

CD99 and polymeric immunoglobulin receptor peptides deregulation in critical COVID-19: a potential link to molecular pathophysiology?

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Abstract

Identification of significant changes in urinary peptides may enable improved understanding of molecular disease mechanisms. We aimed towards identifying urinary peptides associated with critical course of COVID-19 to yield hypotheses on molecular pathophysiological mechanisms in disease development. In this multicentre prospective study urine samples of PCR-confirmed COVID-19 patients were collected in different centres across Europe. The urinary peptidome of 53 patients at WHO stages 6-8 and 66 at WHO stages 1-3 COVID-19 disease was analysed using capillary electrophoresis coupled to mass spectrometry. 593 peptides were identified significantly affected by disease severity. These peptides were compared with changes associated with kidney disease or heart failure. Similarities with kidney disease were observed, indicating comparable molecular mechanisms. In contrast, convincing similarity to heart failure could not be detected. The data for the first time showed deregulation of CD99 and polymeric immunoglobulin receptor peptides and of known peptides associated with kidney disease, including collagen and alpha-1-antitrypsin. Peptidomic findings were in line with the pathophysiology of COVID-19. The clinical corollary is that COVID-19 induces specific inflammation of numerous tissues including endothelial lining. Restoring these changes, especially in CD99, PIGR and alpha-1-antitrypsin, may represent a valid and effective therapeutic approach in COVID-19, targeting improvement of endothelial integrity.

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Statement of significance of the study

Deeper understanding of molecular mechanisms underlying development of severe COVID-19 holds promise for improving therapeutic options and ultimately patients' outcome. Urinary peptidomic analysis allows identification of disease-associated peptides and thus provides information of disease-associated pathways, shedding light on molecular pathophysiology. Previous studies demonstrated value of urinary peptidomics in detecting peptides significantly associated with COVID-19, and supported their value in predicting outcome of COVID-19 patients. This study aims at identifying urinary peptides associated with critical course of COVID-19 to yield hypotheses on yet unknown and known pathophysiological mechanisms in disease development. The level of 593 urinary peptides was found to be significantly different between patients with mild (n=66) and severe (n=53) COVID-19. Among identified peptides, a yet unknown COVID-19 specific deregulation of CD99 and polymeric immunoglobulin receptor peptides was observed. Other prominent changes were observed in collagen and alpha-1-antitrypsin derived peptides. Interestingly, the peptide profiles were similar compared with those observed in kidney disease, a prototype of target organ damage with major microvascular involvement, thereby confirming the observation that endothelial damage is a hallmark of COVID-19. Restoring these changes, especially in CD99, PIGR and alpha-1-antitrypsin, may represent a valid and effective therapeutic approach in COVID-19, targeting improvement of endothelial integrity.

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Introduction

Accumulating evidence indicates that in SARS-CoV2-infection endothelial injury, capillary leakage and renal injury ^[1-3] may be a key factor contributing to hospitalisation and mortality ^[4]. Chronic kidney disease (CKD) is connected to endothelial dysfunction ^[5] and dysregulation of the immune system. Acute kidney injury (AKI) shares molecular links to endothelial traits like, thrombotic microangiopathy and severe sepsis with multi organ dysfunction^[6]. As a result of these facts, pathophysiological mechanisms of COVID-19, especially those linked to protein degradation, are expected to be detectable, possibly even enriched in urine peptides. This assumption was supported initially by a preliminary study that enabled identification of urinary peptides significantly affected by SARS-CoV-2 infection, and by COVID-19 disease severity^[7]. As a result of these promising findings, a prospective multicenter study was initiated. First results from this study based on 327 subjects demonstrated a significant predictive value of urinary peptides, opening the path forward for a strategy to combine accurate prediction with targeted, personalized intervention ^[8].

To characterize the molecular impact of the severity of COVID-19, we performed a comprehensive analysis of urinary peptides of COVID-19 patients using capillary electrophoresis-coupled mass spectrometry (CE-MS) and compared the resulting peptide data from patients with critical and mild courses of COVID-19. In a second step, we investigated additional peptidomics data retrieved from the urine peptide database ^[9] to assess changes observed in (non-COVID-19) patients with diabetic kidney disease (DKD) representing CKD, in AKI, and in heart failure (HF) versus controls. The changes observed in these diseases were subsequently compared to the changes observed in severe COVID-19.

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Materials and Methods

Patients

Urine samples from 119 SARS-CoV-2 infected patients were collected in the "Prospective Validation of a Proteomic Urine Test for Early and Accurate Prognosis of Critical Course Complications in Patients with SARS-CoV-2 Infection" (CRIT-Cov-U) study, described previously ^[7]. Samples were prospectively collected within 2 days after a positive real-time reverse transcriptase-polymerase chain reaction (PCR) test. Both hospital and community-based samples were collected. Patients were followed up for at least 21 days or until hospital discharge or death, whichever occurred first. Disease severity was classified based on the World Health Organisation (WHO) ^[10] scale as mild (WHO stages 1-3), intermediate (WHO stages 4-5), or critical (WHO stages 6-8). Samples were stratified according to the highest WHO grade reported during the follow up. No significant differences in kidney function between the mild and critical group were observed. This study was approved by the local ethics committee (Saxon Chamber of Physicians, registry number #EK-BR-88/20-1), with a waiver of informed consent and by the Institutional Review Boards of all participating centres.

Proteome analysis

Samples were analysed by CE-MS and the generated data processed as described before, including assignment of sequences ^[9,11]. In short, urine aliquots were thawed and 700 µl mixed with 700 µl of 2 M urea, 10 mM NH₄OH containing 0.02 % SDS. Subsequently, samples were ultrafiltered using a Centrstat 20 kDa cut-off centrifugal filter device (Satorius, Göttingen, Germany). The filtrate was desalted using a PD 10 gel filtration column (GE

Healthcare Bio Sciences, Uppsala, Sweden), and stored at 4°. Before CE-MS analysis, the samples were re-suspended in 10 µL HPLC-grade H₂O and injected (with 2 psi for 99 sec, resulting in injection volumes of ~280 nL) onto a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, CA) coupled with a Micro-TOF MS (Bruker Daltonic, Bremen, Germany). A solution of 20% acetonitrile (Sigma-Aldrich, Taufkirchen, Germany) in HPLC-grade water (Roth, Karlsruhe, Germany) supplemented with 0.94% formic acid (Sigma-Aldrich) was used as running buffer. The electrospray ionization interface from Agilent Technologies (Palo Alto, CA) was set to a potential of -4.0 to -4.5 kV. Spectra were recorded over an *m/z* range of 350-3000 and accumulated every 3 sec.

The CE-MS data were analyzed using the MosaFinder software^[9]. Only signals observed in a minimum of 3 consecutive spectra with a signal-to-noise ratio >3 were considered. Data were calibrated utilizing internal standards as reference for mass and migration time by applying global and local linear regression, respectively. Reference signals of 29 abundant peptides were used as internal standards for calibration using linear regression. This procedure was shown to be an easy and reliable method to address both analytical and dilution variances in a single calibration step^[12]. All detected peptides are deposited, matched, and annotated in a Microsoft SQL database allowing further statistical analysis.

Extraction of additional CE-MS datasets

Additional CE-MS datasets were extracted from the urinary proteome database^[9]. This database includes >80.000 datasets processed and normalized as described above. This approach results in a highly comparable dataset, with no detectable batch effects^[11,13].

Extracted were datasets of 119 control subjects without COVID-19 disease matched to the full cohort of COVID-19 patients for age, sex, blood pressure and eGFR (based on the CKD-EPI formula). Furthermore, CE-MS data from patients with diabetic kidney disease, DKD (n=571) and 374 controls matched for age, sex, blood pressure and diabetes duration ^[14], 773 datasets from HF patients and 773 matched (for age, sex, blood pressure, kidney function, and diabetes) controls ^[15], and 94 datasets from patients with AKI (KDIGO grade 2 and 3) and 348 controls (KDIGO grade 0), matched for age and sex were also extracted from the database.

Flow cytometry

Fluorescence-activated cell sorting (FACS) was performed for COVID-19 patients with moderate disease (WHO stage 1-4, n=6), COVID-19 patients with severe/critical disease (WHO stage 5-8, n=12), and healthy staff members (n=10). Further, we included human immunodeficiency virus (HIV) infected patients with (n=17) and without lymphopenia (n=5) as control to exclude non-COVID-19 effects due to lymphopenia. All samples from patients and controls for flow cytometry analysis were consecutively collected between 2nd December and 12th December 2020.

Initially, lymphocyte counts were measured on a particle counter (Sysmex XN-2000, Norderstedt, Germany). The percentage values of studied subsets obtained by cytometric reading were converted into absolute values according to absolute number of lymphocytes count. Immunophenotyping of lymphocytes was performed by 8-color cytometry with monoclonal antibodies including anti-CD99 BV421 (BD Biosciences) on BD FACSLytic™ flow cytometer (BD Biosciences). Applied fluorochroms as well as gating strategy for

identification of lymphocytes (CD45+SSC_{low}), T cells (CD3+), B cells (CD19+), and NK cells (CD16+/56+) is shown **Supplementary Figure 1**. Staining of 100 µl EDTA blood was performed for 15 min at room temperature in the dark with monoclonal antibodies (BD Biosciences). Subsequently, red blood cells were lysed with BD FACS™ Lysing Solution (BD Biosciences) for 10 min, washed with 2 ml PBS. The pelleted cells were re-suspended in 300 µl PBS for analysis. To ensure consistent results, BD FACSuite™ software was utilized for assay generation and data acquisition. Automated cytometer setup, assay setup, and performance tracking was performed with a daily acquisition of BD CS&T™ IVD beads according to the supplier's instructions. Threshold for CD99 positivity was generated by a „Fluorescence minus one control“ (assay without anti-CD99). CD99- granulocytes and CD99+ monocytes were used as reference populations (see scattergrams with CD99 on y-axis, **Supplementary Figure 1**).

Statistical methods, definition of biomarkers

Peptides levels across groups as well as flow cytometry data were compared using the Wilcoxon rank sum test. False discovery rate was assessed by the method described by Benjamini and Hochberg ^[16]. To estimate the relationship between the changes in urinary peptides of different conditions, regression analysis was calculated using MedCalc version 12.7.5.0 (MedCalc Software; Mariakerke, Belgium).

Results

Proteomics

Urinary peptide data of patients with critical COVID-19 course (n=53) were compared to data from mild COVID-19 courses (n=66). Selected clinical/demographic data that are known to impact the urinary peptidome are given in **Table 1**. The statistical analysis returned 593 urinary peptides that were significantly associated with COVID-19 severity (listed in the **Supplementary Table 1**). To set these observations in context with healthy subjects, we extracted CE-MS data from 119 control subjects without COVID-19 disease from the urinary proteome database, matched to the full cohort of COVID-19 patients for age, sex, blood pressure and eGFR (**Table 1**). These data, and the changes observed in mild and critical COVID-19 in comparison to the controls are also listed in **Supplementary Table 1**.

The distribution of the 593 peptides is presented in **Figure 1**, which also depicts the distribution of these peptides in matching controls. As evident from **Figure 1**, the peptides associated with critical COVID-19 disease course show very similar distribution in patients with mild disease course and in matching controls from subjects not infected with COVID-19. Among peptides de-regulated in critical COVID-19, fragments of CD99, polymeric immunoglobulin receptor (PIGR), different types of collagens, alpha-1-antitrypsin, beta-2-microglobulin, fibrinogen, and protein S100-A9 were prominently found.

To assess similarity to other disease, the changes observed when comparing mild to critical disease course were subsequently compared to peptide changes observed in other disease included in the human urinary peptide database^[9]: DKD patients (n=571) were compared to 374 controls matched for age, sex, blood pressure and diabetes duration^[14], 773 datasets from HF patients were compared to 773 matched (for age, sex, blood pressure, kidney

function, and diabetes) controls^[15], and 94 patients with AKI (KDIGO grade 2 and 3) were compared to 348 controls (KDIGO grade 0), matched for age and sex ^[17,18]. For each peptide, fold change in disease vs. controls was calculated. The results are shown in **Figure 2**.

Reductions of urinary CD99 and PIGR peptides (negative correlations) were apparently exclusive in severe COVID-19 and not detectable in DKD, AKI and HF. Significant positive correlations were found between critical COVID-19 and DKD (**Figure 2A**, $R^2 = 0.41$) and AKI (**Figure 2B**, $R^2=0.30$) but not between critical COVID-19 and HF (**Figure 2C**). The substantial degree of similarity between critical COVID-19 and kidney diseases is also evident from the heatmap shown in **Figure 2D** (for non-collagen protein fragments) and **Figure 2E** (for collagen protein fragments), depicting the urinary excretion pattern of the 100 most significant (p-values $< 3.0 \times 10^{-7}$) critical COVID-19 associated peptides.

Flow cytometry

To further investigate the observed reduction of CD99 peptides in critical COVID-19, we assessed CD99 expression on lymphocytes from COVID-19 patients with moderate or severe disease course. Since critical COVID-19 is associated with lymphopenia, samples from HIV-infected patients with and without lymphopenia were used as controls. A significant decrease of CD99 expressing lymphocytes was detectable in patients with critical COVID-19, which was not detected in the control HIV-infected patients (**Figure 3**). The representative cytometric analysis by an 8-color panel including CD99 and an overview of immune perturbation in COVID-19 are shown in **supplementary figure 1**, respectively.

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Discussion

Changes in urinary peptides are specific for distinct pathologies and allow discrimination of different disease aetiologies as demonstrated previously ^[19], but also enable definition of shared changes displaying frequently involved processes like endothelial integrity, fibrosis, or inflammation.

The exclusive and for the first time described highly significant and consistent reduction of CD99 peptides in severe/critical COVID-19 may indicate reduction of endothelial integrity and interference with transendothelial migration of monocytes, neutrophils, and T-cell recruitment. Loss of CD99 is expected to compromise tight junctions, possibly resulting in exposure of collagen, which would lead to thrombotic complications found highly specific for COVID-19 clinical courses ^[20]. Analysis of lymphocyte subsets confirmed the reduction of CD99 in critical COVID-19 patients. In agreement with the proteomics data, in mild COVID-19 we observed an increase of CD99 expression on lymphocytes in comparison to uninfected controls, while in critical COVID-19 a significant decrease of CD99 was observed. These consistent changes indicate a novel potential therapeutic approach, targeting CD99. These exclusive findings of CD99 depletion in severe COVID-19 warrant further mechanistic studies on the role of this mechanism, for instance using recombinant CD99 or knock-out approaches in cell culture or animal experiments. Two FDA-approved compounds are available, clofarabine and cladribine, which target CD99 function ^[21]. Both drugs inhibit dimerization of CD99 which would likely have a negative impact on the COVID-19 progression. However, these drugs may be of value in initial experiments to investigate the role of CD99 in COVID-19.

Like CD99, PIGR has not been investigated in the context of COVID-19. PIGR was reported reduced in chronic obstructive pulmonary disease ^[22], a risk factor for COVID-19 severity.

Further investigations of PIGR in COVID-19 patients via e.g. immunohistochemistry appear justified to confirm the observations in this study, and potentially also indicate opportunities for therapeutic intervention.

Deregulation of collagen homeostasis, found in severe COVID-19 patients here, is a well described feature in kidney disease ^[23]. The interconnection of COVID-19 and kidney injury is now known for several months, initially identified via autaptic findings ^[3]. Here, for the first time by urinary peptide signature, we provide evidence that these diseases share common features in collagen homeostasis deregulation. The most prominent changes in collagen fragments showed even stronger decrease in critical COVID-19 patients compared to kidney disease patients (**Figure 2**). Similarities at molecular level between kidney disease and critical COVID-19 patients were also observed in the regulation of alpha-1-antitrypsin, in both cases up-regulated in urine, likely the result of increased degradation. Of note: alpha-1-antitrypsin deficiency is described as major risk factor for critical COVID-19 ^[24].

Our data also indicate specific association of critical COVID-19 with kidney disease. The molecular changes observed in HF reveal no similarity ^[25].

It is surprising that the very prominent changes observed in urinary peptides were not described in other studies that investigated the impact of COVID-19 disease course on the proteome ^[26,27]. However, these studies in general were of low power, and, in addition, investigated blood as a potential source of biomarkers. Given the high variability that is a result of blood sampling, in part due to activation of proteases in the coagulation cascade ^[28] and the low number of subjects involved in the studies reported, the comparison between the results may be interpreted as supporting approaches that exploit more stable biospecimen (as e.g. urine) and that are based on a large number of independent samples, to provide sufficient statistical power.

In conclusion, the data presented here strongly suggest that severe COVID-19 is associated and may in part be the result of distinct molecular changes of endogenous proteins. Restoring these changes, especially in CD99, PIGR and alpha-1-antitripsin, may represent a valid and effective therapeutic approach in COVID-19, targeting improvement of endothelial integrity.

Disclosure

HM the co-founder and co-owner of Mosaiques Diagnostics. JS, TH and AL are employees of Mosaiques-Diagnostics GmbH.

Data availability

Raw data from the CE-MS analysis from 119 samples from COVID-19 patients are available on Zenodo: <https://zenodo.org/record/4774897>.

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Figure legends

Figure 1 Graphic depiction of the distribution of the 593 urinary peptides in compiled control subjects (controls), patients with mild COVID-19 disease (WHO 1-3) and patients with critical COVID-19 disease course (WHO 6-8). The molecular mass (0.8–15 kDa, on a logarithmic scale) is plotted against normalized migration time (18–45 min). Signal intensity is encoded by peak height and colour.

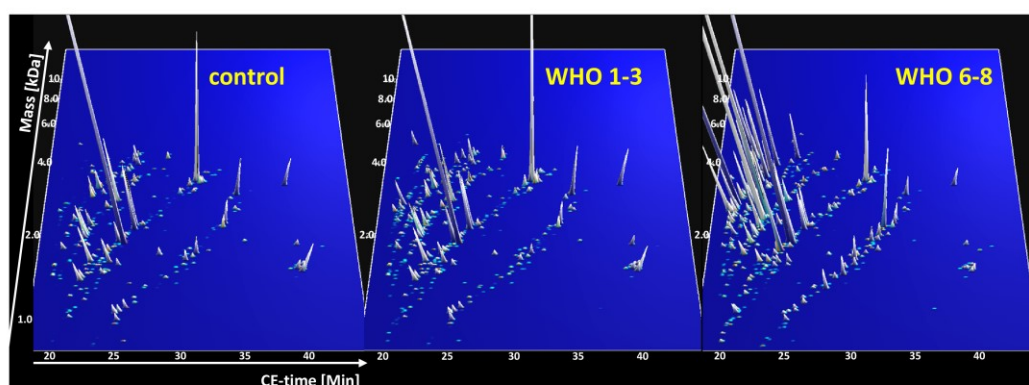


Figure 2: Association of changes observed in urinary peptides of critical COVID-19 patients and patients with diabetic kidney disease (DKD), acute kidney injury (AKI) and heart failure (HF). Regression plots of fold changes of all 593 critical COVID-19 specific urinary peptides (calculated critical vs. mild COVID-19) in comparison to changes observed in DKD patients (A), AKI patients (B) and HF patients (C) (calculated as disease vs. controls). Urinary excretion pattern of the 100 most significant (with p-values $< 3.0 \times 10^{-7}$) critical COVID-19 associated peptides is depicted as a heatmap for non-collagen (D) and for collagen protein fragments (E).

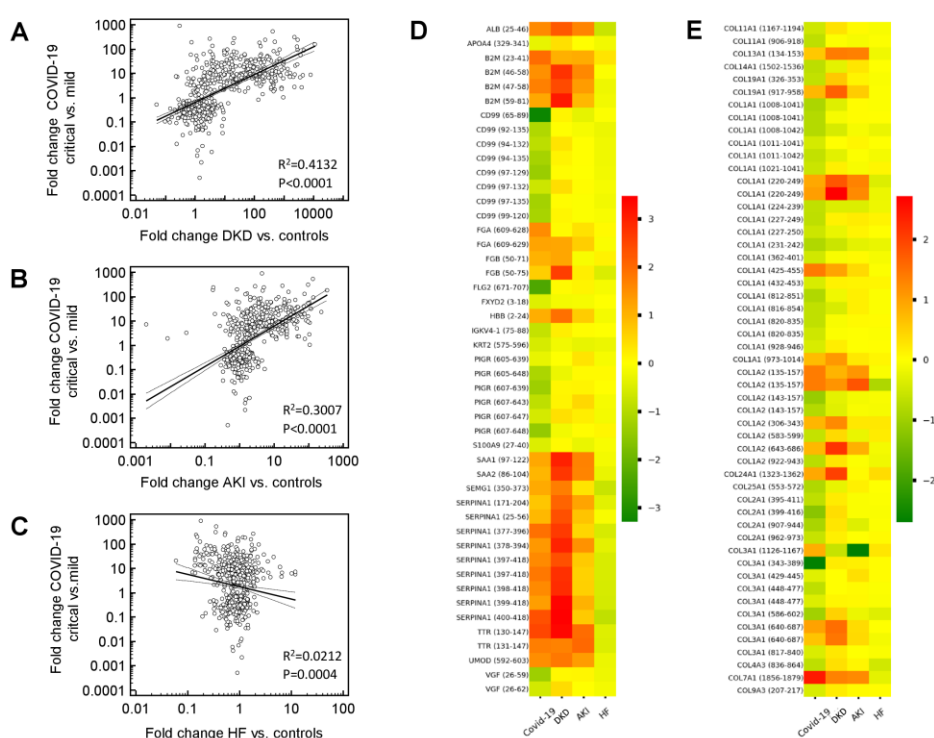
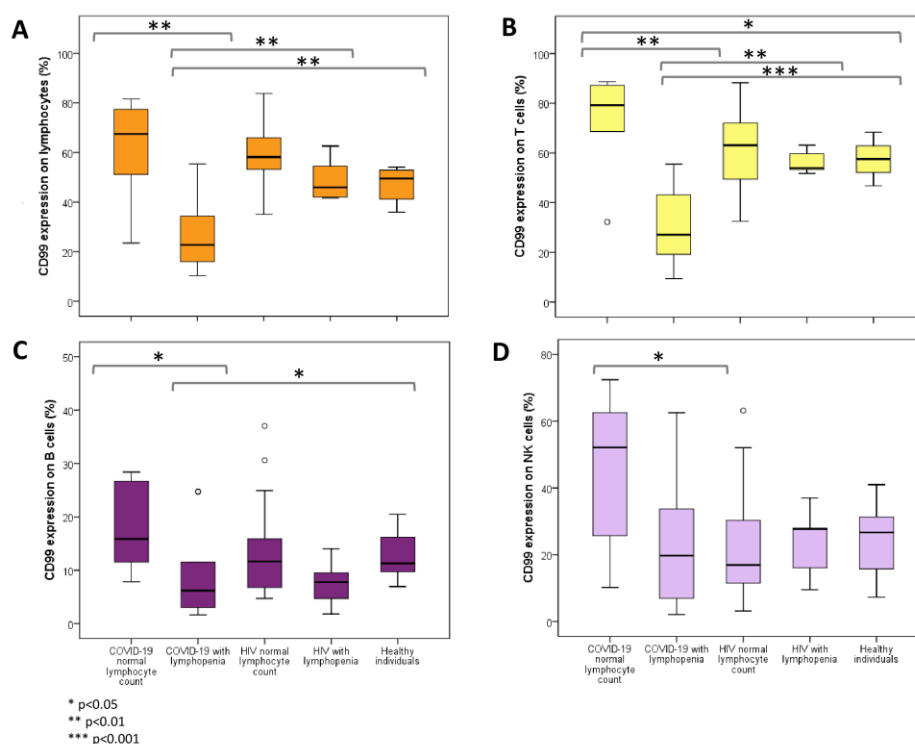


Figure 3: Relative CD99 expression of lymphocytes and of selected lymphocyte subpopulations. To evaluate CD99 expression on lymphocytes and lymphocytes subsets, lymphocytes (orange), T (yellow), B (dark purple) and NK cells (light purple) were selected by positivity of CD45, CD3, CD19, and CD16/56, respectively, using gating strategy depicted in supplementary figure 1. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$



Supplementary Figure 1: Representative cytometric analysis by an 8-color panel including staining of CD99. A) Healthy patient with a lymphocyte count of 2800/ μ l. B) COVID-19 patient with lymphocyte count of 500 per μ l. CD99 expression was measured on total lymphocytes (CD45+SSC-low), T (CD3+), B (CD19+) and NK cells (CD16/56+). The header in the scattergrams represents the investigated subpopulation. As gating reference for CD99 relative expression, neutrophils served as negative-staining and monocytes as positive-staining populations. Mono = monocytes, Neutr = neutrophils, Lymph = lymphocytes.

Table 1 Clinical and demographic characteristics of the COVID-19 patients investigated and matching controls. Median values [95% CI] or number (%) are given. P-values are given for comparison between all patients with COVID-19 and controls. Mann-Whitney test was applied for continuous variables and Chi-squared test for categorical variables.

Characteristics	Mild COVID-19 (WHO 1-3), n=66	Critical COVID-19 (WHO 6-8), n=53	COVID-19, n=119	Controls, n=119	p-value
Age – yr	49.00 [41.57- 56.00]	66.00 [62.00- 70.10]	58.00 [56.00 - 63.00]	61.00 [59.00- 62.67]	$p = 0.3002$
Female sex – no. (%)	34 (52%)	11 (21%)	45 (38%)	48 (40%)	$p = 0.7905$
DBP – mm Hg	80.00 [77.25-80.00]	70.00 [65.03-75.00]	77.00 [72.00- 80.00]	79.00 [77.33- 80.00]	$p = 0.0703$
SBP – mm Hg	123.00 [120.00- 130.00]	122.00 [116.00-130.97]	123.00 [120.00-130.00]	128.00 [125.00- 132.00]	$p = 0.0510$
eGFR – ml/min/1.73m ²	77.00 [64.96-97.11]	89.00 [63.28- 115.70]	81.50 [68.59-101.61]	79.97 [77.14 - 82.80]	$p = 0.7929$

Supplementary Table 1 List of the 593 peptides significantly changed between mild and critical COVID-19 disease course. Listed is the peptide sequence, the uniprot accession and parental protein, the relative abundance in mild (WHO 1-3) and severe (WHO 6-8) COVID -19, the fold-change and the p-value after adjustment for multiple testing. In addition, the distribution of the peptides in matched non SARS-CoV-2 infected controls, in diabetic patients with preserved kidney function and in patients with diabetic kidney disease (DKD), in patients with and without heart failure (HF) and in patients with and without acute kidney injury (AKI), as well as the respective fold-changes.

