# STAT3 eye-opener in COVID-19

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## ABSTRACT

Among the elevated inflammatory mediators, blood Interleukin-6 (IL-6) level is highly correlated with COVID-19 mortality and predicts the need for mechanical ventilation. IL-6 is the major activator of signal transducers and activators of transcription-3 (STAT3) during inflammatory responses. The primary objective of the study is to explore STAT3 activation in different subsets of circulating cells derived from 25 COVID-19 patients (patients) admitted to an Internal Medicine department compared to 25 healthy donors (HD). Higher percentages of classical and intermediate monocytes were observed in patients, compared to HD (respectively P<0.01 and P<0.001). The active form of STAT3, phosphorylated STAT3 (pSTAT3) was significantly higher in patients, compared to HD, both in non-classical and in intermediate monocytes (P<0.001). Low-density neutrophils (LDN) count was significantly higher in patients compared to HD (P<0.001). The pSTAT3 was significantly higher in LDN derived from patients (P<0.05). High pSTAT3 was significantly directly related to d-dimer and inversely related to vitamin D levels (P<0.01). This study gives information about STAT3 activation and the possible role of Vitamin D as a tool against COVID-19.

## Introduction

The COVID-19 pandemic occurred in Wuhan, China, and promptly spread globally, leading to devastating pressure on healthcare systems that required substantial hygienic and containment measures. Severe Acute Respiratory Coronavirus-2 (SARS-CoV-2) has been identified as acute respiratory distress syndrome and sometimes multi-organ failure, leading to death.[1]

Among the higher inflammatory mediators, blood Interleukin-6 (IL-6) level is highly linked with COVID-19 mortality and predicts the need for mechanical ventilation.[2] IL-6 is the major activator of signal transducers and activators of transcription-3 (STAT3) during inflammatory responses.[3] The binding of IL-6 to its receptor complex leads to phosphorylation of Janus kinase with consequent rapid (15-60 min) phosphorylation, dimerization, and nuclear translocation of STAT3 [phosphorylated STAT3 (pSTAT3)].[3] STAT3 then binds to specific response elements in the promoter regions of cytokine-responsive genes and consecutively participates in the transcriptional activation in response to IL-6.

Different subsets of monocytes and neutrophils were found to play an immunosuppressive role in numerous diseases.[4] Monocytes can be subdivided into immature classical (CD14++CD16-) and more differ-

entiated inflammatory transitional/intermediate (CD14+CD16+) and non-classical (CD14- CD16++) subsets. Neutrophils are distributed into high-density neutrophils and low-density neutrophils (LDN) based on their density difference.[5]

Recently, significantly reduced non-classical and intermediate monocytes were found in acute patients with severe COVID-19 symptoms. Conversely in patients with moderate symptoms non-classical and intermediate monocytes were increased.[6]

Moreover, it has been demonstrated that LDN neutrophils correlate with disease severity in COVID-19 patients.[7]

The primary objective of the study is to explore STAT3 activation in circulating cells derived from COVID-19 patients (patients) compared to healthy donors (HD). Therefore, associations between COVID-19-related clinical parameters and pSTAT3 expression are evaluated.

## Materials and Methods

### Ethical approval

All procedures have been conducted in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The Ethical Committee (Protocol Number 2746CESC) has approved the study. The study has been posted to Clinicaltrials.gov (number: NCT04412382). Informed consent was obtained from all individual participants included in the study.

### Study type, setting, population and description of the protocol

The study started in autumn 2020. Ages eligible for the study were 18 years to 100 years old.

#### Study population

25 COVID-19 patients aged ≥18 years were enrolled. 25 medical doctors and nurses working in the same hospital constituted the HD group.

#### Exclusion Criteria (for both groups)

Age <18 years, pregnancy, known autoimmune diseases or cancer.

#### Exclusion Criteria (for the control group)

COVID-19 swabs positivity and/or the presence of IgM and IgG antibodies (the serological picture was known from the samples taken by the Health Surveillance System of the Hospital).

### Diagnostic tests

Venous blood samples were collected from each subject for routine examination on admission: haemoglobin and white blood cell count, serum creatinine, sodium, potassium, urea, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, glucose, C-reactive protein (CRP) and d-dimer (measured with standard methods). Arterial blood samples were collected on admission and on discharge to test the PaO2/FiO2 ratio (the calculated ratio of arterial oxygen partial pressure to fractional inspired oxygen). Peripheral deep vein thrombosis was detected using a high-resolution B-mode ultrasound equipped with a 7.5-to 12-MHz probe. Electrocardiogram, chest X-ray, and computed tomography (if needed) were assessed for COVID-19 patients. Cytokine IL-6 was analyzed from the subjects' plasma (Human IL-6 Quantikine ELISA Kit 96 (R&D).

### Flow cytometry

To determine the intracellular levels of pSTAT3, whole peripheral blood was incubated with FcR Blocking reagent (Miltenyi Biotec, Paris, FR) followed by the addition of anti-human FITC-conjugated CD56 (BD Bioscience, San Jose, CA, USA; clone NCAM16.2), PerCP-Cy5.5-conjugated CD3 (BD Bioscience, San Jose, CA, USA; clone UCHT1), PE.Cy7-conjugated CD19 (eBiosciences, ThermoFisher Scientific, Waltham, MA, USA; clone HIB19), Allophycocyanin (APC).H7-conjugated CD14 (BD Bioscience, San Jose, CA, USA; clone MφP9), Brilliant Violet 421™-conjugated CD15 (BD Bioscience, San Jose, CA, USA; clone W6D3) antibodies and Aqua LIVE/DEAD dye (ThermoFisher Scientific, Waltham, MA, USA). Red blood cells were lysed using Cal-Lyse™ Lysing Solution (ThermoFisher Scientific, Waltham, MA, USA) in accordance with the manufacturer's instructions. Cells were then fixed and permeabilized with Foxp3/Transcription Factor Staining Buffer Set (eBiosciences, # 00-5523-00), following the manufacturer's instructions. For the intracellular staining, APC-conjugated p-STA3 (pTyr705) (eBioscience, ThermoFisher Scientific, Waltham, MA, USA; clone LUVNKLA) was used. All steps were performed in ice. Samples were acquired with fluorescent-activated cell sorting Canto II (BD, Franklin Lakes, NJ, USA) using the gating strategy. Data were analyzed with FlowJo software (Tree Star, Inc., Ashland, OR, USA). To quantify the amount of monocytes and monocyte subsets (defined as classical, CD14high CD16low/dim; intermediate CD14int CD16+; non classical CD14low/dim CD16high), peripheral blood was incubated with FcR Blocking reagent (Miltenyi Biotec, Paris, FR) followed by the addition of: anti-human PE-conjugated CD56 (BD Bioscience, San Jose, CA, USA; clone NCAM16.2), FITC-conjugated-CD16 (Bi-

olegend, San Diego, CA, USA; clone 3G8), PerCP-Cy5.5- conjugated CD3 (BD Bioscience, San Jose, CA, USA; clone UCHT1), PE.Cy7-conjugated HLA-DR (eBiosciences, ThermoFisher Scientific, Waltham, MA, USA; clone L243), APC.H7- conjugated CD14 (BD Bioscience, San Jose, CA, USA; clone MφP9), Brilliant Violet 421™- conjugated PD-L1 (BD Bioscience, San Jose, CA, USA; clone MIH1) antibodies and Aqua LIVE/DEAD dye. Red blood cells were lysed using Cal-Lyse™ Lysing Solution in accordance with the manufacturer's instructions.

### Cell isolation

Cells were isolated from ethylenediaminetetraacetic acid-treated tubes (BD Biosciences, NJ, USA) and freshly separated by Ficoll-Hypaque (GE Healthcare, Uppsala, Sweden) gradient. Peripheral blood mononuclear cells (PBMCs) were counted and the monocyte fraction (CD14+) was further isolated by CD14-microbeads (Miltenyi Biotec, Paris, FR), following the manufacturer's instructions. From the CD14- fraction the CD66+LDNs were isolated by the sequential addition of anti-CD66b-FITC conjugated antibody (BD Biosciences, NJ, USA) and microbeads anti-FITC (Miltenyi Biotec, Paris, FR), following manufacturers' instructions. The normal density neutrophils CD66b+ were isolated from the red blood cell layer by dextran density gradient followed by the anti-CD66b-FITC conjugated antibody and microbeads anti-fluorescein isothiocyanate, as described for LDNs. The purity of each fraction was evaluated by flow cytometry analysis.

### Real time real-time-polymerase chain reaction

Total RNA from PBMCs was isolated by TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). The amount and purity of isolated RNA was analyzed by the ND-1000 Spectrophotometer (NanoDrop Technologies). cDNA was prepared using the Super-Script® VILO cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Real-time-polymerase chain reaction (RT-PCR) was run using 2x SYBR Green master mix (ThermoFisher Scientific, Waltham, MA, USA). All samples were normalized using glyceraldehyde 3-phosphate dehydrogenase endogenous control primers. Post-qRT-PCR analysis to quantify relative gene expression was performed by the comparative Ct method $(2{-}\Delta\Delta Ct)$.

Primers hSTAT3: Forward 5'-GCTACAGCAGCTTGACACACG-3'.Reverse 5'-CTCTTGCAGGAAGCGGCTAT-3'

hIL-6: Forward 5'-GTGTGAAAGCAGCAAAGAGGC-3'.Reverse 5'-CACCAGGCAAGTCTCCTCAT-3'

### Statistical analyses

Student t-test (parametric groups) and Wilcoxon-Mann-Whitney test (nonparametric groups) were used to determine statistical significance between patients and HD groups. Pearson correlations were used to analyze correlations between %STAT3 and clinical values. Values were considered significant at $P{\leq}0.05$. Values are reported as mean±SD. All analyses were performed using Graph Pad Prism (version 8.4.2).

## Results

Baseline clinical characteristics, laboratory, and instrumental data of COVID-19 patients and HD are described in Table 1. Table 1 includes also IL-6 plasma quantification.

The study population was equally distributed (males and females). The mean age was 73 [standard deviation (SD) 10] for COVID-19 patients and 35 (SD 5) for HD. The hospitalization period in days was 20 (SD 5). Two patients died (8%). The more frequent comorbidities were hypertension and diabetes. Patients were treated with Remdesivir (60%), but most of the patients were administered steroids (96%) and experienced different types of oxygen supply, as depicted in Table 1.

Blood collection was taken at admission before starting therapy.

Chest X-ray examinations showed an interstitial involvement in 4 patients (13%) and a consolidation pattern in the majority of the patients (83%).

Inflammation markers (plasma levels of CRP, procalcitonin, and IL-6) were significantly higher in COVID-19 patients compared to HD. Serum vitamin D was significantly lower in patients (without supplementation). No supplementation therapy was taken by HD.

Figure 1 shows the mRNA expression of IL-6 and STAT3 in PBMCs of all patients and HD considered in this study. The fold increase was not significantly different.
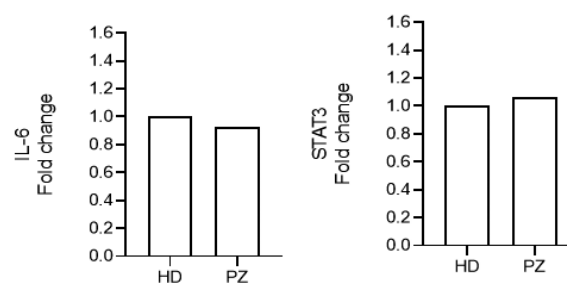


**Figure 1. mRNA expression of interleukin-6 and signal transducers and activators of transcription-3 in peripheral blood mononuclear cells of COVID-19 patients and healthy donors. HD, healthy donors; Pz, COVID-19 patients.**

Figures 2-4 show HD and patient monocyte subsets and pSTAT3 expression.

Significantly different proportions of classical, intermediate, and non-classical monocyte subsets were observed between patients and HD. Higher percentages of classical and intermediate monocytes were observed in patients, compared to HD (respectively $P<0.01$, and $P<0.001$), as shown in Figure 3.

Non-classical monocytes were more frequent in HD (see Figure 5).

The pSTAT3 (percentage and MFI) (Figure 3) were significantly higher in patients, compared to HD, both in non-classical and in intermediate monocytes ($P<0.001$).

Then, we have focused our attention on neutrophils (Figure 5). LDN count was significantly higher in pa-

**Table 1. Demographics and clinical characteristics of COVID-19 patients (n=25) and of healthy donors (n=25).**

| Demographics | COVID-19 patients | HD |
|---|---|---|
| Age (years) | 73±10 | 35±5 |
| Hospitalisation period (days) | 20±9 | - |
| Female gender (%) | 14 (56) | 14 (56) |
| Smoker (current or former) (%) | 3 (12) | - |
| Comorbidities | | |
| Diabetes (%) | 15 (60) | - |
| Ischemic heart disease (%) | 5 (20) | - |
| Cerebrovascular disease (%) | 5 (20) | - |
| Hypertension (%) | 15 (60) | - |
| Dislipidemia (%) | 10 (40) | - |
| Obesity (%) | 3 (12) | - |
| Cancer (%) | 3 (12) | - |
| Chronic obstructive pulmonary disease (%) | 3 (12) | - |
| Previous medication | | |
| Medications for hypertension (%) | 15 (60) | - |
| Aspirin (%) | 15 (60) | - |
| Statin (%) | 15 (60) | - |
| In-hospital treatment | | |
| Steroid (%) | 24 (96) | - |
| Remdesivir (%) | 15 (60) | - |
| Antibiotics (%) | 23 (92) | - |
| Respiratory status | | |
| Mechanical ventilation (%) | 19 (76) | - |
| High flow oxygen (%) | 8 (32) | - |
| Low flow oxygen (%) | 2 (8) | - |
| Room air (%) | 1 (4) | 25 (100) |
| P/F | 133±70 | 400±5 |
| Venous thromboembolism (%) | 1 (4) | 0 |
| Chest X-ray | | |
| Normal (%) | 1 (4) | - |
| Intersrtitial signs (%) | 2 (8) | - |
| Consolidations (%) | 22 (88) | - |
| Clinical outcome | | |
| Hospitalisation period (days) for discharged patients | 20±5 | - |
| Death (%) | 2 (8) | 0 |
| Main laboratory findings | | |
| Hemoglobin, g/dL | 13±2 | 13.5±1 |
| White blood cell 10⁹/L | 9.5 | 5.0±1.0 |
| Platelets 10⁹/L | 209.000±50.000 | 300.000±40.000 |
| C-reactive protein, mg/L | 130±70 | 1±1 |
| Procalcitonin, ng/mL | 1.6±1 | 0.1±0.1 |
| Total cholesterol, mg/dL | 153±20 | 130±20 |
| LDL cholesterol, mg/dL | 87±10 | 66±10 |
| HDL cholesterol, mg/dL | 41±10 | 40±10 |
| Triglycerides, mg/dL | 125±10 | 120±10 |
| D-dimer (µg/L) | 1500±900 | 200±30 |
| Vitamin D (ng/mL) | 15±10 | 70±10 |
| IL-6 pg/mL | 30±29 | 3±1 |

HD, healthy donors; HDL, high-density lipoproteins; IL-6, interleukin-6; LDL, low-density lipoproteins; P/F, PaO2/FiO2 ratio, the calculated ratio of arterial oxygen partial pressure to fractional inspired oxygen. Data are expressed as n (%) or mean±standard deviation.

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tients compared to HD (P<0.001). The pSTAT3 percentage and MFI was significantly higher in LDN derived from patients (P<0.05).

Then we correlated patients' monocytes pSTAT3 expression to clinical and laboratory findings.

Plasma IL-6 was significantly correlated to pSTAT3 expression, both in classical and intermediate monocytes (P<0.001). No correlations were found between pSTAT3 expression and clinical outcome: death or discharge, different oxygen supply up to intubation, and chest X-ray result. This may be due to the clinical char-

acteristics of the sample: they were all admitted to our sub-intensive care unit, they were all in severe or almost severe conditions with the need for high doses of oxygen supply.

Consolidations were the typical chest X-ray results for 88% of them. Levels of CRP were high in all patients.

We focused our attention on d-dimer and vitamin D values, with the aim to investigate possible relationships to pSTAT3.

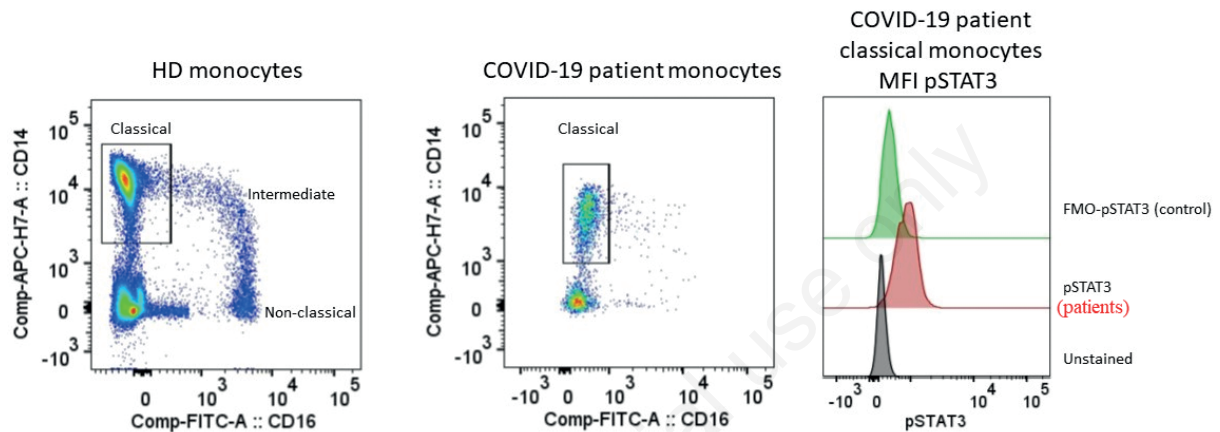As depicted in Figure 6, high levels of d-dimer were



**Figure 2. Fluorescence-activated cell sorting for healthy donors and COVID-19 patient monocytes. HD, healthy donors; MFI, mean fluorescence intensity; MFO, fluorescence minus one.**
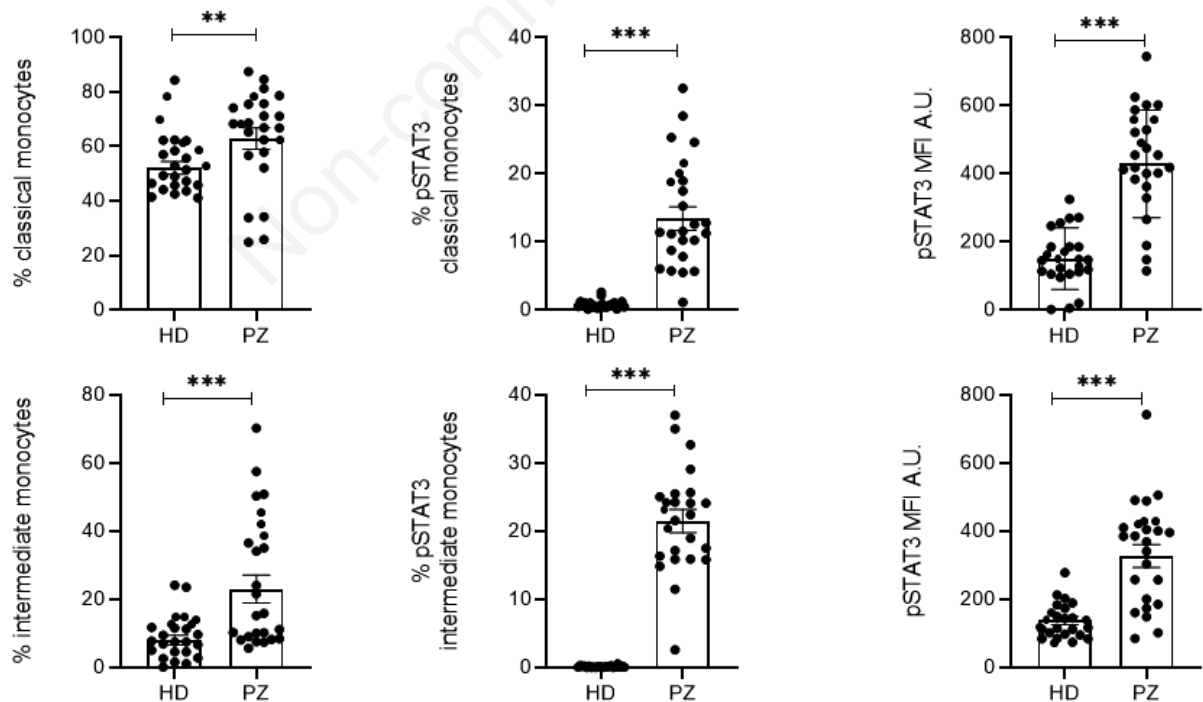


**Figure 3. Monocyte subsets (classical and intermediate) and phosphorylated signal transducers and activators of transcription-3 expression (percentage and mean fluorescence intensity). HD, healthy donors; MFI, mean fluorescence intensity; PZ, COVID-19 patients; *P<0.05; **P<0.01; ***P<0.001.**

significantly related to high percentages of pSTAT3 in intermediate monocytes (P<0.01). On the other hand, low levels of vitamin D were significantly related to

high percentages of pSTAT3 (P<0.01) (Figure 7). The same results were found also for classical monocytes.

## Discussion

Our data highlight the differential involvement of myeloid cell subsets in the pathogenesis of COVID-19. Classical and intermediate monocytes prevalence is higher in COVID-19 patients compared to HD.

The original classification of monocytes into classical (in humans: CD14high, CD16; in mice: Ly6Chigh), intermediate (in humans: CD14high, CD16low), and non-classical (in humans CD14low, CD16high; in mice: Ly6Clow) has been focused also in the area of COVID-19.[8]

Previous reports have confirmed this fact. Gatti *et al.*[6] found considerably reduced non-classical and intermediate monocytes in acute patients with severe symptoms. Conversely, in patients with moderate symptoms non-classical monocytes were increased.
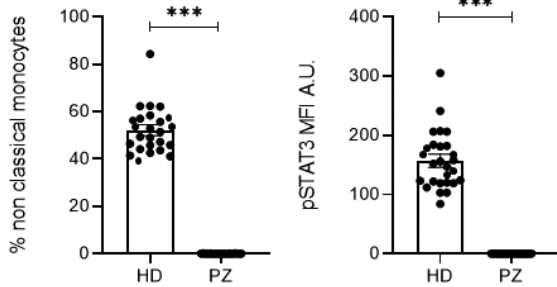


**Figure 4. Non-classical monocytes and phosphorylated signal transducers and activators of transcription-3 (percentage and mean fluorescence intensity). HD, healthy donors; MFI, mean fluorescence intensity; PZ, COVID-19 patients; ***P<0.001.**
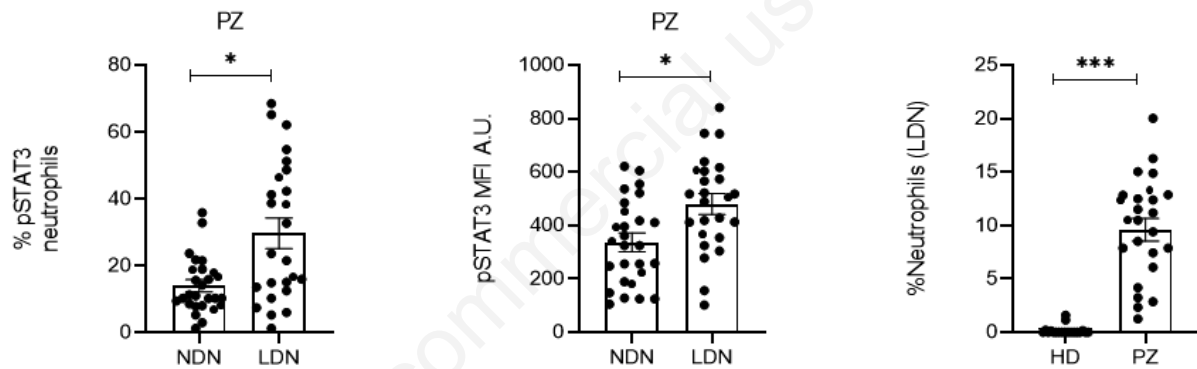


**Figure 5. Phosphorylated signal transducers and activators of transcription-3 and mean fluorescence intensity in low and normal density neutrophils. Percentage of low-density neutrophils in COVID-19 patients and healthy donors. HD, healthy donors; LDN, low-density neutrophils; MFI, mean fluorescence intensity; NDN, normal density neutrophils; PZ, COVID-19 patients; *P<0.05; ***P<0.001.**
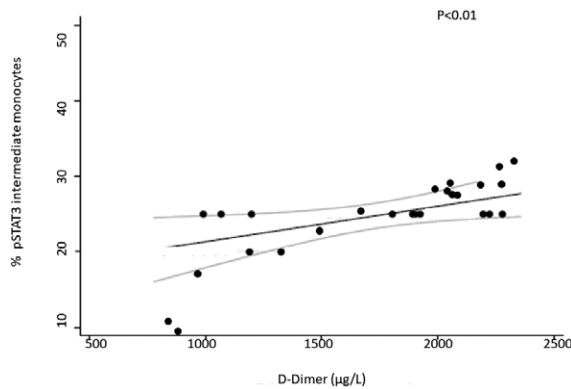


**Figure 6. Intermediate monocytes phosphorylated signal transducers and activators of transcription-3 percentage and d-dimer values in patients.**
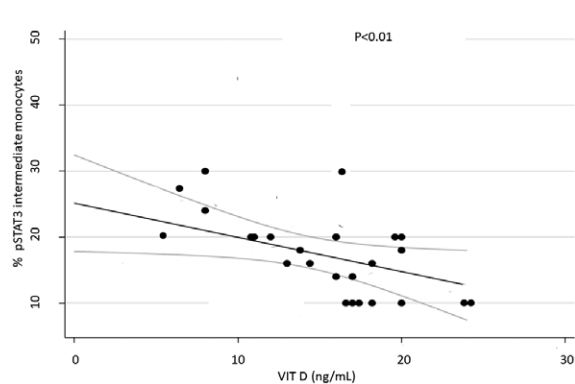


**Figure 7. Intermediate monocytes phosphorylated signal transducers and activators of transcription-3 percentage and vitamin D values in patients. VIT D, vitamin D.**

Our sample, constituted by severely ill patients, resembles the first picture.

The second finding concerns neutrophils. LDN count was significantly higher in patients compared to HD and pSTAT3 was significantly higher in LDN derived from patients.

It has been shown that LDN number correlates to disease severity and contributes to COVID-19-associated coagulopathy.[7] The inflammatory response contributing to COVID-19 associated coagulopathy has been partly elucidated in the study by Morrissey et al.[7] to which we will return later in the discussion.

The major findings of our study concern the role of STAT3.

STAT proteins were originally defined as latent cytoplasmatic transcription factors that need phosphorylation for nuclear retention and activation.

STAT3 was originally recognized as an acute phase response factor that is activated after stimulation, particularly by IL-6. STAT3-deficient T cells show severely impaired IL-6-induced proliferation due to the lack of IL-6-mediated prevention of apoptosis of T cells.[9]

STAT3 is critical for regulating an inflammatory cytokine response. Our results confirm the fact that plasma IL-6 was significantly correlated to pSTAT3 expression both in classical and intermediate monocytes. The role of STAT3 in contributing to the pathogenesis of severe COVID-19 is under investigation.[10]

Among the elevated inflammatory mediators, the blood IL-6 level is highly correlated with disease mortality when COVID-19 survivors and non-survivors are compared.[11]

The IL-6 amplifier is an amplification mechanism for the production of IL-6 and a variety of other cytokines and chemokines through a synergic interaction between STAT3 and nuclear factor-kappa B (NF-kB). NF-kB also plays a key role in inflammatory diseases including cytokine storm syndromes, autoimmune diseases, and cancer,[12] providing a link for STAT3 and NF-kB in these disorders. NF-kB acts as a "rapid-acting" primary transcription factor to regulate many cellular responses. The hallmark of NF-kB activation is the production of IL-6 and the ultimate consequence of NF-kB signaling is the activation of inflammatory genes including adhesion molecules and chemotaxins. The interactions among IL-6, NF-kB, and STAT3 have been considered as major players in the cytokine storm derived from COVID-19 infection.[13]

In our patients (severe cases of COVID-19), the over-stimulation of STAT3 is confirmed.

There is no difference in gene expression of both IL-6 and STAT3 in patients and HD. This fact could be due to the severity of the disease and the adequate production of the active form (pSTAT3) at the beginning of symptoms in the recent past.

Viral components induce signal transducer and activator of transcription 1 (STAT1) dysfunction and compensatory hyperactivation of STAT3. STAT3 upregulates plasminogen activator inhibitor-1 (PAI-1) through five signaling pathways mediated by miR-34a, CRP, p53, transforming growth factor-β, or hyaluronan fragments. PAI-1 is upregulated in aged individuals and in those suffering from hypertension, obesity, or diabetes, which are risk factors for COVID-19. The positive feedback loop established between STAT3 and PAI-1 may lead to an escalating cycle of activation. STAT3 and PAI-1 begin a catastrophic cascade of events resulting in combinations of coagulopathy/thrombosis macrophage production of cytokines and chemokines. In fact, several studies suggest that PAI-1 and STAT3 interact to promote coagulopathy and thrombosis in COVID-19.[14]

The correlation between pSTAT3 and d-dimer may be the expression of this fact because d-dimer is a coagulopathy indicator. Up to now, many studies have confirmed the occurrence of several thrombotic complications in COVID-19 infection (both venous thromboembolism and arterial thrombotic complications).[15]

Furthermore, reports of micro and macro thrombotic phenomena such as microangiopathy, pulmonary embolism have been frequently reported, which has led to a careful evaluation procedure for anti-thrombotic prophylaxis and/or coagulation in COVID-19 patients.[16]

Morrissey et al.,[7] who found increased LDN percentage in COVID-19 patients compared to HD, refers to neutrophil extracellular traps (NETs) as main contributors to COVID-19 coagulopathy.

LDN have been shown to produce more NETs. Other studies have focused attention on NETs as potential drivers for a solution to COVID-19.[17] Recent studies have focused attention on the fact that NETs components have been detected in the sera of COVID-19 patients.[18] Additionally, the presence of NETs in the lungs of COVID-19 patients (post-mortem specimens) has been recently reported. NETs were found in the airway compartment and neutrophil-rich inflammatory areas of the interstitium, while NET-prone primed neutrophils were present in arteriolar microthrombi, supporting the hypothesis that NETs may represent contributors to severe pulmonary COVID-19 complications.[19]

NF-kB is involved in the generation of NETs because it is involved in the inflammatory response mediated by NETs.[20]

Finally, we have correlated pSTAT3 to vitamin D. These results deserve particular attention for their novelty. Previous reports, before the COVID-19 pandemic, analyzed the possible relationships between STAT3 and vitamin D levels.

The active form of vitamin D, 1,25-(OH)2-D3, also called calcitriol, binds the vitamin D receptor (VDR) in the cytoplasm of the cell.[21] This causes a conforma-

tional change so VDR can bind its retinoid X receptor binding partner, and this heterodimer moves to the nucleus to promote or repress transcription of definite genes.[21] This pathway turns off activated T cells by decreasing inflammatory cytokine production and STAT phosphorylation. It has been demonstrated that vitamin D caused robust upregulation of VDR protein levels with a significant decrease in STAT1 and STAT3 tyrosine phosphorylation. In several areas of medical research, vitamin D has been shown to decrease the activation of STAT1 and STAT3 phosphorylation decreasing inflammatory cytokine output. It has been shown in hematological diseases and in kidney cancer.[22]

Research in the area of vitamin D and STAT3 demonstrated that the loss of muscle mass in slow muscles in the absence of vitamin D signaling is due to elevated levels of pSTAT3 that leads to an increase in myostatin signaling which in turn decreases protein synthesis and fiber size.[23]

Significant inverse correlations were found in 20 European countries between the mean serum vitamin D concentrations and the number of COVID-19 cases, as well as with mortality [24].

The severity of hypovitaminosis D appeared to relate to the prognosis and to be associated with greater mortality risk.

Two experimental studies reported higher 14-day survival rates in fragile older adults with COVID-19 receiving regular and preferentially recent vitamin D supplementation.[25,26]

Two different pathways through which vitamin D could reduce the cytokine storm and enhance the antiviral response have been recently proposed.[27]

1,25 D is a biologically active form of vitamin D that blocks tumor necrosis factor induced NF-kB activation. The 1,25 D binds with VDR and promotes the IkB kinase (IKK) activation. In fact, VDR enhances the interaction between this one and IKKB, which prevents the phosphorylation of IKKB and the formation of active IKK. Therefore, the degradation of IkB is blocked, resulting in preventing the translocation of NF-kB to the nucleus. Therefore, the transcription and the expression of NF-kB target genes responsible for the cytokine storm are suppressed. In addition, 1,25 D enhances the interferon (IFN)-a induced Janus kinase (JAK)-STAT signaling pathway. IFN-a activates JAK1 and TYK2 signaling, which subsequently phosphorylates and activate the downstream targets STAT1 and STAT2. The 1,25 D binds with its receptor and induces the dissociation from STAT1. STAT1 becomes available for phosphorylation and formation of active transcription factor complex interferon-stimulated genes (ISG)F3. The translocation of ISGF3 to the nucleus activates the transcription of ISGs, which provide antiviral activity and reduce the SARS-CoV-2 load in cells.

Also before the COVID-19 pandemic, vitamin D was known for its anti-inflammatory properties. In fact, it appeared to reduce the production of some cytokines, including IL-6, in patients with active inflammation.[28] Now, a recent meta-analysis showed that of the 11 studies included in the analysis, all of them identified a significant effect of vitamin D administration on IL-6.[29]

A limitation of our study is that of the sample size. Nevertheless, samples have been drawn specifically for research purposes (not retrieved from the clinical laboratory samples), which differs from the methods of other studies completed during the first phase of the COVID-19 pandemic. This fact has to be considered as a guarantee of the quality of the results. Future research should be focused on a larger panel of actors implicated in STAT3 signaling, first of all NF-kB and the role of NETs.

## Conclusions

In severe COVID-19 patients, non-classical and intermediate monocytes together with LDN are characterized by high pSTAT3. High pSTAT3 was significantly directly related to d-dimer and inversely related to vitamin D levels. This study gives information about possible vitamin D supplementation as a tool against COVID-19. This issue is under investigation in current clinical trials.[30] Although there is a need for additional research on the relationship between vitamin D supplementation, IL-6, STAT3, and COVID-19 outcomes, these findings support a role for vitamin D prescription, both prophylactic and potentially as a specific therapy.

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