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SHORT COMMUNICATION

Serum-derived extracellular vesicles: Novel biomarkers reflecting the disease severity of COVID-19 patients

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Abstract

COVID-19 is characterized by a wide spectrum of disease severity, whose indicators and underlying mechanisms need to be identified. The role of extracellular vesicles (EVs) in COVID-19 and their biomarker potential, however, remains largely unknown. Aiming to identify specific EV signatures of patients with mild compared to severe COVID-19, we characterized the EV composition of 20 mild and 26 severe COVID-19 patients along with 16 sex and age-matched healthy donors with a panel of eight different antibodies by imaging flow cytometry (IFCM). We correlated the obtained data with 37 clinical, prerecorded biochemical and immunological parameters. Severe patients' sera contained increased amounts of $CD13^+$ and $CD82^+$ EVs, which positively correlated with IL-6-producing and circulating myeloid-derived suppressor cells (MDSCs) and with the serum concentration of proinflammatory cytokines, respectively. Sera of mild COVID-19 patients contained more $HLA\text{-}ABC^+$ EVs than sera of the healthy donors and more $CD24^+$ EVs than severe COVID-19 patients. Their increased abundance negatively correlated with disease severity and accumulation of MDSCs, being considered as key drivers of immunopathogenesis in COVID-19. Altogether, our results support the potential of serum EVs as powerful biomarkers for COVID-19 severity and pave the way for future investigations aiming to unravel the role of EVs in COVID-19 progression.

KEYWORDS
biomarkers, COVID-19, extracellular vesicles, IFCM, MDSC, serum

1 | INTRODUCTION

Since December 2019, infection with SARS-CoV-2 virus, causing COVID-19 disease, spread across the world and gave rise to the biggest pandemic since the Spanish flu in 1918, resulting in more than 6,300,000 deaths in 28 months (WHO Coronavirus). COVID-19 is characterized by a wide spectrum of clinical severity, from asymptomatic through mild to severe disease symptoms. Mild COVID-19 cases are defined by fever, cough, sore throat, nasal congestion, headache, fatigue, myalgia, diarrhea, nausea/vomiting, loss of smell/taste but without evidence of hypoxia or pneumonia (Diaz et al., 2021). Severe COVID-19 cases are hallmarked by uncontrolled immune response, development of acute respiratory distress syndrome (ARDS), sepsis, thromboembolism and multiorgan failure (Grant et al., 2020; Zhou et al., 2020). In severely affected patients by immune dysregulation is reflected in cytokine release syndrome (Moore & June, 2020), lymphocytopenia and neutrophilia (Chen et al., 2020). Lymphocytopenia in severe cases of COVID-19 strongly affects different subsets of lymphocytes (Dan et al., 2021), and we found previously that poor T cells response in those patients is followed by dysregulation of autophagy and massive expansion of myeloid derived

suppressor cells (MDSCs) (Tomić et al., 2021). Therefore, severe COVID-19 is characterized by both hyperinflammation and strong immunosuppression (immune paralysis), driving dysfunctional anti-viral response (Mehta et al., 2020; Ritchie & Singanayagam, 2020; Tian et al., 2020). In line with this, several studies (Reizine et al., 2021; Rowlands et al., 2021; Sacchi et al., 2020) including our own (Tomić et al., 2021), showed that MDSCs are one of the major drivers of COVID-19 pathogenesis. These cells were demonstrated to be the strongest producers of both IL-6 and IL-10, in severe COVID-19 (Tomić et al., 2021). However, despite intensive investigations, the knowledge about underlying mechanisms of COVID-19 immunopathogenesis remains scarce. We neither gained elaborated knowledge about causes resulting in the often abrupt progression from mild to severe COVID-19 forms in susceptible individuals, nor have defined any biomarkers being able to identify patients prone to COVID-19-induced immune dysregulation or able to indicate disease progression at a very early time point, before the appearance of severe disease symptoms.

Recently, the COVID-19 pathology was linked to extracellular vesicles (EVs) (Hassanpour et al., 2020); however, studies of the EVs' potential as biomarkers of COVID-19 progression are still scarce. In this context, the activity of the coagulation promoting Tissue Factor (TF, CD142) in EV preparations was found to correlate with the severity of the SARS-CoV-2 infection (Balbi et al., 2021). The EV-associated TF activity was increased in severe vs. mild COVID-19 cases, and correlated with leukocyte numbers and inflammation parameters, including the D-dimer concentration (Guervilly et al., 2021). Furthermore, proteomic analysis revealed that EV preparations from COVID-19 patients plasma contain numerous molecules known to control immune responses, inflammation and activation of the coagulation and complement pathways as well as SARS-CoV-2 RNA (Barberis et al., 2021). Additionally, alterations in the molecular composition of EVs prepared from the plasma of COVID-19 patients have been detected up to 3 months following COVID-19 recovery (Mao et al., 2021), including proteins involved in coagulation, immune response and organ function. Like in many other inflammatory diseases, EVs may not only serve as biomarkers but also provide therapeutic options for severe COVID-19 patients (Börger et al., 2020). In addition to EVs released by mesenchymal stromal cells, CD24 containing EVs of engineered HEK cells appear as promising therapeutic agents for sever COVID-19 patients (Börger et al., 2020; Samara & Belle, 2021, Shapira et al., 2022).

However, EVs are very heterogenous and this heterogeneity cannot be dissected with conventional technologies such as molecular analyses of EV preparation homogenates or by particle quantification such as nanoparticle tracking analysis (NTA) (Dragovic et al., 2011; Koliha et al., 2016; Kowal et al., 2016; Sokolova et al., 2011). Thus, it is a current challenge in the EV field to improve our EV analysis methodology and define methods allowing complex analysis at the single EV level (Giebel & Helmbrecht, 2017). In this context, we have recently qualified imaging flow cytometry (IFCM) as a next generation EV characterization platform (Gögens et al., 2019). Aiming to optimize the IFCM methodology for the single EV analysis especially for exosome sized, small EVs (sEVs), we have set up protocols allowing antibody labelling of sEVs in their original liquid environment, for example, in plasma or sera of peripheral blood, without the need for any up-front or subsequent preparation procedures. With the improved protocols, we can now analyze antibody labelled sEVs directly following labelling without the need of any washing procedure, thus minimizing material losses and method induced biases in EV-subtype recoveries (Droste et al., 2021; Staubach et al., 2022; Tertel, Bremer et al., 2020; Tertel, Görgens et al., 2020; Tertel et al., 2022).

By using an IFCM approach, we have now characterized the serum EV content of severe and mild COVID-19 patients, and age/sex matched healthy donors with a panel of eight different preselected antibodies and correlate the obtained data with that of conventional inflammation markers and the frequency of key peripheral blood immune cells in respective COVID-19 patients, reported previously (Tomić et al., 2021). We have identified EV signatures reflecting the COVID-19 severity that provide diagnostic potentials for the identification of patients being prone to severe disease progression and that may indicate severe disease progression from an early time on, hopefully allowing identification of a time window for successful therapeutic interventions in suppressing realization of the severe disease forms.

2 | METHODS

2.1 | Patients and serum samples

The study was approved by the Ethical Boards of the KBC Zemun (No. 157/1, from 24.05.2020). Informed consent was obtained from patients and healthy donors according to the Declaration of Helsinki. Only patients with SARS-COV-2 infection confirmed by qPCR of nasopharyngeal swabs were included in this study. Malignancy and autoimmune diseases were the exclusion criteria. Twenty patients displayed mild and 26 patients severe COVID-19 symptoms without the need of mechanical ventilation at the day of blood-drawing. Disease severity was determined according to clinical parameters, WHO recommendations (Diaz et al., 2021) and KBC Zemun therapy protocol. Samples of 16 healthy sex- and age-matched individuals were included as healthy control samples. None of the individuals of the healthy control group had a recognized SARS-CoV-2 infection, before, and all of them showed negative results in anti-SARS-CoV-2 IgG and IgM antibody ELISA tests (BioVendor, Brno, Czech Republic) (Tomić et al., 2021). Also, they were free of symptoms and still negative in the anti-SARS-CoV-2 antibody test in the period of 1 month after sampling. According to NIH Early Detection Research Network (EDRN) Standard Operating Procedure (Tuck et al., 2009), after coagulation, which was allowed to proceed for 35–45 min at room temperature (RT), sera were

clarified by centrifugation at 1300× *g* for 20 min at RT and stored at -80°C until usage. Patients and control sera were processed according to the same protocol. The sera concentration of CRP was determined by immunoturbidimetry assay (Analyzer A25 Biosistems, Barcelona, Spain), fibrinogen by spectrophotometry (Ultrospec 2000 UV/Visible Spectrophotometer) and D-Dimer by Chemiluminescence ImmunoAssay (PATHFAST, Etten-Leur, Netherlands), according to routine clinical laboratory methods. Haematological analyses were performed on clinical Haematological Analyzer (Orphee Mythic 18, Geneva, Switzerland).

2.2 | Imaging flow cytometry analyses

Sera were thawed at RT and further clarified by brief centrifugation at 10,000× *g* for 10 min at RT to remove cryo-precipitate. 10 μl aliquots of supernatant were stained with fluorochrome-conjugated anti-human monoclonal antibodies (mAbs:fluorochrome): CD9:PE, CD41:Alexa fluor 488(AF488), HLA-ABC:FITC, CD82:PE, CD61:FITC, CD24:PE, phosphatidylserine (PS):AF488 and CD13:PE (details are provided in Supplemental Table S1). After incubation for 1 h at RT, samples were diluted to 100 μl with PBS (1:10 dilution). According to the MiFlowCyt-EV criteria (Welsh et al., 2020), beside buffer only and buffer plus antibody control, fluorochrome-conjugated isotype antibodies and detergent lysis with 1.25% NP-40 (solved in PBS) were used as controls (Supplemental Table S1 and Supplemental Figure S2).

All samples were analyzed in technical duplicates on the AMNIS ImageStreamX Mark II Flow Cytometer (AMNIS/Luminex, Seattle, WA, USA) using the built-in autosampler for U-bottom 96-well plates (Corning Falcon, cat 353077). Acquisition times were 5 min per well at 60× magnification and a low flow rate (0.3795 ± 0.0003 μl/min, directly determined by the system) with the removed beads option deactivated as described previously (Gögens et al., 2019; Tertel, Bremer et al., 2020). The data were analyzed as described previously (Tertel, Görgens et al., 2020). Additional settings are provided in Supplemental Tables S2 and S3.

2.3 | Flow cytometry

Immunophenotyping of patients and healthy donors' white blood cells (WBC) was performed exactly as described previously (Tomić et al., 2021). Briefly, 100 μl of whole blood samples collected in EDTA tubes (BD Biosciences, New Jersey, USA) were incubated with TrueStain FcX (Biolegend, San Diego, CA, USA) for 15 min prior to incubation with primary monoclonal antibodies for 30 min at 4°C. The following anti-human mAbs:fluorochrome were used: CD14:FITC, CD16:PE, CD15:PerCP-Cy5.5, HLA-DR:APC-Cy7, CD45:APC, PD1:APCcy7, IL-6:PE, CD66b:APC, CD56:FITC, streptavidin:PECy7, IL10:PE, CD123:biotin, CD11b:PE-Cy7, CD3:PE, CD4:PerCPcy5.5, CD8:Pecy7 and CD19:PecCPcy5.5, according to recommendations of manufacturers, as listed in Supplemental Table S4 (contains antibodies clones, Cat. No., manufacturers and concentrations used). Red blood cells were lysed using lysis buffer (0.15 M NH_4Cl/4 mM $NaHCO_3$(Sigma-Aldrich, Darmstadt, Germany) in MiliQ water for 5 min at room temperature. Thereafter, samples were washed twice with phosphate buffered saline (PBS) pH 7.2 containing 2% FCS and 0.01% Na-azide (both from Sigma-Aldrich, Darmstadt, Germany). Additionally, peripheral blood mononuclear cells (PBMCs) from the same donors were isolated from 6 ml of peripheral blood by density gradient (Pancoll human, PAN-Biotech, Aidenbach, Germany), followed by phenotype analysis. For intracellular staining, the cells were fixed and permeabilized by using the flow cytometry fixation and permeabilization kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. After permeabilization, anti-cytokine antibodies were added and incubated for 45 min. At least 50,000 cells were acquired and gated within stable sample flow (Time/FSCa), singlets (FSC-A/FSC-H) and cell-specific FSC/SSC properties. The gating strategies for determining T, NK, NKT, B, Mo-MDSC, PMN-MDSC, monocytes and plasmacytoid dendritic cells from WBC and PBMC are shown in Supplemental Figure S1. The samples were analyzed on a BD LSR II flow cytometer (BD Biosciences, San Jose, CA, USA) at the day of sampling and analyzed using the FCS Express 4 software (De Novo software, Pasadena, CA, USA). The number of specific cell subsets were determined from the percentage of specific cell population by flow cytometry, and the number of cells from haematological analysis, as described previously (Tomić et al., 2021).

2.4 | Quantification of cytokines and SARS-CoV-2 antibodies

The levels of IgG and IgM antibodies specific for SARS-CoV-2 were determined using ELISA assay (BioVendor, Brno, Czech Republic) according to manufacturer's instructions. The sera concentration of IL-10, IL-6, MCP-1, IL-8 and IL-18 were determined in sera samples using the LEGENDPlex, Human Th Cytokine Panel and Human Inflammatory Panel (both 13-plex; BioLegend, San Diego, CA), according to manufacturer's instructions.

2.5 | Statistical analyses

IFCM data were analyzed with Graph Pad Prism (version 8.4). A test for normal distribution was performed followed by a Kruskal-Wallis test to determine differences between cohorts and Dunn-Bonferroni test for multiple comparison correction. Outliers were selected with ROUT (Q = 1%) method from GraphPad. Correlation matrix and correlogram were computed and plotted using RStudio software v1.2.5042. Spearman rank correlation coefficient was estimated to determine the association between the 37 parameters (clinical and immunological) and eight immunological EVs parameters, collected from total 37 patients (patients data in Supplemental Table S5) using *corr.test* function (Revelle, 2010). Categorical data (sex, disease severity, mechanical ventilation, outcome) were transformed to appropriate numerical variables for analysis purpose (sex: female and male were converted to numbers 1 and 2, respectively; disease severity: mild-1 and severe-2; mechanical ventilation: no required-1, required-2; outcome: recovered-1 and exitus letalis-2). *Corrplot* package (Wei et al., 2017) was used for visualization of the results.

Linear discriminant analysis (LDA) Effect Size (LEfSe) was performed using Galaxy-based LEfSe workflow (https://huttenhower.sph.harvard.edu/galaxy/) (Segata et al., 2011) in order to discover biomarkers associated with severity of disease. Metadata table consisting of 39 different immunological, biochemical and clinical parameters (biomarkers) was transformed to floating point values following per-sample normalization 1 M. Results are obtained using Kruskall-Wallis test for differentially distributed biomarkers in different classes, "mild" and "severe," following LEfSe with default parameters.

3 | RESULTS

3.1 | COVID-19 patient cohort

Forty-six patients admitted to KBC Zemun, Belgrade from May to July 2020 with confirmed qPCR for COVID-19 (original strain of SARS-CoV-2), but without history of autoimmune or malignant illness, were included in the study. According to clinical parameters and WHO recommendations (Diaz et al., 2021), 20 patients who showed mild symptoms and required no mechanical ventilation were categorized as mild COVID-19 patients. Twenty-six patients who displayed an increased respiration frequency, dyspnea, SpO2 <93%, PaO2/FlO2 <300 and/or at least 50% increased infiltration of lungs within 24–48 h, were considered severe COVID-19 patients. Additionally, 16 healthy donors who were age/sex matched with COVID-19 patients, were included in the study. In follow-up monitoring of COVID-19 patients, complications appeared in the group of severe COVID-19 patients and the mortality in this group was 30.7% (8/26), whereas all patients with mild COVID-19 recovered successfully. Detailed clinical parameters and therapy protocols during the hospitalization of these patients were described previously (Tomić et al., 2021).

3.2 | Specific subsets of circulatory EVs are distinctly increased in severe or mild COVID-19

In order to compare the EV profile of the peripheral blood among COVID-19 patients of different disease progression stages, we analyzed their serum EV content after staining with a panel of different antibodies by IFCM, all of which had been qualified for the single EV analysis by IFCM upfront (Figure 1A). In detail, we used antibodies against the tetraspanins CD9 and CD82, the integrins CD41 and CD61, phosphatidylserine (PS), HLA-class I antigens and CD13 and CD24. Of note, all epitopes are known to be expressed on different blood cell types; for example, CD9, CD41, and CD61 are abundantly expressed on platelets (Aatonen et al., 2014), whose activity is frequently dysregulated in COVID-19 patients often resulting in thrombotic events (Aatonen et al., 2014; Althaus et al., 2021; Denorme et al., 2020; Zahran et al., 2021). Additionally, PS was demonstrated to be associated with EVs with pro-coagulant properties (Tripisciano et al., 2017), which was described as critical in the abrupt progression of COVID-19 (Cizmecioglu et al., 2021; Grant et al., 2020; Taghiloo et al., 2021). We analyzed vesicular HLA-ABC (MHC Class I) on surface of EVs, considering that this molecule is present on many subpopulations of EVs and it is generally involved in anti-viral response (Nguyen et al., 2020). CD13, CD24, and CD82 are involved in inflammatory processes (Barkal et al., 2019; Lu et al., 2020; Yeung et al., 2018). CD13 is known receptor for several corona viruses (Devarakonda et al., 2021; Nomura et al., 2004; Pöhlmann et al., 2006; Qi et al., 2020) and associated with several EVs types, including ones from, that is, synoviocytes, endometrial epithelial cells, microglia, urine, plasma, leukocytes and umbilical cord-derived mesenchymal stromal cells (Erdbrügger et al., 2021; Lin et al., 2020; Morgan et al., 2016; Priya et al., 2021; Potolicchio et al., 2005; Romanov et al., 2018; Tőkés-Füzesi et al., 2018; Yoshihara et al., 2021). The tetraspanin CD82 is a constituent of tetraspanin-enriched membrane domains (TEMs, which represent the places of entry/egress of some viruses) but also responsible for immune cells recruitment, migration (Yeung et al., 2018) and acts as a costimulatory molecule for T cells (Earnest et al., 2017; Florin & Lang, 2018; ProteinAtlas: CD82; Sims et al., 2018; Yáñez-Mó et al., 2009). Finally, we analyzed CD24, which was described as a negative regulator of inflammation, a part of novel CD24/Sig-10 innate immune checkpoint that regulates inflammation caused by DAMPs (Barkal et al., 2019) and is a protein found on several EV types, including EVs within urine, blood, tears, serum, plasma, saliva, lymphatic exudate, B cells and neutrophils (Altevogt

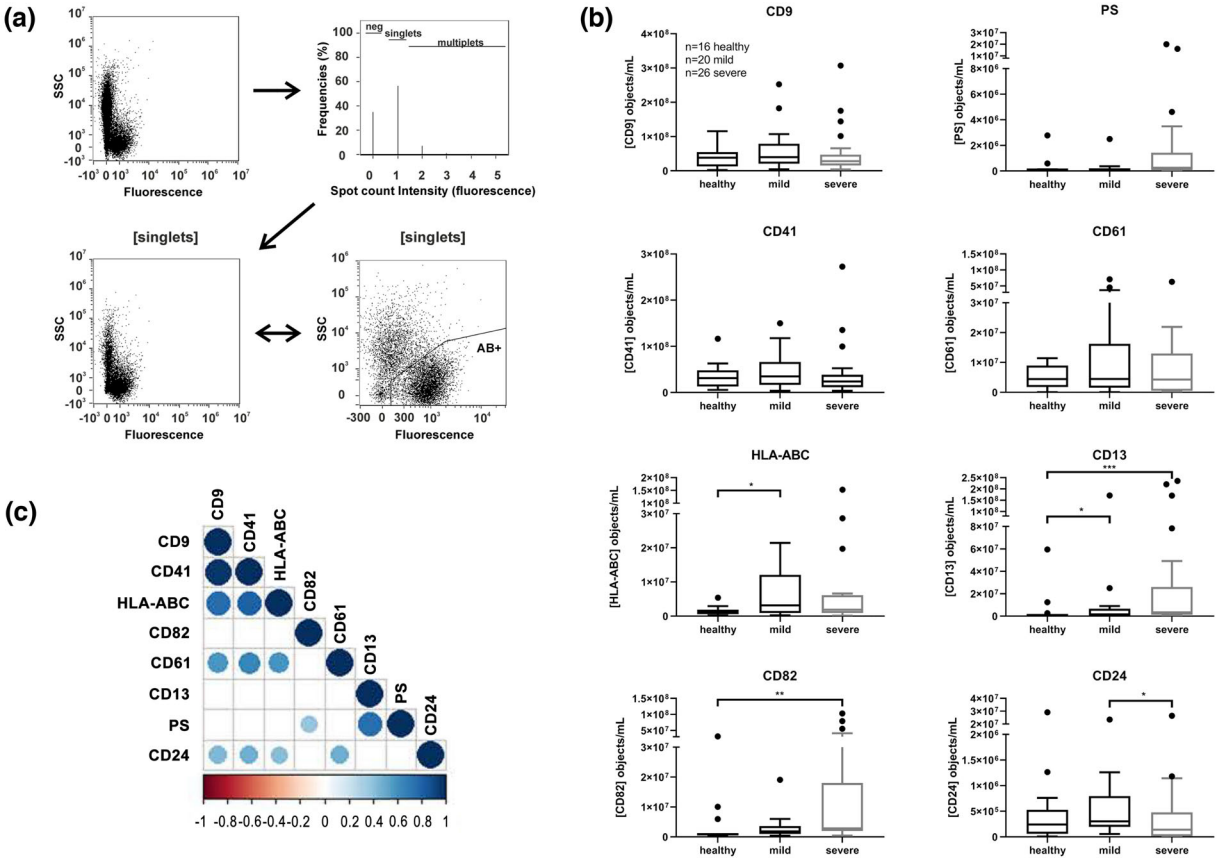

FIGURE 1 Imaging flow cytometry analysis of EVs from sera of healthy donors ($n = 16$) and patients with mild ($n = 20$) or severe ($n = 26$) form of COVID-19. (A) gating strategy for EVs is shown from a representative experiment; (B) Concentration of serum extracellular vesicles (EVs) positive for specific EV-associated proteins is shown as Boxplots with median and Tukey Wiskers for each group without removing outliers (see also Supplement Figure 3). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$ as indicated (Kruskal-Wallis test, Dunn-Bonferroni posttest); (C) The significant ($^{*}p < 0.05$) correlations between subtypes of EVs are shown as determined by Spearmen correlation test, and the colour and size of the circles represent the level of correlation coefficient

et al., 2021; Ayre et al., 2015; Bonifay et al., 2022; Conzelmann et al., 2020, Ekström et al., 2022; Grigor'eva et al., 2016; Keller et al., 2007; Liu et al., 2022; Park et al., 2021; Sornov et al., 2019).

Upon analyzing the samples by IFCM, we observed detergent sensitive EV populations with all antibodies with varying abundancies. No statistically significant differences were observed among the abundance of the $CD9^+$, $CD41^+$, and $CD61^+$ EV populations in the sera of mild and severe COVID-19 patients or in sera of healthy donors, respectively (Figure 1B). PS^+ EV populations, although not reaching statistical significance, appeared slightly larger in the sera of COVID-19 patients than in healthy control. Sera of mild COVID-19 patients contained significantly higher concentrations of HLA-ABC^+ EVs, which was not observed in the sera of severe patients (Figure 1B). In contrast, severe COVID-19 patients displayed statistically significant elevation of $CD13^+$ EVs in sera compared to healthy donors, which was not observed in mild patients. IFCM analyses demonstrated that the concentration of $CD82^+$ EVs in sera of severe COVID-19 patients were significantly elevated, as compared to healthy donors. The sera levels of $CD24^+$ EVs in mild patients were higher than those of both healthy controls and severe COVID-19 patients. The difference in $CD24^+$ EVs sera concentrations were significant between the mild and severe COVID-19 patients (Figure 1B). Observed statistical significances were not changed upon removing outliers (Supplemental Figure S3). Moreover, the levels of $CD24^+$ EVs correlated positively with all other EV-associated proteins, except with CD82, CD13 and PS, which were increased in severe COVID-19 patients (Figure 1C).

3.3 | Distinctive EV populations correlate with immunological, haematological and biochemical parameters of COVID-19

In order to evaluate EVs carrying specific proteins as potential indicators of COVID-19 disease progression, we next correlated the obtained EVs concentrations in sera of patients and healthy controls with the most common and the most significant immunological, haematological and biochemical parameters by multiple correlation analysis (Figure 2A).

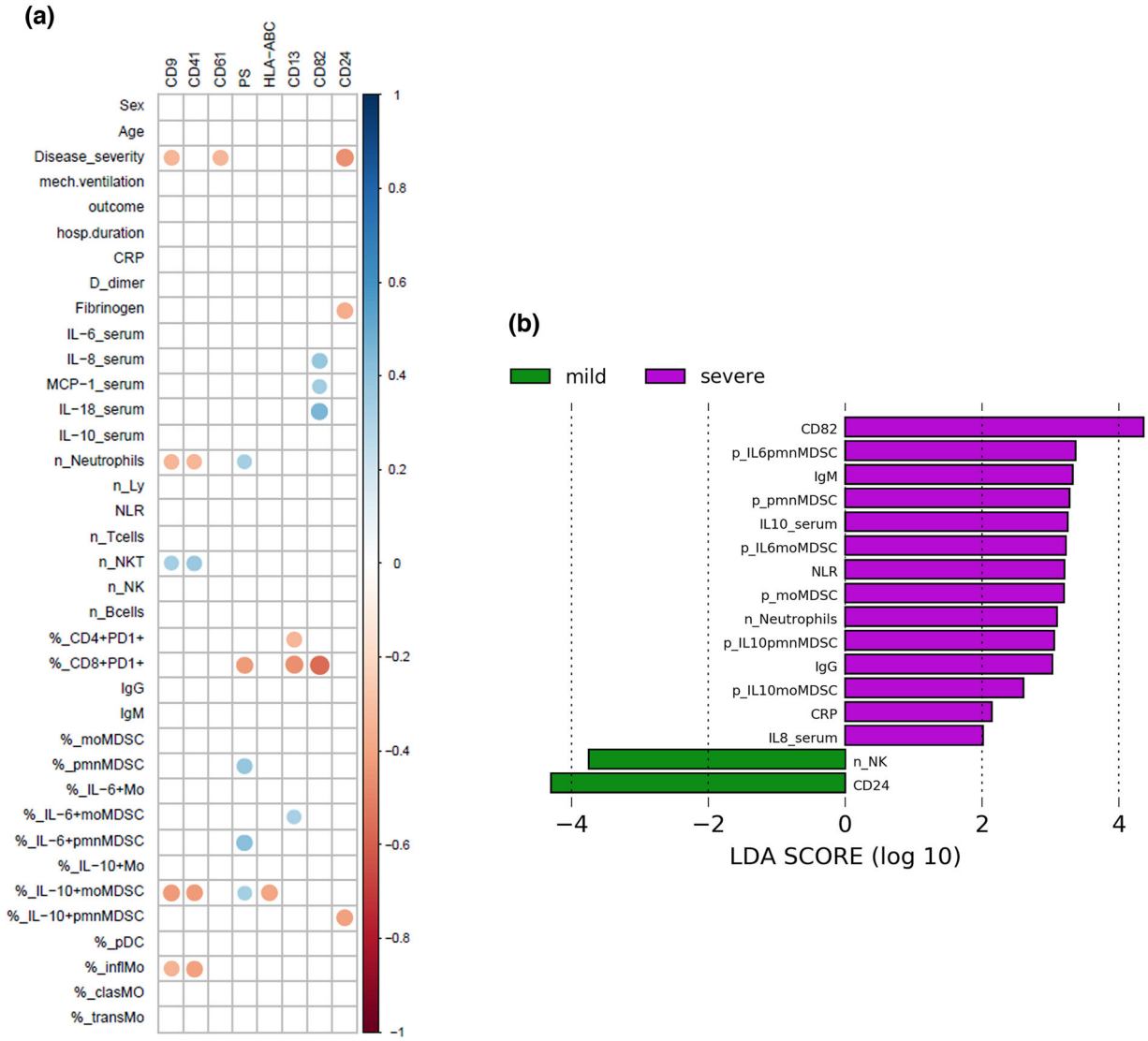

FIGURE 2 Correlation and LEfSe analysis of EVs subtypes data with haematological, immunological and biochemical parameters of COVID-19. (A) A correlation plot shows correlation between EVs immunological parameters and clinical and immunological parameters of mild ($n = 18$) and severe ($n = 19$) COVID-19 patients Spearman rank correlation coefficient was estimated to determine the association between the 37 parameters (clinical and immunological) and 8 immunological EVs parameters, collected from total 37 patients. Only significant comparisons ($p < 0.05$) between variables being compared are shown with circles size and colour corresponding to Spearmen's rank coefficient, as indicated. n_, number; Mo- monocytes; pmn- polymorphonucler; inf- inflammatory; clas- classical; trans-transitory. (B) LEfSe analysis plot of biomarkers associated with disease severity. Linear discriminant analysis (LDA) Effect Size (LEfSe) was performed using Galaxy-based LEfSe workflow to discover biomarkers associated with severity of disease. Results are obtained using Kruskall-Wallis test for differentially distributed biomarkers in different classes, "mild" and "severe," following LEfSe with default parameters. Green color indicates biomarkers enriched in patients with mild disease symptoms, and purple indicates biomarkers associated with severe symptoms. The bar column length represents logarithmic discriminant analysis (LDA) score higher than 2. Only markers able to discriminate between mild and severe conditions are shown. p-percentage; n-number

Parameters for correlations were selected based on previous results showing their specific change with progression of COVID-19. Briefly, several inflammatory markers were shown to be highly increased in severe COVID-19, including CRP, D-Dimer, fibrinogen, IL-6, IL-18, IL-8, MCP-1 (Costela-Ruiz et al., 2020; Ponti et al., 2020), which was observed in our previous study as well (Tomić et al., 2021). Additionally, we previously described that lymphocytopenia in severe COVID-19 significantly affected the number T, NK, NKT cells, but not B cells or production of SARS-COV-2 specific IgM and IgG antibodies. Importantly, severe COVID-19 patients also displayed an increased proportion of $PD1^+$ T cells, lower proportion of plasmacytoid dendritic cells (pDCs) and a large expansion of polymorphonuclear (pmn)-MDSC and mononuclear (mo)-MDSC, the latter of which were the predominant producers of IL-6 and IL-10 in severe COVID-19 (Falck-Jones et al., 2021; Tomić et al., 2021).

As shown in Figure 2A correlogram, the concentrations of $CD9^+$ EVs correlated negatively with indicators of COVID-19 severity, that is, the number of neutrophils, frequency of IL-10 producing moMDSC, and inflammatory monocytes. On the other

hand, the concentration of $CD9^+$ EVs positively correlated with the number of NKT cells in blood. In concordance with that, $CD9^+$ EVs also correlated negatively with disease severity itself.

Platelet derived $CD41^+$ EVs, had correlation profile almost identical to $CD9^+$ EVs, except for the lack of correlation with disease severity. Interestingly, in contrast to $CD41^+$ EVs, no correlations were found for $CD61^+$ EVs except the negative correlation with disease severity.

As for PS^+ EVs, their level were positively correlated with indicators of severe COVID-19: number of neutrophils, both total frequency of pmn-MDSC and their IL-6 producing subpopulation as well as with IL-10 producing moMDSC. Accordingly, these EVs were also negatively correlated with the percentage of $PD1^+CD8^+$ lymphocytes.

In line with the finding that $CD13^+$ EVs and $CD82^+$ EVs are increased in severe form of COVID-19, these EVs were both positively correlated with indicators of severe COVID-19, that is, serum levels of inflammatory cytokines and the frequency of IL-6 producing moMDSC, respectively. In line with that, both of these EVs subtypes were negatively correlated with the percentage of $PD1^+CD8^+$ lymphocytes.

Conversely, HLA-ABC^+ EVs and $CD24^+$ EVs were negatively correlated with IL-10 producing both mo-MDSC and pmn-MDSC, respectively, while $CD24^+$ EVs displayed further negative correlations with disease severity and fibrinogen levels, which is all in accordance to their increase in mild COVID-19.

In line with recent report of Ramasamy et al. (2020) regarding the role of immunosenescence in response to SARS-CoV-2, that is, the severity of COVID-19, we also analysed the subgroup of patients aged below 70 years. As shown in Supplemental Figure S3, no major qualitative difference was observed in comparison to the original cohort including patients over 70 years old.

3.4 | LEfSe analysis reveal EVs with best biomarker potential for COVID-19 progression

To determine which of the analyzed immunological markers have the highest discriminative value between mild and severe form of COVID-19 and thus could be used as a potential biomarker, we performed LEfSe analysis (Figure 2B). This analysis revealed that $CD82^+$ EVs, followed by subsets of MDSC, number of neutrophils and pro-inflammatory markers (IL-8 and CRP), as well as IL-10, are good indicators for severe COVID-19. In contrast, $CD24^+$ EVs and the number of NK cells in blood were the best indicators for mild COVID-19. The analysis also showed that $CD82^+$ EVs and $CD24^+$ EVs display the highest biomarker potential for severe and mild COVID-19, respectively. Interestingly, in this cohort, EVs out-competed many classical inflammatory markers including CRP, which to a certain degree discriminates mild and severe COVID-19 cases, and also fibrinogen and D-dimer, both of which did not allow discrimination of mild and severe cases in our cohort.

4 | DISCUSSION

Here, we evaluated the potential of circulating EVs as indicators of disease severity in COVID-19. For the first time in research of COVID-19, we performed IFCM analyses, a state-of-the-art technique for direct detection and characterization of EVs in patients' sera without the need for any upfront preparation procedure (Gögens et al., 2019; Tertel, Bremer et al., 2020). By investigating the EV contents in sera of mild and severe COVID-19 patients in comparison to that of age and sex matched healthy donors we found that the abundancy of $CD13^+$ and $CD82^+$ EV populations correlated with the severe form of the disease, while the abundancy of $CD24^+$ and HLA-ABC^+ EV populations was higher in the sera of patients with mild COVID-19 forms than in those with severe symptoms.

Indeed, LefSe analysis achieved the highest biomarker value for $CD24^+$ EVs as a promising marker discriminating patients with mild and severe COVID-19 symptoms. The identification of $CD24^+$ EVs as potential biomarkers is especially interesting, since CD24 is known to exert anti-inflammatory functions (Barkal et al., 2019) and is used as a marker for certain B cell subtypes, including regulatory B cells and B cells with class switch activities (Fu et al., 2020) whose presence has been correlated with COVID-19 symptom duration (Newell et al., 2021). Furthermore, therapeutic application of CD24 loaded HEK-EVs (Shapira al., 2022) has been reported to suppress severe COVID-19 forms effectively in a registered Phase I clinical trial (Samara & Belle, 2021) (ClinicalTrials.gov: NCT04747574). Thus, the occurrence of $CD24^+$ EVs in the sera of COVID-19 patients with mild symptoms and the lack of those EVs in patients with severe symptoms might be linked causally. It will be interesting to learn, whether $CD24^+$ EVs also provide prognostic potentials. To explore this a prospective cohort study is required. Unfortunately, we only had access to blood samples taken from hospitalized COVID-19 patients at one time point. Thus, future work on blood samples of a novel COVID-19 patient needs to demonstrate, whether patients lacking $CD24^+$ EVs at early stages are more prone to develop severe forms of COVID-19.

Also confirmed by unsupervised LefSe analysis, our findings imply that a subpopulation of EVs defined by the presence of CD82 is a good indicator of severe form of the disease. Indeed, increased numbers of $CD82^+$ EVs might be connected to the role of CD82 in clustering of MHC molecules (Kropshofer et al., 2002) on antigen presenting cells (APCs) (Dan et al., 2021; Giamarellos-Bourboulis et al., 2020; Tomić et al., 2021), which are down regulated in severe COVID-19 patients. It has been

proposed that their reduction is a consequence of increase shedding of MHC molecules via EVs (Lindenbergh & Stoorvogel, 2018). Connected to the interaction of MHC molecules and CD82, such EVs might be dually marked by the presence of MHC molecules and by CD82.

We also identified $CD13^+$ EVs as potential markers whose presence coincides with immune dysregulation and the appearance of peripheral blood mo-MDSC during disease progression in COVID-19 patients. Although an increase of soluble CD13 has been reported to in COVID-19 patients (Tsou et al., 2020), we, to the best of our knowledge, report for the first time $CD13^+$ EVs as a promising biomarker in COVID-19.

Although not reaching statistical significance, a tendency of PS^+ EVs increase was observed in severe COVID-19 patients, reflecting a previous observation of increased numbers of platelet-derived PS^+ EVs in severe COVID-19 patients (Rausch et al., 2021; Zaid et al., 2020). However, lack of difference in abundance of $CD9^+$ EVs, $CD41^+$ EVs and $CD61^+$ EVs among the three analyzed groups—as all these EV-associated proteins are especially expressed on platelets—indicates that production of platelet EVs is not dysregulated in COVID-19. This apparent contradiction prompts further exploration of platelets-derived EVs heterogeneity in COVID-19.

Compared to a few studies of EVs subpopulations in COVID-19 biomarker discovery (Balbi et al., 2021; Barberis et al., 2021; Cappellano et al., 2021; Kudryavtsev et al., 2021; Lam et al., 2021; Mao et al., 2021; Zahran et al., 2021; Zaid et al., 2020) we analyzed unique set of circulating EVs subtypes and correlated their sera concentrations with an array of immune and clinical markers of COVID-19 including specific ones as are IL-10 and IL-6-producing MDSCs. Correlations with these cells, directly involved in immunopathogenesis of COVID-19 and potential therapeutic targets, give unique insight into intricate processes of COVID-19, pathology, which EVs are an important part of.

In summary, our results demonstrate the power of single EV analyses for the discovery of EV-based biomarkers. According to LEfSe analysis, the presence and quantity of $CD82^+$ EVs and $CD24^+$ EVs obtained higher scores as indicators for severe COVID-19 than some of previously suggested parameters including numbers of circulating neutrophils and MDSCs or as the blood concentration of CRP and various proinflammatory cytokines.

AUTHOR CONTRIBUTIONS

Maja Kosanović, Bernd Giebel and Sergej Tomić designed research study, analyzed results and wrote the manuscript; Tobias Tertel performed IFCM experiments and related statistical analysis and graphs; Sergej Tomić and Nataša Ilić, performed immune cells phenotypisation. Dejan Stevanović collected and interpreted clinical data and acquired informed consents. Jelena Đokić and Dušan Radojević performed statistical data analysis, correlations and graphical illustrations. All authors revised the manuscript and gave final approval for publication.

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CONFLICT OF INTEREST

BG is a scientific advisory board member of Innovex Therapeutics SL and Mursla Ltd and a founding director of Exosla Ltd. All other authors declare none conflict of interests.

REFERENCES

Aatonen, M T., Ã–Hman, T., Nyman, T A., Laitinen, S., Grönholm, M., & Siljander, P. R.-M. (2014). Isolation and characterization of platelet-derived extracellular vesicles. *Journal of Extracellular Vesicles*, *3*(1), 24692.

Altevogt, P., Sammar, M., Hüser, L., & Kristiansen, G. (2021). Novel insights into the function of CD24: A driving force in cancer. *Int J Cancer*, *148*(3), 546–559.

Althaus, K., Marini, I., Zlamal, J., Pelzl, L., Singh, A., Häberle, H., Mehrländer, M., Hammer, S., Schulze, H., Bitzer, M., Malek, N., Rath, D., Bösmüller, H., Nieswandt, B., Gawaz, M., Bakchoul, T., & Rosenberger, P. Antibody-induced procoagulant platelets in severe COVID-19 infection. *Blood*, *137*(8), 1061–1071.

Ayre, D. C., Elstner, M., Smith, N. C., Moores, E. S., Hogan, A. M., & Christian, S. L. (2015). Dynamic regulation of CD24 expression and release of CD24-containing microvesicles in immature B cells in response to CD24 engagement. *Immunology*, *146*(2), 217–233.

Börger, V., Weiss, D. J., Anderson, J. D., Borràs, F. E., Bussolati, B., Carter, D. R. F., Dominici, M., Falcón-Pérez, J. M., Gimona, M., Hill, A. F., Hoffman, A. M., De Kleijn, D., Levine, B. L., Lim, R., Lötvall, J., Mitsialis, S. A., Mongui-Tortajada, M., Muraca, M., Nieuwland, R., … Giebel, B. (2020). International society for extracellular vesicles and international society for cell and gene therapy statement on extracellular vesicles from mesenchymal stromal cells and other cells: Considerations for potential therapeutic agents to suppress coronaviru. *Cytotherapy*, *22*(9), 482–485.

Balbi, C., Burrello, J., Bolis, S., Lazzarini, E., Biemmi, V., Pianezzi, E., Burrello, A., Caporali, E., Grazioli, L. G., Martinetti, G., Fusi-Schmidhauser, T., Vassalli, G., Melli, G., … Barile, L. (2021). Circulating extracellular vesicles are endowed with enhanced procoagulant activity in SARS-CoV-2 infection. *EBioMedicine*, *67*, 103369.

Barberis, E., Vanella, V. V., Falasca, M., Caneapero, V., Cappellano, G., Raineri, D., Ghirimoldi, M., De Giorgis, V., Puricelli, C., Vaschetto, R., Sainaghi, P. P., Bruno, S., Sica, A., Dianzani, U., Rolla, R., Chiocchetti, A., Cantaluppi, V., Baldanzi, G., Marengo, E., & Manfredi, M. (2021). Circulating exosomes are strongly involved in SARS-CoV-2 infection. *Frontiers in Molecular Biosciences*, *8*, 632290.

Barkal, A. A., Brewer, R. E., Markovic, M., Kowarsky, M., Barkal, S. A., Zaro, B. W., Krishnan, V., Hatakeyama, J., Dorigo, O., Barkal, L. J., & Weissman, I. L. (2019). CD24 signalling through macrophage Siglec-10 is a target for cancer immunotherapy. *Nature*, *572*(7769), 392–396.

Bonifay, A., Robert, S., Champagne, B., Petit, P. R., Eugène, A., Chareyre, C., Duchez, A. -. C., Vélier, M., Fritz, S., Vallier, L., Lacroix, R., … Dignat-George, F. (2022). A new strategy to count and sort neutrophil-derived extracellular vesicles: Validation in infectious disorders. *Journal of Extracellular Vesicles*, *11*(4), e12204.

Cappellano, G., Raineri, D., Rolla, R., Giordano, M., Puricelli, C., Vilardo, B., Manfredi, M., Cantaluppi, V., Sainaghi, P. P., Castello, L., De Vita, N., Scotti, L., Vaschetto, R., Dianzani, U., & Chiocchetti, A. (2021). Communication circulating platelet-derived extracellular vesicles are a hallmark of sars-cov-2 infection. *Cells*, *10*(1), 1–10.

Chen, G., Wu, D., Guo, W., Cao, Y., Huang, D., Wang, H., Wang, T., Zhang, X., Chen, H., Yu, H., Zhang, X., Zhang, M., Wu, S., Song, J., Chen, T., Han, M., Li, S., Luo, X., Zhao, J., & Ning, Q. (2020). Clinical and immunological features of severe and moderate coronavirus disease 2019. *Journal of Clinical Investigation*, *130*(5), 2620–2629.

Cizmecioglu, A., Akay Cizmecioglu, H., Goktepe, M. H., Emsen, A., Korkmaz, C., Esenkaya Tasbent, F., Colkesen, F., & Artac, H. (2021). Apoptosis-induced T-cell lymphopenia is related to COVID-19 severity. *Journal of Medical Virology*, *93*(5), 2867–2874.

Conzelmann, C., Groß, R., Zou, M., Krüger, F., Görgens, A., Gustafsson, M. O., El Andaloussi, S., Münch, J., & Müller, J. A. (2020). Salivary extracellular vesicles inhibit Zika virus but not SARS-CoV-2 infection. *Journal of Extracellular Vesicles*, *9*(1), 1808281.

Costela-Ruiz, V. J., Illescas-Montes, R., Puerta-Puerta, J. M., Ruiz, C., & Melguizo-Rodríguez, L. (2020). SARS-CoV-2 infection: The role of cytokines in COVID-19 disease. *Cytokine & Growth Factor Reviews*, *54*, 62–75.

Dan, J. M., Mateus, J., Kato, Y., Hastie, K. M., Yu, E. D., Faliti, C. E., Grifoni, A., Ramirez, S. I., Haupt, S., Frazier, A., Nakao, C., Rayaprolu, V., Rawlings, S. A., Peters, B., Krammer, F., Simon, V., Saphire, E. O., Smith, D M., Weiskopf, D., … Crotty, S. (2021). Immunological memory to SARS-CoV-2 assessed for up to 8 months after infection. *Science (80-.)*, *371*(6529), eabf4063.

Denorme, F., Manne, B. K., Portier, I., Petrey, A. C., Middleton, E. A., Kile, B. T., Rondina, M. T., & Campbell, R. A. (2020). COVID-19 patients exhibit reduced procoagulant platelet responses. *Journal of Thrombosis and Haemostasis*, *18*(11), 3067–3073.

Devarakonda, C. K. V., Meredith, E., Ghosh, M., & Shapiro, L. H. (2021). Coronavirus receptors as immune modulators. *Journal of Immunology*, *206*(5), 923–929.

Diaz, J., Appiah, J., Askie, L., Baller, A., Banerjee, A., Barkley, S., Bertagnolio, S., Hemmingsen, B., Bonet, M., & Cunningham, J. (2021). *COVID-19: Clinical management living guidance*. World Health Organization, 81.

Dragovic, R. A., Gardiner, C., Brooks, A. S., Tannetta, D. S., Ferguson, D. J. P., Hole, P., Carr, B., Redman, C. W. G., Harris, A. L., Dobson, P. J., Harrison, P., & Sargent, I. L. (2011). Sizing and phenotyping of cellular vesicles using Nanoparticle Tracking Analysis. *Nanomedicine*, *7*(6), 780–788.

Droste, M., Tertel, T., Jeruschke, S., Dittrich, R., Kontopoulou, E., Walkenfort, B., Börger, V., Hoyer, P. F., Büscher, A. K., Thakur, B. K., & Giebel, B. (2021). Single extracellular vesicle analysis performed by imaging flow cytometry and nanoparticle tracking analysis evaluate the accuracy of urinary extracellular vesicle preparation techniques differently. *International Journal of Molecular Sciences*, *22*(22), 12436.

Earnest, J. T., Hantak, M. P., Li, K., Mccray, P. B., Perlman, S., & Gallagher, T. (2017). The tetraspanin CD9 facilitates MERS-coronavirus entry by scaffolding host cell receptors and proteases. *PLoS Pathog*, *13*(7), e1006546.

Ekström, K., Crescitelli, R., Pétursson, H. I., Johansson, J., Lässer, C., & Olofsson Bagge, R. (2022). Characterization of surface markers on extracellular vesicles isolated from lymphatic exudate from patients with breast cancer. *BMC Cancer*, *22*(1), 50.

Erdbrügger, U., Blijdorp, C. J., Bijnsdorp, I. V., Borràs, F. E., Burger, D., Bussolati, B., Byrd, J. B., Clayton, A., Dear, J. W., Falcón-Pérez, J. M., Grange, C., Hill, A. F., Holthöfer, H., Hoorn, E. J., Jenster, G., Jimenez, C. R., Junker, K., Klein, J., Knepper, M. A., … Martens-Uzunova, E. S. (2021). Urinary extracellular vesicles: A position paper by the urine task force of the international society for extracellular vesicles. *Journal of Extracellular Vesicles*, *10*(7), e12093.

Falck-Jones, S., Vangeti, S., Yu, M., Falck-Jones, R., Cagigi, A., Badolati, I., Björn, Ö., Lautenbach, M. J., Åhlberg, E., Lin, A., Lepzien, R., Szurgot, I., Lenart, K., Hellgren, F., Maecker, H., Sälde, J., Albert, J., Johansson, N., Bell, M., … Smed-Sörensen, A. (2021). Functional monocytic myeloid-derived suppressor cells increase in blood but not airways and predict COVID-19 severity. *Journal of Clinical Investigation*, *131*(6), e144734.

Florin, L., & Lang, T. (2018). Tetraspanin assemblies in virus infection. *Frontiers in Immunology*, *9*(MAY), .

Fu, B., Wang, D., Shen, X., Guo, C., Liu, Y., Ye, Y., Sun, R., Li, J., Tian, Z., & Wei, H. (2020). Immunomodulation induced during interferon-α therapy impairs the anti-HBV immune response through CD24+CD38hi B cells. *Frontiers in Immunology*, *11*, 591269.

Görgens, A., Bremer, M., Ferrer-Tur, R., Murke, F., Tertel, T., Horn, P A., Thalmann, S., Welsh, J A., Probst, C., Guerin, C., Boulanger, C M., Jones, J C., Hanenberg, H., Erdbrügger, U., Lannigan, J., Ricklefs, F. L., El-Andaloussi, S., & Giebel, B. (2019). Optimisation of imaging flow cytometry for the analysis of single extracellular vesicles by using fluorescence-tagged vesicles as biological reference material. *Journal of Extracellular Vesicles*, *8*(1), 1587567.

Giamarellos-Bourboulis, E. J., Netea, M. G., Rovina, N., Akinosoglou, K., Antoniadou, A., Antonakos, N., Damoraki, G., Gkavogianni, T., Adami, M. -. E., Katsaounou, P., Ntaganou, M., Kyriakopoulou, M., Dimopoulos, G., Koutsodimitropoulos, I., Velissaris, D., Koufargyris, P., Karageorgos, A., Katrini, K., Lekakis, V., … Koutsoukou, A. (2020). Complex immune dysregulation in COVID-19 patients with severe respiratory failure. *Cell Host & Microbe*, *27*(6), 992–1000.e3.

Giebel, B., & Helmbrecht, C. (2017). Methods to analyze EVs. *Methods in Molecular Biology*, 2017;*1545*, 1–20.

Grant, M. C., Geoghegan, L., Arbyn, M., Mohammed, Z., McGuinness, L., Clarke, E. L., & Wade, R. G. (2020). The prevalence of symptoms in 24,410 adults infected by the novel coronavirus (SARS-CoV-2; COVID-19): A systematic review and meta-analysis of 148 studies from 9 countries. *PLoS One*, *15*(6 June), e0234765.

Grigor'eva, A. E., Tamkovich, S. N., Eremina, A V., Tupikin, A. E., Kabilov, M. R., Chernykh, V. V., Vlassov, V. V., Laktionov, P. P., & Ryabchikova, E. I. (2016). Exosomes in tears of healthy individuals: Isolation, identification, and characterization. *Biochemistry Supplement Series B: Biomedical Chemistry*, *10*(2), 165–172.

Guervilly, C., Bonifay, A., Burtey, S., Sabatier, F., Cauchois, R., Abdili, E., Arnaud, L., Lano, G., Pietri, L., Robert, T., Velier, M., Papazian, L., Albanese, J., Kaplanski, G., Dignat-George, F., & Lacroix, R. (2021). Dissemination of extreme levels of extracellular vesicles: Tissue factor activity in patients with severe COVID-19. *Blood Advances*, *5*(3), 628–634.

Hassanpour, M., Rezaie, J., Nouri, M., & Panahi, Y. (2020). The role of extracellular vesicles in COVID-19 virus infection. *Infection, Genetics and Evolution*, *85*, 104422.

Keller, S., Rupp, C., Stoeck, A., Runz, S., Fogel, M., Lugert, S., Hager, H. - D., Abdel-Bakky, M. S., Gutwein, P., & Altevogt, P. (2007). CD24 is a marker of exosomes secreted into urine and amniotic fluid. *Kidney International*, *72*(9), 1095–1102.

Koliha, N., Wiencek, Y., Heider, U., Jüngst, C., Kladt, N., Krauthäuser, S., Johnston, I. C. D., Bosio, A., Schauss, A., & Wild, S. (2016). A novel multiplex bead-based platform highlights the diversity of extracellular vesicles. *Journal of Extracellular Vesicles*, *5*, 29975.

Kowal, J., Arras, G., Colombo, M., Jouve, M., Morath, J. P., Primdal-Bengtson, B., Dingli, F., Loew, D., Tkach, M., & Théry, C. (2016). Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(8), E968–E977.

Kropshofer, H., Spindeldreher, S., Röhn, T. A., Platania, N., Grygar, C., Daniel, N., Wölpl, A., Langen, H., Horejsi, V., … Vogt, A. B. (2002). Tetraspan microdomains distinct from lipid rafts enrich select peptide-MHC class II complexes. *Nature Immunology*, *3*(1), 61–68.

Kudryavtsev, I., Kalinina, O., Bezrukikh, V., Melnik, O., & Golovkin, A. (2021). The significance of phenotyping and quantification of plasma extracellular vesicles levels using high-sensitivity flow cytometry during covid-19 treatment. *Viruses*, *13*(5), 767.

Lam, S. M., Zhang, C., Wang, Z., Ni, Z., Zhang, S., Yang, S., Huang, X., Mo, L., Li, J., Lee, B., Mei, M., Huang, L., Shi, M., Xu, Z., Meng, F. P., Cao, W. J., Zhou, M.-J., Shi, L., Chua, G. H., … Shui, G. (2021). A multi-omics investigation of the composition and function of extracellular vesicles along the temporal trajectory of COVID-19. *Nature Metabolism*, *3*(7), 909–922.

Lin, M. J., Li, S., Yang, L. J., Ye, D.-Y., Xu, L.-Q., Zhang, X., Sun, P.-N., & Wei, C.-J. (2020). Plasma membrane vesicles of human umbilical cord mesenchymal stem cells ameliorate acetaminophen-induced damage in HepG2 cells: a novel stem cell therapy. *Stem Cell Res Ther*, *11*(1), 225.

Lindenbergh, M. F. S., & Stoorvogel, W. (2018). Antigen presentation by extracellular vesicles from professional antigen-presenting cells. *Annual Review of Immunology*, *36*, 435–459.

Liu, H., Yuan, W., Pang, Q., Xue, C., & Yan, X. (2022). Single-particle analysis of tear fluid reveals abundant presence of tissue factor-exposing extracellular vesicles with strong coagulation activity. *Talanta*, *239*, 123089.

Lu, C., Amin, M. A., & Fox, D. A. (2020). CD13/Aminopeptidase N is a potential therapeutic target for inflammatory disorders. *Journal of Immunology*, *204*(1), 3–11.

Mao, K., Tan, Q., Ma, Y., Wang, S., Zhong, H., Liao, Y., Huang, Q., Xiao, W., Xia, H., Tan, X., Luo, P., Xu, J., Long, D., & Jin, Y. (2021). Proteomics of extracellular vesicles in plasma reveals the characteristics and residual traces of COVID-19 patients without underlying diseases after 3 months of recovery. *Cell Death & Disease*, *12*(6), 541.

Mehta, P., Mcauley, D. F., Brown, M., Sanchez, E., Tattersall, R. S., & Manson, J. J. (2020). COVID-19: Consider cytokine storm syndromes and immunosuppression. *Lancet*, *395*(10229), 1033–1034.

Moore, J. B., & June, C. H. (2020). Cytokine release syndrome in severe COVID-19. *Science (80-.)*, *368*(6490), 473–474.

Morgan, R. L., Behbahani-Nejad, N., Endres, J., Amin, M. A., Lepore, N. J., Du, Y., Urquhart, A., Chung, K. C., & Fox, D. A. (2016). Localization, shedding, regulation and function of aminopeptidase N/CD13 on fibroblast like synoviocytes. *PLoS One*, *11*(9), e0162008.

Newell, K. L., Clemmer, D. C., Cox, J. B., Kayode, Y. I., Zoccoli-Rodriguez, V., Taylor, H. E., Endy, T. P., Wilmore, J. R., … Winslow, G. M. (2021). Switched and unswitched memory B cells detected during SARS-CoV-2 convalescence correlate with limited symptom duration. *PLoS One*, *16*(1), e0244855.

Nguyen, A., David, J. K., Maden, S. K., Wood, M. A., Weeder, B. R., Nellore, A., & Thompson, R. F. (2020). Human leukocyte antigen susceptibility map for severe acute respiratory syndrome coronavirus 2. *Journal of Virology*, *94*(13), e00510–20.

Nomura, R., Kiyota, A., Suzaki, E., Kataoka, K., Ohe, Y., Miyamoto, K., Senda, T., & Fujimoto, T. (2004). Human coronavirus 229E binds to CD13 in rafts and enters the cell through caveolae. *Journal of Virology*, *78*(16), 8701–8708.

Park, J., Park, J. S., Huang, C. H., Jo, A., Cook, K., Wang, R., Lin, H. -. Y., Van Deun, J., Li, H., Min, J., Wang, L., Yoon, G., Carter, B S., Balaj, L., Choi, G. S., Castro, C M., Weissleder, R., & Lee, H. (2021). An integrated magneto-electrochemical device for the rapid profiling of tumour extracellular vesicles from blood plasma. *Nature Biomedical Engineering*, *5*(7), 678–689.

Pöhlmann, S., Gramberg, T., Wegele, A., Pyrc, K., van der Hoek, L., Berkhout, B., … Hofmann, H. (2006). Interaction between the spike protein of human coronavirus NL63 and its cellular receptor ACE2. *Advances in Experimental Medicine and Biology*, *581*, 281–284.

Ponti, G., Maccaferri, M., Ruini, C., Tomasi, A., & Ozben, T. (2020). Biomarkers associated with COVID-19 disease progression. *Critical Reviews in Clinical Laboratory Sciences*, *57*(6), 389–399.

Potolicchio, I., Carven, G J., Xu, X., Stipp, C., Riese, R J., Stern, L J., & Santambrogio, L. (2005). Proteomic analysis of microglia-derived exosomes: Metabolic role of the aminopeptidase CD13 in neuropeptide catabolism. *Journal of Immunology*, *175*(4), 2237–2243.

Priya, R., Jain, V., Akhtar, J., Chauhan, G., Sakhuja, P., Goyal, S., Agarwal, A. K., Javed, A., Jain, A. P., Polisetty, R. V., Sirdeshmukh, R., Kar, S., & Gautam, P. (2021). Plasma-derived candidate biomarkers for detection of gallbladder carcinoma. *Scientific Reports*, *123AD*(11), 23554.

ProteinAtlas: CD82. https://www.proteinatlas.org/ENSG00000085117-CD82/blood (Last accessed 10.10.2021.)

Qi, F., Qian, S., Zhang, S., & Zhang, Z. (2020). Single cell RNA sequencing of 13 human tissues identify cell types and receptors of human coronaviruses. *Biochemical and Biophysical Research Communications*, *526*(1), 135–140.

Ramasamy, M. N., Minassian, A. M., Ewer, K. J., Flaxman, A. L., Folegatti, P. M., Owens, D. R., Voysey, M., Aley, P. K., Angus, B., Babbage, G., Belij-Rammerstorfer, S., Berry, L., Bibi, S., Bittaye, M., Cathie, K., Chappell, H., Charlton, S., Cicconi, P., Clutterbuck, E. A., … Zizi, D. (2020). Safety and immunogenicity of ChAdOx1 nCoV-19 vaccine administered in a prime-boost regimen in young and old adults (COV002): A single-blind, randomised, controlled, phase 2/3 trial. *Lancet*, *396*(10267), 1979–1993.

Rausch, L., Lutz, K., Schifferer, M., Winheim, E., Gruber, R., Oesterhaus, E. F., Rinke, L., Hellmuth, J. C., Scherer, C., Muenchhoff, M., Mandel, C., Bergwelt-Baildon, M., Simons, M., Straub, T., Krug, A. B., Kranich, J., & Brocker, T. (2021). Binding of phosphatidylserine-positive microparticles by PBMCs classifies disease severity in COVID-19 patients. *Journal of Extracellular Vesicles*, *10*(14), e12173.

Reizine, F., Lesouhaitier, M., Gregoire, M., Pinceaux, K., Gacouin, A., Maamar, A., Painvin, B., Camus, C., Le Tulzo, Y., Tattevin, P., Revest, M., Le Bot, A., Ballerie, A., Cador-Rousseau, B. R., Lederlin, M., Lebouvier, T., Launey, Y., Latour, M., Verdy, C., … Tadié, J. M. (2021). SARS-CoV-2-Induced ARDS associates with MDSC expansion, lymphocyte dysfunction, and arginine shortage. *Journal of Clinical Immunology*, *41*(3), 515–525.

Revelle, W. (2010) psych: Procedures for personality and psychological research [Computer software manual]. https://CRAN.R-project.org/package=psych

Ritchie, A. I., & Singanayagam, A. (2020). Immunosuppression for hyperinflammation in COVID-19: a double-edged sword? *Lancet*, *395*(10230), 1111.

Romanov, Y. A., Volgina, N. E., Dugina, T. N., Kabaeva, N. V., & Sukhikh, G. T. (2018). Human umbilical cord mesenchymal stromal cell-derived microvesicles express surface markers identical to the phenotype of parental cells. *Bulletin of Experimental Biology and Medicine*, *166*(1), 124–129.

Rowlands, M., Segal, F., & Hartl, D. (2021). Myeloid-derived suppressor cells as a potential biomarker and therapeutic target in COVID-19. *Frontiers in Immunology*, 2021, *12*, 2435.

Sacchi, A., Grassi, G., Bordoni, V., Lorenzini, P., Cimini, E., Casetti, R., Tartaglia, E., Marchioni, L., Petrosillo, N., Palmieri, F., D'Offizi, G., Notari, S., Tempestilli, M., Capobianchi, M. R., Nicastri, E., Maeurer, M., Zumla, A., Locatelli, F., Antinori, A., … Agrati, C. (2020). Early expansion of myeloid-derived suppressor cells inhibits SARS-CoV-2 specific T-cell response and may predict fatal COVID-19 outcome. *Cell Death & Disease*, *11*(10), 921.

Samara, A., & Belle, B. D. (2021). Nanomaterials upscaling cell production and advancing exosome-based stem cell therapies. *Frontiers in Nanotechnology*, *3*, 74.

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., & Huttenhower, C. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol*, *12*(6), R60.

Shapira, S., Ben Shimon, M., Hay-Levi, M., Shenberg, G., Choshen, G., Bannon, L., Tepper, M., Kazanov, D., Seni, J., Lev-Ari, S., Peer, M., Boubas, D., Stebbing, J., Tsiodras, S., & Arber, N. (2022). A novel platform for attenuating immune hyperactivity using EXO-CD24 in COVID-19 and beyond. EMBO Molecular Medicine. Portico. https://doi.org/10.15252/emmm.202215997

Sims, B., Farrow, A. L., Williams, S. D., Bansal, A., Krendelchtchikov, A., & Matthews, Q. L. (2018). Tetraspanin blockage reduces exosome-mediated HIV-1 entry. *Archives of Virology*, *163*(6), 1683–1689.

Sokolova, V., Ludwig, A. -. K., Hornung, S., Rotan, O., Horn, P. A., Epple, M., & Giebel, B. (2011). Characterisation of exosomes derived from human cells by nanoparticle tracking analysis and scanning electron microscopy. *Colloids and Surfaces B: Biointerfaces*, *87*(1), 146–150.

Sornov, A. K., Yunusova, N. V., & Shtam, T. A., Proskura, K. V., Zambalova, E. A., Laktionov, P. P., Tamkovich, S. N. (2019). ADAM-10 on the surface of exosomes from breast cancer patients blood: Newly mechanisms tumor dissemination. *Vopr Onkol*, *65*(5), 678–683.

Staubach, S., Tertel, T., Walkenfort, B., Buschmann, D., Pfaffl, M. W., Weber, G., … Giebel, B. (2022). Free flow electrophoresis allows quick and reproducible preparation of extracellular vesicles from conditioned cell culture media. *Extracellular Vesicles and Circulating Nucleic Acids*, *3*(1), 31–48.

Tőkés-Füzesi, M., Ruzsics, I., Rideg, O., Kustán, P., Kovács, G. L., & Molnár, T. (2018). Role of microparticles derived from monocytes, endothelial cells and platelets in the exacerbation of COPD. *International Journal of Chronic Obstructive Pulmonary Disease*, *13*, 3749–3757.

Taghiloo, S., Aliyali, M., Abedi, S., Mehravaran, H., Sharifpour, A., Zaboli, E., Eslami-Jouybari, M., Ghasemian, R., Vahedi-Larijani, L., Hossein-Nattaj, H., Amjadi, O., Rezazadeh, H., Ajami, A., & Asgarian-Omran, H. (2021). Apoptosis and immunophenotyping of peripheral blood lymphocytes in Iranian COVID-19 patients: Clinical and laboratory characteristics. *Journal of Medical Virology*, *93*(3), 1589–1598.

Tertel, T., Bremer, M., Maire, C., Lamszus, K., Peine, S., Jawad, R., Andaloussi, S. E. L., Giebel, B., Ricklefs, F. L., & Görgens, A. (2020). High-resolution imaging flow cytometry reveals impact of incubation temperature on labeling of extracellular vesicles with antibodies. *Cytometry Part A*, *97*(6), 602–609.

Tertel, T., Görgens, A., & Giebel, B. (2020). Analysis of individual extracellular vesicles by imaging flow cytometry. *Methods Enzymol*, *645*, 55–78.

Tertel, T., Schoppet, M., Stambouli, O., Al-Jipouri, A., James, P. F., & Giebel, B. (2022). Imaging flow cytometry challenges the usefulness of classically used extracellular vesicle labeling dyes and qualifies the novel dye Exoria for the labeling of mesenchymal stromal cell-extracellular vesicle preparations. *Cytotherapy*, *24*(6), 619–628.

Tian, W., Zhang, N., Jin, R., Feng, Y., Wang, S., Gao, S., Gao, R., Wu, G., Tian, D., Tan, W., Chen, Y., Gao, G. F., & Wong, C. C. L. (2020). Immune suppression in the early stage of COVID-19 disease. *Nature Communications*, *11*(1), 5859.

Tomić, S., Đokić, J., Stevanović, D., Ilić, N., Gruden-Movsesijan, A., Dinić, M., Radojević, D., Bekić, M., Mitrović, N., Tomašević, R., Mikić, D., Stojanović, D., … Ćolić, M. (2021). Reduced expression of autophagy markers and expansion of myeloid-derived suppressor cells correlate with poor T cell response in severe COVID-19 patients. *Frontiers in Immunology*, *12*, 614599.

Tripisciano, C., Weiss, R., Eichhorn, T., Spittler, A., Heuser, T., Fischer, M. B., & Weber, V. (2017). Different potential of extracellular vesicles to support thrombin generation: Contributions of phosphatidylserine, tissue factor, and cellular origin. *Scientific Reports*, *7*(1), 6522.

Tsou, E. P. S., Sule, G., Rubio, M. G., Asif Amin, M., Zuo, Y., Knight, J., Kanthi, Y., & Fox, D. (2020). Identification of CD13 as a potential cause for SARS-CoV-2-triggered hyperinflammation and thrombosis. *Arthritis Rheumatol*, *72*(SUPPL 10), 143–144.

Tuck, M. K., Chan, D. W., Chia, D., Godwin, A. K., Grizzle, W. E., Krueger, K. E., Rom, W., Sanda, M., Sorbara, L., Stass, S., Wang, W., & Brenner, D. E. (2009). Standard operating procedures for serum and plasma collection: Early detection research network consensus statement standard operating procedure integration working group. *Journal of Proteome Research*, *8*(1), 113–117.

Wei, T., Simko, V., Levy, M., Xie, Y., Jin, Y., Zemla, J., Freidank, M., Cai, J., … Protivinsky, T. (2017). R package "corrplot": Visualization of a correlation matrix. *Statistician*, *56*, 316–324.

Welsh, J A., Van Der Pol, E., Arkesteijn, G. J. A., Bremer, M., Brisson, A., Coumans, F., Dignat-George, F., Duggan, E., Ghiran, I., Giebel, B., Görgens, A., Hendrix, A., Lacroix, R., Lannigan, J., Libregts, S. F. W. M., Lozano-Andrés, E. A., Morales-Kastresana, A., Robert, S., De Rond, L., … Jones, J. C. (2020). MIFlowCyt-EV: A framework for standardized reporting of extracellular vesicle flow cytometry experiments. *Journal of Extracellular Vesicles*, *9*(1), 1713526.

WHO Coronavirus (COVID-19) Dashboard. https://covid19.who.int/(Last accessed 15.12.2021.)

Yáñez-Mó, M. A., Barreiro, O., Gordon-Alonso, M., Sala-Valdés, M., & Sánchez-Madrid, F. (2009). Tetraspanin-enriched microdomains: A functional unit in cell plasma membranes. *Trends in Cell Biology*, *19*(9), 434–446.

Yeung, L., Hickey, M. J., & Wright, M. D. (2018). The many and varied roles of tetraspanins in immune cell recruitment and migration. *Frontiers in Immunology*, *9*(JUL), 1644.

Yoshihara, M., Mizutani, S., Kato, Y., Matsumoto, K., Mizutani, E., Mizutani, H., Fujimoto, H., Osuka, S., & Kajiyama, H. (2021). Recent insights into human endometrial peptidases in blastocyst implantation via shedding of microvesicles. *International Journal of Molecular Sciences*, *22*(24), 13479.

Zahran, A. M., El-Badawy, O., Ali, W. A., Mahran, Z. G., Mahran, E. E. M. O., … Rayan, A. (2021). Circulating microparticles and activated platelets as novel prognostic biomarkers in COVID-19; relation to cancer. *PLoS One*, *16*(2 February), e0246806.

Zaid, Y., Puhm, F., Allaeys, I., Naya, A., Oudghiri, M., Khalki, L., Limami, Y., Zaid, N., Sadki, K., Ben El Haj, R., Mahir, W., Belayachi, L., Belefquih, B., Benouda, A., Cheikh, A., Langlois, M. A., Cherrah, Y., Flamand, L., Guessous, F., & Boilard, E. (2020). Platelets can associate with SARS-CoV-2 RNA and are hyperactivated in COVID-19. *Circulation Research*, *127*(11), 1404–1418.

Zhou, F., Yu, T., Du, R., Fan, G., Liu, Y., Liu, Z., Xiang, J., Wang, Y., Song, B., Gu, X., Guan, L., Wei, Y., Li, H., Wu, X., Xu, J., Tu, S., Zhang, Y., Chen, H., & Cao, B. (2020). Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: A retrospective cohort study. *Lancet*, *395*(10229), 1054–1062.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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