Supplementary Material

# Supplementary Methods

## Selection criteria for COVID-19 patients

Inclusion criteria for the COVID-19 group were: positive RT-PCR assay result from an upper respiratory tract specimen for SARS‑CoV-2; associated abnormalities or infiltrates on chest x-ray/CT scan; active or documented within 48h fever or antipyretic use; and hypoxemia (room-air oxygen saturation <92% or requirement of Supplementary oxygen). Exclusion criteria included: age <18 years; onset of COVID-19 symptoms 14 days before hospitalization; concomitant systemic bacterial or fungal infection; history of immunodeficiency or neutropenia (absolute neutrophil count <1500/mm3); active neoplasm; history of severe pulmonary disease requiring home oxygen therapy or mechanical ventilation; history of current systemic autoimmune or auto-inflammatory disease, or previous therapy with long-term oral corticosteroids, anti-IL-1β , anti-IL-6 or anti-TNFα.

## PBMCs and plasma isolation

20 mL of blood were collected by venipuncture into heparinized tubes, layered on top of 10 mL Ficoll-Paque Plus (Amersham Biosciences) and centrifuged at 1500 rpm for 20 minutes at 24°C. Plasma was removed from the upper layer, PBMCs were removed from the interphase and washed in PBS.

## Cytokines’ analysis

Inflammatory cytokines IL-1β and IL-6 concentration was measured using BD Human Inflammatory Cytokine CBA kit (551811, Becton-Dickinson Biosciences, Belgium), acquired by BD FACS-Calibur flow cytometer (Becton-Dickinson Biosciences, Belgium) and analyzed by FCAP Array software (Becton-Dickinson Biosciences, Belgium).

## mRNA isolation and quantification by qPCR

RNA was extracted using High Pure RNA Isolation Kit (Roche Diagnostics, Switzerland). 1μg of RNA was retrotranscribed using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA). RNA levels were measured by RTqPCR using QuantiMix Easy kit (Biotools, Spain) and Light-Cycler system (Roche Diagnostics, Switzerland) and results normalized to 18S expression. Primer sequences are listed in Supplementary Table 5 and were synthesized by Eurofins Scientific SE (Luxembourg).

## Flow cytometry

PBMCs were treated following a standard protocol using the Transcription Factor Buffer Set (Becton-Dickinson Biosciences). Cells were labeled (30 minutes, 4ºC) with antibodies detailed in Supplementary Table 6. Cells were acquired using BD FACS-Celesta flow cytometer (Becton-Dickinson Biosciences, Belgium), and data were analyzed using FlowJo vX.0.7 software (FlowJo, USA).

## Hypoxia and plasma stimulation model

PBMCs were isolated from blood samples of healthy volunteers. After isolation, 2 x 106 PBMCs/well were cultured in M6 plates in RPMI medium supplemented with 100 U/mL Penicillin and 100 μg/mL Streptomycin. Cells were stimulated with 10% plasma from either HC subjects or COVID-19 patients and cultured for 16 hours in standard conditions (37°C, 21% O2, 5% CO2) or using a specific hypoxic chamber (Oxycycler C42 from [Biospherix](https://www.biospherix.com/cell-culture-equipment/oxycycler-c42), USA) which can maintain 9% O2.

## Platelet isolation and stimulation

O negative blood sample from healthy volunteer was collected in sodium citrate tubes and centrifuged 150g for 10min at 24º C without brake to obtain platelet-rich plasma (PRP), which was diluted 1:5 in Walsh buffer (NaCl 137mM, KCl 2.7mM, NaH2PO4 3.3mM, MgCl2 1mM and HEPES 20mM in distilled water).50μL of diluted PRP were stimulated with purified ADP (Sigma-Aldrich, ref:01905-250MG-F) or 50μL of plasma from HC or COVID-19 patients. Plasmas were untreated or treated with apyrase (0.2U/mL) (Sigma-Aldrich, ref: A6535-200UN) for 20min for ADP hydrolysis. In addition, P2Y12R was blocked on the surface of platelets using the commercialized drug ticagrelor (20μM) (AstraZeneca AB, Brilique) for 40min. Then platelets were stained with PAC-1FITC antibody (Becton-Dickinson, ref: 340507), which recognizes glycoprotein IIb/IIIa (gpIIb/IIIa, αIIbβ3) complex, a marker of activated platelets and analyzed by BD FACS-Calibur flow cytometer (Becton-Dickinson Biosciences, Belgium).

# Supplementary Figures and Tables

## Supplementary Tables

**Supplementary Table 1.** Multivariate logistic regression model including sCD39 and D-dimer as predictors of ICU admission\*

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | B | S.E. | Odds ratio | 95%CI | p-value |
| D-dimer | 0.002 | 0.001 | 1.002 | 1.001 to 1.004 | 0.003 |
| sCD39 | 0.036 | 0.021 | 1.037 | 1.001 to 1.080 | 0.038 |
| Constant | -2.128 | 1.865 | - | - | - |

\*Values are adjusted by sex, age and presence or absence of obesity, hypertension or diabetes. S.E.: standard error, C.I.: confidence interval.

**Supplementary Table 2.** Spearman’s correlation of sCD39 with coagulation markers in influenza A patients

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Spearman’s correlations | TF | CD40L | CRP | Ferritin |
| sCD39 | ρ=0.049P=0.848 | ρ=-0.046P=0.349 | ρ=0.198P=0.517 | ρ=0.500P>0.999 |

Correlations were performed by Spearman’s correlation test. Spearman correlation coefficients (ρ) and *P*-values are shown.

**Supplementary Table 3.** Comparison of different inflammatory and pro-thrombotic markers on admission and on day 7 post-admission

|  |  |  |  |
| --- | --- | --- | --- |
|  | At day 1mean ± SEM | At day 7mean ± SEM | Wilcoxon matched-pairs tests P-value |
| sCD39 (pg/mL) | 43.38 ± 3.733 | 42.42 ± 3.070 | 0.9839 |
| CD3+CD4+CD39 MFI | 1464 ± 91.47 | 1387 ± 73.13 | 0.2465 |
| CD3+CD8+CD39 MFI | 1644 ± 108.2 | 1517 ± 90.02 | 0.1572 |
| CD3-CD56+CD16+CD39 MFI | 1959 ± 128.4 | 1994 ± 135.0 | 0.8848 |
| eATP (ng/mL) | 212.3 ± 19.11 | 218.9 ± 17.53 | 0.5858 |
| CD14+ NLRP3 MFI | 1.834 ± 0.07642 | 1.987 ± 0.1336 | 0.3483 |
| C-reactive protein (mg/L) | 78.51 ± 8.939 | 19.56 ± 4.126 | <0.0001\*\*\*\* |
| Ferritin (mg/L) | 894.8 ± 162.1 | 630.5 ± 84.49 | 0.0330\* |
| IL-1β (pg/mL) | 146 ± 87.71 | 31.67 ± 7.990 | 0.0104\* |
| IL-6 (pg/mL) | 15.84 ± 8.703 | 1.412 ± 0.5005 | 0.0095 \*\* |
| TF (pg/mL) | 102.9 ± 6.389 | 98.17 ± 6.138 | 0.2940 |
| CD40L (ng/mL) | 0.7186 ± 0.06912 | 0.9030 ± 0.09906 | 0.1264 |

Comparisons were made by Wilcoxon matched-pairs signed rank test; P-values are shown.

**Supplementary Table 4.** ELISA kits used in the study

|  |  |  |  |
| --- | --- | --- | --- |
| ELISA target | Manufacturer | Reference | Detection limit |
| CD39 | CUSABIO technology, USA | CSB-EL007690 | 23.44 pg/mL |
| TF | CUSABIO technology, USA | CSB-E07913h | 3.12 pg/mL |
| CD40L | Invitrogen, Austria | BMS293 | 0.06 ng/mL |
| ATP | Cloud Clone, USA | CEA349GE | 5.25 ng/mL |
| ADP | MyBioSource, USA | MBS263784 | 0.31 ng/mL |
| ADO | MyBioSource, USA | MBS 2605344 | 0.5 ng/mL |

**Supplementary Table 5.** qPCR Primer sequences used in the study

|  |  |  |
| --- | --- | --- |
| CD39 | Forward primer | TCGCCTCTATGGCAAGGACTAC |
| Reverse primer | TCCAGGATGAAAGCATGGGTCC |
| RIG-I | Forward primer | ATGTGGGCAATGTCATCAAA |
| Reverse primer | GAAGCACTTGCTACCTCTTGC |
| IRF-3 | Forward primer | GGCTCGTGATGGTCAAGGTT |
| Reverse primer | CATGCCCTCCACCAAGTCCT |
| MAVS | Forward primer | CAGGCCGAGCCTATCATCTG |
| Reverse primer | GGGCTTTGAGCTAGTTGGCA |
| HIF-1α | Forward primer | TTCCAGTTACGTTCCTTCGATCA |
| Reverse primer | TTTGAGGACTTGCGCTTTCA |
| CD73 | Forward primer | TATTGCACTGGGACATTCG |
| Reverse primer | CCCATCATCAGAAGTGACTATG |

**Supplementary Table 6.** Flow cytometry anti-human antibodies used in the study

|  |  |  |  |
| --- | --- | --- | --- |
| Target | Fluorochrome | Manufacturer | Reference |
| CD39 | BB515 | BD-Biosciences | 565469 |
| CD3 | BUV395 | BD-Biosciences | 563546 |
| CD4 | PerCP-CyTM5.5 | BD-Biosciences | 566923 |
| CD8 | BV711 | BD-Biosciences | 563667 |
| CD56 | BV605 | BD-Biosciences | 562780 |
| CD16 | BUV737 | BD-Biosciences | 612786 |
| CD19 | BV786 | BD-Biosciences | 563325 |
| CD25 | APC | MACS Miltenyi Biotec | 130-092-856 |
| Foxp3 | PE | BD-Biosciences | 560046 |
| CD14 | APC | Immunostep | 14A-100T |
| NLRP3 | PE | MACS Miltenyi Biotec | 130-111-209 |
| Fixable viability stain | FVS510 | BD-Biosciences | 564406 |

## Supplementary Figures



**Supplementary Figure 1.** CD39 expression in other immune-cell subsets. Analysis of CD39 expression by flow cytometry from total PBMCs of healthy controls and COVID-19 patients on CD4+CD25+Foxp3+ (left panel, HC=9, COV=18), CD14+ (middle, panel, HC=8, COV=28) and CD19+ (right panel, HC=14, COV=48). CD39 expression is represented by MFI (Mean Fluorescence Intensity). Mean differences were analyzed using unpaired Student *t*-test analysis with Welch correction. Error bars: mean ± SEM and *P*-values (P) are shown.



**Supplementary Figure 2.** CD39 expression is related to hypoxia and innate immune response. **(A)** HIF-1α mRNA expression analysis by qPCR in PBMCs from healthy controls (HC, n=17) and COVID-19 patients (COV, n=53). **(B)** RIG-I (left panel, HC, n=20; COV, n=52), MAVS (middle panel, HC, n=15; COV, n=33) and IRF-3 (right panel, HC, n=20; COV, n=44) mRNA expression analysis by qPCR in PBMCs. Mean differences were analyzed using unpaired Student’s *t*-test analysis with Welch correction. **(C)** Association of HIF-1α, RIG-I, MAVS and IRF3 mRNA expression with CD39 mRNA expression and sCD39 plasma concentration in COVID-19 patients. Spearman’s correlation coefficients (ρ) and *P*-values (P) are shown. **(D-E)** PBMCs from healthy subjects were isolated and cultured for 16h, either stimulated or not with 10% COVID-19 plasma and either under normoxia or hypoxia (n=6). **(D)** CD39 mRNA relative expression analysis by qPCR. **(E)** CD39 expression analysis by flow cytometry in CD3+CD4+ T-cells (left panel), CD3+CD8+ T-cells (middle panel) and CD3-CD56+CD16+ NK-cells (right panel) CD39 expression is represented by MFI (Mean Fluorescence Intensity). Comparisons between groups were performed by two-way ANOVA with Bonferroni’s multiple comparison test. Error bars: mean ± SEM. \*: *P*<0.05.



**Supplementary Figure 3.** Dysregulated purinergic nucleotides are involved in NLRP3 overexpression. **(A)** CD73 mRNA relative expression analysis by qPCR in PBMCs from healthy controls (HC, n=14) and COVID-19 patients (COV, n=34). **(B)** Analysis of NLRP3 intracellular expression by flow cytometry in CD14+ monocytes from healthy controls (HC, n=18) and COVID-19 patients (COV, n=47). NLRP3 expression is represented by MFI. Comparison between groups was performed by unpaired Student t-test with Welch correction. Error bars: mean ± SEM. **(C)** Correlation of eATP plasma concentration with NLRP3 expression in CD14+ monocytes (n=47). **(D)** Correlation of NLRP3 expression in CD14+ monocytes with TF plasma level (n=42). Spearman correlation coefficients (ρ) and P-values are shown. **(E)** Flow cytometry quantification of percentage of PAC-1 positive platelets upon challenge with increasing concentrations of ADP or upon addition of Thrombin Receptor Activating Protein (TRAP, 10μM) (n=3). Mean differences were assessed by one-way ANOVA analysis and Tukey’s multiple comparison test. Error bars: Mean ± SEM. \*: P<0.05; \*\*\*\*: P<0.0001 vs ADP 0 μg/mL.