 45% reported being in close contact with a confirmed case, though this may be under-representative, owing to the testing restrictions in effect in Ireland at the time of the study. 30% were healthcare workers. Community-acquired infection, defined for the purpose of this study as a positive diagnosis in the absence of either known close contact with any other confirmed case or recent travel to an area officially designated as high-risk, accounted for 40% of the positive cases described. Comorbidities were common in the study population. Over one quarter of the total cohort had pre-existing lung disease, 40% had hypertension, 20% had diabetes mellitus, 18% had documented coronary artery disease and 23% had chronic kidney disease. Twenty patients (50%) were current or former smokers and 5 were active vapers. COVID-19 patients who required ICU support also had elevated white cell counts, and higher levels of circulating neutrophils, C-reactive protein (CRP), fibrinogen and lactate when compared to $COVID_{stable}$ patients (Table 2). When compared to patients with severe CAP requiring intubation and mechanical ventilation, patients with COVID-19 exhibited markedly lower eosinophil counts and higher levels of ferritin and lactate

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dehydrogenase (LDH). Total white cell count, neutrophil count and CRP were higher in the CAP_{ICU} cohort than in either COVID-19 group.

Characterization of the COVID-19-associated cytokinemia

Circulating levels of IL-1β were elevated in patients with a diagnosis of COVID-19 compared to HC (P<0.0001, Fig.1A), with a significant difference in plasma IL-1β between the $COVID_{stable}$ and $COVID_{ICU}$ groups (13.7+/-5.8pg/mL, 40.8+/-10.4pg/mL, respectively, P<0.0001, Fig. 1A). A significant difference was also observed between patients in the $COVID_{ICU}$ group and those with severe CAP requiring ICU admission (31.8+/-8.2pg/mL, P=0.009, Fig. 1A).

A similar pattern was observed for IL-6, with plasma levels in COVID-19 patients increasing with severity of illness (HC: 0.8+/-1.6pg/mL, $COVID_{stable}$: 45.9+/-24.8pg/mL, $COVID_{ICU}$: 169.4+/-70.7pg/mL, P<0.0001, Fig. 1B). IL-6 levels in the in CAP_{ICU} group (99.4+/-40.5pg/mL) were higher than for $COVID_{stable}$ (P=0.0001) but significantly lower than for $COVID_{ICU}$ (P=0.0005, Fig. 1B). While circulating levels of the anti-inflammatory cytokine IL-10 were higher in $COVID_{stable}$ than in HC (54.7+/-15.7pg/mL, 7.5+/-3.6pg/mL, respectively, P<0.0001, Fig. 1C), a further increase between $COVID_{stable}$ and $COVID_{ICU}$ was not observed ($COVID_{ICU}$: 47.5+/-17.4, P=0.17, Fig. 1C). In contrast, patients in the CAP_{ICU} group exhibited markedly increased IL-10 levels in response to inflammation when compared to $COVID_{ICU}$ patients (86.8+/-14.9pg/mL, P<0.0001, Fig. 1C), indicating that in addition to the rise in pro-inflammatory mediators, concomitant loss of anti-inflammatory protection may also be clinically relevant. In support of this concept, the ratios of IL-6:IL-10 and IL-1β:IL-10 were significantly increased in the $COVID_{ICU}$ cohort (both P<0.0001, Fig. 1D, Fig. S1). Levels of the potent neutrophil chemoattractant IL-8 were also higher in patients positive for COVID-19, with a

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further increase observed between the $COVID_{stable}$ and $COVID_{ICU}$ groups (HC: 1.9+/-2.1pg/mL, $COVID_{stable}$: 48.2+/-24.0pg/mL, $COVID_{ICU}$: 115.5+/-46.4pg/mL, P<0.0001, Fig. 1E). Unlike IL-6 and IL-1β, the IL-8 response to infection was not more pronounced in severe COVID-19 than in severe CAP (121.4+/-65.5pg/mL, P=0.98, Fig. 1E). Additionally, sTNFR1, a surrogate for circulating TNF-α, was markedly increased in both COVID groups, with a significant difference observed between $COVID_{ICU}$ (4.2+/-1.7ng/mL) and both $COVID_{stable}$ and CAP_{ICU}

(2.22+/-0.7ng/mL, P=0.0001 and 3.2+/-0.98ng/mL, P=0.04, respectively, Fig. 1F).

Neutrophils undergo metabolic reprogramming in severe COVID-19 illness

Given that the relationship observed between IL-1β, IL-6 and IL-10 in inflamed $COVID_{ICU}$ patients was consistent with increased HIF-mediated transcription, we searched for evidence of altered immunometabolism in these individuals. Neutrophils were chosen for this purpose, since they produce all of the aforementioned cytokines (17, 18), were increased in the peripheral blood of $COVID_{ICU}$ patients, and are heavily implicated in the pathogenesis of ARDS (12), where HIF-mediated signaling is upregulated (19). PKM2 was significantly increased in $COVID_{ICU}$ neutrophil cytosols compared to HC (P<0.0001, Fig. 2A). Assumption of a tetramer conformation prevents nuclear translocation, thereby impairing interaction with HIF-1α (9). Phosphorylation of PKM2 on Tyrosine 105 prevents PKM2 from attaining a tetramer conformation, and is thus considered indicative of dimer formation. Cytosolic levels of phosphorylated PKM2 were also higher in $COVID_{ICU}$ neutrophil cytosols compared to HC (P<0.0001, Fig. 2B).

In addition to being an important control point in glycolysis, PKM2 has been shown to function as a coactivator of HIF-1α (7), a key mediator of acute inflammation that also exerts a positive

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feedback effect by inducing expression of pro-glycolytic enzymes such as LDH, pyruvate dehydrogenase kinases and the glucose transporter GLUT-1. In a healthy resting cell, HIF-1α is hydroxylated at conserved proline residues by prolyl hydroxylases (PHDs), marking it for ubiquitination and rapid proteasomal degradation. PHDs are oxygen-dependent. Thus, in a state of normoxia, HIF-1α typically displays a short cytosolic half-life, high turnover and low basal levels, but in a state of relative hypoxemia is conserved (8). HIF-1α breakdown is also prevented by cytosolic accumulation of succinate (9), a PHD inhibitor and inflammatory danger signal. Cytosolic succinate was elevated in $COVID_{ICU}$ neutrophils compared to HC neutrophils ($P<0.0001$, Fig. 2C). Consistent with this, cytosolic levels of HIF-1α were also markedly increased in $COVID_{ICU}$ neutrophil cytosols ($P<0.0001$, Figure 2D). The preservation of HIF-1α in the cytosol was reflected at the nucleus, where it was also increased, an effect similarly observed for phosphorylated PKM2 ($P=0.0002$, $P<0.0001$, respectively, Fig. 2D). Cytosolic lactate levels were significantly higher in $COVID_{ICU}$ neutrophils than in HC (12.23+/-1.82 nM vs 2.07+/-0.88 nM, $P<0.0001$, Fig. 1E). Cytosolic lactate:pyruvate (LP) ratio was also increased in $COVID_{ICU}$ neutrophils ($COVID_{ICU}$: 7.24+/-1.16, HC: 2.49+/-0.25, $P<0.0001$, Fig. 1F), confirming that the increase in lactate was due to a fundamental metabolic shift, rather than merely an increase in overall metabolism.

The acute phase response of alpha-1 antitrypsin is insufficient in severe COVID-19 illness

Having shown increased levels of IL-6 and IL-1β in the presence of an inadequate IL-10 response, we investigated whether other endogenous acute phase anti-inflammatories were similarly outstripped in patients with severe COVID-19. Alpha-1 antitrypsin (AAT) is a 52kDa glycoprotein synthesized primarily in the liver. In addition to its role as a serine protease inhibitor, AAT is a potent anti-inflammatory and a key modulator of the acute phase immune

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response in humans (14, 20-23). Stimulation of HepG2 cells with human IL-6 triggered increased production of *AAT* mRNA, and increased secretion of AAT protein (Figure S2A, S2B), and circulating AAT levels were significantly elevated in both COVID-19 and severe CAP (Table 2). Glycosylation of AAT is also altered in response to infection, with increased sialylation of AAT previously shown to enhance the protein's anti-inflammatory effects (24). Immunofixation of plasma from COVID-19 patients by isoelectric focusing gel electrophoresis revealed the presence of the highly sialylated M0 and M1 AAT glycoforms (Fig. 3A). These data indicated that affected individuals were mounting an acute AAT response to increased inflammation. However, this response failed to keep pace with IL-6 in COVID-19 patients who were critically unwell. Despite $COVID_{ICU}$ patients having significantly higher IL-6 levels than patients with severe CAP, no difference in circulating AAT levels was observed between the two groups, leading to a clear difference in the plasma IL-6:AAT ratio between the two groups ($P<0.0001$, Fig. 3B). Similarly, the IL-6:AAT ratio in $COVID_{ICU}$ patients was substantially higher than in $COVID_{stable}$ ($P<0.0001$, Fig. 3B). Further analysis of the $COVID_{ICU}$ cohort demonstrated that, in patients sampled prospectively at 2-day intervals for 6 days following ICU admission, an increase in IL-6:AAT between day 0 and day 6 was associated with poor outcome – defined as death or prolonged ICU stay – whereas a reduction in IL-6:AAT was associated with clinical improvement and discharge to the ward (Fig. 3C, Fig. S4). Specifically, logistic regression analysis demonstrated that an IL-6:AAT ratio ≤ 85.02 on day 6 predicted a good outcome perfectly and a decrease from day 0 to day 6 of ≥ 25.68 predicted a good outcome perfectly. Furthermore, a decrease from day 0 to day 4 of ≥ 3.76 predicted a good outcome perfectly, while a one-unit increase in the change in IL-6:AAT from day 0 to day 2 increased the odds of a poor outcome by 17% (OR=1.17, 95%CI= 1.01 to 1.35, P = 0.039). In patients with

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severe CAP sampled in the same manner, the trends in IL-6:AAT observed were different, with a decrease in IL-6:AAT observed earlier in those who resolved clinically and a flatter IL-6:AAT trajectory in those who went on to poor outcome (Fig. 3C). Therefore, while multiple endogenous anti-inflammatory responses are present in patients with severe COVID-19, they are overwhelmed by an excessive IL-6 burden, a feature of inflammatory dysregulation that distinguishes this condition from others in the ICU setting.

Discussion

Here we define the COVID-19 cytokinemia and the inflammatory phenotype of the critically unwell COVID-19 patient, and show for the first time the presence of immunometabolic reprogramming in patients with severe COVID-19 illness requiring ICU admission.

Differences in laboratory values between COVID-19 patient groups were mirrored by a series of pro-inflammatory cytokines. IL-1β, IL-6, IL-8 and sTNFR1 were all increased in infected patients compared to healthy controls. Furthermore, the $COVID_{ICU}$ cohort could be clearly differentiated from ICU patients with severe CAP and those who were COVID-positive but stable. The most unanticipated differentiating factor between patients with severe COVID-19 and severe CAP, however, was not the degree of increase in pro-inflammatory cytokines, but rather the relatively blunted anti-inflammatory responses of IL-10 and AAT. Indeed, the profound increases observed in the ratios of IL-6:IL-10, IL-1β:IL-10 and IL-6:AAT in severe COVID-19 highlights a distinct inflammatory phenotype, one that may be associated with altered immunometabolism.

Neutrophils from patients with severe COVID-19 displayed increased cytosolic levels of the pro-inflammatory metabolic regulator and glycolytic marker PKM2, with its phosphorylation at Tyr-

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105 also increased. Nuclear translocation of the phosphorylated PKM2 was evident in the $COVID_{ICU}$ group, as was an increase in both cytosolic and nuclear levels of HIF-1α.

While there are several well-described circumstances in which the latter phenomenon may arise in other cell types, such as in neoplastic cells or in muscle cells during intense exertion, there are two in particular that apply to circulating neutrophils. The first, hypoxemia, is intuitive, and is pertinent here. Patients with COVID-19 requiring ICU admission displayed profound hypoxemia when sampled immediately prior to intubation. The second involves build-up of HIF-1α following its stabilization by succinate in the cytosol (9). Succinate was significantly elevated in neutrophil cytosols from $COVID_{ICU}$ patients. Cytosolic accumulation of succinate is not exclusive to hypoxia – it can also be triggered by infection and severe inflammation (9, 15, 25), both of which are observed in the $COVID_{ICU}$ cohort.

While the metabolic rewiring of neutrophils described in these patients supports the concept that the cytokinemia observed is driven by circulating immune cells, it is certainly possible that cytokine leakage from a permeabilized lung also plays a role. In this regard, we are informed by prior experiments delineating the mechanism of IL-6 release into the serum following administration of adenovirus to the airway (26), in which a loss of the integrity of the respiratory epithelial barrier, coupled with de novo synthesis by airway neutrophils, led to passage of IL-6 into the local tissue and, subsequently, the systemic circulation. It should also be noted that other immune cell types besides neutrophils, such as macrophages and monocytes, are likely to contribute to the cytokine burden observed. Similarly, the elevations seen in circulating lactate and LDH (Table 2) in $COVID_{ICU}$ patients are likely to be driven by increased glycolytic activity in multiple cell types, although cell breakdown in the context of critical illness may also play a contributing role. Furthermore, the predilection for increased glycolytic activity varies across

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different tissues and cells. Tumor cells preferentially employ glycolysis and HIF-1α to drive angiogenesis, for example, while skeletal muscle typically only reverts to glycolysis as its predominant source of ATP after an anaerobic threshold is reached.

Physiologically, it has been suggested that the ARDS observed in patients with severe Covid-19 differs from "typical" ARDS, with relatively preserved compliance (27, 28), though this is not universally accepted (29). We observed that our patients had abnormal compliance, with a median dynamic compliance of 33.7ml/cmH_2O (interquartile range 30.1-43.0). From an inflammatory perspective, comparing the COVID-19 cytokine profile described here to data from previous studies in ARDS and sepsis is made more difficult by the heterogeneity of these conditions and differences in the cohorts studied. This applies to several factors, including severity and duration of illness, timing of sampling, socioeconomic and demographic factors, diagnostic criteria used, treatments available at the time of study commencement, and underlying etiology. Methodological variation also exists between studies, from the assay type used to the sample type assayed, to the processing, handling and storage of samples (30-34). This is of particular relevance when interpreting biomarker data from large clinical trials or biobanks. Indeed, a recent study by Stapleton, et al., compared baseline plasma cytokine levels from current ARDS randomized control trial (RCT) patients to baseline values from similar historical controls from previous ARDS RCTs, and found that modern-day values were generally substantially lower, most notably the level of IL-6 (35). While the cytokine levels describe in our manuscript are closer to the numbers described by Stapleton, et al., than to many of these historical studies, the balance of these cytokines in COVID-19 is different, in particular the IL-10 response to infection relative to both IL-1β and IL-6.

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The data provide some insight regarding potential therapeutic options, some of which have already been licensed for use in humans. However, it is apparent that limitations to each approach exist. Drugs such as tocilizumab, anakinra and infliximab inhibit the action of specific cytokines, but do not offer broad spectrum anti-inflammatory cover. Steroid therapy induces an indiscriminate pancytokinaemia, with suppression of anti-inflammatory and pro-resolution cytokines such as IL-10 in addition to those that are pro-inflammatory. This may explain why recent attempts to use steroids in this population have proven unsuccessful (36).

Targeting metabolism represents another means of modulating inflammation. Most of the pyruvate generated by glycolysis is converted to acetyl CoA via pyruvate dehydrogenase, or to lactate via LDH. As the transcription factor driving both LDH and pyruvate dehydrogenase kinase (PDK), HIF-1α dictates the fate of pyruvate and holds the key to cellular metabolic balance (37). Dichloroacetate, a PDK inhibitor has been shown to redirect the conversion of pyruvate away from lactate and back towards acetyl-CoA and oxidative phosphorylation by catalyzing pyruvate decarboxylation, and has been successfully administered to humans (38).

Other potential strategies, such as the maintenance of PKM2 in a tetramer conformation by molecules such as TEPP-46 (5), or the use of itaconate as an anti-inflammatory and anti-oxidant (39) hold promise but have yet to progress to the clinical arena.

The IL-6:AAT ratio in $COVID_{ICU}$ was more than twice that seen in CAP_{ICU}, and the progression of IL-6 relative to AAT over time matched clinical trajectory in patients with severe COVID-19. In this regard, supplementation of the acute AAT response with exogenous AAT may merit consideration, since it has been shown to modulate the production and activity of the key pro-inflammatory cytokines described here (14, 20, 24, 32, 40), while preserving the production of

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IL-10 (41). Indeed, we have recently shown that abrupt cessation of AAT augmentation therapy for patients with hereditary AAT deficiency results in marked increases in levels of these specific pro-inflammatory cytokines, loss of IL-10 and subsequent progression to respiratory failure (42).

Although one or more of the above-mentioned therapies may yet prove to be beneficial in severe COVID-19 illness, it is important that the urgency surrounding the current pandemic does not prompt hasty engagement in treatment strategies that may, while well-intentioned, do more harm than good. It is our shared responsibility to emphasize the importance of applying therapies to vulnerable patients only when there is sufficient preclinical data to support their advancement to expedited, properly conducted clinical trials. Identifying potential targets, as we have done here, stands to reduce the number of inappropriate off-label therapies being administered and inform the selection of candidate therapies for robust evaluation against defined outcomes in the clinical trial setting.

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Figure legends

Figure 1. The COVID-19 cytokinemia.

(A) Plasma was obtained from healthy control subjects (HC; n=15), people with COVID-19 infection who required hospitalization but were stable at ward level ($COVID_{stable}$; n=20), severely unwell COVID-19 patients requiring intubation and mechanical ventilation ($COVID_{ICU}$; n=20) and patients with severe CAP in ICU (CAP_{ICU}; n=15). IL-1β levels were elevated in $COVID_{stable}$ compared to HC, with an increase observed between the $COVID_{stable}$ and $COVID_{ICU}$ groups. IL-1β levels were higher in $COVID_{ICU}$ than CAP_{ICU}. (B) IL-6 levels were elevated in $COVID_{stable}$ compared to HC, with an additional increase in $COVID_{ICU}$. IL-6 levels were higher in CAP_{ICU} than in $COVID_{stable}$, but significantly lower than in $COVID_{ICU}$. (C) IL-10 was higher in $COVID_{stable}$ than HC. IL-10 in CAP_{ICU} was higher than both COVID-19 groups. (D) IL-6:IL-10 was higher in $COVID_{ICU}$ than $COVID_{stable}$ and CAP_{ICU}. (E) IL-8 was increased in $COVID_{stable}$ plasma, with a further rise in $COVID_{ICU}$. No difference between $COVID_{ICU}$ and CAP_{ICU} was observed. (F) Levels of sTNFR1 were higher in $COVID_{stable}$ than HC with a further increase in $COVID_{ICU}$. Levels were higher in $COVID_{ICU}$ than CAP_{ICU}. *P <0.05, **P <0.001. IL = interleukin; sTNFR1 = soluble tumor necrosis factor receptor 1.

Figure 2. Neutrophil immunometabolism is altered in severe COVID-19 illness.

(A) Neutrophils were isolated from the peripheral blood of healthy control subjects (HC; n = 8), and people with severe COVID-19 illness requiring intubation and mechanical ventilation (COVID; n = 8), and cytosolic fractions obtained. PKM2 was significantly increased in COVID neutrophil cytosols compared with HC (P <0.0001). (B) Cytosolic levels of phosphorylated PKM2, indicative of PKM2 dimer formation, were also significantly increased in COVID

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compared with HC (P <0.0001). (C) Cytosolic succinate levels were higher in COVID neutrophils than in healthy control subjects (fold increase 10.41 +/- 1.97, P <0.0001). (D) Cytosolic HIF-1α was higher in COVID neutrophil cytosols than in HC (P <0.0001). Nuclear levels of HIF-1α and PKM2 were also increased in neutrophils from the same infected patients (both P <0.0001). (E) Cytosolic lactate was higher in circulating COVID neutrophils than in HC (P <0.0001). (F) Cytosolic lactate:pyruvate (LP) ratio was similarly increased (P <0.0001). *P <0.05, **P <0.001. DU = densitometric units; PKM2 = pyruvate kinase M2; HIF-1α = hypoxia-inducible factor 1-α.

Figure 3. The acute phase response of AAT is overwhelmed in severe COVID-19 illness.

AAT is a 52kDa glycosylated protein synthesized primarily in the liver. (A) Immunofixation of plasma from COVID-19 patients following isoelectric focusing gel electrophoresis demonstrated the presence of the highly sialylated M0 and M1 AAT glycoforms indicative of an attempt to mount a response to inflammation. (B) Plasma IL-6:AAT ratios were significantly higher in patients who required ICU support ($COVID_{stable}$: 19.00+/-8.41, $COVID_{ICU}$: 92.05+/-35.61, CAP_{ICU}: 35.26+/-13.77, P=0.0002). (C) Sequential plasma samples were obtained from 16 $COVID_{ICU}$ patients (indicated in red), 8 of whom resolved sufficiently within 10 days of entering ICU to be discharged to the ward, and 8 of whom had a poor outcome (death or prolonged ICU stay). A progressive increase in IL-6:AAT was observed in $COVID_{ICU}$ patients who had a poor outcome, while a decrease in IL-6:AAT was seen in $COVID_{ICU}$ patients who recovered. By comparison, CAP_{ICU} patients (indicated in blue; good outcome: n=8, poor outcome: n=7) did not exhibit the same trend.

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Tables

Table 1. Clinical characteristics of the two COVID-19 cohorts

	$COVID_{stable}$ (n=20)	$COVID_{ICU}$ (n=20)	Total (n=40)
Age in years	56.6 +/- 17.3	54.3 +/- 18.2	55.5 +/- 17.7
Male/female	12/8	13/7	25/15
Days since onset of symptoms	7.00 +/- 0.58	7.05 +/- 0.81	7.03 +/- 0.74
Symptoms at admission			
Fever	17 (85)	18 (90)	35 (88)
Dyspnea	11 (55)	15 (75)	26 (65)
Cough	11 (55)	14 (70)	25 (60)
Sputum production	5 (25)	5 (25)	10 (25)
Myalgia	8 (40)	7 (35)	15 (38)
Sore throat	5 (25)	4 (20)	9 (23)
Nasal congestion	1 (5)	0 (0)	1 (3)
Headache	6 (30)	5 (25)	11 (28)
Fatigue	13 (65)	14 (70)	13 (68)
Anorexia	5 (25)	6 (30)	11 (28)
Nausea	5 (25)	5 (25)	10 (25)
Vomiting	0 (0)	1 (5)	1 (3)
Diarrhea	4 (20)	3 (15)	7 (18)
Chest pain	6 (30)	6 (30)	6 (30)
Anosmia	4 (20)	3 (15)	7 (18)
Circumstances surrounding infection			
Recent travel to high-risk area	4 (20)	2 (10)	6 (15)
Close contact with infected person	8 (40)	10 (50)	18 (45)
Community-acquired	8 (40)	8 (40)	16 (40)
Comorbidities			
Hypertension	8 (40)	8 (40)	16 (40)
Coronary artery disease	4 (20)	3 (15)	7 (18)
Diabetes mellitus	4 (20)	4 (20)	8 (20)
Obesity	13 (65)	14 (70)	27 (68)
Chronic lung disease	6 (30)	5 (25)	11 (28)
Chronic kidney disease	5 (25)	4 (20)	9 (23)
Smoking history			
Current	6 (30)	6 (30)	12 (30)
Former	4 (20)	4 (~~+~~20)	8 (20)
Never	10 (50)	10 (50)	20 (50)
Vaping history			
Current	3 (15)	2 (10)	5 (13)
Former	0 (0)	0 (0)	0 (0)
Never	17 (85)	18 (90)	35 (88)

Data presented as mean +/- SD or absolute number (percentage of group total).

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Table 2. Laboratory findings at study entry

	$COVID_{stable}$ (n=20)		**$COVID_{ICU}$ (n=20)**		**CAP_{ICU} (n=15)**	
	Mean (SD)	Median	Mean (SD)	Median	Mean (SD)	Median
White cell count (4.0-11.0)	8.29 (2.27)	7.99	13.12 (5.36)	12.22	16.31 (5.77)	16.00
Neutrophils 2.0-7.5)	7.85 (3.18)	6.95	11.17 (5.17)	10.29	14.11 (5.12)	12.41
Lymphocytes (1.0-4.0)	1.55 (0.66)	1.46	1.09 (0.34)	1.03	1.88 (0.89)	1.80
Monocytes (0.2-1.0)	0.65 (0.25)	0.58	0.41 (0.25)	0.38	0.84 (0.43)	(0.66)
Eosinophils (0.04-0.4)	0.04 (0.02)	0.02	0.01 (0.01)	0.000	0.1 (0.07)	0.07
Platelets (140-400)	266 (56.4)	253	228 (54.2)	236	226 (52.2)	230
Hemoglobin (13.0-17.5 g/dL)	13.7 (1.08)	13.1	12.9 (1.44)	12.8	13.0 (1.39)	12.9
C-reactive protein (0-5 mg/L)	62.1 (61.7)	47	232 (104.5)	192	253 (101.4)	272
Aspartate aminotransferase (0-40 U/L)	51 (24.0)	38	63 (21.4)	66	59 (23.2)	53
Alanine aminotransferase (0-41 IU/L)	59 (40.3)	43	47 (16.1)	49	49 (15.8)	46
Gamma-glutamyltransferase (0-59 IU/L)	74 (57.6)	57	94 (52.8)	90	62 (38.9)	60
Bilirubin (0-21 µmol/L)	8 (2.8)	9	10 (4.3)	9	8 (3.1)	8
Albumin (35-52 g/L)	37 (4.82)	37	33 (5.76)	34	34 (4.32)	34
Fibrinogen (1.90-3.50 g/L)	3.98 (0.72)	4.00	5.14 (1.13)	4.70	4.22 (0.36)	4.10
Ferritin* (30-400 ng/mL)	1710 (863)	1473	1812 (1276)	1599	773 (421)	796
Lactate (0.5-1.0 mmol/L)	0.9 (0.3)	0.9	2.9 (1.3)	2.9	2.8 (1.2)	2.8
Lactate dehydrogenase† (135-225 U/L)	304 (105.8)	267	806 (102.2)	787	332 (98.4)	320
Alpha-1 antitrypsin (0.90-1.80 g/L)	2.10 (0.51)	2.16	2.89 (0.49)	2.85	2.85 (0.39)	2.85

**data available for 39 COVID-19 patients and 13 CAP patients; †data available for 37 COVID-19 patients and 13 CAP patients. All cell counts are $x10^9$/L.*

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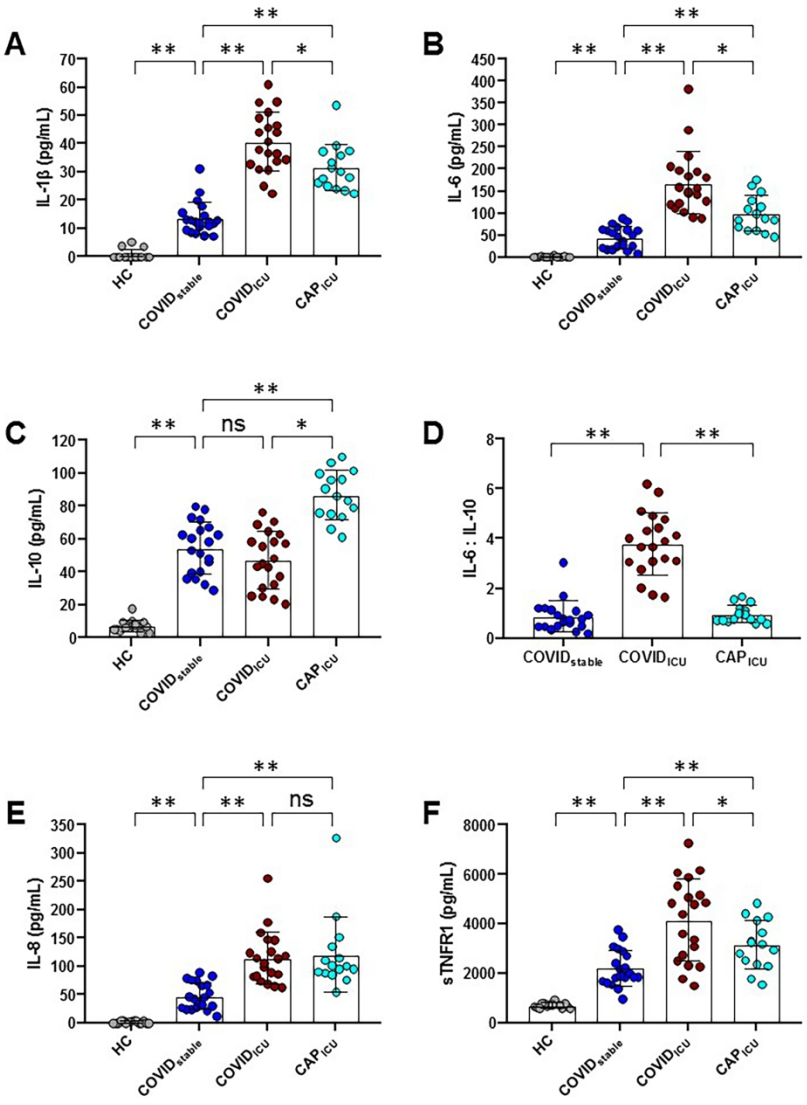

Figure 1. The COVID-19 cytokinemia.

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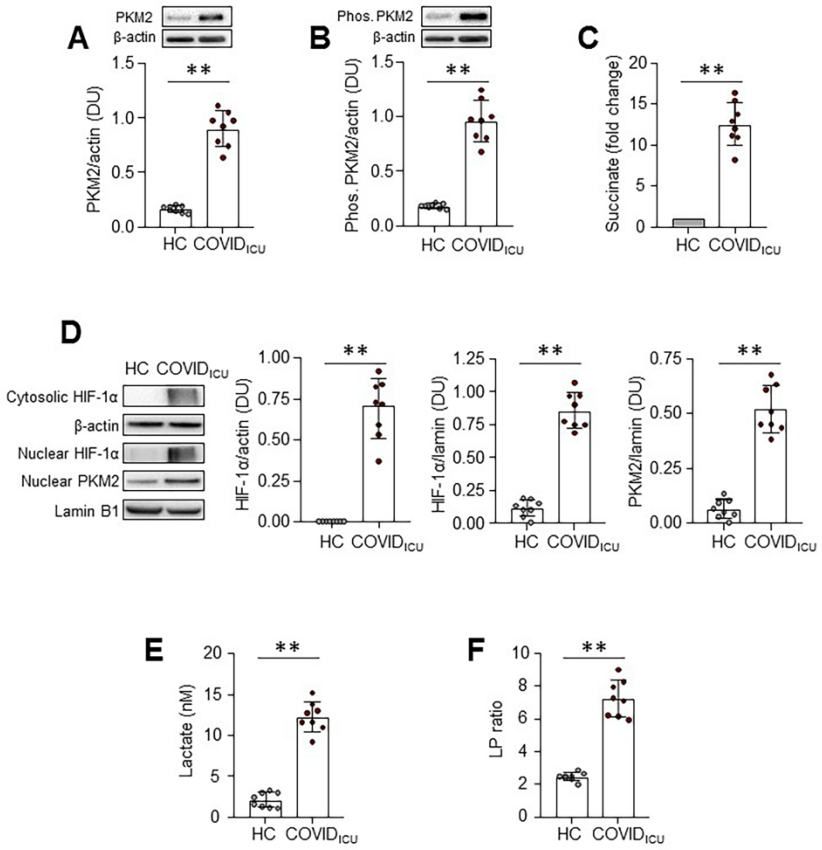

Figure 2. Neutrophil immunometabolism is altered in severe COVID-19 illness.

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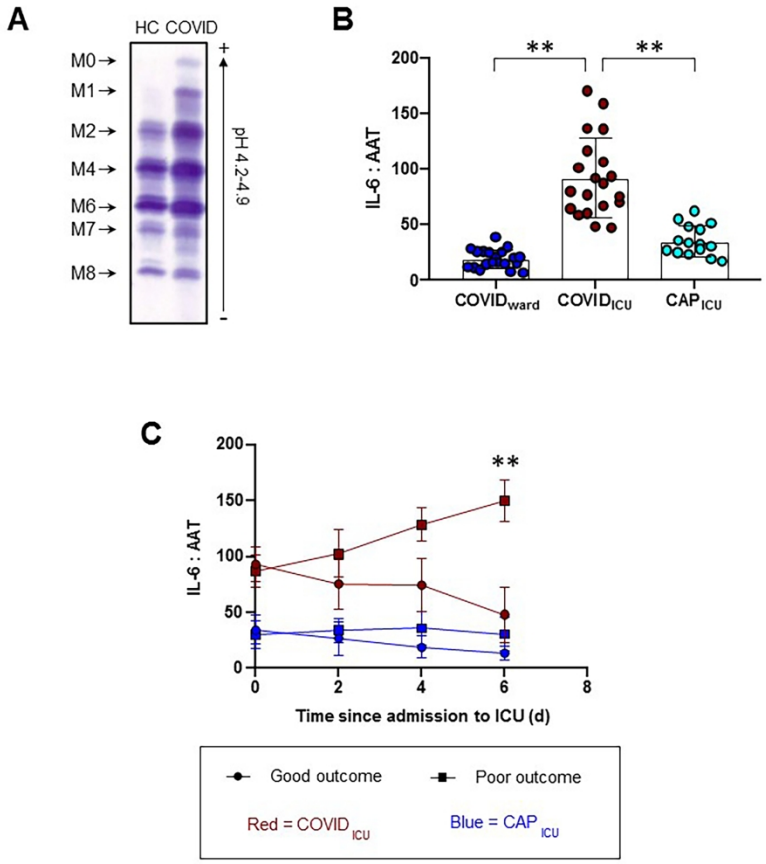

Figure 3. The acute phase response of AAT is overwhelmed in severe COVID-19 illness.

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Online Data Supplementary

Legends:

Figure S1. IL-1β:IL-10 ratio is increased in severe COVID-19 illness.

Plasma was obtained from $COVID_{stable}$ (n=20), $COVID_{ICU}$ (n=20) and CAP_{ICU} (n=15). IL-1β:IL-10 was significantly increased in $COVID_{ICU}$ (0.97+/-0.39) compared to $COVID_{stable}$ (0.27+/-0.12) and CAP_{ICU} (0.37+/-0.07), both $P<0.0001$).

Figure S2. Production of AAT by primary hepatocytes is increased in response to IL-6.

(A) AAT qRT-PCR results of primary hepatocytes (HepG2 cell line) following exposure to human IL-6 (50pg/ml) for 48 h. AAT gene expression was significantly increased in the presence of IL-6 at both time points (n=3, $P<0.05$). (B) The level of AAT secreted from HepG2 cells at 48 h following treatment with IL-6 was increased 1.5-fold (n=3, $P<0.05$).

Figure S3. Individual trends in circulating levels of AAT and IL-6 over time in the $COVID_{ICU}$ cohort.

(A) Circulating levels of AAT over time in ICU are displayed for the $COVID_{ICU}$ patients included in Fig. 3C. Patients who went on to good outcome are indicated in blue, while those who went on to poor outcome are in red. (B) Plasma IL-6 for the same individuals.

Figure S4. Individual IL-6:AAT ratio values over time in the $COVID_{ICU}$ cohort.

The IL-6:AAT ratio for each patient in the $COVID_{ICU}$ group included in Fig. 3C at each time point is depicted. Patients who went on to good outcome are indicated in blue, while those who went on to poor outcome are indicated in red. We also investigated whether outcome would

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associate with a worsening or improving IL-6:AAT ratio, if at all, taking good outcome and poor outcome as ordinal variables, and the slope of the IL-6:AAT ratio as a continuous variable, For each patient who experienced a bad outcome, their IL-6:AAT ratio worsened – a negative slope of ratio over time – while each patient who had a good outcome had a positive slope. The R^2 value when Pearson's correlation was applied to the data set was 0.9.

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Tables

Table S1. Protease inhibitor cocktail constituents

Protease inhibitor (1µl/ml)	Description
Tosyl-L-lysyl-chloromethane hydrochloride	Serine protease inhibitor
Pepstatin A	Inhibitor of aspartyl proteases
Pefabloc	Serine protease inhibitor
Ethylenediaminetetraacetic acid	Metallopeptidase inhibitor
Leupeptin	Inhibitor of cysteine, serine and threonine peptidases
Sodium fluoride	Phosphatase inhibitor
Sodium orthovanadate	Phosphatase inhibitor
Phenylmethylsulfonyl fluoride	Serine protease inhibitor

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Table S2. Details of key resources used.

Reagent or resource	Supplier/source	Identifier
Antibodies		
PKM2	Cell Signaling Technology	#3198
phospho-PKM2 (Tyr105)	Cell Signaling Technology	#3827
HIF-1α	Cell Signaling Technology	#36169
β-actin	Cell Signaling Technology	#4267
Lamin B1	Cell Signaling Technology	#13435
Anti-rabbit IgG (HRP-linked)	Cell Signaling Technology	#7074
Anti-mouse IgG (HRP-linked)	Cell Signaling Technology	#7076
Critical Commercial Assays		
IL-1β ELISA (sensitivity: 1 pg/ml)	R&D	#DLB50
IL-6 ELISA (sensitivity: 0.7 pg/ml)	R&D	#D6050
IL-8 ELISA (sensitivity: 7.5 pg/ml)	R&D	#D8000C
IL-10 ELISA (sensitivity: 3.9 pg/ml)	R&D	#D1000B
sTNFR1 ELISA (sensitivity: 1.2 pg/ml)	R&D	#DRT100
Succinate assay	Abcam	#ab204718
Lactate assay	Abcam	#ab65330
Pyruvate assay	Abcam	#ab65342
Software and hardware		
Graphpad Prism 8.0	www.graphpad.com	sales@graphpad.com
SpectraMax M3 plate reader	Molecular Devices	N/A
Chemi Doc MP System	Bio-Rad	#17001402
Image Lab	Bio-Rad	https://www.selectscience.net/products/image-lab-software-(170-9690)/?prodID=115956
Hydrasys electrophoresis platform	Sebia	#PN1200
Other		
Nuclear extract kit	Active Motif	#40010
Hydragel 18 A1AT Isofocusing kit	Sebia	#PN4357

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Table S3. Clinical features of the $COVID_{ICU}$ cohort at time of ICU admission

Parameter	Value
Temperature >38°C	8 (66)
Heart rate >100 beats per minute	7 (58)
Respiratory rate >20 breaths per minute	12 (100)
SaO_2 <80% or requiring FiO_2 ≥60%	11 (92)
PaO_2	7.15 +/- 1.41
Acute confusion	3 (25)
Mean arterial pressure	86.25 +/- 13.16
qSOFA score	1.33 +/- 0.65
PaO_2:FiO_2 on arrival to ICU	157 +/- 67

Data presented as absolute number (%) or mean +/- SD; FiO_2: fraction of inspired oxygen; PaO_2: partial pressure of arterial oxygen in kPa; mean arterial pressure and PaO_2:FiO_2 in mmHg; qSOFA: quick sequential organ failure assessment.

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Table S4. Clinical characteristics of the HC cohort

Number	15
Age in years	39.24 +/- 13.16
Male/female	9/6
Smoking history	
Current	0 (0)
Former	2 (13)
Never	13 (87)
Vaping history	
Current	0 (0)
Former	0 (0)
Never	15 (100)

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Table S5. Clinical characteristics of the CAP_{ICU} cohort

Number	15
Age in years	59.66 +/- 17.82
Male/female	9/6
Airway microbiology	
Streptococcus pneumoniae	8 (53)
Haemophilus influenzae	3 (20)
Staphylococcus aureus	3 (20)
Pseudomonas aeruginosa	1 (7)
Positive respiratory viral swab	5 (33)
Comorbidities	
Hypertension	7 (47)
Coronary artery disease	7 (47)
Diabetes mellitus	2 (13)
Obesity	6 (40)
Chronic lung disease	6 (40)
Chronic kidney disease	6 (40)
Smoking history	
Current	5 (33)
Former	7 (47)
Never	3 (20)
Vaping history	
Current	1 (7)
Former	0 (0)
Never	14 (93)

Data presented as absolute number (%) or mean +/- SD; Some patients had more than one organism identified, therefore % totals for respiratory pathogens exceed 100%; Of those with S. aureus, two had recent influenza.

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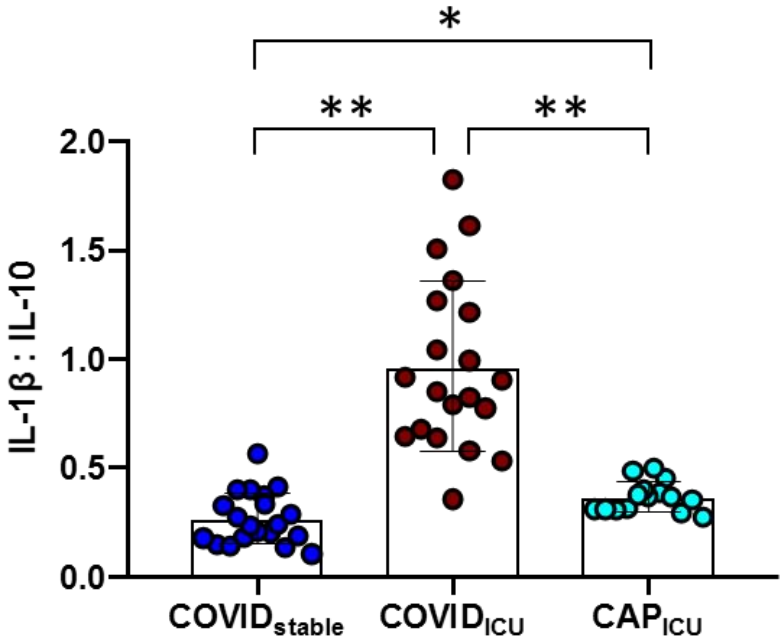

Figure S1.

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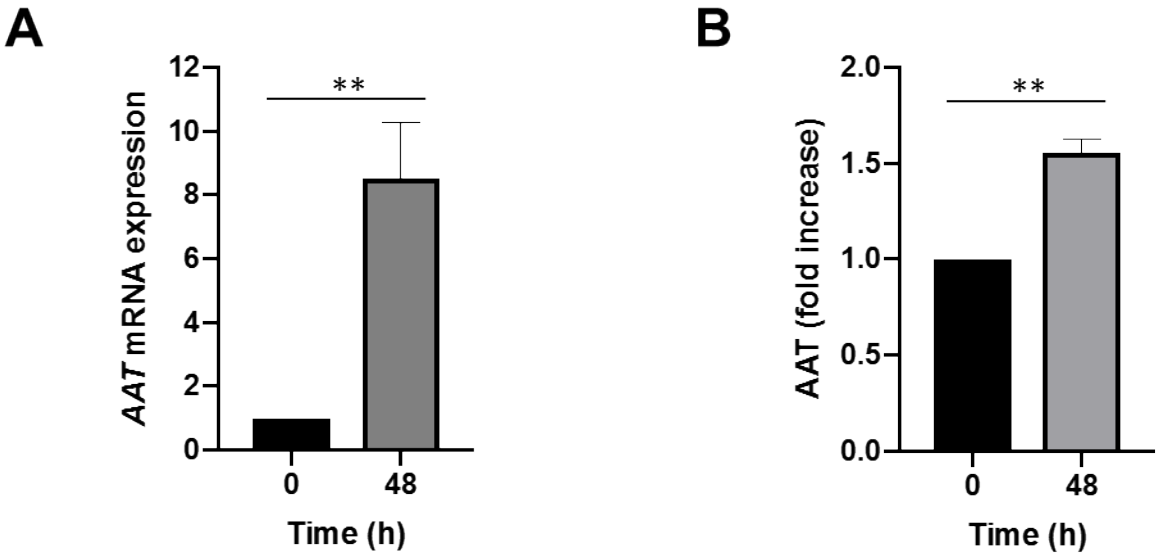

Figure S2.

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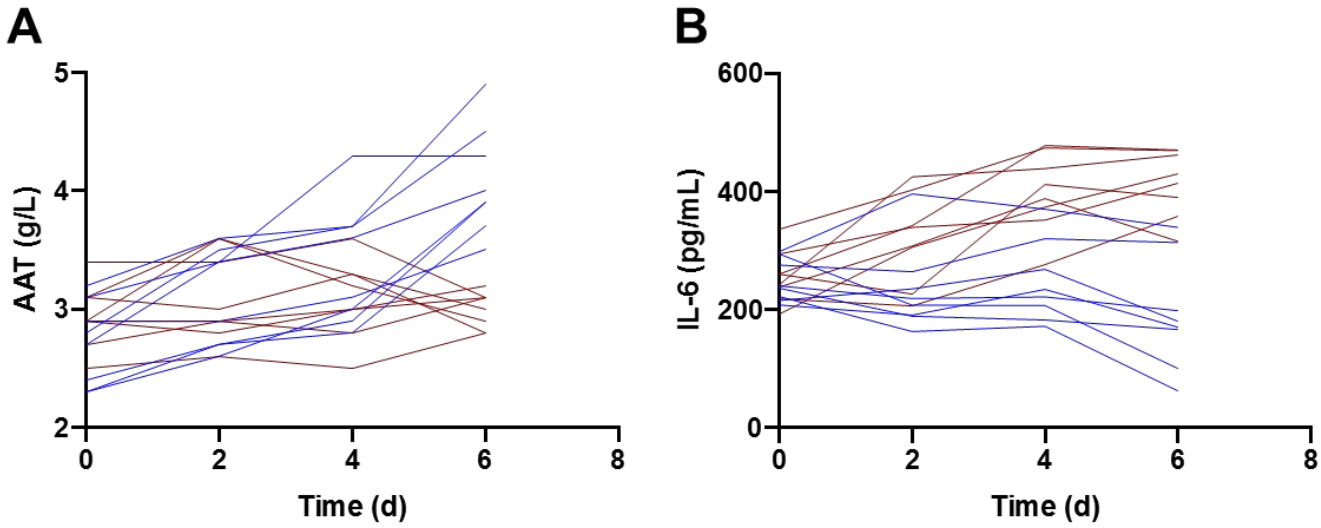

Figure S3.

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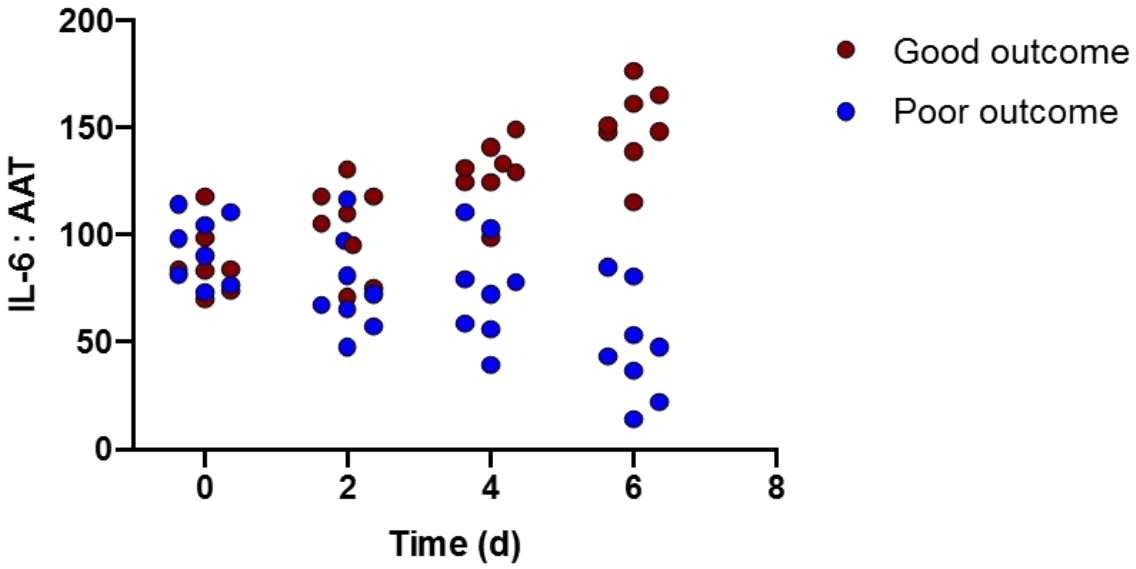

Figure S4.

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