

Article

# Complex immuno-metabolic profiling reveals activation of cellular immunity and biliary lesion in patients with severe COVID-19

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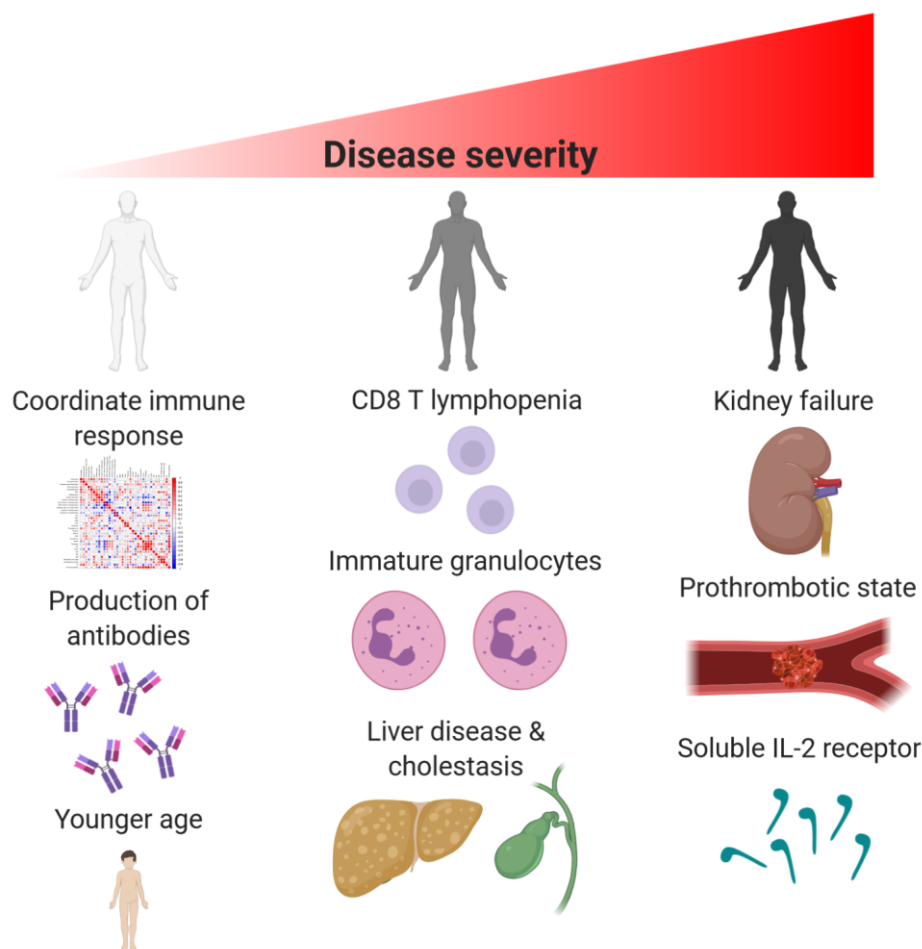
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**Abstract:** The aim of this study was to assess the key laboratory features displayed by coronavirus disease 2019 (COVID-19) inpatients which associated with mild, moderate, severe and fatal course of the disease and, through longitudinal follow-up, to understand the dynamics of COVID-19 pathophysiology. All SARS-CoV-2 positive patients admitted to the University Hospital in Motol between March and June 2020 were included in this study.

Severe course of COVID-19 was associated with elevation of proinflammatory markers, efflux of immature granulocytes into peripheral blood, activation of CD8 T cells, which infiltrate lungs, and transient liver disease. In particular, the elevation of serum gamma-glutamyl transferase (GGT) and histological signs of cholestasis were highly specific for patients with severe disease. In contrast, patients with fatal course of COVID-19 failed to upregulate markers of inflammation, showed dyscoordination of immune response and progressed towards acute kidney failure.

COVID-19 is a disease with multi-organ affinity characterized by activation of innate and cellular adaptive immunity. Biliary lesion with elevation of GGT and organ-infiltration of IL-6 producing cells are defining characteristic for patients with fulminant disease.



**Keywords:** COVID-19; SARS-CoV-2; immunity; T cells; IL-6; GGT; hepatopathy; biliary lesion; acute kidney injury; liver disease

## 1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a novel human coronavirus that has caused a swiftly spreading disease named COVID-19, defined as pandemic by the World Health Organization in February 2020(1). As of June 24th, 2020, a total of 9,2 million cases of COVID-19 have been reported globally, including over 477 thousand deaths(2). Most people (about 80%) who acquire COVID-19 experience mild to moderate symptoms and recover without special treatment(3), however a subgroup of patients develops severe disease with high mortality rate, which is hallmarked by severe respiratory distress syndrome, sepsis, coagulation disorder or even multiple organ failure(4–7).

Although the exact pathogenesis of the virus-induced damage is not yet known, several mechanisms have been proposed. The surface spike protein of SARS-CoV-2 binds to angiotensin-converting enzyme-2 (ACE2) receptor(8–10) expressed in alveolar epithelia of lungs, kidneys, hepatocytes, the epithelial cells of the bile ducts, vascular endothelium and other cells(11–14). Other potential SARS-CoV-2 receptors, such as CD147 or CD26, have also been identified, and found to be expressed in immune cells(15). Accordingly, SARS-CoV-2 organotropism extends beyond the respiratory tract(16). Endothelitis, alveolar damage and thrombotic microangiopathy have been described in lungs and kidneys, accompanied by infiltration of mononuclear cells and macrophages(12,17,18).

An efficient, well-coordinated host immune response is a crucial first line antiviral defense. In severe COVID-19 patients, several studies have documented various degrees of immune dysregulation affecting both innate and adaptive immunity, which may result in immune-mediated tissue

injury(19,20). The recruitment and activation of immune cells, particularly neutrophils, is accompanied by an exuberant release of pro-inflammatory cytokines and chemokines, a so called “cytokine storm”(4,21,22). Along with simultaneous decrease in monocytes, eosinophils and basophils(20,23), a marked lymphopenia and functional exhaustion of CD8 T cells and NK cells was associated with severe course of the disease(24–27).

Various prognostic markers for increased severity and mortality in adult COVID-19 disease have been proposed in several heterogeneous studies, including male sex, higher age, pre-existing lung, cardiac, renal and liver disease, hypertension and obesity(28–31). Individually, laboratory abnormalities have been reported in COVID-19 patients, including elevation of inflammatory markers, liver enzymes, abnormal renal function tests, elevated serum soluble IL-2 receptor (sIL2R) and IL-6. Coagulopathy associated with elevated D-dimers has also been frequently observed amongst severe COVID-19 patients(4,32).

The current clinical knowledge-pool for the research of COVID-19 disease relies on largely heterogeneous cohort studies of various scales and individual objectives. Thus, we chose to prospectively follow all patients with verified SARS-CoV-2 infection admitted to our hospital, constructing a rich dataset featuring clinical information such as disease severity, treatment and outcome, and a complex high-parametric laboratory profile of all patients spanning metabolic, hematologic and immune parameters followed longitudinally over the course of the disease.

## 2. Patients and methods

### 2.1 Patient cohort and study design

All patients admitted to the University Hospital in Motol, Prague, Czech Republic, between March and May 2020 who tested positive for the presence of SARS-CoV-2 RNA in nasopharyngeal swab using reverse real time polymerase chain reaction (rtPCR) were included in this study. Patients were retrospectively divided into subcohorts based on severity of disease course as follows: patients with moderate course of the disease had clinical signs of pneumonia (cough, auscultation) and verified infiltration on chest X-ray or computed tomography; patients with severe course of the disease required mechanical ventilation; patients with mild course of the disease did not fulfill any of the criteria above but had positive SARS-CoV-2 nasopharyngeal swab rtPCR; and patients with fatal course of the disease died during the course of the study. Summary of the overall cohort including cohort size, age and sex and basic clinical characteristics of each subcohort are included in Table 1.

This study was carried out in accordance with the recommendations of the Ethical Committee of the second Faculty of Medicine, Charles University in Prague and University Hospital in Motol, Czech Republic. The protocol was approved by the Ethical Committee. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

### 2.2 Laboratory parameters

Routine in-house methods were used for evaluation of all laboratory parameters included in this study. Details concerning individual parameters are available from authors upon request.

For evaluation of serum anti-SARS-CoV-2 antibodies, the EDI™ Novel Coronavirus COVID-19 IgM (or IgG) ELISA Kits (EDI Epitope Diagnostics, Inc., San Diego, CA, USA) were used and the data was acquired using the QUANTA-Lyser 3000 (Inova Diagnostics, San Diego, CA, USA).

### 2.3 Lymphocyte subsets

Lymphocyte subsets were evaluated using flow cytometry. Full blood was drawn into EDTA-coated tubes, then stained according to manufacturer's instructions using the DryFlowEx ASC Screen Kit and DryFlowEx ACT T Screen Kit and the EXCELLYSE I lysing kit (all from EXBIO, Prague, Czech Republic). Data was acquired on BD LSR II Fortessa (BD Biosciences, Franklin Lakes, NJ) and analysed with FlowJo software (version 10; TreeStar, Ashland, Oregon).

## 2.4 Immunohistochemistry

Tissue samples were fixed in neutral buffered 4% formaldehyde and embedded in paraffin. For immunohistochemistry 3 micrometer thin histological sections were used. Anti-CD8 antibody (clone C8/144B, Dako, dilution 1:200, pre-treatment: heating in buffer pH9 in water bath). Anti-IL-6 antibody (monoclonal antibody against recombinant full-length protein corresponding to Human IL-6 aa 29-212, clone is not specified by antibody producer, Abcam, dilution 1:2000, pre-treatment: heating in buffer pH6 in water bath). Detection was performed using a one-step micropolymeric non-Biotin system (BioSB) with peroxidase complex and DAB (3,3'-diaminobenzidine tetra-hydrochloride). The nuclei were counterstained with hematoxylin.

A sample from lung transplantation donor lungs was used as a healthy control for the lung necropsy. A sample from healthy liver biopsy was used as a healthy control for the liver necropsy.

## 2.5 Statistical analysis

In the boxplots used throughout the manuscript, boxes depict 25th and 75th percentiles (1st and 3rd quartile), whiskers depict 2.5-97.5th percentiles and student's t-tests with Holm's multiple comparison adjustment were used for assessment of differences between groups. In correlation graphs in Fig 4 and the correlation matrices in Fig 5, Spearman correlation was used.

Statistical analyses and generation of graphs were performed in the statistical language and environment R, version 3.6.3, using the "ggplot2", "ggpubr" and "corrplot" packages, GraphPad Prism software (version 8.0.1; GraphPad Software, San Diego, CA, USA) and Microsoft Excel 2016 (Microsoft, Redmond, WA, USA). Simplified Presentation of Incredibly Complex Evaluations (SPICE) plots shown in Fig 2 were constructed using the SPICE 6 software(33).

## 3. Results

### 3.1 Clinical course of the disease

Of the 37 patients included in this study, 10 (27 %) had mild course of COVID-19 characterized by few clinical symptoms, particularly fever, myalgia, arthralgia or general malaise. 13 patients (35 %) had moderate course of the disease characterized chiefly by cough, dyspnea and necessity of oxygen therapy, however, they did not require mechanical ventilation. 7 patients (19 %) suffered from a severe course of the disease which necessitated admission to intensive care unit, mechanical ventilation and in several cases developed into systemic inflammation with multi-organ failure. Finally, 7 patients (19 %) suffered a fatal course of the disease, after an average of  $6.8 \pm 10.4$  days (mean  $\pm$  SD) days following the admission to the hospital.

The specific characteristics of the cohort, found in Table 1, verify the trend of severe course of the disease mostly in the elderly, while the younger patients experience predominantly mild symptoms.

**Table 1.** Cohort description

Cohort	Size <sup>1</sup>	Age [years] <sup>2</sup>	Pneumonia on X-ray	Oxygen therapy	Mechanical ventilation	Dialysis	Hydroxychloroquin	Azithromycin	Other therapy
<b>All patients</b>	37 (17 F, 20 M)	0,2 – 96,7 (60,6 $\pm$ 27,6)	24 (65 %)	21 (57 %)	10 (27 %)	6 (16 %)	15 (43 %)	11 (30 %)	-
<b>Mild</b>	10 (6 F, 4 M) (27 %)	0,2 – 80,0 (29,8 $\pm$ 22,6)	0 (0 %)	1 (10 %)	0 (0 %)	1 (10 %)	0 (0 %)	0 (0 %)	-
<b>Moderate</b>	13 (6 F, 7 M) (35 %)	44,4 – 96,7 (76,8 $\pm$ 13,6)	13 (100 %)	11 (85 %)	0 (0 %)	1 (8 %)	7 (54 %)	4 (31 %)	1 steroids
<b>Severe</b>	7	8,5 – 73,6 (52,0 $\pm$ 19,0)	6 (86 %)	3 (43 %)	7 (100 %)	2 (28 %)	6 (85 %)	4 (57 %)	3 steroids

	(3 F, 4 M)								2 tocilizumab
	(19 %)								1 remdesivir
<b>Fatal</b>	7	56,7 – 94,8	5	6	3	2	2	3	1 Cytosorb
	(2 F, 5 M)	(83,4 ± 12,5)	(71 %)	(85 %)	(43 %)	(28%)	(28%)	(43 %)	2 steroids
	(19 %)								

<sup>1</sup> F = female, M = male.

<sup>2</sup> Age range shown as mean ± standard deviation.

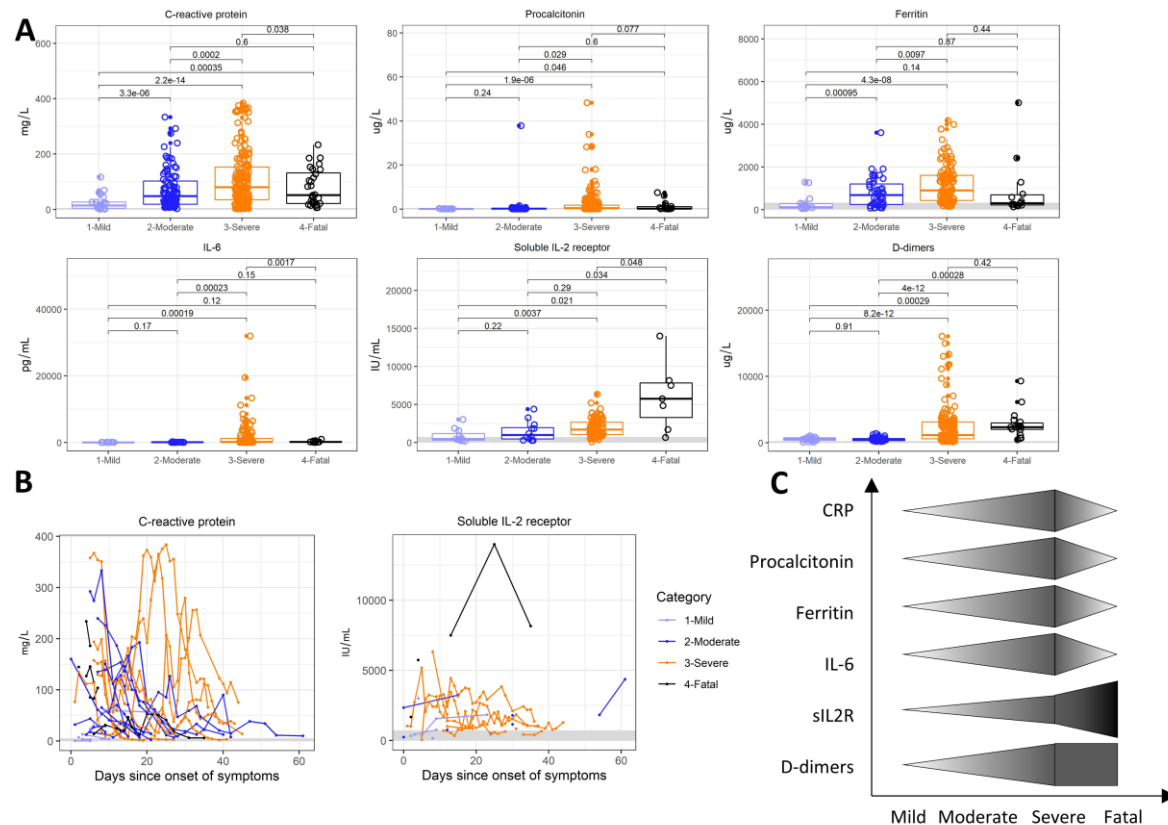
### 3.2 Inflammation

While patients with mild course of the disease only rarely showed overt elevation of inflammatory markers such as the C-reactive protein (CRP), procalcitonin or ferritin (Fig 1A), CRP and ferritin were markedly elevated in the moderate, severe and fatal subcohorts. High serum IL-6 levels reaching thousands of pg/mL and high procalcitonin were characteristic for severe patients who required mechanical ventilation and had multi-organ involvement.

Interestingly, patients with fatal course of COVID-19 failed to display inflammatory response at similarly high levels, which may have contributed to their eventual demise, but averaged exceedingly elevated serum soluble IL-2 receptor (sIL2R) levels.

CRP was highest at the beginning of the disease and decreased rapidly in the first 20 days since onset of symptoms (Fig 1B) but flared up with multi-organ involvement in the delayed phase of severely ill patients. In contrast, sIL2R remained mostly constant throughout the course of the disease, regardless of the intermittent elevations in CRP levels.

Thus, we observed a gradual increase of CRP, procalcitonin, ferritin and serum IL-6 corresponding with the severity of the disease, but their relative failure to upregulate in patients with fatal course, who have instead shown high sIL2R and D-dimers (Fig 1C).



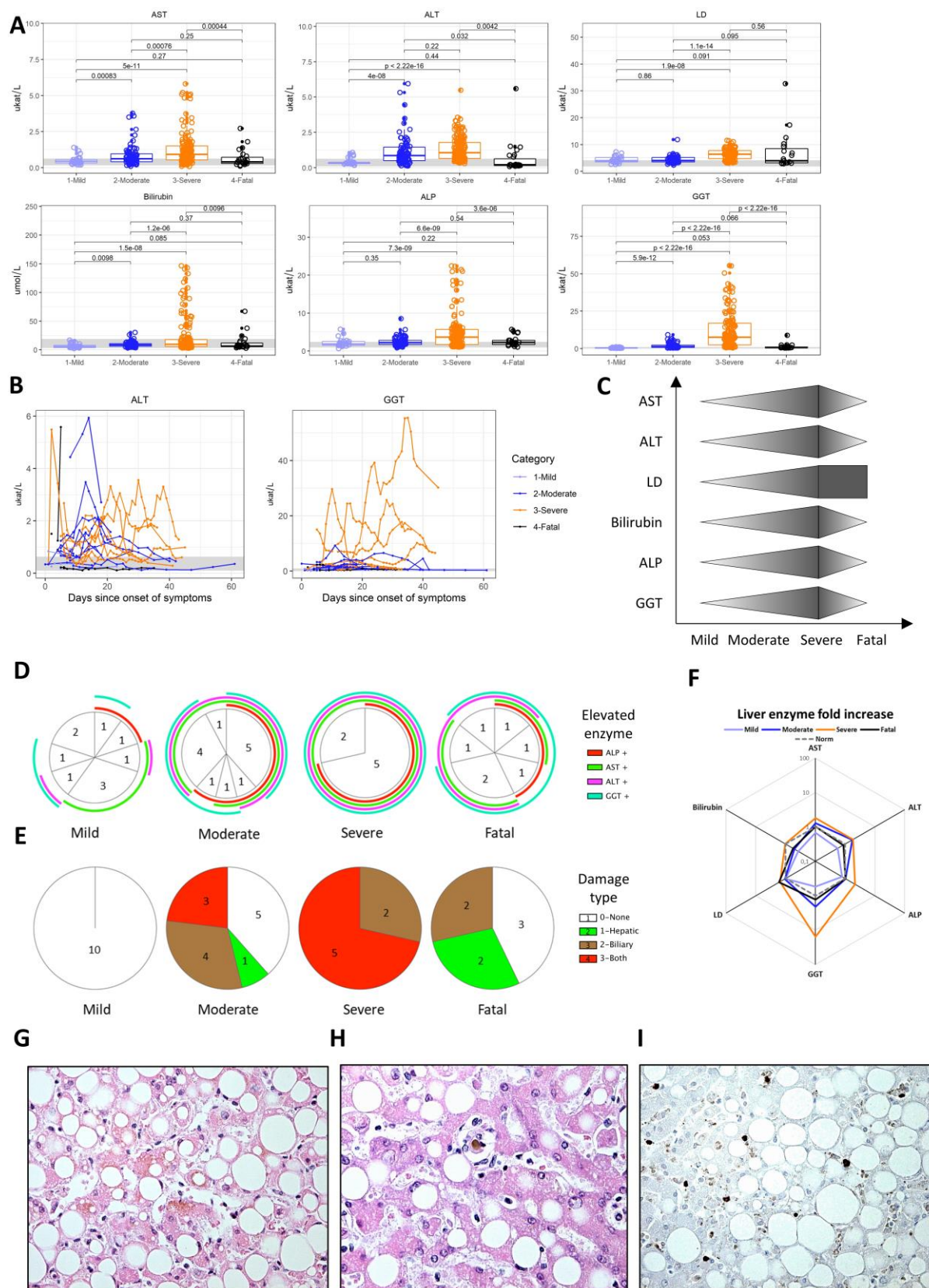
**Figure 1 Inflammation** (A) C-reactive protein, procalcitonin, ferritin, IL-6, soluble IL-2 receptor and D-dimers in patients with mild, moderate, severe and fatal course of the disease. (B) Temporal changes in C-reactive protein and soluble IL-2 receptor levels in patients with mild, moderate, severe and fatal course of the disease over time. (C) Comparison of trends between individual parameters shown in A). Boxes depict median, 1st and 3rd quartile, whiskers show 2.5 and 97.5th percentile. Each symbol represent a unique measurement. Multiple measurements at different timepoints are included from all patients. Values shown are student's t test p values with Holm's multiple comparison adjustment. Where available, healthy reference range for adult males is shown in light gray.

### 3.3 Hepatopathy

The hepatotropic affinity of SARS-CoV-2 and its hepatopathic qualities have been demonstrated previously(34). Correspondingly, we observed the elevation of liver enzymes, , i.e. aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LD) or bilirubin throughout our cohort (Fig 2A). The elevation of ALT peaked around day 15 since the onset of symptoms and then gradually subsided (Fig 2B).

Additionally, we also registered a significant elevation of gamma-glutamyl transferase (GGT) and alkaline phosphatase (ALP) that is most pronounced in patients with severe COVID-19 (Fig 2A) and has a more delayed onset than ALT, past day 20 on average (Fig 2B). Indeed, while even patients with mild course of the disease showed some elevation of liver enzymes above healthy age and sex-matched reference range (Fig 2D), patients with severe course displayed a consistent elevation of all four enzymes in 5/7 patients and fulfilled the more stringent laboratory criteria for either biliary lesion (defined as the elevation of GGT or ALP > 2x healthy age and sex-matched reference range values) or both biliary and hepatic (AST or ALT > 3x ref.) damage (Fig 2E). Of all the enzymes, the elevation of GGT was most significant and characteristic for severe but non-fatal COVID-19, surmounting the healthy age and sex-matched reference range 15 times on average (Fig 2F).

Cholestasis, the collateral feature of biliary injury, was indeed apparent in liver autopsy from a patient with fatal course of COVID-19 (Fig 2G), including a clot in the biliary tract (Fig 2H), along with substantial steatosis despite no previous history of liver disease. A discrete production of IL-6 was detected in the liver (Fig 2I), which was not present in the liver of a non-COVID-19 control (Sup Fig 1B), suggesting a role for IL-6 in tissue inflammation and the resulting damage.



**Figure 2 Hepatopathy** (A) AST, ALT, lactate dehydrogenase (LD), bilirubin, ALP and GGT levels in patients with mild, moderate, severe and fatal course of the disease. (B) Temporal changes in ALT and GGT levels in patients with mild, moderate, severe and fatal course of the disease over time. (C) Comparison of trends among individual parameters shown in A. (D) Elevated enzymes (above healthy age and sex-matched reference range) encountered in each subcohort over the course of the disease,

showing number of patients with each combination of elevated enzymes. (E) Hepatic damage (defined as AST or ALT > 3 × healthy age and sex-matched reference) and/or biliary damage (defined as GGT or ALP > 2 × healthy reference) encountered in each subcohort over the course of the disease. (F) Fold increase in each marker of biliary or hepatic lesion, compared to upper limit of healthy age and sex-matched reference range. (G) Hematoxylin-eosin staining of a COVID-19 patient liver necropsy (40x magnification) revealing marked macrovesicular steatosis and intracellular cholestasis of hepatocytes. (H) Hematoxylin-eosin staining of a COVID-19 patient liver necropsy (40x magnification) revealing a clot in the biliary tract. (I) Immunohistochemical staining of IL-6 in COVID-19 patient liver necropsy (40x magnification) showing infiltrate of IL-6-producing cells within sinusoids and interstitium of the liver parenchyma. Boxes depict median, 1st and 3rd quartile, whiskers show 2.5 and 97.5th percentile. Each symbol represent a unique measurement. Multiple measurements at different timepoints are included from all patients. Values shown are student's t test p values with Holm's multiple comparison adjustment. Where available, healthy reference range for adult males is shown in light gray.

### 3.4 Immune response

Activation of innate immunity is the body's first-line defense against all types of infectious pathogens, including viruses, although the functional integrity of adaptive immune cells such as cytotoxic CD8 T cells and NK cells is the principal component for final clearance of viral infections.

Similarly to previous studies(4), we show a stark elevation of neutrophils in patients with moderate and especially severe course of the disease, with a significantly elevated proportion of immature granulocytes (Fig 3A). Of note, eosinophils were also elevated in several patients with severe course of the disease.

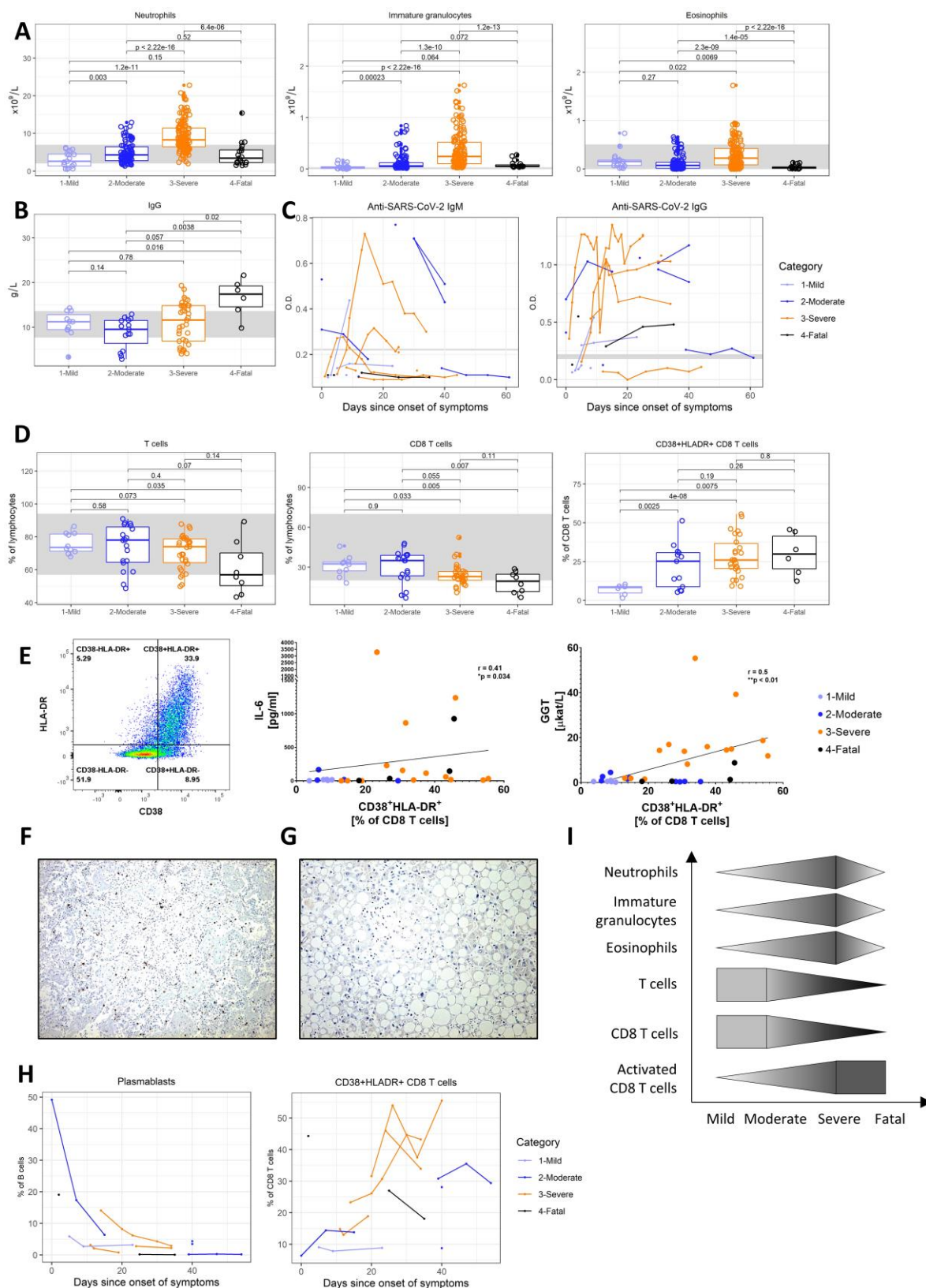
We did not note a major difference in total serum IgG levels between the subcohorts (Fig 3B), however, patients with fatal course of COVID-19 showed significant IgG hypergammaglobulinemia.

Temporal development of specific anti-SARS-CoV-2 antibodies was apparent throughout the course of the disease. Specific IgM antibodies appeared in the first 10 (5-15) days since onset of symptoms and disappeared after day 15 (13-24), although in some patients they remained present for over 30 days (Fig 3C). Virtually concurrent IgM and IgG seroconversion was apparent in all patients and IgG antibodies showed better persistence and even gradual increase over time.

Lymphopenia is a well-described negative prognostic factor associated with severe course of COVID-19(4). As part of the lymphopenia in our patients, we specifically noted the decrease of T cells and CD8 T cells in patients with severe and fatal disease course (Fig 3D). These CD8 T cells were highly activated, co-expressing the surface markers CD38 and HLA-DR and correlated significantly with serum IL-6 levels and the marker of biliary damage, GGT (Fig 3E). While numerous CD8 T cells were found to infiltrate the lungs with histologic signs of interstitial pneumonia in one patient who died from respiratory insufficiency, no such infiltration was found in his liver (Fig 3F), despite the cholestasis and steatosis shown in Fig 2G.

Whereas the humoral immune response contracted within the first 20 days since onset of the disease with fast decrease of plasmablasts detected in the peripheral blood (CD45+CD19+CD27hiCD38hi), the activation of CD8 T cells persisted for over 40 days (Fig 3G). The trends of immune response to COVID-19 are summarized in Fig 3H.





**Figure 3 Immunity** (A) Neutrophil, immature granulocyte and eosinophil counts in patients with mild, moderate, severe and fatal course of the disease. (B) Serum IgG levels in patients with mild, moderate, severe and fatal course of the disease. (C) Temporal changes in anti-SARS-CoV-2 specific IgM and IgG antibodies in patients with mild, moderate, severe and fatal course of the disease over time. (D) Proportion of T cells, CD8 T cells and activated (CD38+HLADR+) CD8 T cells in patients with mild,

moderate, severe and fatal course of the disease. (E) Flow cytometry gating strategy for activated CD38+HLA-DR+ CD8 T cells and their correlation to serum IL-6 and GGT levels, with linear regression trendlines shown and Spearman correlation  $r$  and  $p$  values. (F) Immunohistochemical staining of CD8+ cells in a COVID-19 patient lung necropsy (4x magnification) showing cytotoxic T cells within the interstitium and capillaries of interalveolar septa, as well as within alveolar spaces. (G) Immunohistochemical staining of CD8+ cells in a COVID-19 patient liver necropsy (20x magnification) showing lack of CD8 positive cells. (H) Dynamics of plasmablast and activated CD38+HLA-DR+ CD8 T cell populations in patients with mild, moderate, severe and fatal course of the disease over time. (I) Comparison of trends between individual parameters shown in A). Boxes depict median, 1st and 3rd quartile, whiskers show 2.5 and 97.5th percentile. Each symbol represent a unique measurement. Multiple measurements at different timepoints are included from all patients. Values shown are student's  $t$  test  $p$  values with Holm's multiple comparison adjustment. Where available, healthy reference range for adult males is shown in light gray.

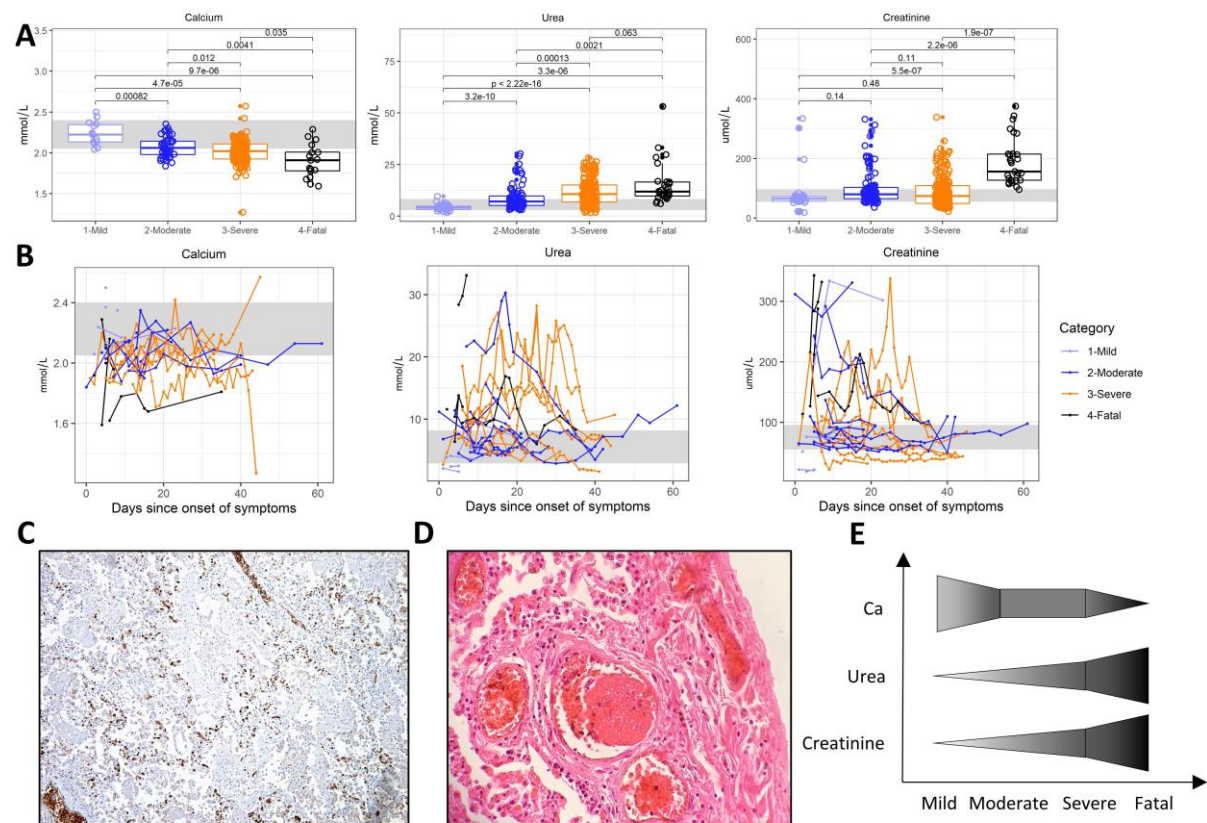
### 3.5 Kidney and lung damage

Most markers of inflammation, immune response and liver damage presented in patients with fatal course of COVID-19 so far seem mostly on par with those seen in patients with moderate disease, suggesting a weaker response to the infection compared to severely ill patients, nevertheless ultimately resulting in patient death.

Other key characteristics of patients with fatal course of the disease seen in our study were the mineral disbalance, particularly hypocalcaemia, and renal insufficiency with elevated serum urea and creatinine (Fig 4A). Although elevated urea and creatinine were also present in some moderately and severely ill patients, these tended to normalize eventually (Fig 4B).

Although serum IL-6 was not particularly high in fatally ill patients (Fig 1A), there was a substantial production of IL-6 in the lungs, driven by interstitially positioned leukocytes (Fig 4C). The pneumonia and acute respiratory distress syndrome were accompanied by numerous thrombi (Fig 4D) along with high plasma D-dimers.

The trends of calcemia and markers of kidney failure in COVID-19 are summarized in Fig 4E.



**Figure 4 Kidney failure and lung damage (A)** Calcium, urea and creatinine levels in patients with mild, moderate, severe and fatal course of the disease. **(B)** Dynamics of calcium, urea and creatinine levels in patients with mild, moderate, severe and fatal course of the disease over time. **(C)** Immunohistochemical staining of IL-6 in COVID-19 patient lung necropsy (4x magnification) showing IL-6-producing cells within interstitium and capillaries of interalveolar septa and within the lumen of larger vessels. **(D)** Hematoxylin-eosin staining of COVID-19 patient lung necropsy (20x magnification) showing interstitial pneumonia and the detail of a venous thrombus. **(E)** Comparison of trends between individual parameters shown in A). Boxes depict median, 1st and 3rd quartile, whiskers show 2.5 and 97.5th percentile. Each symbol represent a unique measurement. Multiple measurements at different timepoints are included from all patients. Values shown are student's t test p values with Holm's multiple comparison adjustment. Where available, healthy reference range for adult males is shown in light gray.

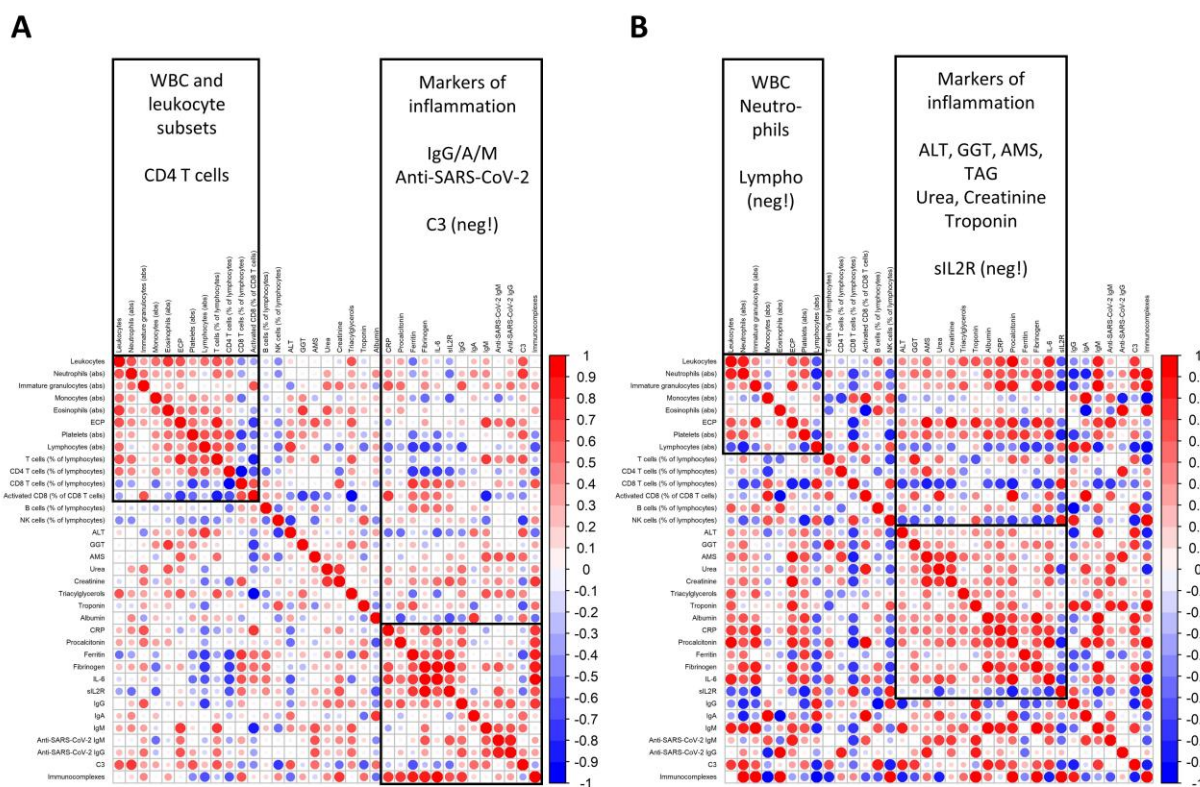
### 3.6 Dyscoordination of the immune response

As demonstrated above, common trends arise when studying the immune response against the SARS-CoV-2 virus and the different facets of its pathogenicity against humans, as summarized in trend graphs of figures 1-4.

To characterize the complexity of differences between patients with efficient, well-coordinated response to the infection and, therefore, only mild course of the disease, and patients with fatal course of COVID-19, we constructed correlation matrices of selected laboratory parameters (Fig 5).

In patients with mild course of COVID-19 (Fig 5A), we show a cluster of positively intercorrelated hematological parameters such as overall leukocyte count, neutrophils, immature neutrophils but also, interestingly lymphocytes and T cells. Markers of inflammation such as CRP, procalcitonin, IL-6, and sIL2R correlated positively with humoral immune response – serum IgG, IgA, IgM and specific anti-SARS-CoV-2 antibodies, clearly showing a well-orchestrated immune response of both the innate and humoral adaptive immunity.

In contrast, patients with fatal COVID-19 (Fig 5B) display a negative correlation between leukocytes and lymphocytes and their inflammatory markers rise with markers of organ failure – liver enzymes, amylase, GGT, urea and creatinine – instead.



**Figure 5 Discoordination of immune response** (A) Heatmap showing Spearman correlation coefficients between selected parameters mapping inflammation, immune response and metabolic parameters in patients with mild course of the disease. (B) Heatmap showing Spearman correlation coefficients between selected parameters mapping inflammation, immune response and metabolic parameters in patients with fatal course of the disease. Size and opacity of individual circles represent the Spearman correlation R value between each pair of parameters. Positive correlation is shown in shades of red, negative correlation in shades of blue. No sorting algorithm was used, parameters are ordered manually based on thematic groups.

#### 4. Discussion

COVID-19 is a multifaceted disease with a striking stratification of the severity spectrum. Aspiring to contribute to the current knowledge pool, our report describes a representative cohort of COVID-19 patients hospitalized during the pandemic in a large Czech hospital. The distribution of mild, moderate, severe and fatal courses of the disease aligns with previously described cohorts(3,7,22).

Similar to others, we observed a correlation between a set of inflammatory markers, CRP, procalcitonin, ferritin and serum IL-6 and additionally note that fatal cases failed to mount the corresponding elevation of these parameters, suggesting either an exhaustion or suppression of these key inflammatory components. Instead, patients with fatal course of the disease showed high sIL2R and D-dimers. Although unspecific, sIL2R as a marker of T cell activation has been shown to identify patients with multi-organ sarcoidosis(35) in similar fashion to our patients with fatal COVID-19. The elevation of D-dimers accompanies a hypercoagulation state that manifests as macro and microvascular thrombotic complications in severe COVID-19 patients(12,17,36) and has been implied as an independent marker of increased mortality(4,32). Indeed, here we show venous thrombi in the lungs of a deceased COVID-19 patient. Furthermore, by directly demonstrating the presence of IL-6 producing cells and CD8+ T cells in the lungs we document a cellular inflammation-related mechanism of lung damage beyond the systemic cytokine storm.

Abnormalities in white blood count, i.e. lymphopenia with marked neutrophilia, are now well-established features of severe COVID-19 that we confirm in our cohort(20,24). Additionally, we describe a marked shift towards immature granulocyte forms, becoming more pronounced with increasing severity, and a stark decline in both the mature neutrophil and their precursor counts in the fatal courses. The expansion of developing neutrophils in patients with severe COVID-19 was recently identified through single cell RNA sequencing(37) and their reduction may imply a primary failure to efficiently recruit these innate immune responders.

Moreover, the severe and fatal cases were accompanied with profound T cell, and particularly CD8 T cell depression, yet with cellular phenotype suggestive of their activation. This reflects the observations that T cells express one of the SARS-CoV-2 receptors CD147(15), rendering the T cells susceptible to viral entry, and that the infection is associated with the reduction of naive CD8 T cell percentage(38). The lymphopenia observed in COVID-19 may, in part, arise as a result of the IL-2 signaling inhibition due to increased soluble IL-2 receptor seen in ours and other cohorts(39). Taken together, T lymphocyte damage is likely an important aspect of clinical deterioration in COVID-19.

Hepatopathy has been reported in 16-53% of symptomatic patients with COVID-19(6,40). Although severe liver dysfunction has been described, the liver injury appears to be mild and transient in majority of patients, with the median transaminase level remaining lower than twice the upper reference(34,41), which corresponds well with our mild cohort. The elevation of GGT, a marker of cholangiocyte injury, has been reported in COVID-19 only scarcely so far(34,42). Interestingly, in our severe, but not fatal subgroup we observed an excessive increase of GGT, strikingly disproportionate to the increase of ALT and AST. The progression to severe disease has been previously associated with predominantly hepatic (elevated ALT and AST) or mixed hepatic and biliary (elevated GGT and ALP) type of liver injury(42). In our severe cohort, biliary or mixed biliary and hepatic damage was found in the majority of patients. Therefore, we suggest that in COVID-19-related hepatopathy with biliary injury the predominant elevation of GGT may represent a new independent negative prognostic marker.

Although the hepatopathy and cholestasis present in our cohort of patients may be, at least in part, of hypoxemic or drug-induced origin, the permissiveness of hepatocytes and cholangiocytes to

SARS-CoV-2 entry has also been documented(11,43), therefore a direct viral-induced injury to these cells is feasible. To the best of our knowledge, no direct evidence for pro-inflammatory cytokines involvement in the hepatopathy in COVID-19 has been reported. The infiltration of IL-6 producing cells into liver sinusoids and interstitium may accelerate the production of other markers of inflammation. However, their relative scarcity and the lack of infiltrating CD8 T cells suggests that immune cells, unlike in the lungs, are not the main drivers of pathology in COVID-19 liver disease, despite the correlation between activated CD8 T cells and serum GGT levels.

An overwhelming evidence thus points to the multi-organ affinity of the virus, extending also to the kidneys(16). Indeed, our finding of elevated markers of kidney damage in patients with more severe course of the disease echoes the data from China, where high creatinine and acute kidney injury were risk factors for in-hospital death(44,45). The renal pathophysiology is, however, likely multifactorial, involving hypoxemic, hypovolemic, thrombotic and medication-induced insults.

A comprehensive mapping of markers of immune and metabolic response in our cohort illustratively documented its dyscoordinated orchestration, highlighted in comparison between the mild and fatal cases. While systems biology approaches may be helpful in deciphering the pathophysiology of COVID-19, especially due to its multi-organ affinity, limitations imposed by heterogeneous cohorts, temporal changes in examined parameters and interindividual variability due to comorbidities and medication should be borne in mind. These are indeed the main limitations of ours and most other published studies on COVID-19.

## 5. Conclusions

In summary, we demonstrated the complexity of immune and metabolic disturbances in COVID-19 patients. Our experiments contribute to the current understanding of the nature of SARS-CoV-2-driven immunopathology and tissue injury, particularly the systemic inflammation, lymphopenia with T cell activation and organ infiltration. We observed that severe COVID-19-related hepatopathy may be associated with marked biliary lesion hallmarked by stark elevation as GGT and suggest the enzyme may represent an additional negative prognostic marker.

**Supplementary Materials:** The following are available online at [www.mdpi.com/xxx/s1](http://www.mdpi.com/xxx/s1), Figure S1: Healthy control biopsy

**Author Contributions:** Conceptualization, A.K., M.B., A.S.; analysis, A.K., J.B., J.Z.; investigation and resources, Z.P., I.Z., P.V., L.F.C.M., A.G., J.S., M.T., T.V.; writing and review, A.K., M.B., Z.P., A.S.. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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