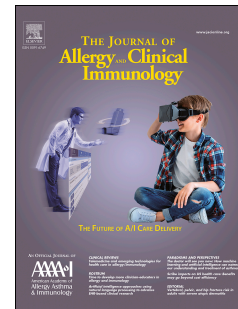


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Pre-existing comorbidities shape the immune response associated with severe COVID-19

Stefanie Kreutmair, MD, Manuel Kauffmann, MSc, Susanne Unger, PhD, Florian Ingelfinger, PhD, Nicolás Gonzalo Núñez, PhD, Chiara Alberti, PhD, Donatella De Feo, MD, PhD, Sinduya Krishnarajah, PhD, Ekaterina Friebe, PhD, Can Ulutekin, MSc, Sepideh Babaei, PhD, Benjamin Gaborit, MD, PhD, Mirjam Lutz, Nicole Puertas Jurado, Nisar P. Malek, MD, Siri Göpel, MD, Peter Rosenberger, MD, Helene A. Häberle, MD, Ikram Ayoub, MSc, Sally Al-Hajj, PhD, Manfred Claassen, PhD, Roland Liblau, MD, PhD, Guillaume Martin-Blondel, MD, PhD, Michael Bitzer, MD, Antoine Roquilly, MD, PhD, Burkhard Becher, PhD



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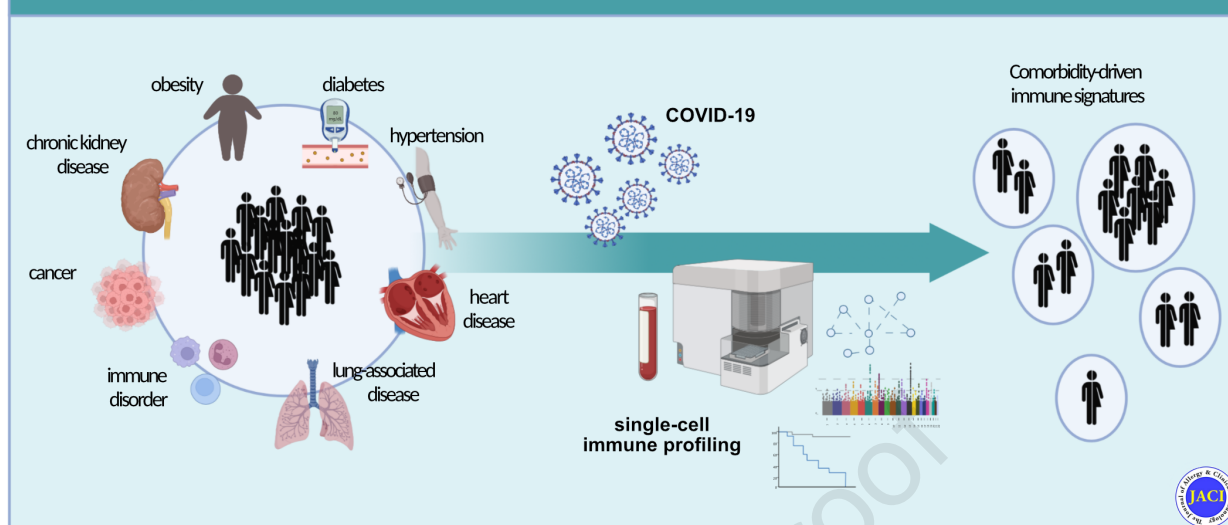
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TITLE

Pre-existing comorbidities shape the immune response associated with severe COVID-19

AUTHORS

Stefanie Kreutmair, MD^{1,2,*}, Manuel Kauffmann, MSc^{1,3}, Susanne Unger, PhD^{1,3}, Florian Ingelfinger, PhD^{1,3}, Nicolás Gonzalo Núñez, PhD^{1,3}, Chiara Alberti, PhD¹, Donatella De Feo, MD, PhD¹, Sinduya Krishnarajah, PhD¹, Ekaterina Friebe, PhD¹, Can Ulutekin, MSc¹, Sepideh Babaei, PhD⁴, Benjamin Gaborit, MD, PhD⁵, Mirjam Lutz¹, Nicole Puertas Jurado¹, Nisar P. Malek, MD^{4,6}, Siri Göpel, MD^{4,7}, Peter Rosenberger, MD⁸, Helene A. Häberle, MD⁸, Ikram Ayoub, MSc⁹, Sally Al-Hajj, PhD⁹, Manfred Claassen, PhD^{4,11}, Roland Liblau, MD, PhD^{9,11}, Guillaume Martin-Blondel, MD, PhD^{9,10,11}, Michael Bitzer, MD^{4,6,11}, Antoine Roquilly, MD, PhD^{5,11}, Burkhard Becher, PhD^{1,11,12,*}

AFFILIATIONS

1 Institute of Experimental Immunology, University of Zurich, 8057 Zurich, Switzerland

2 German Cancer Consortium (DKTK) and German Cancer Research Center (DKFZ), Heidelberg, Partner Site Freiburg, 79106 Freiburg, Germany

3 These authors contributed equally

4 Department Internal Medicine I, Eberhard-Karls University, 72076 Tuebingen, Germany

5 Université de Nantes, CHU Nantes, EA3826 Thérapeutiques Anti-Infectieuses, Institut de Recherche en Santé 2 Nantes Biotech, Service d'Anesthésie Réanimation chirurgicale, Hôtel Dieu, 44093 Nantes, France

6 Center for Personalized Medicine, Eberhard-Karls University, 72076 Tuebingen, Germany

7 German Centre for Infection Research (DZIF), Partner Site Tuebingen, 72076 Tuebingen, Germany

8 Department of Anesthesiology and Intensive Care Medicine, Eberhard-Karls University, 72076 Tuebingen, Germany

9 Toulouse Institute for Infectious and Inflammatory diseases, Université de Toulouse, Centre National de la Recherche Scientifique, Institut National de la Santé et de la Recherche Médicale, UPS, 31024 Toulouse, France

10 Department of infectious and tropical diseases, Toulouse University Hospital, 31059 Toulouse, France

11 These authors contributed equally

12 Lead contact

* Correspondence to: becher@immunology.uzh.ch or kreutmair@immunology.uzh.ch

DECLARATION OF INTERESTS

The authors declare no competing financial interests.

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ABSTRACT

Background

Comorbidities are risk factors for the development of severe COVID-19. However, to which extent an underlying comorbidity influences the immune response to SARS-CoV-2 remains unknown.

Objective and Methods

High-throughput, high-dimensional single-cell-mapping of peripheral blood leukocytes and algorithm-guided analysis were employed to investigate the complex interrelations of comorbidities, the immune response and patient outcome in COVID-19.

Results

We discovered characteristic immune signatures associated not only with severe COVID-19, but also with the underlying medical condition. Different factors of the metabolic syndrome (obesity-hypertension-diabetes) affected distinct immune populations, thereby additively increasing the immune dysregulatory effect when present in a single patient. Patients with disorders affecting the lung or heart together with factors of metabolic syndrome clustered

together, while immune disorder and chronic kidney disease displayed a distinct immune profile in COVID-19. Particularly SARS-CoV-2-infected patients with pre-existing chronic kidney disease were characterized by the highest number of altered immune signatures of both lymphoid and myeloid immune branches. This overall major immune dysregulation could be the underlying mechanism for the estimated odds ratio of 16.3 for severe COVID-19 in this burdened cohort.

Conclusion

The combinatorial systematic analysis of COVID-19 patient immune signatures, comorbidities, and patient outcomes provides the mechanistic immunological underpinnings of comorbidity-driven patient risk and uncovered comorbidity-driven immune signatures.

KEY MESSAGES

- The immune response associated with severe COVID-19 displayed comorbidity-specific characteristics
- The metabolic syndrome combined impaired innate immunity and adaptive immune dysregulation as possible underlying mechanism for worse COVID-19 outcome
- Chronic kidney disease prior to SARS-CoV-2 infection was associated with increased risk for severe COVID-19 and was linked to an overall dysregulated lymphoid and myeloid compartment
- The mechanistic underpinnings of comorbidity-driven patient risk serve as a basis for precision medicine

CAPSULE SUMMARY

The comorbidity-specific immune alterations in severe COVID-19 are yet unknown. Using single-cell-cytometry and algorithm-guided-analysis, Kreutmair et al. extract immune profiles characterizing the comorbidity-specific immune response to SARS-CoV-2 as mechanistic immunological underpinnings of comorbidity-driven patient risk.

KEYWORDS

COVID-19, SARS-CoV-2, immune response, spectral flow cytometry, comorbidity, metabolic syndrome, chronic kidney disease, heart disease, immune disorder, algorithm-guided analysis

ABBREVIATIONS

ARDS (acute respiratory distress syndrome), cDC2s (conventional dendritic cells type 2), CKD (chronic kidney disease), CM (central memory), COPD (chronic obstructive pulmonary

disease), COVID-19 (coronavirus disease 2019), DCs (dendritic cells), EM (effector memory), HD (heart disease), ID (immune disorder), LAD (lung-associated disorder), MFI (mean fluorescence intensity), OR (odds ratio), NK (Natural Killer), PBMCs (peripheral blood mononuclear cells), PCA (principal component analysis), pDCs (plasmacytoid dendritic cells), SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2), TEMRA (T effector memory CD45RA⁺), T2DM (type 2 diabetes mellitus), WHO (World Health Organization)

INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic is continuously unfolding, with more than 5 million deaths to date following infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)¹. The majority of people infected with SARS-CoV-2 show mild symptoms and some are even asymptomatic². Nevertheless, a relevant share of COVID-19 patients do not only develop fever, cough and shortness of breath, but experience complications of pneumonia, respiratory failure and severe acute respiratory distress syndrome (ARDS)^{3–5}. As seriously ill COVID-19 patients regularly suffer from multiorgan failure, affecting such as the cardiovascular system and kidneys next to the lungs, the medical history - and specifically comorbidities - plays a pivotal role in COVID-19 disease severity.

It is vital to identify patients at higher risk to develop severe COVID-19. This is important for the prevention of SARS-CoV-2 infections, therapeutic interventions once COVID-19 develops as well as COVID-19 vaccination strategy. Several comorbidities and old age are established risk factors for severe COVID-19^{6,7}. The most common comorbidities in hospitalized COVID-19 patients are hypertension, diabetes and obesity⁷, followed by cardiovascular and respiratory system disease⁶. Chronic kidney disease, cancer and different immune disorders also have been associated with worse disease outcomes^{8–11}. However, there are contradictory data on whether a specific comorbidity increases the risk for severe COVID-19 and to which extent.

Severe cases of COVID-19 are mediated by immunopathology¹², specifically emergency myelopoiesis and a dysregulated, exhausted T and NK cell compartment^{13–15}. Moreover, the uncontrolled production of cytokines – so called cytokine storm - is a main driver of the immunopathogenesis observed in severe COVID-19 cases¹⁶. It is unknown, to which extent an underlying comorbidity and therewith the preceding immune status of an individual influences the immune response to SARS-CoV-2. As new therapeutics for COVID-19 patients enter the market, understanding the underlying comorbidity-driven immunopathological mechanisms in the immune response to SARS-CoV-2 is crucial for correct patient selection and individualized and optimized therapy.

Here, we analyzed our previously generated high-dimensional single-cell spectral flow dataset¹³ for the role of specific comorbidities in the immune response of patients affected by COVID-19. This enabled us to link the single cell immune signature of COVID-19 patients to specific comorbidities. The combinatorial systematic analysis of patient immune signatures, comorbidities and patient outcomes sheds light onto the mechanistic underpinnings of comorbidity-driven patient risk.

METHODS

Samples from COVID-19 patients and healthy donors

Clinical routine data and blood samples for peripheral blood mononuclear cell (PBMC) isolation and cryopreservation were collected at the University Hospital Tuebingen (Germany), the Toulouse University Hospital (France, in the frame of the COVID-BioToul biobank, ClinicalTrials.gov Identifier: NCT04385108) and the Nantes University Hospital (France) (Table S1A), as previously reported¹³. In brief, longitudinal collection of PBMC samples of COVID-19 patients was performed (Table S1A, S1B). Patients included in this study were recruited between 04/2020 and 11/2020 (1st and second waves, Wuhan and Alpha clades), therefore before the vaccine and monoclonal antibody era. The grading of COVID-19 patients was executed according to the maximum severity of disease during the study based on the WHO ordinal scale¹⁷, the appropriate severity grade was then allocated to all related samples of one patient. Healthy control blood samples were recruited at the Blood Transfusion Center (Etablissement Français du Sang, Nantes, France) (Table S1A). Obesity was defined by body mass index > 30. Chronic kidney disease included chronic renal insufficiency, history of kidney transplant and dialysis. Hypertension was based on prior diagnosis (systolic (≥ 140 mmHg) and/or diastolic (≥ 90 mmHg) blood pressure at rest, measured on at least two different days) and assigned to the patient independently of current blood pressure/ antihypertensive therapy. The category of heart disease included chronic cardiac disease, coronary artery disease, (ischemic) cardiomyopathy, cardiac arrhythmia, Fallot cardiopathy and history of myocardial infarction. Lung-associated disorders included bronchial asthma, lung cancer, chronic bronchitis and smoking. Diabetes was assigned for patients diagnosed with type 2 diabetes mellitus. Immune disorder was assigned for patients diagnosed with rheumatologic disorder (such as rheumatoid arthritis), polymyalgia rheumatica, systemic lupus erythematosus or patients receiving immunosuppressive therapy after organ transplant. The category of cancer included patients diagnosed with prostate cancer, melanoma, non-small cell lung cancer and gastric cancer prior to COVID-19, independent of current anti-cancer therapy.

Spectral flow cytometry data

Spectral flow cytometry data used for this study are available from Mendeley Data at <http://dx.doi.org/10.17632/ffkvft27ds.2> (DOI: 10.17632/ffkvft27ds.2)¹³. In brief, PBMC samples were acquired on the spectral flow cytometer Cytex Aurora (Cytex Biosciences) using a surface panel, a panel focused on cytokine production in lymphoid cells (PMA/ Ionomycin stimulated) and one for cytokines in myeloid cells (R848 stimulated). Quality control, transformation, normalization and downstream analysis were performed using FlowJo (TreeStar) and R environment, as previously reported¹³.

Statistical analysis and visualization

Effect sizes were determined with a non-parametric Wilcoxon signed-rank test combined with a rankbiserial correlation. These tests were performed using the `wilcox.effsize` function from the `rstatix` package in R. Effect sizes above 0.3 (for severe vs. mild COVID-19 comparison) or 0.2 (for comorbidity vs. other samples), were considered relevant for further analysis. 0.3 is considered to be a medium strength effect. Frequencies of immune populations and median expression were compared with the non-parametric Mann-Whitney-Wilcoxon test from the `ggpubr` package in R, including the Benjamini-Hochberg correction using the `stats` package. The upset plots were made using the `Complex UpSet` package in R. The PCA plots were made using `fviz_pca` function from the `factoextra` package and the forest plots using the `forestplot` package in R. For the correlogram, Pearson's r correlation coefficients were computed using the `Hmisc` package and the resulting correlation matrix was visualized using the `corrplot` package. For correlation measurements, we used a linear regression model by applying the `lm()` and `summary()` functions. All other plots were drawn using `ggplot2`. The significance of the statistical tests between groups is represented by * for p values < 0.05 , ** for p values < 0.01 , *** for p values < 0.001 and **** for p values < 0.0001 .

RESULTS

Cohort characteristics

Three independent cohorts of in total 57 COVID-19 patients (121 peripheral blood mononuclear cell (PBMC) samples) across Germany (Tuebingen) and France (Toulouse and Nantes) were analyzed in a longitudinal manner, together with 21 healthy controls (Table S1A and S1B). Although we initially anticipated center-specific batch effects in our multi-center study, this was not the case¹³. COVID-19 patients were categorized based on the World Health Organization's (WHO) ordinal scale (World Health Organization, 2020) subdividing mild (WHO scale 1-4) and severe (WHO scale 5-8) disease (Fig. 1A, Table S1A, S1B and S1C). The COVID-19 patient cohort consisted of 40% females and 60% males, with the latter presenting a higher rate of severe COVID-19 (Fig 1B). Apart from male gender and in line with previous reports, patients older than 65 years were more likely to develop severe COVID-19 (Fig 1C). Older age is associated with accumulation of comorbidities, as also represented by our study cohort (Fig S1A): patients suffering from type 2 diabetes mellitus (T2DM), hypertension or heart disease (HD) were significantly older than patients without any comorbidity (Fig 1D), whereas this was not the case for obesity, lung-associated disorders (LAD, smoking, chronic bronchitis, lung cancer and bronchial asthma), chronic kidney disease (CKD), immune disorders (ID) and cancer (Fig S1B). Comparing patients with and without comorbidities, the COVID-19 severity was significantly higher in burdened patients (Fig 1E, Table S1C). Notably, 30 patients (52.6% of cohort) presented with more than one type of comorbidity. Co-occurrence of specific comorbidities is visualized in Figure 1F: most frequent combinations involve hypertension, obesity and T2DM, factors of the so-called metabolic syndrome (Fig 1F, S2A-G).

Patients with chronic kidney disease demonstrate an increased risk for severe COVID-19

To investigate the influence of specific pre-existing medical conditions on the development of severe COVID-19, we compared all patients suffering from a specific comorbidity against patients without any comorbidity and estimated the odds ratios (ORs) for severe COVID-19 (Fig 2A). All comorbidity groups demonstrated a tendency to more severe COVID-19, with CKD presenting significantly increased risk (OR = 16.25, 95% CI: 1.77 – 148.84). To minimize age being a confounding factor, we further compared patients with each comorbidity against all other COVID-19 patients and once again estimated ORs for severe COVID-19 for each comorbidity (Fig 2B). CKD still revealed a significant co-occurrence with an OR of 7.92 (95% CI: 1.36 – 46.18) for severe COVID-19, whereas obese patients showed a strong trend in the

same direction. Notably, in both analyses, patients with LAD had the lowest estimated OR for severe COVID-19 of all comorbidities in our cohort.

COVID-19 patients display severity- and comorbidity-specific immune profiles

Since several comorbidities are associated with dysregulation of the immune system^{7,18–25}, we questioned as to whether the immune dysregulation seen in comorbidities influence the immune response to SARS-CoV-2 infection and whether severity of COVID-19 can be linked to the immune signature of pre-existing medical conditions. To do so, we made use of the detailed immune profiles, which we had generated from PBMC samples (day 0-96 after hospital-admission due to COVID-19) using high-parametric single-cell spectral flow cytometry¹³. The dataset provides information about the differentiation and activation status of lymphocyte and myeloid subpopulations (T cells, NK cells, B cells, monocytes, dendritic cells) as well as their cytokine polarization profiles. The generated dataset resulted in hundreds of immune features, such as frequency of plasmacytoid dendritic cells (pDCs), mean fluorescence intensity (MFI) of PD-1 in CD4⁺ effector memory (EM) T cells or percentage of IFN- γ positive cells among CD4⁺ central memory (CM) T cells. To take immune alteration at different time points into account, we treated all samples as individual and combined all time points of the longitudinal dataset. We further enriched our data with clinical information, specifically comorbidities, to link the immune response in COVID-19 not only to severity but also to a pre-existing medical condition (Fig 3A). Of note, we did not find any significant changes in lymphocyte and granulocyte count specific to a comorbidity group (Fig S1C,D).

To gain an initial overview into the global immune profiles of COVID-19 patients with specific comorbidities, we performed a principal component analysis (PCA). The median values for each immune feature per sample were integrated, the resulting dataset was then grouped by comorbidity (Fig 3B, Table S1D). Since patients belong to multiple comorbidity groups, similar values on the PC can also stem from an overlap of comorbidities in patients. Interestingly, COVID-19 patients with pre-existing CKD, LAD and cancer were separated from the other comorbidities (Fig 3B), suggesting a more distinct immune profile of COVID-19 patients suffering from these comorbidities.

To identify immune signatures which are specifically relevant to severe COVID-19 and in addition associated with a specific comorbidity, the list of immune features was pre-filtered by their Wilcoxon effect size: a statistical measure which combines both fold change and statistical significance (0.1 - 0.3 small effect, > 0.3 intermediate and large effect)^{26,27}. Firstly, we computed the effect size of immune signatures from a specific comorbidity vs. all other COVID-19 samples, thereby extracting all immune alterations associated with a specific pre-existing medical condition (Fig 3C). Secondly, the effect size per immune feature of severe vs.

mild COVID-19 samples was estimated (Fig 3D) and only severe COVID-19-associated immune features were now used for PCA analysis (Fig 3E, Table S1D). Interestingly, CKD and ID were now clearly separated, while all other comorbidities clustered together (Fig 3E). Hypertension, T2DM and obesity - factors of the metabolic syndrome - as well as HD and LAD appeared closely together, suggesting a more similar severe COVID-19-associated immune profile in those patients.

To further investigate the effect of age on all immune features associated with severe COVID-19, we performed a detailed correlation analysis. To gain an overall overview, for each feature, computed Pearson's r coefficients values were visualized in a heatmap plot (Fig S2H). Candidate correlating features were furthermore linearly correlated with age (Fig S2I). Interestingly, although being significant, all R^2 values turned out to be below 0.1, highlighting there is no biologically relevant correlation between age and the expression value/ frequency of analyzed immune features. Of note, only three out of all severe COVID-19-associated immune features showed differences that were sex dependent (Fig S3A, S3B). Although samples from male patients in most comorbidity groups demonstrated a higher severity grade than samples from female COVID-19 patients (Fig S3C), we cannot exclude sex as potential confounding factor.

In conclusion, immune disorder and chronic kidney disease appear to have the most distinct effects on the SARS-CoV-2 immune response, while comorbidities of the metabolic syndrome, cancer as well as lung-associated and heart disorders show a similar pattern.

The metabolic syndrome combines immune alterations of both innate and adaptive immune branches

Pre-filtered immune features, which exhibited a “comorbidity effect size” above 0.2 (Fig 3C) and a “COVID-19 severity effect size” above 0.3 (Fig 3D), were selected for further in-depth analysis (Fig 4-6, Fig S4-7, Table S1E). The lower “comorbidity effect size” filter cut-off was used to include all potentially relevant immune features for next step analysis. To calculate the exact association of severe COVID-19-associated immune features with a specific comorbidity, we introduced a Mann-Whitney-U-test with Benjamini-Hochberg correction comparing pre-filtered immune features of COVID-19 patients with and without individual comorbidity.

Within our study cohort, only four COVID-19 patients suffered from cancer co-occurring with other comorbidities (Fig S4A). In depth analysis of pre-filtered immune features (Fig S4B) did not show severe COVID-19-associated signatures which were specific for cancer (Fig S4C), most likely due to limited power.

In case of obese COVID-19 patients (Fig 4D, S5A), GM-CSF expression in CD4⁺ T effector memory CD45RA⁺ (TEMRA) cells, as well as PD-1 expression in CD4⁺ T regulatory cells and

CTLA-4 expression in CD4⁺ EM T cells was significantly higher compared to non-obese COVID-19 patients (Fig 4D). COVID-19 patients with hypertension likewise demonstrated an impaired CD4⁺ T cell compartment with higher PD-1 expression in CD4⁺ CM and T regulatory cells (Fig 4E). Furthermore, hypertensive COVID-19 patients displayed reduced frequencies of CD4⁺ CD8⁻ (TCR $\gamma\delta$ enriched) T cells. Interestingly, this subset shows higher expression of CD38 as a sign of activation and reduced expression of CD161, resembling impaired innate activity and pointing to a dysregulated unconventional T cell profile associated with hypertension (Fig 4E). In contrast, COVID-19 patients suffering from T2DM were not only characterized by a T cell signature (higher expression of CD38 in CD4⁺ CD8⁻ (TCR $\gamma\delta$ enriched) T cells), but in addition showed lower expression of the co-stimulatory signal CD86 in conventional dendritic cells type 2 (cDC2s) (Fig 4F).

To summarize, severe COVID-19-associated immune alterations concentrated in the unconventional and CD4⁺ T cell compartment in patients with obesity and hypertension, pointing to increased activation/ exhaustion and impaired innate activity. In contrast, T2DM was linked to impaired myeloid co-stimulation.

Heart disease and lung-associated disorders are associated with hyperactivated and exhausted T and NK cells

The pre-filtered immune signatures associated with severe COVID-19 as well as either HD or LAD concentrated in the T and Natural Killer (NK) cell compartment (Fig 5, S6). In comparison to healthy controls and COVID-19 patients without HD, we identified higher expression of the activation marker CD38 in CD4⁺ CD8⁻ (TCR $\gamma\delta$ enriched) and CD4⁺ EM T cells in COVID-19 patients suffering from HD (Fig 5C). The immune checkpoint PD-1 was significantly higher in CD4⁺ CXCR3⁺ CCR6⁺ (T helper (Th) 1/17 enriched) T cells (Fig 5D) and higher expression of CD95 was found in CD56^{high} (cytokine producing) and CD56^{dim} CD16⁺ (cytotoxic) NK cells (Fig 5E). The latter NK immune signature was also found as characteristic for patients with LAD (Fig 5F). Of note, all these immune features characteristic for HD and LAD were shared across patients suffering from chronic kidney disease (Fig S2D-F, Table S1E).

In short, HD and LAD are associated with hyperactivated and exhausted T and NK cell subsets.

The immune response in COVID-19 patients with chronic kidney disease and immune disorders displays major dysregulations affecting all immune branches

When compared to all other COVID-19 study patients, the immune response in individuals with CKD was characterized by the highest number of immune alterations associated with severe disease (Fig 6A-G, S7A-F, Table S1E). Although no COVID-19 patient suffered solely from CKD without any of the other comorbidities (Fig S2F, S7A), several immune signatures

appeared to be unique in CKD: lower expression of HLA-DR in DCs and monocytes (Fig 6B, S7B) as well as a reduced frequency of CD56⁺ CD4⁻ CD8⁻ (Natural Killer T-like) T cells among all T cells (Fig 6C). These specific features did not significantly appear in any of the other comorbidity groups. Further supporting such overall altered immune response in COVID-19 patients with CKD, we also identified reduced expression of CD86 in myeloid subsets (Fig 6D, S7C), significantly higher expression of the activation and exhaustion marker CD38 and PD-1 on several unconventional and conventional T cell subsets (Fig 6E, 6F, S7D, S7E), higher expression of CD95 in cytotoxic and cytokine producing NK cell subpopulations (Fig 6G) as well as reduced expression of HLA-DR in CD56^{low} CD16⁻ NK cells (Fig S7F) – all of these features pointing towards a massive deregulation affecting both the innate and adaptive immune compartments.

Similar to CKD, COVID-19 patients with pre-existing ID presented significant immune features affecting both the myeloid and lymphoid compartments - although limited in number (Fig 6H-K, S7G-I). Next to signs of hyperactivation and exhaustion (higher expression of PD-1 and CD38 in CD4⁺ T cell subsets; Fig 6I, S7G, S7H), impaired innate immunity seems to play a role in the SARS-CoV-2 immune response in ID patients, represented by reduced HLA-DR in CD56^{low} CD16⁻ NK cells (Fig 6J). While these features were also seen in CKD patients, reduced frequencies of pDCs among all monocytes/ DCs turned out to be unique to the ID cohort (Fig 6K).

In summary, the immune profile associated with both severe COVID-19 and CKD as well as IDs was characterized by an overall dysregulation of all immune branches (Fig 7), specifically an hyperactivated exhausted T cell compartment and compromised innate immunity. These results were distinct from the comorbidity-driven immune signatures of HD and LAD - associated with T and NK cell hyperactivation/ exhaustion - and of cancer, hypertension as well as obesity, which solely affect the T cell compartment (Fig 7).

DISCUSSION

There are several large COVID-19/comorbidity studies out there reporting risk calculations between comorbidities and COVID-19 severity. However, none of them take into account the immune profile as a potential underlying mechanism for the variable outcomes. Our study is not primarily geared to reveal which comorbidities dictate COVID-19 outcomes. Instead, here the combinatorial systematic analysis of patient immune signatures, their comorbidities and outcomes sheds light onto the mechanistic underpinnings of comorbidity-driven patient risk. In general, the identified features comprised increased T and NK cell activation and exhaustion as well as signs of impaired antigen presentation – with a comorbidity-specific immune profile for each subgroup, respectively.

Obesity has been confirmed as a risk factor for severity and death in COVID-19^{28,29} – in line with our results demonstrating that obese COVID-19 patients were 3.1 times more likely to experience a severe disease course than non-obese patients. Overall, obesity could lead to increased risk for severe COVID-19 through immune cell and hormone deregulation, which then lead to decreased host defense and a higher chance for hyperinflammation^{30,31}. Insulin signaling has immunostimulatory effects on T cells, inducing growth, proliferation and production of cytokines in T cells. All these pathways are critical to T cell function and thus host defense and their dysregulation leads, in case of obesity, to a pro-inflammatory condition²⁹. In line with this, we detected higher GM-CSF expression in CD4⁺ CM T cells as a characteristic signature in our obese cohort. This feature was distinct to non-obese patients, but did not differ from healthy controls. Thus in this cohort it might not play a major role as early pathogenic driver of the hyperinflammation seen in severe COVID-19^{13,32}. Higher expression of CTLA-4 on CD4⁺ EM T cells could be part of the chronic inflammatory state found in obese individuals as a potential negative feedback loop²⁹.

Our analysis resulted in hypertension demonstrating an odds ratio of 2.1 for severe COVID-19, consistent with previous studies presenting hypertension to be significantly associated with adverse outcomes in COVID-19³³. A recent study using single-cell RNA sequencing showed a distinct inflammatory predisposition of immune cells from airway samples of patients with COVID-19 and hypertension, which correlated with COVID-19 severity²⁰. Our data add results of the circulating immune system comprising decreased frequencies of altered CD4⁺ CD8⁺ (TCR $\gamma\delta$ enriched) T cells - most likely due to their migration to the lung³⁴ - characterizing impaired early response to viral infection³⁵.

Next to obesity and hypertension, another factor of the metabolic syndrome, T2DM, has been associated with worse outcomes in COVID-19^{31,36}. Our study estimates an odds ratio of 1.9 for diabetic patients developing severe COVID-19. Hyperglycemia and insulin resistance promote synthesis of pro-inflammatory cytokines, oxidative stress and the production of

adhesion molecules that mediate tissue inflammation. Poorly controlled diabetes has been linked to inhibited lymphocyte proliferative response and impaired monocyte, macrophage and neutrophil functions^{37,38}. Interestingly, although being related to obesity and hypertension through the metabolic syndrome, in our study focusing on severe COVID-19 associated immune signatures, T2DM was not only characterized by unconventional T cell dysregulation but reduced expression of the costimulatory receptor CD86 in cDC2s. One could hypothesize, that COVID-19 patients suffering from several comorbidities which are part of the metabolic syndrome might display dysregulation of several immune branches, thereby explaining their highly increased need for mechanical ventilation and ICU admissions, as well as COVID-19 mortality³⁹.

While previous reports describe patients with heart disease (HD) as well as chronic obstructive pulmonary disease (COPD) and smoking being more likely to develop severe COVID-19^{7,40,41}, our analysis was not able to confirm significance for this assumption when compared to all patients without this specific comorbidity. Removing all samples from patients having asthma, which has been hypothesized to have even a protective effect through an increase in Th2 cells and decreased expression of the SARS-CoV-2 entry receptor ACE2 in lung tissue^{42–44}, did not change these results of the edited LAD group (data not shown). Of note, increased ACE2 expression was observed in the bronchial epithelium of obese COVID-19 patients with COPD⁴⁵.

Both HD and LAD in our study cohort were characterized by alterations of the T and NK cell compartment. Specifically, HD patients demonstrated signs of T cell hyperinflammation and exhaustion, consistent with reported inflammatory signatures associated with HD in patients without SARS-CoV-2 infection⁴⁶. All described immune features in HD patients were shared with CKD. Interestingly, the PCA analysis showed HD located as the closest comorbidity to CKD, which supports the similarity in effects on the immune response to SARS-CoV-2 between these two comorbidities.

CKDs are the most prominent risk factors for COVID-19 mortality^{8,47}. We confirmed an increased risk for severe COVID-19 in patients with CKD in our study cohort with an OR of 7.9 (95% CI: 1.4 – 46.2). Normal kidney function contributes to immune homeostasis by filtering circulating cytokines and immunogenic pathogen components, thereby limiting inflammation – important functions especially in COVID-19 as the cytokine storm plays a key role in disease progression. In advanced kidney disease, reduced filtering capacity leads to increased innate immune cell activation, which in turn leads to an increased cytokine and reactive oxygen species production, further increasing inflammation²⁴. Additionally, lymphocyte number and function are decreased in patients with CKD, making them immunocompromised^{24,48}. Together with a deregulated renin-angiotensin-system including the coronavirus entry receptor ACE2⁴⁹, the effects of CKD on the immune system leads to increased inflammation and host

susceptibility for infections, especially SARS-CoV-2^{24,50,51}. Our data resembles a similar condition after SARS-CoV-2 infection: increased expression of PD-1, CD38 and CD95 on several T and NK cell subsets points to an hyperactivated and exhausted T and NK cell compartment^{13,52,53}. Furthermore, reduced expression of HLA-DR and CD86 in monocytes and DCs points to impaired antigen presentation in these individuals. Of note, the amount of significantly changed and CKD-specific immune features was higher than in any other comorbidity cohort, thereby supporting an immune profile of COVID-19 patients with CKD characterized by an overall dysregulation both in the lymphoid and myeloid compartment leading to such a severe disease. Next to CKD, alterations in both immune branches were also associated with pre-existing immune disorders, but not to this extent.

Several risk assessments of comorbidities and severe COVID-19 have been performed, but there is a striking lack of investigative studies examining the underlying immunological mechanisms. Our study uncovers novel associations between specific comorbidities and the severe COVID-19-associated immune response, presenting characteristic immune landscapes depending on the underlying medical condition of a patient. These results enrich the database of COVID-19 immunopathology and provide a source for more individualized treatments of COVID-19 patients based on their comorbidities. Using therapies targeting the immunological changes specific to severe COVID-19 and even a patient's comorbidities could be a promising tool to alleviate the severity of this disease.

LIMITATIONS OF THE STUDY

We used a “real-life” cohort of COVID-19 patients, thus the sample numbers for COVID-19 patients with rare comorbidities was low and reduced the power of the analysis for these cohorts. Furthermore, as patients regularly suffered from multiple comorbidities, the effects could be biased. To take immune alteration at different time points into account, we treated all samples as individual and combined all time points of the longitudinal dataset, resulting in different numbers of samples per patient within the dataset. Due to the retrospective design of this study, we cannot completely exclude further confounding factors such as sex. Despite these challenges, this study is the first of its kind, analyzing comorbidities of COVID-19 patients and their association to specific immune signatures, providing novel insights into the underlying immunopathology in COVID-19 patients with different medical histories.

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AUTHOR CONTRIBUTIONS

Conceptualization, B.B., S.Kreutmair, M.K., S.U., F.I., N.G.N.; Methodology, S.Kreutmair, M.K., S.U., N.G.N. and F.I.; Formal Analysis, S.Kreutmair, M.K., S.U., N.G.N., F.I., C.U.A.; Investigation, S.Kreutmair, M.K., S.U., N.G.N., F.I., C.A., D.D.F.; Resources, B.B., A.R., M.B., G.M.-B., R.L., M.C.; Sample and patient data collection and processing, S.Kreutmair, M.K., B.G., I.A., S.A.-H., J.N., M.B., G.M.-B., M.L., N.P.J., N.P.M., S.G., P.R., H.A.H.; Data Curation, S.Kreutmair, M.K., S.U., N.G.N., F.I., C.A., D.D.F., E.F.; Writing – Original Draft, S.Kreutmair, M.K. and B.B.; Writing – Review & Editing, S.Kreutmair, M.K., S.U., F.I., N.G.N., C.A., D.D.F., C.U.A., S.Krishnarajah, E.F., S.B., B.G., M.L., N.P.J., N.P.M., S.G., P.R., H.A.H., I.A., S.A.-H., J.N., M.C., R.L., G.M.-B., M.B., A.R. and B.B.; Visualization, S.Kreutmair, M.K. and F.I.; Funding Acquisition and Supervision, M.C., R.L., G.M.-B., M.B., A.R., and B.B.

SUPPLEMENTAL MATERIAL

20220430_Kreutmair_et_al_Suppl table:

Table S1A: Sample list and according information

Table S1B: Time points of sampling

Table S1C: COVID-19 severity grades

Table S1D: Immune features contributing to PCA

Table S1E: Immune features

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FIGURE LEGENDS**Figure 1:**

A: Schematic of study cohort.

B: Proportion of female and male patients developing mild or severe COVID-19.

C: Proportion of patients younger and older than 65 years developing mild or severe COVID-19.

D: Violin plot showing the age of patients suffering from the indicated comorbidity vs. patients without this comorbidity. Box plots depicting median and 25th and 75th percentile of age.

E: Violin plot showing the COVID-19 severity of patients suffering from at least one comorbidity vs. patients without comorbidity.

F: UpSet plot to visualize co-occurrence of comorbidities in individual COVID-19 patients. Black dots connected by black lines represent the different comorbidity combinations. Bar plots on top display the number of patients per comorbidity-combination. Bar plots on the left display the number of patients per comorbidity. Pie charts below show the proportion of severe and mild COVID-19 patients in each comorbidity combination (shown for all combinations with more than 2 patients).

Significant p values are depicted using an asterisk (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ and **** = $p < 0.0001$, Mann-Whitney test, BH correction).

Figure 2:

A: Forest plot of estimated odds ratios for development of severe COVID-19. Comparison of patients with specific comorbidity against patients without any comorbidity.

B: Forest plot of estimated odds ratios for development of severe COVID-19. Comparison of patients with a specific comorbidity against patients without this specific comorbidity.

Figure 3:

A: Schematic of the experimental approach.

B: Principal component analysis of the total immune compartment on the basis of all extracted immune features grouped by comorbidity. CKD = chronic kidney disease. LAD = lung-associated disorder. HD = heart disease. ID = immune disorder. T2DM = type 2 diabetes mellitus.

C: Comorbidity effect size (ES) filter. Dot plot displaying the ES of every immune feature calculated for indicated comorbidity vs. without indicated comorbidity. Each dot represents

one immunological feature, colored for the affected immune compartment. ES of 0.2 represents the filter cut-off for further in-depth analysis of immune features.

D: COVID-19 severity ES filter. Dot plot displaying the ES of every immune feature calculated for severe vs. mild COVID-19. Each dot represents one immunological feature, colored for the affected immune compartment. ES of 0.3 represents the filter cut-off for further in-depth analysis of immune features.

E: Principal component analysis of the total immune compartment on the basis of all severe COVID-19-associated (ES severe vs. mild COVID-19 > 0.3) immune features grouped by comorbidity. CKD = chronic kidney disease. LAD = lung-associated disorder. HD = heart disease. ID = immune disorder. T2DM = type 2 diabetes mellitus.

Figure 4:

A-C: Dot plot displaying ES of severe vs. mild COVID-19 (x axis) against ES of indicated comorbidity vs. without indicated comorbidity (y axis). Each dot represents one immunological feature, colored for the affected immune compartment. The black box highlights immune features for further in-depth analysis.

D-F: Median frequency or median expression and 25th and 75th percentiles of the indicated parameters in indicated FlowSOM-generated immune cell clusters. Comparison of obesity, no obesity and healthy controls in **(D)**, hypertension, no hypertension and healthy controls in **(E)**, T2DM, no T2DM and healthy controls in **(F)**.

Significant p values are depicted using an asterisk (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ and **** = $p < 0.0001$, Mann-Whitney test, BH correction).

Figure 5:

A-B: Dot plot displaying ES of severe vs mild COVID-19 (x axis) against ES of indicated comorbidity vs. without indicated comorbidity (y axis). Each dot represents one immunological feature, colored for the affected immune compartment. The black box highlights immune features for further in-depth analysis.

C-F: Median expression and 25th and 75th percentiles of the indicated parameters in indicated FlowSOM-generated immune cell clusters. Comparison of heart disease, no heart disease and healthy controls in **(C-E)**, and lung-associated disorder, no lung-associated disorder and healthy controls in **(F)**.

Significant p values are depicted using an asterisk (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ and **** = $p < 0.0001$, Mann-Whitney test, BH correction).

Figure 6:

A: Dot plot displaying ES of severe vs. mild COVID-19 (x axis) against ES of indicated comorbidity vs. without indicated comorbidity (y axis). Each dot represents one immunological feature, colored for the affected immune compartment. The black box highlights immune features for further in-depth analysis.

B-G: Median frequency or median expression and 25th and 75th percentiles of indicated parameters in the indicated FlowSOM-generated immune cell clusters. Comparison of chronic kidney disease, no chronic kidney disease and healthy controls.

H: Dot plot displaying ES of severe vs. mild COVID-19 (x axis) against ES of indicated comorbidity vs. without indicated comorbidity (y axis). Each dot represents one immunological feature, colored for the affected immune compartment. The black box highlights immune features for further in-depth analysis.

I-K: Median frequency or median expression and 25th and 75th percentiles of the indicated parameters in indicated FlowSOM-generated immune cell clusters. Comparison of immune disorder, no immune disorder and healthy controls.

Significant p values are depicted using an asterisk (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ and **** = $p < 0.0001$, Mann-Whitney test, BH correction).

Figure 7:

A: Dot plot displaying the ES of every severe COVID-19-associated immune feature calculated for indicated comorbidity vs. without indicated comorbidity. Each dot represents one immunological feature, colored for the affected immune compartment. ES of 0.2 represents the filter cut-off for further in-depth analysis of immune features.

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Figure 1

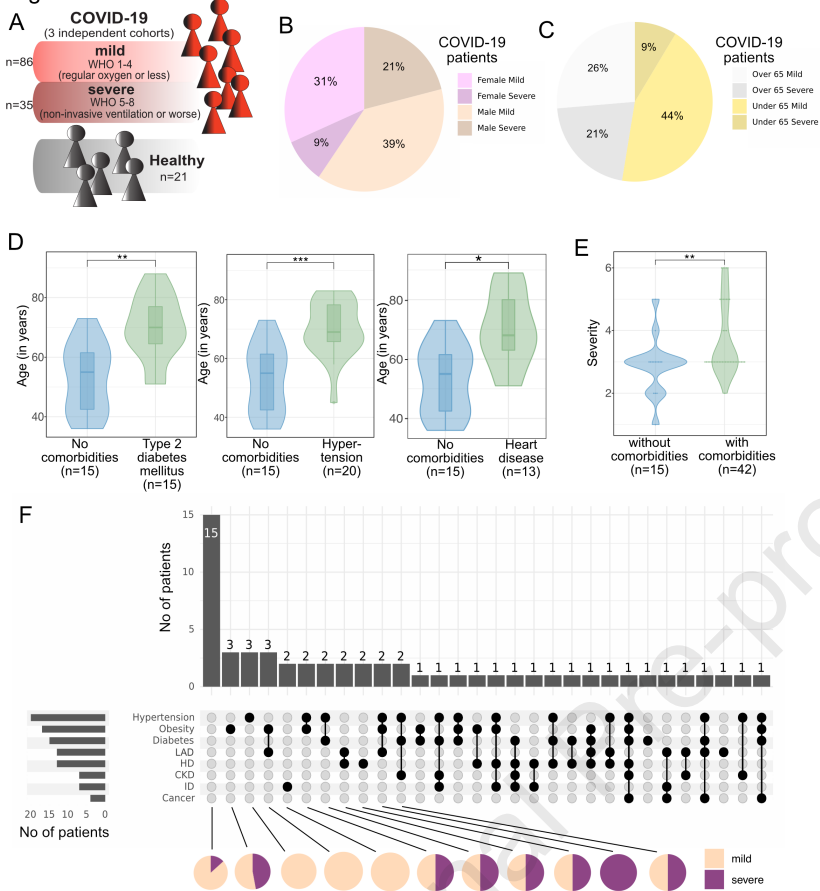
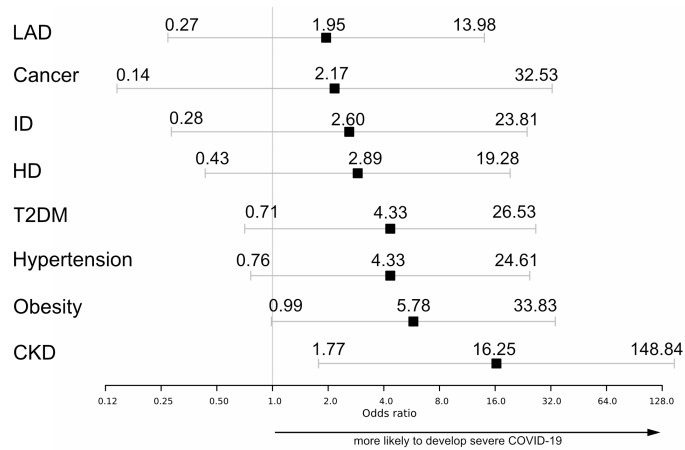


Figure 2

A patients with specific comorbidity against patients without any comorbidity



B patients with specific comorbidity against patients without specific comorbidity

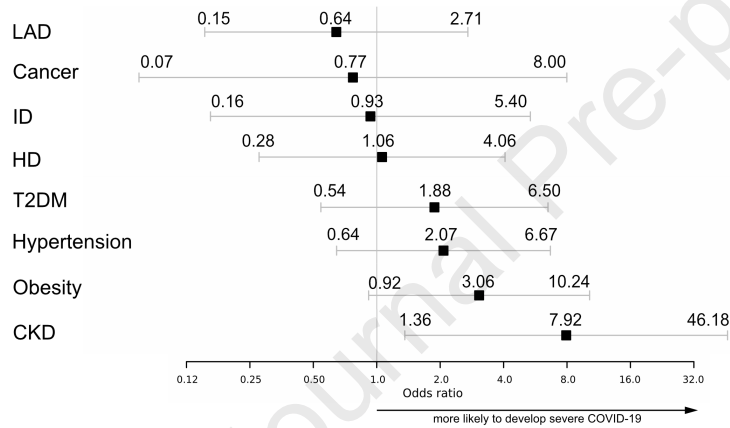


Figure 3

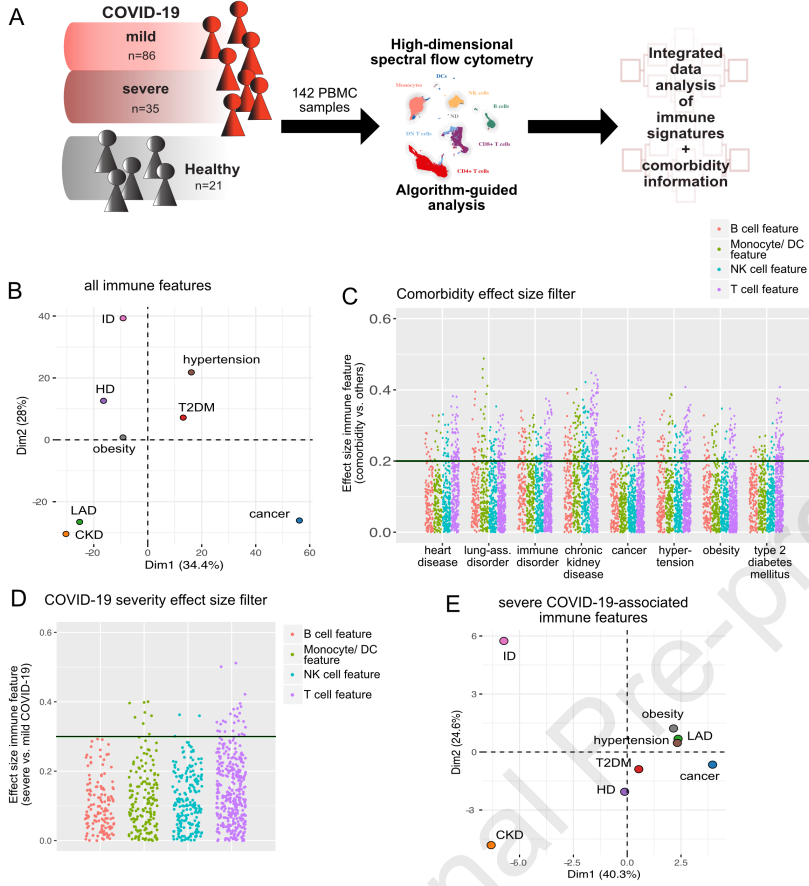


Figure 4

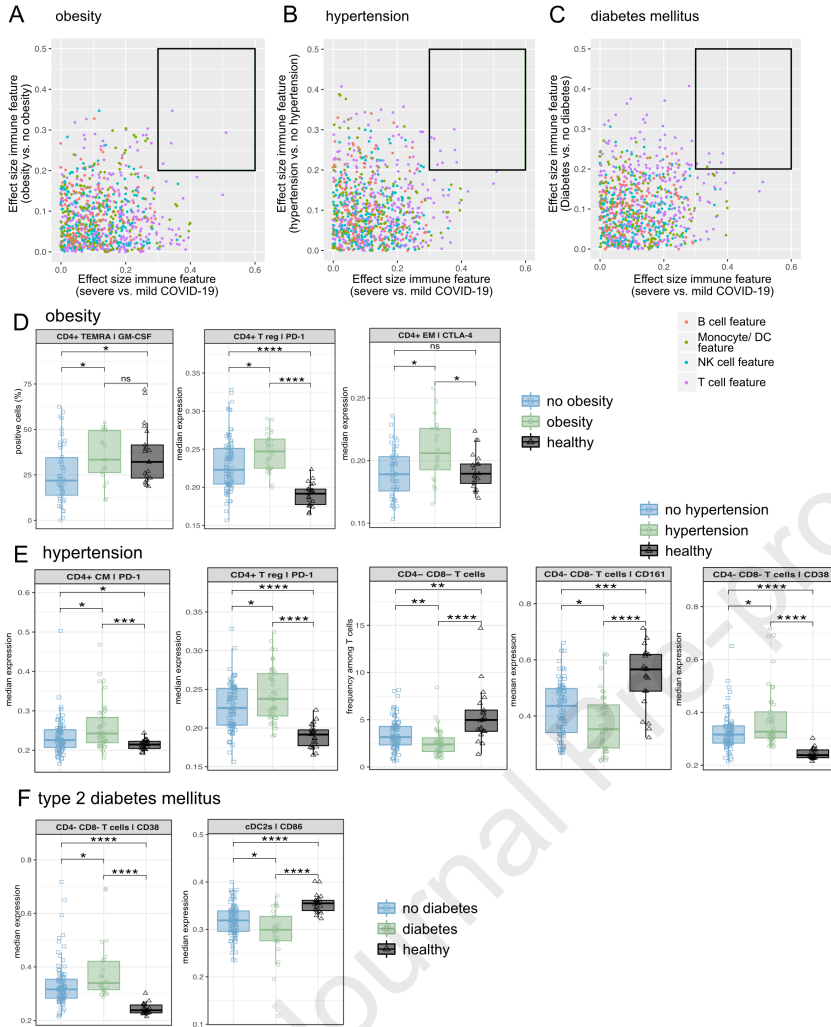


Figure 5

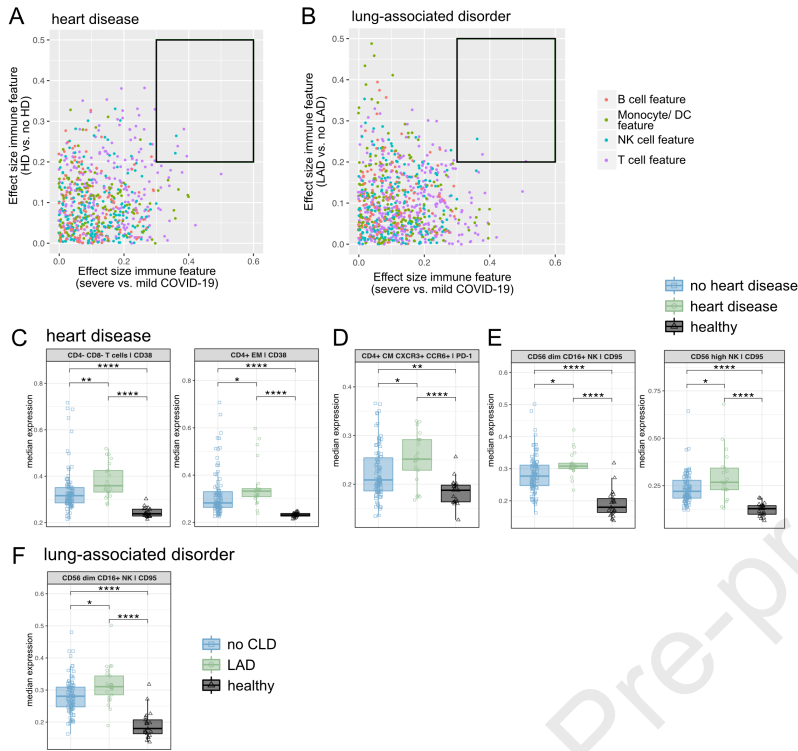


Figure 6

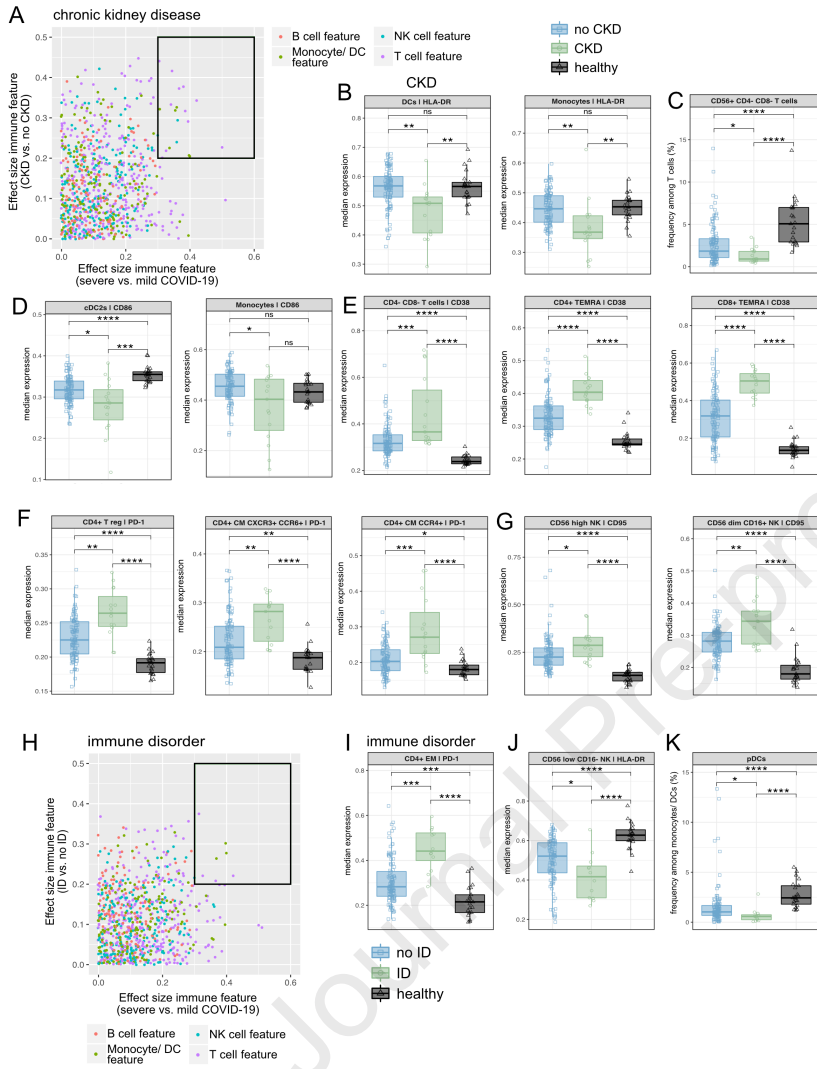


Figure 7

A

