A granulocytic signature identifies COVID-19 and its severity

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Summary:

Unsupervised mapping of leukocyte surface markers identified a granulocytic COVID-19 signature comprising eosinophil and basophil CRTH2 downregulation, increased counts of $CD15^{+}CD16^{+}$ neutrophils, and decreased granulocytic CD11b expression, while PDL1 checkpoint expression in basophils and eosinophils was associated with severity.

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Footnote page

Conflict of interest: TM, JMB and FM are employees of Beckman Coulter Life Sciences. JV has received speaker and consultancy fees from Meda Pharma, Mylan, Sanofi, Thermo Fisher, Beckman Coulter, outside this work. ML received fees as speaker from MSD, Edwards LifeScience and as consultant from Aguettant, Amomed and Gilead. DO is cofounder and shareholder of Imcheck Therapeutics, Emergence Therapeutics and Alderaan. The other authors declare no conflict of interest.

Funding statement: This work was supported by a grant from the French National Research Agency (Agence Nationale de la Recherche, ANR Flash COVID, grant IMMUNO-COVID)

Trial registration. ANSM (France) ID-RCB: 2020-A00756-33

Meeting(s) where the information has previously been presented: none

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Abstract

Background An unbiased approach of SARS-CoV-2-induced immune dysregulation has not been undertaken so far. We aimed to identify previously unreported immune markers able to discriminate COVID-19 patients from healthy controls and to predict mild and severe disease.

Methods An observational, prospective, multicentric study was conducted in patients with confirmed COVID-19: mild/moderate (n=7) and severe (n=19). Immunophenotyping of whole blood leukocytes was performed in patients upon hospital ward or intensive care unit admission and in healthy controls (n=25). Clinically relevant associations were identified through unsupervised analysis.

Results Granulocytic (neutrophil, eosinophil and basophil) markers were enriched during COVID-19 and discriminated between mild and severe patients. Increased counts of $CD15^{+}CD16^{+}$ neutrophils, decreased granulocytic expression of integrin CD11b, and Th2-related CRTH2 downregulation in eosinophils and basophils established a COVID-19 signature. Severity was associated with the emergence of PDL1 checkpoint expression in basophils and eosinophils. This granulocytic signature was accompanied by monocyte and lymphocyte immunoparalysis. Correlation with validated clinical scores supported pathophysiological relevance.

Conclusion Phenotypic markers of circulating granulocytes are strong discriminators between infected and uninfected individuals as well as between severity stages. COVID-19 alters the frequency and functional phenotypes of granulocyte subsets with the emergence of CRTH2 as a disease biomarker.

Keywords SARS-CoV-2; COVID-19; neutrophil; eosinophil; basophil; CRTH2; immune checkpoint; CD11b; CD16; PD-L1

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Introduction

The hallmark of COVID-19, the infection caused by the severe acute respiratory syndrome coronavirus 2 (SARS CoV-2), is the occurrence, in 10-20% of patients, of a sudden deterioration 7-10 days after the onset of symptoms, increasing the risk of acute respiratory distress syndrome, of intensive care unit need and ultimately of death [1]. Studies exploring the immune response suggested that SARS-CoV-2 may induce unique patterns of immune dysregulation [2-3]. To our knowledge, a systematic approach of SARS-CoV-2-induced immune dysregulation at the phenotype level has not been undertaken so far. Single-cell RNA sequencing of peripheral blood mononuclear cells evidenced phenotypic remodeling affecting innate and adaptive populations [4]. Our aim was to establish a comprehensive, unsupervised map of circulating immune cells in COVID-19 patients using a first-in-class flow cytometry approach for rapid whole-blood assessment. The primary objective was the identification of immunophenotypic patterns most accurately associated with COVID-19 diagnosis and severity. Among the large number of phenotypic markers of circulating immune cells modulated by SARS-CoV-2, those related to granulocyte lineage (neutrophils, basophils and eosinophils) were strong discriminators between infected and uninfected individuals as well as between different degrees of disease severity. Beside SARS-CoV-2 associated lymphopenia, changes in frequency and activation of granulocyte subsets may be predictive of clinical worsening during COVID-19.

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Methods

Study design

This open multicenter prospective observational study was conducted in the intensive care unit (ICU) of North Hospital of Marseille and the COVID-19 ward unit of European Hospital of Marseille.

Patients and controls

Patients admitted to ward and ICU with confirmed SARS-CoV-2 infection were included in the study if they fulfilled the criteria: i) age 18 or older and ii) a positive SARS-CoV-2 reverse transcriptase-polymerase chain reaction (RT-PCR) in nasopharyngeal swabs or tracheal aspiration. Exclusion criteria were preexisting treatments interfering with immune functions, pregnancy and missing clinical or laboratory data.

Demographic, clinical and laboratory data (arterial blood gas analysis, complete blood count, biochemistry, virology) including SARS-CoV-2-related symptoms, date of disease onset, organ support, and medications were collected for each patient upon admission to ICU or conventional ward. The same data were collected on the day of blood sampling. At day 28 after COVID-19 diagnosis, the duration of mechanical ventilation, length of ICU and hospital stays, and ICU and hospital mortality rates were also recorded. The Simplified Acute Physiology Score II (SAPS II) [5], the Sepsis-related Organ Failure Assessment (SOFA) [6], the National Early Warning Score 2 (NEWS2) [7], and the World Health Organization (WHO) progression scale [8-9] were calculated at admission and on the day of blood sampling. Patients were classified as mild/moderate (WHO grade 4 and 5, hereafter termed “mild”) depending on the presence of oxygen supply, while those receiving high-flow oxygen

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therapy (WHO grade 6) or invasive mechanical ventilation (WHO grade 7-9) were considered as severe.

Samples of healthy blood donors (HBD) group, serving as controls, were received from Etablissement Français du Sang (EFS), Marseille, France.

Study approval

The study was conducted in accordance with the Declaration of Helsinki and the French law on research involving humans. It was registered with the French ANSM under the reference ID-RCB: 2020-A00756-33 and received approval from the national review board Comité de Protection des Personnes Ile de France XI (20027-60604, March 25th 2020). The patients were informed and agreed to participate to this study. Patient enrollment took place from March 30 to April 8th, 2020. HBD samples were obtained through an institutional agreement between EFS and UMR-D258 MEPHI.

Flow cytometry

All antibodies and reagents were from Beckman Coulter (Villepinte, France). Blood (4 mL) was collected by venipuncture on EDTA-anticoagulated tubes, stored and delivered at room temperature to the Immunology laboratory. Multiparametric flow cytometry was used for immune cell enumeration and immune phenotyping less than 24 hours after blood collection. Each immune phenotyping panel (**Table 1**) was provided in a pre-mix dry antibody cocktail completed in some cases by the addition of liquid conjugates prior to sample addition. Staining of leukocytes for enumeration was performed by the addition of 100 µl whole blood to the IM Count tube followed by 15 min incubation at room

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temperature. Lysis of red blood cells was achieved with 2 ml Versalyse (Beckman Coulter) followed by a 15 min incubation prior to acquisition. Immune phenotyping followed a similar protocol except for the incubation (20 and 10 minutes, respectively, in the dark). Lysed cells were washed with 3 ml PBS and the cell pellet re-suspended in 0.5 ml PBS 1X, 0.1% formaldehyde. Acquisition was done with a Navios flow cytometer (Beckman Coulter).

Data analysis and statistics

Multiparameter flow cytometry data files were analyzed using the Kaluza software, version 2.1 (Beckman Coulter). Parameters were exported to JMP 14.2.0 software (SAS) for statistical analysis. The response screening platform of JMP, not only yielding a p value but also a false discovery rate (FDR) corrected value, was then used to identify the parameters with the highest discriminative capabilities. Most discriminating parameters were ranked according to the LogWorth of their FDR corrected p-values. Multivariate analyses following the principal component analysis (PCA) approach were also conducted with JMP. Non-parametric Wilcoxon Rank Sum tests, equivalent to Mann Whitney tests, were also performed by JMP to compare parameter levels across different subgroups of individuals. The X^2 with Yate correction, Fisher's exact test, t test, Mann Whitney test and Wilcoxon test were used to compare clinical and laboratory variables between the mild and severe groups as appropriate. Statistical significance was defined as $p<0.05$.

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Results

Demographic characteristics of the study population

During the study period, 55 confirmed COVID-19 cases were referred to the participating centers. Among them, 19 patients were admitted to the ICU (severe group) and seven to the conventional ward (mild group). Twenty-five HBD served as control group (**Supplementary Figure 1**). Demographic data are presented in **Table 2**. Differences were observed between the mild and the severe group. Elevated body mass index and hypertension were more frequent in the severe group than in the mild group (p = 0.005 and 0.03, respectively). Severity scores, including the WHO progression score and the SOFA score, were significantly higher in the severe group than in the mild group. C-reactive protein was increased in the severe group as compared with the mild group whereas eosinophils and monocytes were significantly decreased between patient groups (**Table 2**). Lymphopenia, defined as a lymphocyte count of less than 1 giga/L, was found in 85% of COVID-19 patients comprising 71% of the mild group and 89% of the severe group, a non-significant difference (**Table 2**).

Controls versus COVID-19 patients

An unsupervised analysis of circulating leukocyte subsets and immune phenotypic markers yielded more than 100 significant discriminators between COVID-19 patients and controls, with FDR p-values less than 0.05. Further analysis was arbitrarily restricted to the 25 most discriminant markers (**Fig. 1a**). PCA of these 25 markers effectively discriminated COVID-19 patients from controls (**Fig. 1b**). Eleven of 25 were granulocyte-associated markers, followed by lymphocyte, NK and dendritic cell (DC) variables (**Fig. 1a**). Enrichment in granulocyte-associated markers affected the three granulocytic lineages: neutrophils, eosinophils, and basophils. There was a significant increase in the frequency of $CD15^{+}$ granulocytes (mainly

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comprising neutrophils), an increase in the frequency of $CD15^{+}CD16^{+}$ neutrophil subset, and a decrease in the frequency of basophils in COVID-19 patients, as compared with controls (**Fig. 2 a-b**). Two prominent function-associated membrane antigens were modulated in COVID-19 patients as compared to HBD: CD11b (αM subunit of integrin CD11bCD18, also known as complement receptor 3, CR3), whose expression was decreased at the surface of neutrophils and basophils (**Fig. 2 c**), and CRTH2 (CD294), a receptor for prostaglandin D2 (PGD2), whose expression was decreased on basophils and eosinophils (**Fig. 2 d**). Hence, SARS-CoV-2 infection was characterized by changes in frequency of granulocyte subsets and alteration of their functional phenotypes with the emergence of CRTH2 as a biomarker for COVID-19.

Effect of disease severity

We wondered if the granulocyte signature displayed specific changes associated with disease severity (**Fig. 3a**). Unsupervised analysis followed by PCA of the best markers discriminating between mild and severe COVID-19 patients (**Fig. 3b**) evidenced the predominance of granulocytic markers (8 out of 19 with a FDR p value less than 0.05). Some of the markers discriminating COVID-19 patients from HBD also discriminated mild from severe patients. Neutrophil subset frequency was one of these shared markers. The frequency of $CD15^{+}$ granulocytes and $CD15^{+}CD16^{+}$ neutrophils was significantly increased in the severe group ($p = 0.002$), while the levels of expression of both CD15 and CD16 were decreased in the severe group as compared with the mild group (**Fig. 4a**). Another shared marker was eosinophil CRTH2 expression, which was profoundly decreased in the severe group (**Fig. 4b**). Hence, COVID-19 severity was associated with a more profound imbalance of granulocyte subsets and functional markers of the disease.

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However, severe disease was associated with the emergence of specific markers. Severe patients differed from mild ones with respect to functional markers of eosinophils and basophils. At the surface of both basophils and eosinophils, the expression of checkpoint inhibitors such as PDL1 was significantly higher in the severe group than in the mild group (**Fig. 4c-d**). Such prominent changes in surface expression of functional granulocytic markers prompted us to ask whether granulocyte alterations correlated with clinical scores. We found that both WHO and SOFA scores correlated positively with innate immune checkpoints such as PDL1 expression on basophils and eosinophils, and negatively with neutrophil CD11b and eosinophil CRTH2 expression (**Fig. 5**). The level of correlation between immunophenotypic markers and clinical scores was similar to that of clinical scores between them (WHO versus SOFA: R^2 = 0.567).

Partners of the granulocyte signature

The granulocytic signature of COVID-19 was not isolated since it was associated with a decreased representation of $CD4^+$ T cells, $CD8^+$ T cells, and plasmacytoid dendritic cells (**Supplementary Fig. 2**). The upregulation of checkpoint inhibitors was not restricted to the granulocyte lineage: PDL1 expression on monocytes and NK cells, and PD1 expression on T cells were also increased in the severe group (**Supplementary Fig. 3**).

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Discussion

This study was undertaken as a holistic description of immune cells and markers from COVID-19 whole blood samples. Alterations of lymphocyte subsets have been widely reported [4;10-11]. Here, a multiparametric flow cytometry approach using whole blood samples allowed us to assess the features of the cells involved in the innate response, beyond the lymphocyte response. As opposed to monocytes and lymphocytes, granulocyte investigation requires freshly isolated whole blood samples. A combination of dry antibody panels optimized for whole blood investigation and the detection of rare events [12] enabled the simultaneous study of more than 100 phenotypic markers. This unbiased approach showed that changes in the frequency of granulocyte subsets and alteration of their functional phenotypes characterize patients during the course of COVID-19.

In previous studies, the neutrophil-to-lymphocyte ratio was used to predict the degree of disease severity in patients with early-stage COVID-19 [13-14]. Eosinopenia was reported in severe patients [15-16] and was also present in our study population. We show here that the increase in neutrophil counts is characterized by the emergence of cells involved in the inhibition of immune responses. At the neutrophil level, the increase in absolute numbers was due to $CD15^{+}CD16^{+}$ neutrophils, which may have pro-inflammatory properties [17]. Neutrophils express predominantly the glycophosphatidyl inositol-linked CD16b isoform, also known as low affinity IgG receptor FcγRIIIb, which acts as a suppressive FcγR receptor [18]. Low fucosylation of anti-SARS-CoV-2 antibodies [19] suggests that increased $CD16^{+}$ neutrophils in severe patients might contribute to persistent inflammation through synergistic mechanisms.

Neutrophils from COVID-19 patients also expressed lower levels of CD11b as compared to HBD. CD11b is a subunit of the αMβ2 (CD11bCD18) integrin involved in intercellular adhesion, transmigration, fibrinogen adhesion, and neutrophil-T cell crosstalk during infection [20-22]. CD11b

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has been found to play a critical role in the resolution of inflammation process [23]. Although neutrophil CD11b is mobilized from intracellular stores to the cell surface upon activation, alterations of circulating neutrophil CD11b expression are reported in autoimmune conditions, e.g. low levels of neutrophil CD11b in rheumatoid arthritis [24] and strong associations with systemic lupus erythematosus in genome-wide studies [20]. Thus, altered neutrophil CD11b expression may contribute to the autoimmune and hypercoagulable status reported in COVID-19 and its severe prognosis [25].

At the surface of basophils and eosinophils, we found a high expression of immune checkpoint PD-L1. Immune checkpoints are regulatory molecules involved in tissue repair at the end of an immune response [26], prevent immune-driven diseases but can also be subverted by pathogens, including viruses, to reduce the clearance of pathogens during infectious processes [26-28]. Immune checkpoint studies have addressed mainly T lymphocytes, resulting in a relative lack of information on granulocyte immune checkpoint regulation and clinical implications [27,29]. Upregulation of neutrophil checkpoint molecules including PD-L1 has been associated with poor outcomes in sepsis patients [29,30]. Our results show that SARS-CoV-2-induced immune dysregulation and immunoparalysis target the first steps of the virus encounter with granulocytic first-line defenses.

Downregulation of basophil and eosinophil CRTH2 (CD294), a high-affinity receptor of prostaglandin D [31], suggests that SARS-CoV-2 infection might be associated with an inhibition of Th2-polarized immune responses and decreased chemotaxis of CRTH2+ cells. CRTH2, a central activator of eosinophils, basophils and Th2-type responses in allergy and hypereosinophilic asthma, might bear the explanation for decreased ACE2 expression and apparent protection conferred against SARS-CoV-2 infection and severity by such conditions [32-34]. Conversely, CRTH2 deficiency was associated with pulmonary fibrosis in a mouse model [35], suggesting a role for CRTH2 and Th2 downregulation in the pathophysiology of post-COVID-19 pulmonary fibrosis. The combination of increases in $CD15^{+}CD16^{+}$ neutrophil frequency, checkpoint inhibitor expression and reduction in

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basophil and eosinophil CRTH2 suggest that the granulocyte signature may serve as a reliable biomarker for COVID-19 diagnosis and severity assessment.

The strength of our study is the translational dimension. We explored the association between immune status and disease severity assessed with validated scores in two distinct, well-characterized patient groups. There were marked clinical differences between the mild and the severe group, notably the recourse to invasive mechanical ventilation required in 89% of the latter. We found a continuum between the decreased counts and surface marker expression of immune cells and the disease severity, suggesting an association between disease severity and the impairment of the immune response. Taken together, our data show an early and deep impairment of the immune response, and question the use of drugs that could alleviate the immune response in COVID-19 patients, especially in the most severe forms requiring intensive care unit admission.

Several limitations must be acknowledged. The small size of the study population precludes definitive conclusions. However, our results are homogenous in each subgroup, and consistent with other published COVID-19 cohorts. Second, the timing of blood sampling differed between mild and severe groups, however it reflected immune status at turning points in disease progression: at diagnosis and upon progression to severity.

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In conclusion, we show here that immune exhaustion during SARS-CoV-2 infection markedly affects first line immune cells: neutrophils, eosinophils, and basophils as evidenced by increased expression of inhibitory checkpoints, decreased expression of adhesion molecules and decreased expression of CRTH2. These findings provide further clues for dysregulated induction of adaptive immune responses and the observed risk mitigation in allergic patients. The predominance of inhibitory systems may preclude the efficiency of viral clearance mechanisms. Further pathophysiological investigations of this new COVID-19 granulocytic signature are required in order to better understand and manage this disease.

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Author contributions: JLM, ML, PH, DO, PM and JV designed the study. AB, ABD, MM, SM, YS conducted experiments and acquired data. ML, JAS, AL enrolled the patients, collected, and analyzed demographic, clinical and laboratory data. JMB, TM, FM provided antibody panels. JLM, ML, JAS, AL, JV, JMB, TM, FM, SM analyzed experimental data. ML, JLM, JV, JAS, AL, MM wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgments: Dr Isabelle Arnoux for technical assistance with flow cytometry.

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Figure legends

Figure 1. Best immunophenotype discriminants between controls and COVID-19 patients. (A) Ranking of the 25 most discriminant variables between controls and COVID-19 patients resulting from an unsupervised analysis of immunophenotypic markers. Population frequency is expressed in percentage of gated populations and marker expression is expressed as the Median Fluorescence Intensity (MFI) of those markers on a defined cell subset.

(B) Representation of the principal component analysis results obtained with the 25 most discriminant markers. COVID-19 patients (red dots) and controls (black dots) are well separated with no overlap (left panel). The contribution of each parameter to each cluster is displayed on the right panel.

Figure 2. Granulocyte immunophenotypic markers in controls and COVID-19 patients.

Box plots summarizing (A) the differences observed in basophils and $SS^{hi}CD15^{+}$ granulocytes frequency, and the differential expression of (B) CRTH2 on basophils and eosinophils and (C) CD11b on basophils and SShiCD15+ granulocytes observed between controls and COVID-19 patients.

Figure 3. Best immunophenotype discriminants between mild/moderate and severe COVID-19 patients. (A) Ranking of the most discriminant variables (FDR<0.05) between mild/moderate and severe COVID-19 patients resulting from an unsupervised analysis of immunophenotypic markers. Population frequency is expressed in percentage of gated populations and marker expression is expressed as the Median Fluorescence Intensity (MFI) of those markers on a defined cell subset. (B) Representation of the principal component analysis results obtained with the most discriminant

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markers (FDR<0.05). Mild/moderate patients (blue triangles) and severe SARS-CoV-2 (red dots) are well separated with no overlap (left panel). The contribution of each parameter to each cluster is displayed on the right panel.

Figure 4. Granulocyte immunophenotypic markers in mild/moderate and severe COVID-19 groups. Box plots summarizing the differential expression of (A) CD16 on $SS^{hi}CD15^{+}$ granulocytes (B) CRTH2 on eosinophils, (C) PDL1 on eosinophils, and (D) PDL1 on basophils, observed between the mild/moderate and severe COVID-19 patients.

Figure 5. Correlation of granulocytic immune markers and severity scores. In SARS-CoV-2 patients group, the correlation between the most discriminant flow cytometry markers of COVID-19 granulocytic signature and two standard measures of clinical severity scale, i.e. WHO and SOFA, is displayed. Blue triangles: mild/moderate COVID-19 patients; red dots: severe COVID-19 patients.

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Table 1. Dry antibody panels for whole blood flow cytometry.

7-AAD, 7-aminoactinomycin D (viability marker); AF, AlexaFluor; APC, allophycocyanin; CB, counting beads; ECD, Phycoerythrin-Texas Red; FITC, fluorescein isothiocyanate; KrO, Krome Orange; Lin, lineage; NK, Natural Killer; PB, Pacific Blue; PC, phycoerythrin cyanin; PE, phycoerythrin.

Variables	**PB**	**KrO**	**FITC/AF 488**	**PE**	**EC**	**PC 5.5**	**PC7**	**APC**	**AF 700**	**APC-AF750**
Count			CD45	CB		7-AAD				
Basic		CD45	CD16	CD56	CD19		CD14	CD4	CD8	CD3
Granulocytes	CD15	CD45	CD294		CD16	CD33	CD11b	PD-L1	Lin	CD62L
T cell subsets	CD57	CD45	CD45RA	CCR7	CD28	PD1	CD27	CD4	CD8	CD3
Regulatory T cells	Helios	CD45	CD45RA	CD25		CD39	CD4	FoxP3		CD3
B cells	IgM	CD45	IgD	CD21	CD19		CD27	CD24		CD38
Innate lymphoid cells		CD45	CD294	CD1a/CD3/ CD14/CD16 /CD19/CD34/ CD94/ TCRαβ/ TCRγδ/ CD123/FcεRI		CD117	NKp46	CD127		CD161
NK subsets	CD57	CD45	CD16	NKp46/ NKp30			KIR2DL2L3	NKG2a	CD56	CD3/CD14/ CD19
NK checkpoint 1	CD16	CD45	CD54	NKp46	CD137	PD-1	CD274	NKG2A	CD56	CD3/ CD14/ CD19/ CD66b
NK checkpoint 2	TIM-3	CD45	CD16	NKp46	LAG-3	HLA-DR	CD69	NKG2D	CD56	CD3/ CD14/ CD19
Dendritic cells	HLA-DR	CD45	CD16	Lin		CD1c	CD11c	Clec9A	CD123	

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Table 2. Demographic, clinical and laboratory data of enrolled patients.

Variables	Healthy donors n = 25	COVID-19 patients n = 26	P	Mild group n = 7	Severe group n = 19	P
Characteristics						
Male	10 (38)	20 (73)	**0.007**	5 (71)	15 (79)	0.69
Age, median (IQR 25-75), years	45 (31-54)	66 (57-74)	**<0.0001**	71 (49-75)	65 (57-74)	0.79
BMI, median (IQR 25-75), kg/m^2	-	28 (25-33)	-	24 (23-26)	29 (27-36)	**0.005**
Co-morbidities			-			
Coronary disease	-	7 (27)	-	1 (14)	6 (32)	0.6
Hypertension	-	17 (65)	-	2 (29)	15 (79)	**0.03**
COPD	-	3 (12)	-	0	3 (16)	0.5
Stroke	-	0	-	0	0	-
Smoker	-	5 (19)	-	0	5 (26)	0.3
Active cancer	-	3 (12)	-	0	3 (16)	0.5
Immunodepression	-	3 (12)	-	1 (14)	2 (11)	1
Chronic kidney disease	-	0	-	0	0	-
Liver disease	-	2 (8)	-	1 (14)	1 (5)	0.5

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Diabetes	-	10 (38)	-	2 (29)	8 (42)	0.7
At hospital admission						
Clinical features						
Temperature, median (IQR 25-75), °C	-	38 (37.4-39)	-	37 (36.6-39)	38.5 (38-39)	0.08
MAP, median (IQR 25-75), mmHg	-	790 (72-91)	-	87 (73-94)	78 (69-85)	0.2
Heart rate, median (IQR 25-75), bpm	-	95 (80-107)	-	90 (67-95)	100 (80-122)	0.2
Respiratory rate, median (IQR 25-75), cpm	-	28 (18-34)	-	17 (16-22)	32 (27-35)	**0.002**
Oxygen low flow	-	1 (4)	-	1 (14)	0	0.3
Non-invasive ventilation	-	2 (8)	-	0	2 (11)	1
Mechanical ventilation	-	17 (65)	-	0	17 (89)	**<0.0001**
WHO progression scale, median (IQR 25-75)	-	6 (4-6)	-	4 (4-4)	6 (5-6)	**<0.0001**
SpO2, median (IQR 25-75), %	-	94 (90-95)	-	96 (95-97)	93 (84-95)	**0.003**
SpO2/FiO2 ratio, median (IQR 25-75)	-	186 (154-407)	-	457 (452-462)	176 (142-194)	**0.0001**

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PaO2/FiO2 ratio, median (IQR 25-75)	-	135 (108-193) (n=22)	-	275 (271-476) (n=3)	124 (106-172)	**0.007**
SAPS II, median (IQR 25-75)[a]	-	29 (22-37)	-	21 (13-23)	32 (28-39)	**0.002**
NEWS2 score, median (IQR 25-75)	-	7 (4-10)	-	3 (1-3)	9 (7-10)	**0.0001**
SOFA Score, median (IQR 25-75)	-	4 (2-6)	-	0 (0-4)	4 (3-7)	**0.005**
Use of vasopressors	-	13 (50)	-	0	13 (68)	**0.008**
Laboratory data						
Serum Aspartate-Amino-Transferase, median (IQR 25-75), UI/l	-	51 (38-67) (n=25)	-	38 (31-52) (n=6)	54 (43-71)	**0.04**
Serum Alanine-Amino-Transferase, median (IQR 25-75), UI/l	-	33 (22-52) (n=24)	-	24 (14-50) (n=5)	39 (23-52)	0.2
Serum creatinine, median (IQR 25-75), µmol/l	-	72 (62-98)	-	65 (58-71)	78 (63-100)	0.3
C-Reactive Protein, median (IQR 25-75), mg/l	-	110 (64-164)	-	17 (12-59)	150 (88-181)	**0.0003**

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Red blood cells, median (IQR 25-75), G/L	-	4.4 (4.2-4.7)	-	4.4 (4.2-4.8)	4.4 (4.2-4.8)	0.5
Platelets, median (IQR 25-75), G/L	-	212 (168-263)	-	214 (171-307)	210 (159-259)	0.8
White blood cells, median (IQR 25-75), G/L	-	6.3 (4.3-7.9)	-	5.7 (4.2-6.5)	6.3 (4.3-10)	0.2
Neutrophils, median (IQR 25-75), G/L	-	4.3 (3.3-5.4)	-	3.8 (2.9-4.5)	4.8 (3.4-6.5)	0.15
Basophils, median (IQR 25-75), G/L	-	0.01 (0.01-0.02)	-	0.01 (0.01-0.03)	0.01 (0.01-0.02)	0.98
Eosinophils, median (IQR 25-75), G/L	-	0 (0-0.01)	-	0.01 (0-0.04)	0 (0-0)	**0.002**
Lymphocytes, median (IQR 25-75), G/L	-	0.8 (0.6-1.1)	-	1 (0.4-1.6)	0.8 (0.6-1)	0.7
Lymphopenia[b]	-	22 (85)	-	5 (71)	17 (89)	0.28
Monocytes, median (IQR 25-75), G/L	-	0.4 (0.2-0.5)	-	0.5 (0.4-0.7)	0.3 (0.2-0.5)	**0.02**
Neutrophil-to-lymphocyte ratio, median (IQR 25-75)	-	4.9 (3.6-8.2)	-	3.9 (1.8-9.3)	5.3 (4-7.9)	0.2
Platelet-to-lymphocyte	-	237	-	212	241 (192-	0.7

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ratio, median (IQR 25-75)		(183-346)		(147-475)	344)	
Laboratory data at blood sample collection						
Red blood cells, median (IQR 25-75), G/L	-	3.7 (3.1 – 4.2)	-	4.4 (4.1-5)	3.3 (3-3.9)	**0.002**
Platelets, median (IQR 25-75), G/L	-	318 (243-429)	-	236 (150-278)	412 (302-458)	**0.003**
White blood cells, median (IQR 25-75), G/L	-	9.5 (6.4-12.3)	-	5.2 (3.9-5.8)	11 (8.3-14)	**0.0002**
Neutrophils, median (IQR 25-75), G/L	-	7.7 (4.4-9.1)	-	3.6 (2.8-4)	8.9 (7-12)	**0.0001**
Basophils, median (IQR 25-75), G/L	-	0.04 (0.02-0.05)	-	0.02 (0.01-0.02)	0.05 (0.03-0.06)	**0.002**
Eosinophils, median (IQR 25-75), G/L	-	0.08 (0.02-0.12)	-	0.02 (0.01-0.03)	0.09 (0.03-0.16)	**0.03**
Lymphocytes, median (IQR 25-75), G/L	-	1.1 (0.7-1.7)	-	0.8 (0.5-1.7)	1.2 (0.7-1.7)	0.52
Lymphopenia[b]	-	16 (62)	-	5 (71)	11 (57)	0.67
Monocytes, median (IQR 25-75), G/L	-	0.7 (0.5-1)	-	0.5 (0.4-0.7)	0.7 (0.6-1.2)	**0.05**

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Neutrophil-to-lymphocyte ratio, median (IQR 25-75)	-	6.3 (3.6-10.7)	-	3.5 (1.6-5.9)	8.9 (5.1-11.8)	**0.02**
Platelet-to-lymphocyte ratio, median (IQR 25-75)	-	276 (174-435)	-	188 (140-312)	297 (201-459)	0.13
Covid-19 Infection's history						
Respiratory symptoms at hospital admission			-			
Cough	-	18 (69)	-	4 (57)	14 (74)	0.6
Dyspnea	-	19 (73)	-	3 (43)	16 (84)	0.06
Systemic symptoms at admission						
Fever	-	23 (88)	-	7 (100)	16 (84)	0.5
Diarrhea	-	8 (31)	-	2 (29)	6 (32)	1.0
Myalgia	-	14 (54)	-	3 (43)	11 (58)	0.8
Anosmia, dysgeusia	-	6 (23)	-	1 (14)	5 (26)	0.65
Duration of symptoms before hospital admission, median (IQR 25-75), days	-	5 (2-7)	-	7 (2-16)	5 (2-7)	0.4
Time between onset of symptoms and RT-PCR, median (IQR 25-75), days	-	4 (2-8)		4 (2-16)	4 (1-7)	0.46
Duration between	-					**0.02**

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symptom onset and blood sample collection, median (IQR 25-75), days		16 (11-20)		8 (5-18)	18 (15-20)	
Duration between hospital admission and blood sample collection, median (IQR 25-75), days	-	11 (3-14)		2 (1-2)	13 (10-16)	**0.0001**
Time between RT-PCR and blood sample collection, median (IQR 25-75), days	-	11 (4-15)		2 (2-3)	13 (10-16)	**0.0001**
Treatment						
Hydroxychloroquine	-	14 (54)		0	14 (74)	**0.004**
Lopinavir - Ritonavir	-	4 (15)		0	4 (21)	0.55
Azithromycin	-	22 (85)		5 (71)	17 (89)	0.3
Follow-up						
In-hospital mortality	-	5 (19)		0	5 (26)	0.3
Day 28 mortality	-	3 (12)		0	3 (16)	0.5
Length of stay in hospital, median (IQR 25-75), days	-	25 (17-33)		12 (4-17)	28 (24-38)	**0.0004**
Oxygen administration free days, median (IQR 25-75), days	-	3 (0-15)		28 (20-28)	0 (0-6)	**<0.0001**

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Data are expressed as N (%) of participants unless otherwise indicated.

Abbreviations: BMI, Body Mass Index; COPD, Chronic obstructive pulmonary disease; MAP, mean arterial pressure; bpm, beats per minute ; cpm, cycle per minute ; WHO, World Health Organization ; SpO2, pulse oximetry ; SpO2/FiO2, ratio of pulse oximetry to the fraction of inspired oxygen ; PaO2/FiO2 ratio, ratio of partial of arterial oxygen partial to the fraction of inspired oxygen ; NEWS2, National Early Warning Score 2 ; SAPS II, Simplified Acute Physiology Score II; SOFA, Sepsis-related Organ Failure Assessment; RT-PCR, Reverse Transcriptase Polymerase Chain Reaction; SD, Standard Deviation.

[a] The SAPS II ranges from 0 to 163, with higher scores indicating higher risk of mortality. A patient with a score of 30 has an estimated mortality risk of 10 %.

[b] Lymphopenia was defined as a lymphocyte count below 1500 G/L.

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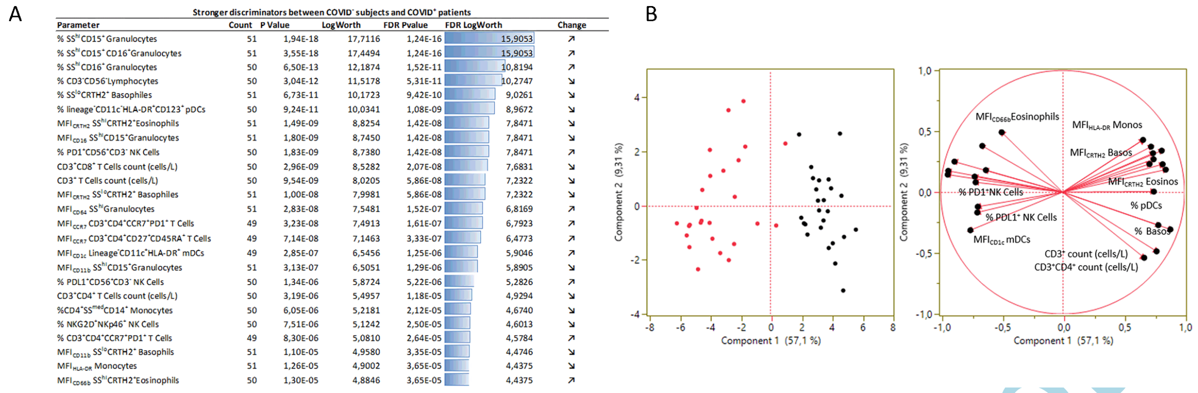
Figure 1



A

Stronger discriminators between COVID⁻ subjects and COVID⁺ patients

Parameter	Count	P Value	LogWorth	FDR Pvalue	FDR LogWorth	Change
% $SS^{hi}CD15^+$ Granulocytes	51	1,94E-18	17,7116	1,24E-16	15,9053	↗
% $SS^{hi}CD15^+CD16^+$ Granulocytes	51	3,55E-18	17,4494	1,24E-16	15,9053	↗
% $SS^{hi}CD16^+$ Granulocytes	50	6,50E-13	12,1874	1,52E-11	10,8194	↗
% $CD3^-CD56^-$ Lymphocytes	50	3,04E-12	11,5178	5,31E-11	10,2747	↘
% $SS^{lo}CRTH2^+$ Basophiles	51	6,73E-11	10,1723	9,42E-10	9,0261	↘
% $lineage^-CD11c^-HLA\text{-}DR^+CD123^+$ pDCs	50	9,24E-11	10,0341	1,08E-09	8,9672	↘
MFI_{CRTH2} $SS^{hi}CRTH2^+$ Eosinophils	51	1,49E-09	8,8254	1,42E-08	7,8471	↘
MFI_{CD16} $SS^{hi}CD15^+$ Granulocytes	51	1,80E-09	8,7450	1,42E-08	7,8471	↘
% $PD1^+CD56^+CD3^-$ NK Cells	50	1,83E-09	8,7380	1,42E-08	7,8471	↗
$CD3^+CD8^+$ T Cells count (cells/L)	50	2,96E-09	8,5282	2,07E-08	7,6831	↘
$CD3^+$ T Cells count (cells/L)	50	9,54E-09	8,0205	5,86E-08	7,2322	↘
MFI_{CRTH2} $SS^{lo}CRTH2^+$ Basophiles	51	1,00E-08	7,9981	5,86E-08	7,2322	↘
MFI_{CD64} SS^{hi} Granulocytes	51	2,83E-08	7,5481	1,52E-07	6,8169	↗
MFI_{CCR7} $CD3^+CD4^+CCR7^+PD1^+$ T Cells	49	3,23E-08	7,4913	1,61E-07	6,7923	↗
MFI_{CCR7} $CD3^+CD4^+CD27^+CD45RA^+$ T Cells	49	7,14E-08	7,1463	3,33E-07	6,4773	↗
MFI_{CD1c} $Lineage^-CD11c^+HLA\text{-}DR^+$ mDCs	49	2,85E-07	6,5456	1,25E-06	5,9046	↗
MFI_{CD11b} $SS^{hi}CD15^+$ Granulocytes	51	3,13E-07	6,5051	1,29E-06	5,8905	↘
% $PDL1^+CD56^+CD3^-$ NK Cells	50	1,34E-06	5,8724	5,22E-06	5,2826	↗
$CD3^+CD4^+$ T Cells count (cells/L)	50	3,19E-06	5,4957	1,18E-05	4,9294	↘
% $CD4^+SS^{med}CD14^+$ Monocytes	50	6,05E-06	5,2181	2,12E-05	4,6740	↘
% $NKG2D^+NKp46^+$ NK Cells	50	7,51E-06	5,1242	2,50E-05	4,6013	↘
% $CD3^+CD4^+CCR7^+PD1^+$ T Cells	49	8,30E-06	5,0810	2,64E-05	4,5784	↗
MFI_{CD11b} $SS^{lo}CRTH2^+$ Basophils	51	1,10E-05	4,9580	3,35E-05	4,4746	↘
$MFI_{HLA\text{-}DR}$ Monocytes	51	1,26E-05	4,9002	3,65E-05	4,4375	↘
MFI_{CD66b} $SS^{hi}CRTH2^+$ Eosinophils	50	1,30E-05	4,8846	3,65E-05	4,4375	↗

Figure 2

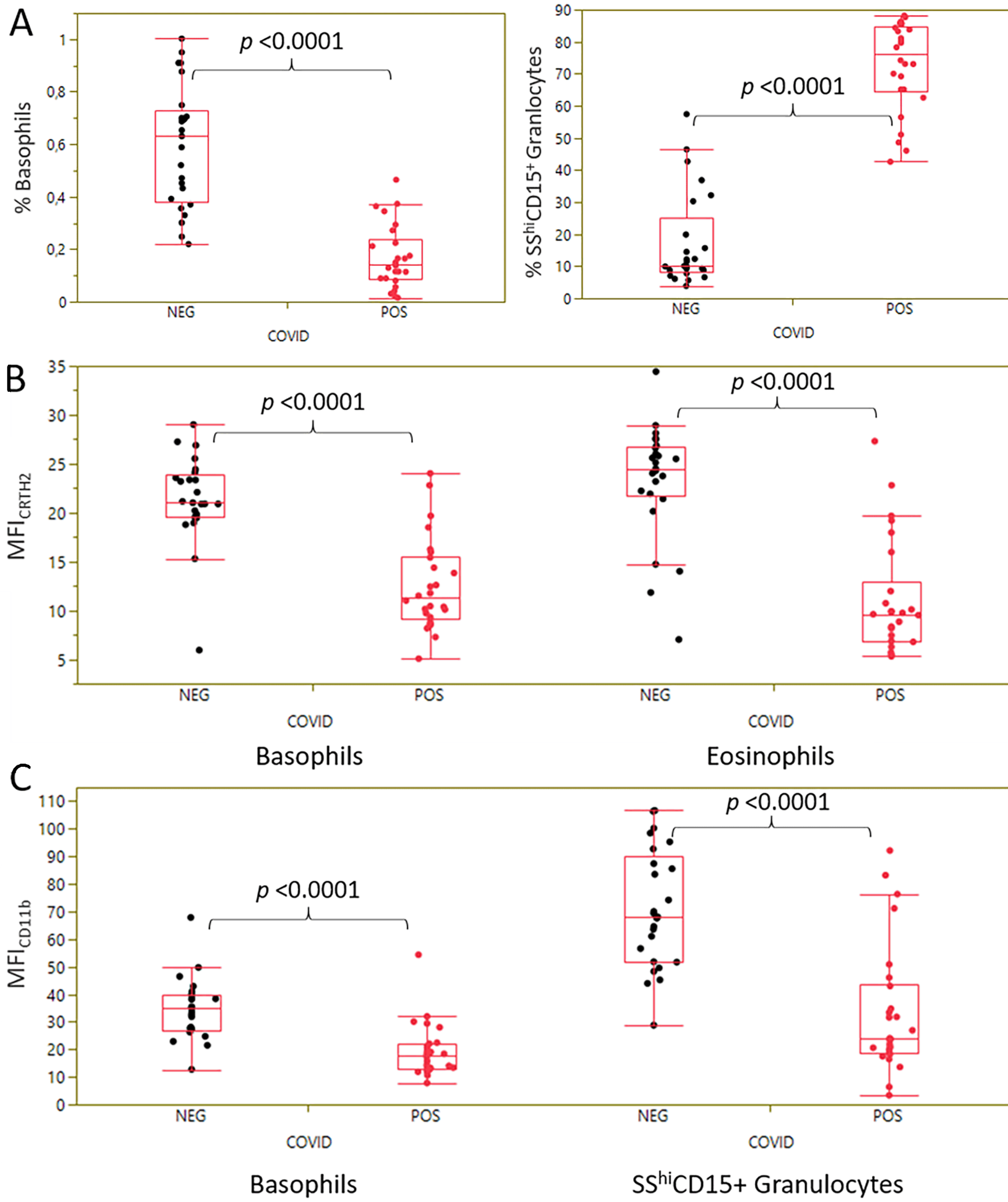


Figure 3

A

Stronger discriminators between mild/intermediate and severely affected patients

Parameter	P Value	LogWorth	FDR Pvalue	FDR LogWorth	Change
% lineage^{-}CD11c^{-}HLA-DR^{+}CD123^{+} pDCs	5,93E-06	5,2267	2,37E-04	3,6247	↘
MFI$_{HLA-DR}$ Monocytes	4,48E-06	5,3487	2,37E-04	3,6247	↘
%CD4^{+}SS^{med}CD14^{+} Monocytes	3,56E-05	4,4480	9,50E-04	3,0221	↘
% SS^{hi}CD15^{+} Granulocytes	3,62E-04	3,4418	7,23E-03	2,1408	↗
% SS^{lo}CRTH2^{+}PDL1^{+} Basophiles	6,21E-04	3,2068	9,94E-03	2,0027	↗
% CD3^{+}Lymphocytes	1,30E-03	2,8850	1,74E-02	1,7600	↘
% SS^{hi}CD15^{+}CD16^{+}Granulocytes	2,61E-03	2,5834	1,76E-02	1,7550	↗
MFI$_{CRTH2}$ SS^{hi}CRTH2^{+}Eosinophils	2,67E-03	2,5742	1,76E-02	1,7550	↘
MFI$_{CD16}$ SS^{hi}CD15^{+}Granulocytes	3,16E-03	2,5008	1,76E-02	1,7550	↘
% NKG2D^{+}NKp46^{+} NK Cells	1,66E-03	2,7810	1,76E-02	1,7550	↘
MFI$_{CD15}$ SS^{hi}CD15^{+}Granulocytes	2,40E-03	2,6196	1,76E-02	1,7550	↘
% SS^{hi}CD16^{+} Granulocytes	3,23E-03	2,4903	1,76E-02	1,7550	↗
MFI$_{CD4}$ Monocytes	1,81E-03	2,7432	1,76E-02	1,7550	↘
MFI$_{PDL1}$ SS^{hi}CRTH2^{+}Eosinophils	3,00E-03	2,5228	1,76E-02	1,7550	↗
Ratio (MFI$_{CD169}$ Monos)/(MFI$_{CD169}$Lymphos)	3,30E-03	2,4820	1,76E-02	1,7550	↘
% CD3^{-}CD56^{-}Lymphocytes	6,24E-03	2,2051	2,91E-02	1,5366	↘
% PDL1^{+}CD56^{+}CD3^{-} NK Cells	6,54E-03	2,1844	2,91E-02	1,5366	↗
MFI$_{NKp46}$ NKG2D^{+}NKp46^{+}NK Cells	7,81E-03	2,1075	3,29E-02	1,4831	↘
% SS^{hi}CD16^{+} Granulocytes	1,13E-02	1,9451	4,54E-02	1,3431	↗
MFI$_{CD69}$CD45+ Leukocytes	1,66E-02	1,7805	6,31E-02	1,1996	↗
% CD16^{+}CD56^{+}CD3^{-} NK Cells	2,11E-02	1,6767	7,66E-02	1,1160	↘
MFI$_{CD1c}$ Lineage^{-}CD11c^{+}HLA-DR^{+} mDCs	2,99E-02	1,5242	1,04E-01	0,9829	↗
% SS^{lo}CRTH2^{+} Basophiles	4,08E-02	1,3892	1,26E-01	0,9010	↘
MFI$_{CD64}$ Monocytes	3,99E-02	1,3989	1,70E-01	0,7707	↘
MFI$_{CD11b}$ SS^{hi}CD15^{+} Granulocytes	6,46E-02	1,1900	2,50E-01	0,6018	↘
MFI$_{CD11b}$ SS^{lo}CRTH2^{+} Basophils	6,49E-02	1,1881	2,50E-01	0,6018	↘

B

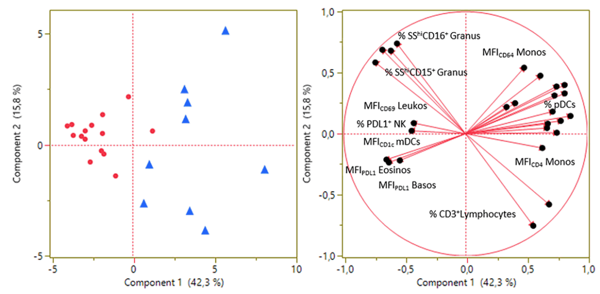


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Figure 4

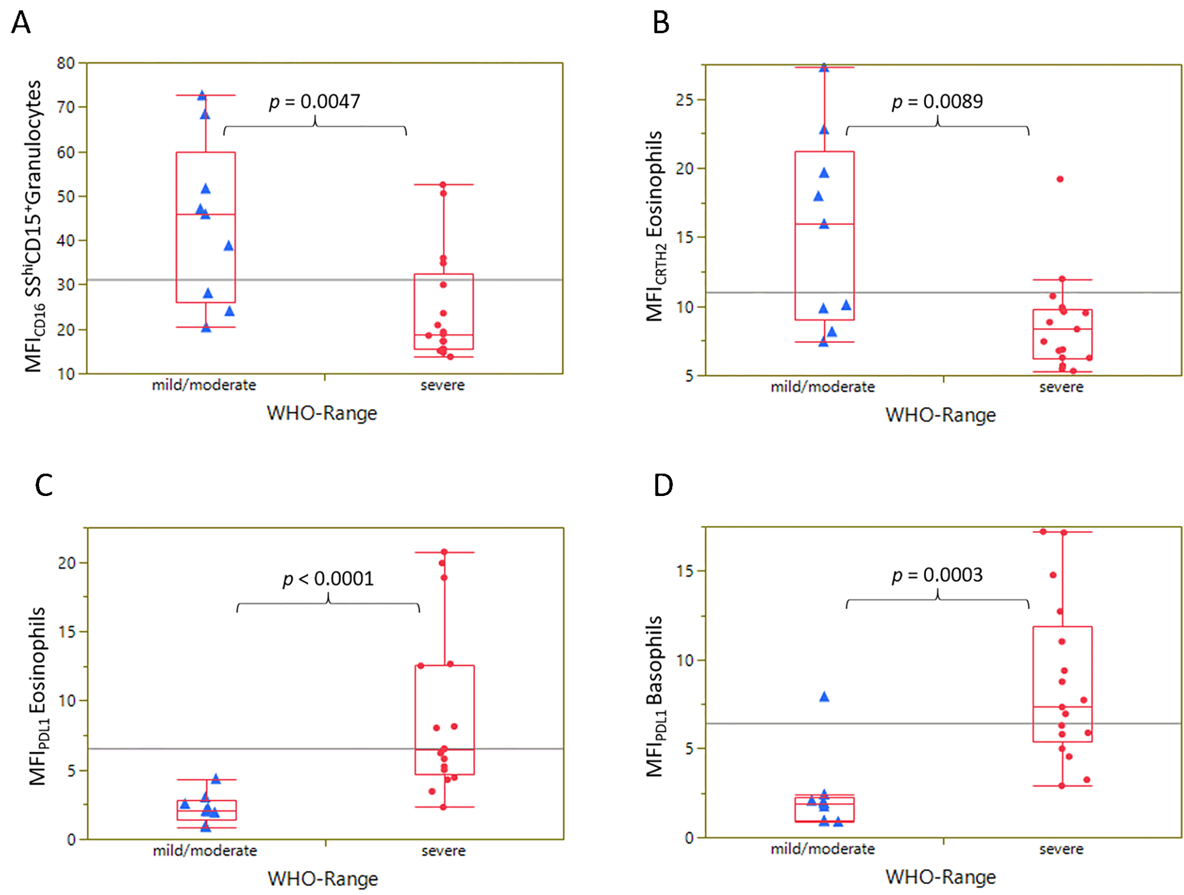

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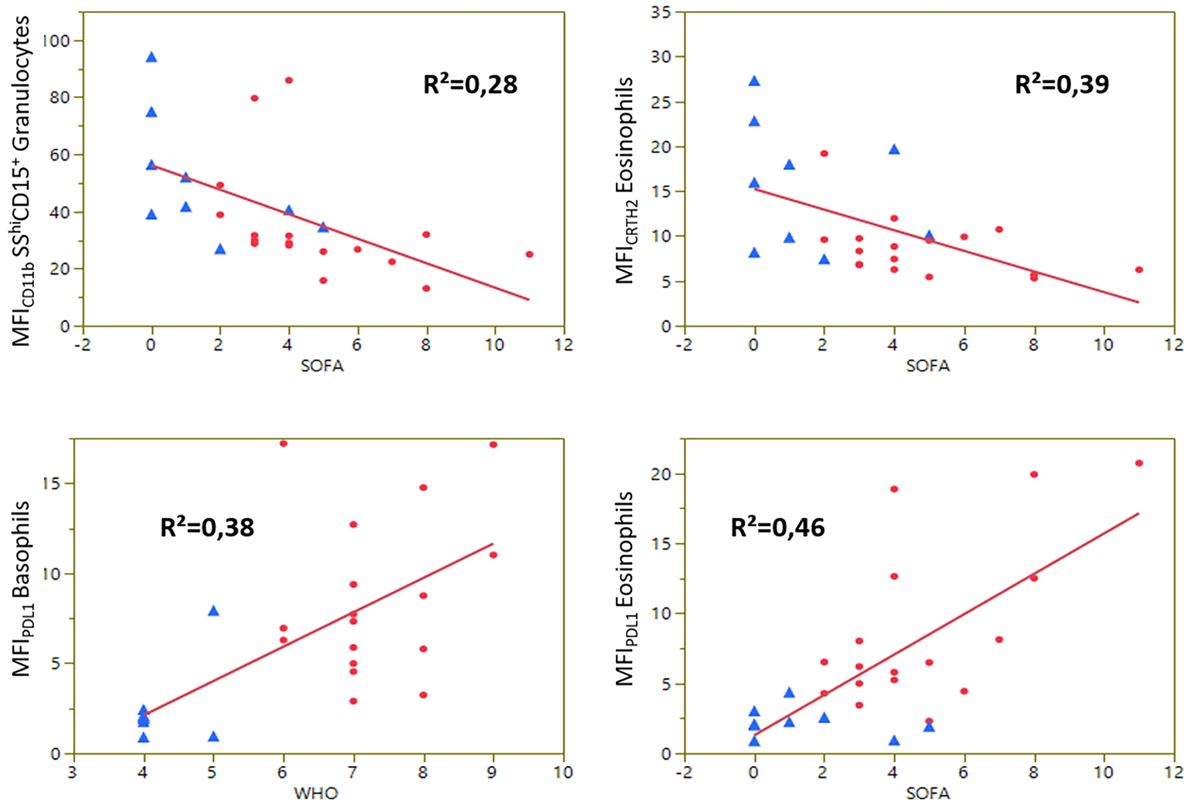
Figure 5



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