

# Thrombopoietin participates in platelet activation in COVID-19 patients



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## Summary

**Background** The pathogenesis of coronavirus disease 2019 (COVID-19) is characterized by enhanced platelet activation and diffuse hemostatic alterations, which may contribute to immunothrombosis/thromboinflammation and subsequent development of target-organ damage.

Thrombopoietin (THPO), a growth factor essential to megakaryocyte proliferation, is known to prime platelet activation and leukocyte-platelet interaction. In addition, THPO concentrations increase in several critical diseases, such as acute cardiac ischemia and sepsis, thus representing a potential diagnostic and prognostic biomarker. Furthermore, several data suggest that interleukin (IL)-6 is one of the most important inflammatory mediators involved in these phenomena, which led to explore the potential therapeutic role of IL-6 inhibitors.

In this prospective cohort study, we aimed to study THPO and IL-6 concentrations in COVID-19 patients at the time of first clinical evaluation in the Emergency Department (ED), and to investigate their potential use as diagnostic and prognostic biomarkers. In addition, we sought to explore the role of THPO contained in plasma samples obtained from COVID-19 patients in priming *in vitro* platelet activation and leukocyte-platelet interaction.

**Methods** We enrolled 66 patients presenting to the ED with symptoms suggestive of COVID-19, including 47 with confirmed COVID-19 and 19 in whom COVID-19 was excluded (Non-COVID-19 patients). As controls, we also recruited 18 healthy subjects.

*In vitro*, we reproduced the effects of increased circulating THPO on platelet function by adding plasma from COVID-19 patients or controls to platelet-rich plasma or whole blood obtained by healthy donors, and we indirectly studied the effect of THPO on platelet activation by blocking its biological activity.

**Findings** THPO levels were higher in COVID-19 patients than in both Non-COVID-19 patients and healthy subjects. Studying THPO as diagnostic marker for the diagnosis of COVID-19 by receiver-operating-characteristic (ROC) statistics, we found an area under the curve (AUC) of 0.73, with an optimal cut-off value of 42.60 pg/mL. IL-6 was higher in COVID-19 patients than in healthy subjects, but did not differ between COVID-19 and Non-COVID-19 patients.

THPO concentrations measured at the time of diagnosis in the ED were also higher in COVID-19 patients subsequently developing a severe disease than in those with mild disease. Evaluating THPO as biomarker for severe COVID-19 using ROC analysis, we found an AUC of 0.71, with an optimal cut-off value of 57.11 pg/mL.

IL-6 was also higher in severe than in mild COVID-19 patients, with an AUC for severe COVID-19 of 0.83 and an optimal cut-off value of 23 pg/mL.

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THPO concentrations correlated with those of IL-6 ( $r=0.2963$ ;  $p=0.043$ ), and decreased 24 h after the administration of tocilizumab, an IL-6 receptor blocking antibody, showing that the increase of THPO levels depends on IL-6-stimulated hepatic synthesis.

*In vitro*, plasma obtained from COVID-19 patients, but not from healthy subjects, primed platelet aggregation and leukocyte-platelet binding, and these effects were reduced by inhibiting THPO activity.

**Interpretation** Increased THPO may be proposed as an early biomarker for the diagnosis of COVID-19 and for the identification of patients at risk of developing critical illness. Elevated THPO may contribute to enhance platelet activation and leukocyte-platelet interaction in COVID-19 patients, thus potentially participating in immunothrombosis/thromboinflammation.

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**Keywords:** COVID-19; Thrombopoietin; Interleukin-6; Platelet activation; Biomarker; Thromboinflammation

### Research in context

#### Evidence before this study

Patients affected by COVID-19 often develop thrombotic complications, which deeply influence disease prognosis. This may be related to systemic hyperinflammation, often referred to as a “cytokine storm”, which leads to disturbance of the coagulation and to activation of platelets. Several data suggest that interleukin-6 is one of the most important soluble mediators involved in these phenomena. Thrombopoietin is a key growth factor for platelet production in the bone marrow, but also facilitates the activation of circulating platelets. Moreover, its concentrations increase in several critical diseases, including cardiac ischemic diseases and severe infections.

#### Added value of this study

Herein, we show that the circulating levels of thrombopoietin are higher in patients with COVID-19 than in Non-COVID-19 patients and healthy subjects, as in severe than in mild COVID-19 patients. The observed increase of thrombopoietin levels probably depends on augmented synthesis in the liver stimulated by interleukin-6, expressing the hyper-inflammatory phase of COVID-19.

Furthermore, plasma from COVID-19 patients, but not from healthy subjects, primes *in vitro* platelet activation, an effect that is decreased by inhibiting the activity of thrombopoietin, thus suggesting that thrombopoietin participates in platelet activation in COVID-19.

#### Implications of all the available evidence

Our findings suggest that thrombopoietin may concur to increase platelet activation in COVID-19 patients, and

that thrombopoietin may be potentially useful as an early biomarker for the diagnosis and for the severity assessment of COVID-19. Our results may contribute to design and plan a personalized and host-directed treatment for COVID-19 patients.

### Introduction

The course of coronavirus disease of 2019 (COVID-19), caused by SARS-CoV-2 infection, is frequently complicated by the occurrence of target-organ damage and thromboembolic events,<sup>1,2</sup> which strongly affect patient's prognosis.<sup>1</sup> Microvascular or frank pulmonary thrombosis have been described in both autopsic<sup>3,4</sup> and imaging studies.<sup>2,5–8</sup>

A relevant role has been attributed to systemic hyperinflammation, often referred to as a “cytokine storm”.<sup>9–12</sup> This phenomenon has been associated with elevated levels of interleukin (IL)-6, IL-1 $\beta$ , and tumor necrosis factor (TNF)- $\alpha$ ,<sup>13</sup> as well as of other pro-inflammatory biomarkers,<sup>1,12,14</sup> together with the occurrence of a peculiar COVID-19-associated coagulopathy. The latter is characterized by unique clinical and laboratory findings,<sup>2,5,15,16</sup> which include prolonged prothrombin time, altered partial activated thromboplastin time, reduced plasma fibrinogen and high D-dimer levels,<sup>14,17,18</sup> suggesting the presence of a hypercoagulable state.<sup>2,5,15,16</sup>

This complex interaction between coagulation and inflammatory pathways, also called thromboinflammation or immunothrombosis,<sup>16,19–25</sup> which involve complement and coagulation factors, cytokines/chemokines, monocytes, neutrophils and neutrophil

extracellular traps, is believed to ultimately lead to enhanced platelet activation and increased risk of microvascular thrombosis.<sup>16,19–23</sup>

Patients with COVID-19 usually have a moderate thrombocytopenia, which can aggravate in the most severe cases, generally related to platelet-consumption.<sup>16,19,26,27</sup> Several studies, however, have also provided evidence that circulating platelets in COVID-19 patients show features of hyper-activation and pro-coagulant state,<sup>19–21,28–32</sup> which include changes in gene expression in pathways associated with protein ubiquitination, antigen presentation, and mitochondrial dysfunction.<sup>28</sup> Accordingly, platelets aggregate faster and at suboptimal thrombin concentration, show increased spreading on fibrinogen and collagen mediated by enhanced MAPK pathway activation and thromboxane generation,<sup>28</sup> have increased basal expression of P-selectin, enhanced coagulation through Factor VIII, and increased release of pro-inflammatory cytokines and chemokines.<sup>28,30,32</sup> Finally, COVID-19 patients also have higher circulating monocyte- and neutrophil-platelet aggregates.<sup>28,31,32</sup> Platelet hyper-activation seems to correlate with the severity of COVID-19.<sup>21</sup>

Our and others research groups have previously shown that thrombopoietin (THPO), a growth factor essential to megakaryocyte proliferation and differentiation in the bone marrow,<sup>33–35</sup> is also able to prime platelet aggregation and platelet-leukocyte adhesion in response to several stimuli.<sup>36–39</sup>

Increased plasma concentrations of THPO have been described in several critical diseases where platelet activation represents a crucial pathogenic mechanism,<sup>36</sup> including unstable angina,<sup>40</sup> coronary artery disease,<sup>41</sup> ischemic stroke,<sup>42</sup> inflammatory bowel disease,<sup>43</sup> burn injury,<sup>44</sup> sepsis,<sup>45</sup> and Severe Acute Respiratory Syndrome (SARS).<sup>46</sup> Interestingly, one of the mechanisms that may sustain increased THPO levels in inflammatory systemic diseases is represented by augmented hepatic synthesis stimulated by IL-6.<sup>47</sup> Furthermore, disease severity is a major determinant of elevated THPO levels in patients affected by sepsis<sup>48</sup> or acute pancreatitis,<sup>49</sup> suggesting that THPO may be proposed as a potential prognostic marker in these pathologic conditions.

In experimental studies, it has been shown that THPO-induced platelet activation is fundamental for the development of organ injury in different models of murine sepsis,<sup>50</sup> and that THPO cooperates with other cytokines, namely TNF- $\alpha$  and IL-1 $\beta$ , in mediating the depression of myocardial contractility induced by serum of patients with septic shock.<sup>51</sup> Finally, a recently published study has demonstrated that THPO levels are significantly elevated in severe COVID-19 patients requiring critical care support, as expression of a platelet hyperactive phenotype.<sup>52</sup>

Based on available evidences, THPO appears as a candidate mediator of platelet hyper-activation and immunothrombosis/thromboinflammation in COVID-19.

In this study, we aimed to investigate the levels of THPO and IL-6 in patients with COVID-19 at the time of diagnosis and first evaluation in the Emergency Department (ED), and to evaluate THPO as an early biomarker for diagnosis and disease severity prognostication in COVID-19 patients. In addition, we studied the potential correlation of THPO with IL-6 levels, and the role of THPO in *in vitro* platelet priming.

## Methods

### Study design

This was a prospective observational cohort study. All patients gave informed oral consent for the participation to the study (signed written consent form was waived due to safety protocols), and all data were immediately de-identified.

### Ethics

The study was approved by the Ethics Committee of our Hospital (“Comitato Etico Interaziendale A.O.U. Città della Salute e della Scienza di Torino - A.O. Ordine Mauriziano - A.S.L. Città di Torino”; n. CS2/139) and conducted in accordance with the ethical standards of the Declaration of Helsinki and its later amendments.

### Patients and case adjudication

Between April 7<sup>th</sup> and 27<sup>th</sup>, 2020, we considered eligible adult patients (age >18 years) who, at triage, screened positive for acute symptoms commonly associated with SARS-CoV2 infection (i.e. fever, dyspnea, new or worsening cough, sore throat, diarrhea, ageusia, anosmia, asthenia). Screening positive patients were cohorted in a dedicated area of the ED, and subsequently evaluated and approached for study enrollment by the treating physician. Patients already intubated at the time of ED arrival were excluded. Other exclusion criteria were: age <18 years, known hematological diseases affecting coagulation, platelet count or THPO production,<sup>53</sup> and known malignancies in active treatment.

For all patients, SARS-CoV-2 detection was performed on nasopharyngeal swab samples at the time of ED admission. Imaging methods, treatment, and ICU admission were decided at the discretion of the attending physicians, independently from participation to this study.

Final case adjudication was performed by two expert physicians who independently assessed all patients' data, including qRT-PCR test results on any respiratory specimen (including bronchoalveolar lavage), medical charts of ED visit(s) and hospital admission(s), laboratory test results, all imaging data (comprehensive of computed tomography) obtained within 28 days from the index visit, and results of 28-day follow-up,

performed either by telephone (if discharged) or in person (for patients still admitted to the hospital).

Adjudication was dichotomic: COVID-19 present or absent (alternative diagnosis). COVID-19 was always considered present in patients with a positive qRT-PCR test result obtained within 5 days from ED presentation. In the other patients, the final diagnosis was established considering all follow-up data. In case of discordant adjudication among the two experts, a third expert adjudicated the final diagnosis.

COVID-19 patients were further classified based on disease severity in two groups: those who developed, during the course of hospitalization, respiratory failure needing mechanical ventilation (either non-invasive or invasive) or septic shock were considered affected by severe COVID-19.

Another control group consisted of healthy volunteers, receiving no medications, who were recruited among laboratory staff members and their friends and relatives. None had shown any evidence of febrile illness or other infectious disease during the previous two weeks. Their hematological indices, and liver and kidney function tests were within normal ranges.

### Biochemical analyses

For molecular diagnosis of SARS-CoV-2 on nasopharyngeal swabs, after purification with QIA symphony DSP virus/pathogen kit (QIAGEN, Venlo, The Netherlands), qRT-PCR was performed using the GeneFinder 2019 nCoVRealAmp Kit (Elitech, Puteaux, France) or the Simplexa COVID-19 Direct kit (Diasorin Molecular, Cypress, California, USA), following manufacturer's instructions.

In all patients enrolled in the ED, THPO and IL-6 levels were determined on blood samples drawn at the admission in the emergency room, before any therapeutic intervention was started.

Blood samples were obtained by clean venipuncture using a 21-gauge infusion set in both patients and healthy subjects.

In a group of 6 COVID-19 patients, THPO and IL-6 were measured before and 24 h after the administration of tocilizumab (8 mg/kg BW *i.v.*, repeated after 12 h), a blocking antibody directed against IL-6 receptor.<sup>54</sup>

To obtain plasma samples, EDTA-anticoagulated tubes were centrifuged at 1600 g for 15 min at 4 °C within 2 h after blood sampling. All plasma samples were stored at -70 °C until analysis.

THPO concentrations were measured in duplicate using an enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, Minnesota), following the manufacturer's instructions.

IL-6 levels in plasma were determined by quantitative immunofluorescence using the automated sqidlite system (SQI Diagnostics Systems Ltd., Toronto, Canada) with a time-to-result of 50 min. Plasma samples

were thawed overnight at 4 °C and diluted 1:1 in assay diluent. 60 µL of diluted serum were loaded onto a custom 96-well microtitre plate that contained a standard curve generated and a high and low positive control derived from the reference standard. The World Health Organization (WHO) reference standard was used for IL-6.

### *In vitro* platelet aggregation

Platelet aggregation in 3.8% trisodium citrate-anticoagulated platelet-rich plasma (PRP) was evaluated as previously described.<sup>37,40</sup> PRP was obtained from healthy subjects by clean venipuncture using a 19-gauge butterfly infusion set, without venous stasis, and incubated with 25 µL of plasma at 37 °C. When evaluating priming activity, epinephrine (EPI), adenosine-diphosphate (ADP) or thrombin (THR) (Helena Laboratories, Beaumont, TX) was added as secondary agonist. For each experiment, the agonist concentration that induced the minimum measurable aggregation was determined; EPI dose range was 0.05–0.2 µM/L, ADP dose range was 0.1–1.0 µM/L, and THR dose range was 0.1–0.3 U/ml. The priming index (PI) was calculated as the response to plasma and agonist together, divided by the sum of individual responses elicited by plasma and agonist.<sup>37,40</sup>

In separate experiments, designed to inhibit the biological effects of THPO, test plasma was incubated with a recombinant human (rh) thrombopoietin receptor (THPOR; 2.5 µg/ml, R&D Systems) or PRP with a neutralizing antibody anti-hTHPOR (Ab αTHPOR; 3 µg/ml, R&D Systems) for 5 min at 37 °C. The mixture of plasma sample and rhTHPOR was then added to PRP and further incubated for 7 min at 37 °C. Finally, the secondary agonist (EPI, ADP, or THR) was added to the test tube containing either the plasma sample pre-incubated with rhTHPOR or the PRP pre-treated with the Ab αTHPOR, and platelet aggregation was evaluated.

The ability of rhTHPOR and Ab αTHPOR to specifically inhibit THPO biological activity was evaluated in preliminary experiments using rhTHPO (1.0 ng/ml, R&D Systems). Briefly, rhTHPO was incubated with the rhTHPOR (2.5 µg/ml) or PRP with the Ab αTHPOR (3 µg/ml), and platelet aggregation was then evaluated as described above.

### Flow cytometry

Leucocyte-platelet aggregates *in vitro* were analyzed by 3-color staining of whole blood samples.<sup>40</sup> For *in vitro* experiments, 100 µL of blood from healthy adult donors were diluted with Tyrodes' HEPES buffered saline (pH 7.4), pre-incubated at 37 °C with 25 µL plasma of patients or control subjects for 5 min, and then stimulated with EPI (4 µM), ADP (0.8 µM), or THR (0.2 U/ml). Cell staining was performed by use of PerCP-Cy5.5-

conjugated anti-CD45 (eBioscience, Thermo Fisher Scientific, Waltham, MA, USA), PE-conjugated anti-CD14 (eBioscience), and PE-Cy7-conjugated anti-CD41 (eBioscience) monoclonal antibodies. Cells were then fixed with 1% paraformaldehyde and erythrocytes were removed by hypotonic lysis.

Samples were analysed on an Attune NxT Flow Cytometer (Thermo Fisher Scientific) using adequate compensation for different fluorochromes. Total leukocytes were identified by their positive staining with anti-CD45, and lymphocyte, granulocyte and monocyte populations were discriminated on the ground of CD45 versus side scatter. The percentage of leukocyte subgroups co-expressing CD45-CD41 (granulocytes-platelets) or CD14-CD41 (monocytes-platelets) over the total population of leukocytes expressing CD45 or CD14 was used as an index of leukocyte-platelet adhesion.<sup>40</sup>

The ability of rhTHPO and Ab  $\alpha$ THPO to specifically inhibit THPO biological activity was evaluated in preliminary experiments using rhTHPO (1.0 ng/ml, R&D Systems). Briefly, rhTHPO was incubated with the rhTHPO (2.5  $\mu$ g/ml) or whole blood with the Ab  $\alpha$ THPO (3  $\mu$ g/ml), and leukocyte-platelet adhesion was then evaluated as described above.

### Cell proliferation assay

The ability of rhTHPO and Ab  $\alpha$ THPO to block rhTHPO biological activity *in vitro* was tested by using the 5-bromo-2'-deoxyuridine (BrdU) Cell Proliferation Assay Kit (Abcam, Cambridge, UK) on the megakaryoblast cell line M-07,<sup>50,55</sup> following manufacturer's instructions. Briefly,  $2 \times 10^4$  cells/well were seeded in 96-well plates and treated with rhTHPO (1.0 ng/ml) for 72 h. In selected experiments, rhTHPO was pre-incubated with rhTHPO (2.5  $\mu$ g/ml) for 1 h, or M-07 cells were pre-treated with the Ab  $\alpha$ THPO (3  $\mu$ g/ml) for 1 h. Finally, optical density was quantified at 450 nm. Every experiment was repeated three times and the readings were taken in triplicates.

### Analysis of data

Data are presented as median (range) or mean  $\pm$  standard error (SE), according to data distribution as assessed by Shapiro-Wilk test. Comparisons between groups were carried-out by Mann-Whitney rank sum test or Kruskal-Wallis one-way analysis of variance on ranks followed by Dunn's multiple comparison test, Wilcoxon matched-pairs signed rank test or unpaired or paired Student's t test, as appropriate.

Relationships between variables were investigated using Spearman correlation test.

We used receiver-operating-characteristic (ROC), specificity, sensitivity, positive predictive value (PPV) and negative predictive value (NPV), and area under the curve (AUC) statistics to test THPO and IL-6 accuracy

as biomarkers for the diagnosis of COVID-19 and for that of severe COVID-19.<sup>56,57</sup>

Performance of THPO and IL-6 in terms of clinical usefulness was also assessed using decision curve analyses (DCA).<sup>58</sup> DCA allow to judge the relative benefits and harms associated with each tests.

A *p* value < 0.05 was considered significant.

All statistics have been performed using GraphPad Prism 7.00 for Windows (GraphPad Software, La Jolla, CA, USA) or STATA software, version 13.1 (Stata Corporation, College Station, Texas, USA).

### Sample-size estimation

No data were available about the diagnostic accuracy of THPO in patients with suspected COVID-19. We hypothesized a null effect of this test (*i.e.* AUC 0.5), and the possibility to detect at least a 25% increase in accuracy, with a power of 80% and an alpha error of 0.05. Based on these assumptions, we calculated a sample size of 47 patients.

We also tested the same hypothesis but with a power of 90% and an alpha error of 0.01. In such a case, the sample size needed was 55 patients.

### Role of the funding source

The funding source had no role in the study design, in the collection, analysis and interpretation of the data, in the writing of the report, and the decision to submit the paper for publication.

## Results

### Patient characteristics

The study population consisted of 66 patients admitted to the ED during the study period with symptoms compatible with COVID-19. Of these, 47 patients were diagnosed with COVID-19 (COVID-19 patients), whereas the diagnosis of COVID-19 was excluded in the remaining 19 patients (Non-COVID-19 patients). We prospectively enrolled unselected patients presenting to the ED with acute symptoms commonly associated with SARS-CoV2 infection, hence a patient cohort representative of the wider patient population potentially affected by COVID-19 during the enrolment period.

The overall in-hospital mortality rate was 10.6% (5/47) in patients with COVID-19, and 5.3% (1/19) in Non-COVID-19 patients. All patients died within 10 days due to respiratory failure/multiple organ failure.

We also recruited 18 healthy subjects, used as controls.

Demographic and clinical characteristics of study subjects are detailed in [Table 1](#).

Within COVID-19 patients, 36 patients (76.6%) were diagnosed as having mild COVID-19, and 11 (23.4%) severe COVID-19.

|                                | Healthy subjects (n = 18) | Non-COVID-19 (n = 19) | COVID-19 (n = 47)       | Mild COVID-19 (n = 36) | Severe COVID-19 (n = 11) |
|--------------------------------|---------------------------|-----------------------|-------------------------|------------------------|--------------------------|
| <b>Age, median (range), yr</b> | 51 (29–93)                | 53 (31–87)            | 52 (22–97)              | 48.5 (22–93)           | 68 (43–97) <sup>§</sup>  |
| <b>Gender, N (%)</b>           |                           |                       |                         |                        |                          |
| Female                         | 11 (61.1)                 | 14 (73.7)             | 30 (63.8)               | 24 (66.7)              | 6 (54.5)                 |
| Male                           | 7 (38.9)                  | 5 (26.3)              | 17 (36.2)               | 12 (33.3)              | 5 (45.5)                 |
| <b>Ethnicity, N (%)</b>        |                           |                       |                         |                        |                          |
| Caucasian                      | 18 (100)                  | 17 (89.5)             | 35 (74.5)               | 24 (66.7)              | 11 (100)                 |
| Afro-american                  | 0                         | 0                     | 2 (4.3)                 | 2 (5.6)                | 0                        |
| Hispanic                       | 0                         | 2 (10.5)              | 9 (19.1)                | 9 (25)                 | 0                        |
| Asian                          | 0                         | 0                     | 1 (2.1)                 | 1 (2.8)                | 0                        |
| <b>Symptoms, N (%)</b>         |                           |                       |                         |                        |                          |
| Fever                          | -                         | 12 (63.2)             | 37 (78.7)               | 27 (75)                | 10 (90.9)                |
| Cough                          | -                         | 11 (57.9)             | 29 (61.7)               | 23 (63.9)              | 6 (54.5)                 |
| Shortness of breath            | -                         | 8 (42.1)              | 24 (51.1)               | 16 (44.4)              | 8 (72.7)                 |
| Sore throat                    | -                         | 2 (10.5)              | 2 (4.3)                 | 2 (5.6)                | 0                        |
| Diarrhea                       | -                         | 6 (31.6)              | 11 (23.4)               | 8 (22.2)               | 3 (27.3)                 |
| Ageusia                        | -                         | 3 (15.8)              | 11 (23.4)               | 8 (22.2)               | 3 (27.3)                 |
| Anosmia                        | -                         | 0                     | 9 (19.1) <sup>*</sup>   | 7 (19.4)               | 2 (18.2)                 |
| <b>Comorbidities, N (%)</b>    |                           |                       |                         |                        |                          |
| Hypertension                   | -                         | 2 (10.5)              | 12 (25.5)               | 6 (16.7)               | 6 (54.5) <sup>§</sup>    |
| Diabetes Mellitus              | -                         | 1 (5.3)               | 3 (6.4)                 | 1 (2.8)                | 2 (18.18)                |
| CAD                            | -                         | 0                     | 6 (12.8)                | 4 (11.1)               | 2 (18.2)                 |
| Atrial fibrillation/flutter    | -                         | 1 (5.3)               | 9 (19.1)                | 5 (13.9)               | 4 (36.4)                 |
| Asthma/COPD                    | -                         | 1 (5.3)               | 4 (8.5)                 | 1 (2.8)                | 3 (27.3) <sup>§</sup>    |
| Cancer                         | -                         | 1 (5.3)               | 2 (4.3)                 | 2 (5.6)                | 0                        |
| Cerebrovascular disease        | -                         | 0                     | 5 (10.6)                | 3 (8.3)                | 2 (18.2)                 |
| CKD                            | -                         | 0                     | 1 (2.1)                 | 0                      | 1 (9.1)                  |
| DVT/PE                         | -                         | 0                     | 0                       | 0                      | 0                        |
| Home discharge                 | NA                        | 15 (78.9)             | 15 (31.9) <sup>**</sup> | 15 (41.7)              | 0 <sup>§§</sup>          |
| Ward Admission                 | NA                        | 3 (15.8)              | 26 (55.3) <sup>**</sup> | 20 (55.6)              | 6 (54.5)                 |
| ICU/HDU Admission              | NA                        | 1 (5.3)               | 6 (12.8)                | 1 (2.8)                | 5 (45.4) <sup>§§§</sup>  |
| MV (NIV/IMV)                   | NA                        | 1 (5.3)               | 5 (10.6)                | 0                      | 5 (45.4) <sup>§§§§</sup> |
| In-hospital Mortality          | NA                        | 1 (5.3)               | 5 (10.6)                | 0                      | 5 (45.4) <sup>§§§§</sup> |

**Table 1: Characteristics of the patients and ED outcome.**

CAD, Coronary Artery Disease; COPD, Chronic Obstructive Pulmonary Disease; CKD, Chronic Kidney Disease; DVT, Deep Vein Thrombosis; PE, Pulmonary Embolism; ED, Emergency Department; ICU, Intensive Care Unit; HDU, High Dependency Unit; MV, Mechanical Ventilation; NIV, Non-invasive Ventilation; IMV, Invasive Mechanical Ventilation; NA, not applicable.

\*  $p < 0.05$ .

\*\*  $p < 0.01$  vs Non-COVID-19.

§  $p < 0.05$ .

§§  $p < 0.01$ .

§§§  $p < 0.001$  vs Mild COVID-19.

Among Non-COVID-19 patients, the final diagnosis was flu-like syndrome in 13, bacterial pneumonia in 3, acute exacerbation of chronic obstructive pulmonary disease in 1, worsening inflammatory bowel disease in 1, and acute gastroenteritis in 1 patient.

No significant differences for age and gender were found between the three study groups.

Age was lower in the mild COVID-19 group compared to the severe COVID-19 group, whereas the ratio female/male was not different between the two groups.

Non-COVID-19 patients had higher white blood cell (WBC), neutrophil, and platelet counts than healthy

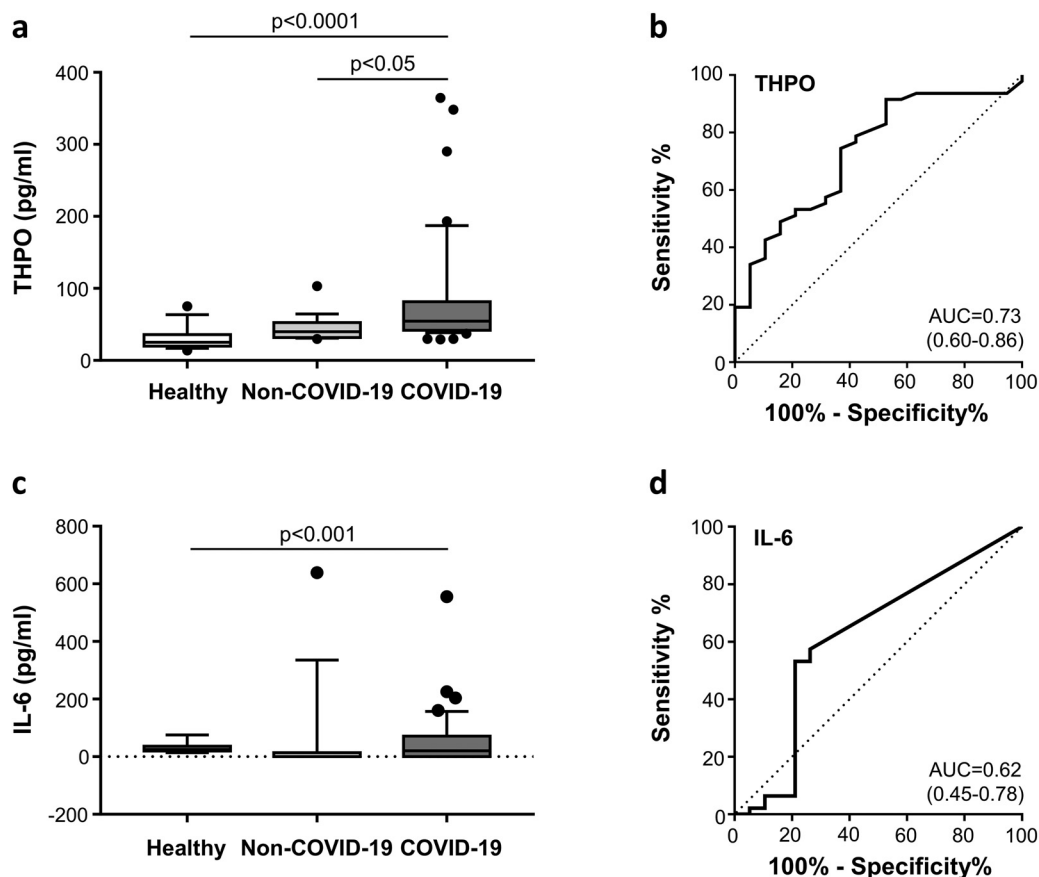
subjects (Table 2). In contrast, COVID-19 patients had lower WBC, lymphocyte, and platelet counts than Non-COVID-19 patients, but they showed increased neutrophil/lymphocyte ratio, leukocyte/lymphocyte ratio, and C-reactive protein values (Table 2).

Severe COVID-19 patients had increased WBC, neutrophil, and platelet counts than mild COVID-19 patients, while lymphocyte count was greatly reduced. Also neutrophil/lymphocyte ratio and leukocyte/lymphocyte ratio, as well as C-reactive protein values were significantly higher in severe compared to mild COVID-19 patients (Table 2).

|   | Healthy (n=18)   | Non-COVID-19 (n=19)           | COVID-19 (n=47)               | Mild COVID-19 (n=36) | Severe COVID-19 (n=11)         |
|---|------------------|-------------------------------|-------------------------------|----------------------|--------------------------------|
| White blood cell count (10 <sup>9</sup> /L) | 5.4 (3.9–6.9)    | 7.8 (4.7–20.3) <sup>†††</sup> | 5.6 (2.4–17.3) <sup>**</sup>  | 5.5 (2.4–10.1)       | 7 (5.5–17.3) <sup>§§</sup>     |
| Neutrophil count (10 <sup>9</sup> /L)       | 3.3 (1.7–4.3)    | 5.5 (1.9–18.2) <sup>††</sup>  | 3.6 (0.8–16.6)                | 3.2 (0.8–9.1)        | 5.3 (2.2–16.6) <sup>§§</sup>   |
| Lymphocyte count (10 <sup>9</sup> /L)       | 1.7 (0.9–2.1)    | 1.9 (0.8–2.8)                 | 1.3 (0.3–4.4)                 | 1.5 (0.3–3.3)        | 1 (0.3–4.4) <sup>§§</sup>      |
| Neutrophil / Lymphocyte (ratio)             | 2.0 (1.0–4.3)    | 2.3 (0.8–23.4)                | 2.9 (0.5–49) <sup>†</sup>     | 2.2 (0.5–1620)       | 19.5 (0.5–1020) <sup>§§</sup>  |
| Leukocyte/Lymphocyte (ratio)                | 3.3 (2.3–5.9)    | 3.6 (2.5–26)                  | 3.9 (1.6–51) <sup>†</sup>     | 3.5 (1.8–28.6)       | 9.7 (1.6–51) <sup>§§</sup>     |
| Platelet count (10 <sup>9</sup> /L)         | 209 (119–365)    | 271 (185–386) <sup>††</sup>   | 190 (105–401) <sup>***</sup>  | 190 (134–401)        | 211 (105–362)                  |
| Mean platelet volume (fL)                   | 10.8 (9.5–12)    | 10.7 (8.7–13.4)               | 10.8 (8.7–20.9)               | 10.6 (8.7–13.6)      | 11.6 (9.8–20.9) <sup>§§</sup>  |
| Platelet distribution width (fL)            | 13.4 (10.6–16.1) | 13.4 (9–17.2)                 | 12.8 (8.4–18.9)               | 12.3 (8.4–18.9)      | 13.3 (9.9–15.9)                |
| C-Reactive Protein (mg/L)                   | 1.0 (0.2–5.9)    | 5.3 (0.3–223)                 | 17.3 (0.3–261) <sup>†††</sup> | 8.7 (0.3–133)        | 66.6 (37.9–261) <sup>§§§</sup> |

**Table 2: Laboratory findings.**

†  $p < 0.05$ .  
 ††  $p < 0.01$ .  
 †††  $p < 0.001$  vs Healthy.  
 \*  $p < 0.05$ .  
 \*\*  $p < 0.01$ .  
 \*\*\*  $p < 0.001$  vs Non-COVID-19.  
 §§  $p < 0.01$ .  
 §§§  $p < 0.001$  vs Mild COVID-19. Data are expressed as median (range).



**Figure 1.** Comparison of plasma levels of THPO and IL-6 in healthy subjects, Non-COVID-19 patients, and COVID-19 patients and relative receiver-operating characteristics (ROC) curves for the diagnosis of COVID-19.

Box-and-whisker plots of THPO (a) and IL-6 (c) plasma concentrations in healthy subjects (n = 18), Non-COVID-19 patients (n = 19), and COVID-19 patients (n = 47).

Data were expressed as median (range), and were analyzed by using Kruskal-Wallis one-way analysis of variance on ranks followed by Dunn’s multiple comparison test.

ROC curves of plasma THPO (b) and IL-6 (d) for the diagnosis of COVID-19. Area under the curve (AUC) values are reported with 95% CI in brackets.

**Circulating THPO and IL-6 levels**

THPO levels were significantly higher in COVID-19 patients than in both Non-COVID-19 patients and healthy subjects (Figure 1a). On the contrary, THPO concentrations did not differ between Non-COVID-19 patients and healthy subjects (Figure 1a).

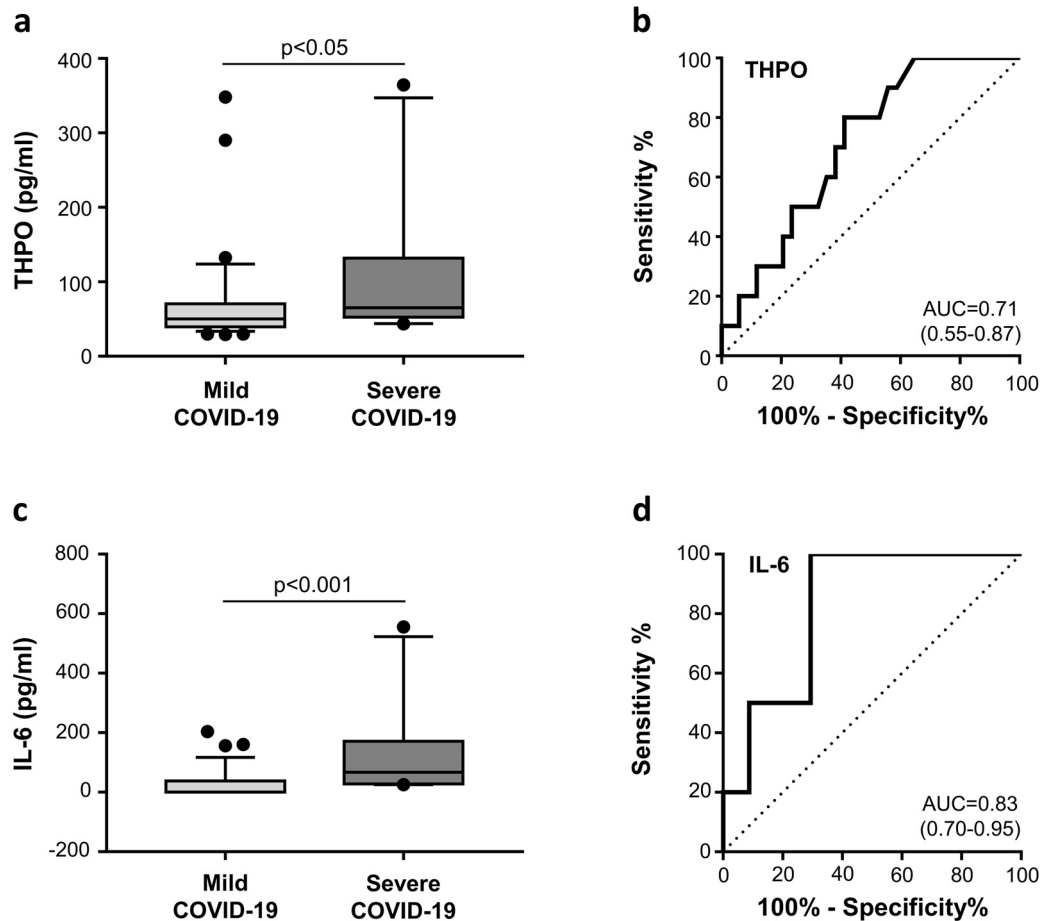
Evaluating THPO as potential diagnostic biomarker for COVID-19 using the ROC statistics, we found an AUC of 0.73 (95% CI, 0.60 to 0.86; Figure 1b). The concentrations of THPO showing the best relationship between sensitivity (74.47%) and specificity (63.16%) was 42.60 pg/ml, with a positive predictive value (PPV) of 83.3% (68.6–93%) and a negative predictive value (NPV) of 50% (29.1–70.9%).

IL-6 concentrations were also higher in COVID-19 patients than in healthy subjects, but did not differ between COVID-19 and Non-COVID-19 patients (Figure 1c). In ROC analysis, IL-6 had an AUC of 0.62

(95% CI, 0.45 to 0.78; Figure 1d) for the diagnosis of COVID-19.

We also aimed to evaluate THPO and IL-6, measured at the time of first diagnosis and clinical evaluation in the ED, as biomarkers for the detection of those patients at higher risk of developing a critical course at the disease. Median THPO levels were higher in severe COVID-19 patients (65.26, 43.51 to 364.40 pg/mL) than in mild COVID-19 patients (50.31, 29.01 to 348.10 pg/mL) (Figure 2a).

ROC analysis of THPO for prediction of severe COVID-19 gave an AUC of 0.71 (95% CI, 0.55 to 0.87). The concentrations of THPO showing the best relationship between sensitivity (70%) and specificity (61.76%) was 57.11 pg/mL, with a PPV of 35% (15.4–59.2%) and a NPV of 87.5% (67.6–97.3%). A cut-off value of 42.60 pg/mL reached a sensitivity of 100%, allowing conclusive rule-out of severe COVID-19.



**Figure 2.** Comparison of THPO and IL-6 plasma levels in mild and severe COVID-19 patients and relative receiver-operating characteristics (ROC) curves for the development of severe COVID-19.

Box-and-whisker plots of THPO (a) and IL-6 (c) plasma concentrations in mild (n = 36) and severe (n = 11) COVID-19 patients.

Data were expressed as median (range), and were analyzed by using Mann-Whitney rank sum test.

ROC curves of plasma THPO (b) and IL-6 (d) for the development of severe COVID-19. Area under the curve (AUC) values are reported with 95%CI in brackets.



IL-6 concentrations were also significantly more elevated in severe than in mild COVID-19 (Figure 2c). ROC analysis of IL-6 for prediction of severe COVID-19 gave an AUC of 0.83 (95% CI, 0.70 to 0.95; Figure 2d). A cut-off value of 23 pg/ml showed a sensitivity of 100% and a specificity of 70.59% for severe COVID-19 (Figure 2d), with a PPV of 50% (28.2–71.8%) and a NPV of 96% (79.6–99.9%).

Decision curve analysis (DCA) of THPO and IL-6 for the diagnosis of COVID-19 (Figure 3a) and of severe COVID-19 (Figure 3b) indicated better clinical utility of THPO in detecting COVID-19 than IL-6, whereas IL-6 proved more useful in assessing infection severity.

Within COVID-19 patients, plasma concentrations of THPO significantly correlated with the levels of IL-6 ( $r = 0.2963$ ;  $p = 0.043$ ). Moreover, an inverse correlation was found between THPO and platelet count ( $r = -0.5148$ ;  $p < 0.001$ ). In contrast, we found no correlation of THPO level with MPV and PDW.

In order to determine whether elevated THPO concentrations in COVID-19 patients were related to increased IL-6 concentration, we measured THPO and IL-6 levels in a sub-group of 6 COVID-19 patients before and 24 h after the administration of tocilizumab, a blocking antibody directed against IL-6 receptor.<sup>54</sup> THPO levels were significantly decreased 24 h after tocilizumab administration (from 62.09, 46.23 to 185.80, to 44.87, 31.72 to 84.30 pg/mL; Figure 4a), whereas those of IL-6 were greatly increased (from 105.20, 0 to 153.50, to 514.90, 166 to 2820 pg/mL; Figure 4b). Whereas the effect of tocilizumab on IL-6 levels was already described,<sup>59,60</sup> our results show that inhibiting IL-6 effects induces a decrease of THPO circulating levels, suggesting that THPO levels in COVID-19 patients mostly depend on IL-6-stimulated increased hepatic synthesis.

#### Effect of plasma from COVID-19 patients on platelet activation *in vitro*

In order to reproduce the potential effect of increased THPO concentrations in COVID-19, we tested *in vitro* the effect of plasma from normal subjects and COVID-19 patients on platelet aggregation in platelet-rich plasma (PRP) from healthy donors.

Plasma from COVID-19 patients did not induce platelet aggregation *per se*, but significantly enhanced aggregation induced by secondary agonists such as EPI, ADP, and THR (Figure 5, panels a-c). This priming effect on platelet aggregation in PRP was seen with all plasma samples examined.

Plasma from healthy subjects did not prime platelet aggregation in PRP (Figure 5, panels a-c). Representative aggregation traces are shown in Supplementary Figure 1.

We have then studied the effects of plasma from normal subjects and COVID-19 patients on monocyte- and granulocyte-platelet binding in whole blood from

healthy donors. Neither plasma from COVID-19 patients nor from healthy subjects increased monocyte- and granulocyte-platelet binding in whole blood *per se* (Figure 5, panels d-i). On the contrary, plasma from COVID-19 patients, but not from healthy subjects, significantly enhanced monocyte-platelet binding induced by EPI, ADP or THR in whole blood, as determined by flow-cytometric analysis (Figure 5, panels d-f), as well as granulocyte-platelet binding (Figure 5, panels g-i).

#### Role of THPO in the priming activity of plasma from COVID-19 patients

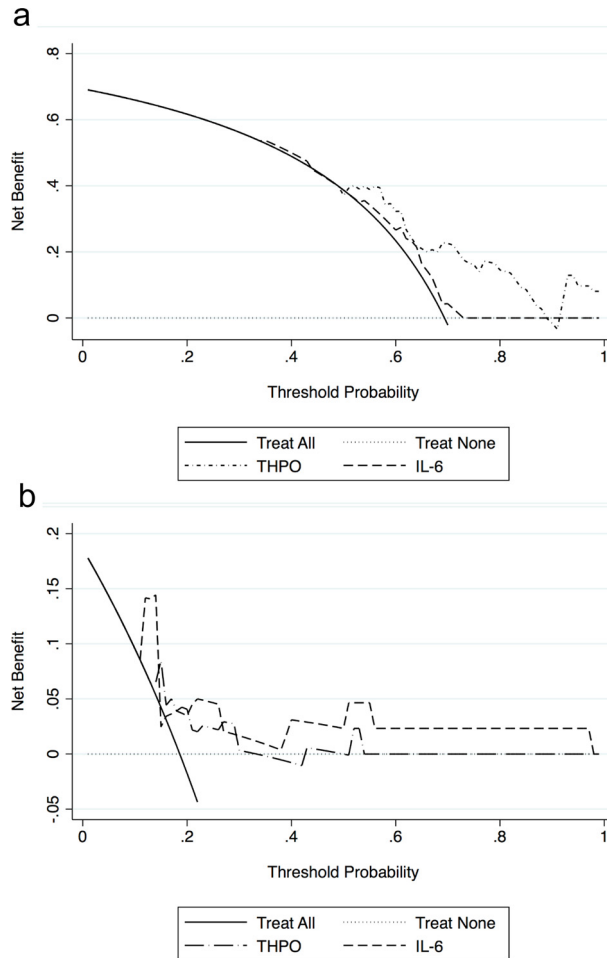
Circulating THPO levels measured *in vivo* correlated with the *in vitro* priming activity exerted by plasma samples on platelet aggregation in PRP. In particular, plasma THPO levels significantly correlated with the priming index induced in PRP by EPI ( $r = 0.6865$ ;  $p = 0.008$ ), ADP ( $r = 0.6041$ ;  $p = 0.0149$ ), and THR ( $r = 0.7637$ ;  $p = 0.0034$ ). These results are coherent with the hypothesis that increased THPO may be implicated in priming platelet activation *in vivo* in COVID-19 patients.

In order to investigate whether elevated circulating levels of THPO may contribute to enhanced platelet activation in COVID-19 patients, we studied *in vitro* the effects of two different inhibitors of THPO biological activity, a rhTHPOR and a neutralizing antibody directed against THPO receptor, Ab  $\alpha$ THPOR, on the priming effect exerted by patient plasma samples both on platelet aggregation in PRP and on leukocyte-platelet adhesion in whole blood from healthy subjects.

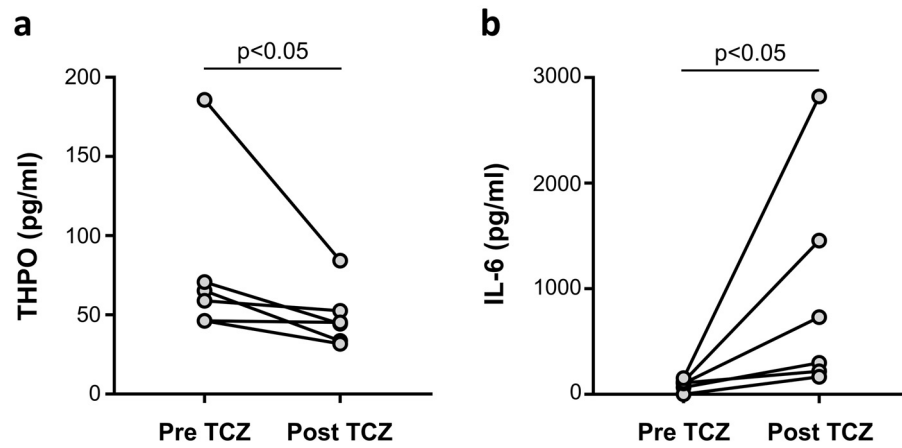
In preliminary experiments, we verified that both inhibitors block the biological activity of rhTHPO *in vitro*, and that they have no effect either on platelet aggregation in PRP (Supplementary Figure 2) and on leukocyte-platelet binding in whole blood (Supplementary Figures 3 and 4). In addition, both inhibitors were able to abolish the stimulatory effect exerted by rhTHPO on cell proliferation in the M-07 cell line, evaluated using a BrdU Incorporation Assay (Supplementary Figure 5).

The pre-incubation of plasma from COVID-19 patients with rhTHPOR reduced the priming effect exerted on platelet aggregation in PRP by EPI (Figure 6a), ADP (Figure 6c), and THR (Figure 6e). On the contrary, the pre-incubation of plasma samples from healthy subjects with rhTHPOR did not modify the effects observed after stimulation with EPI, ADP and THR on platelet aggregation in PRP (Figure 6, panels a-c).

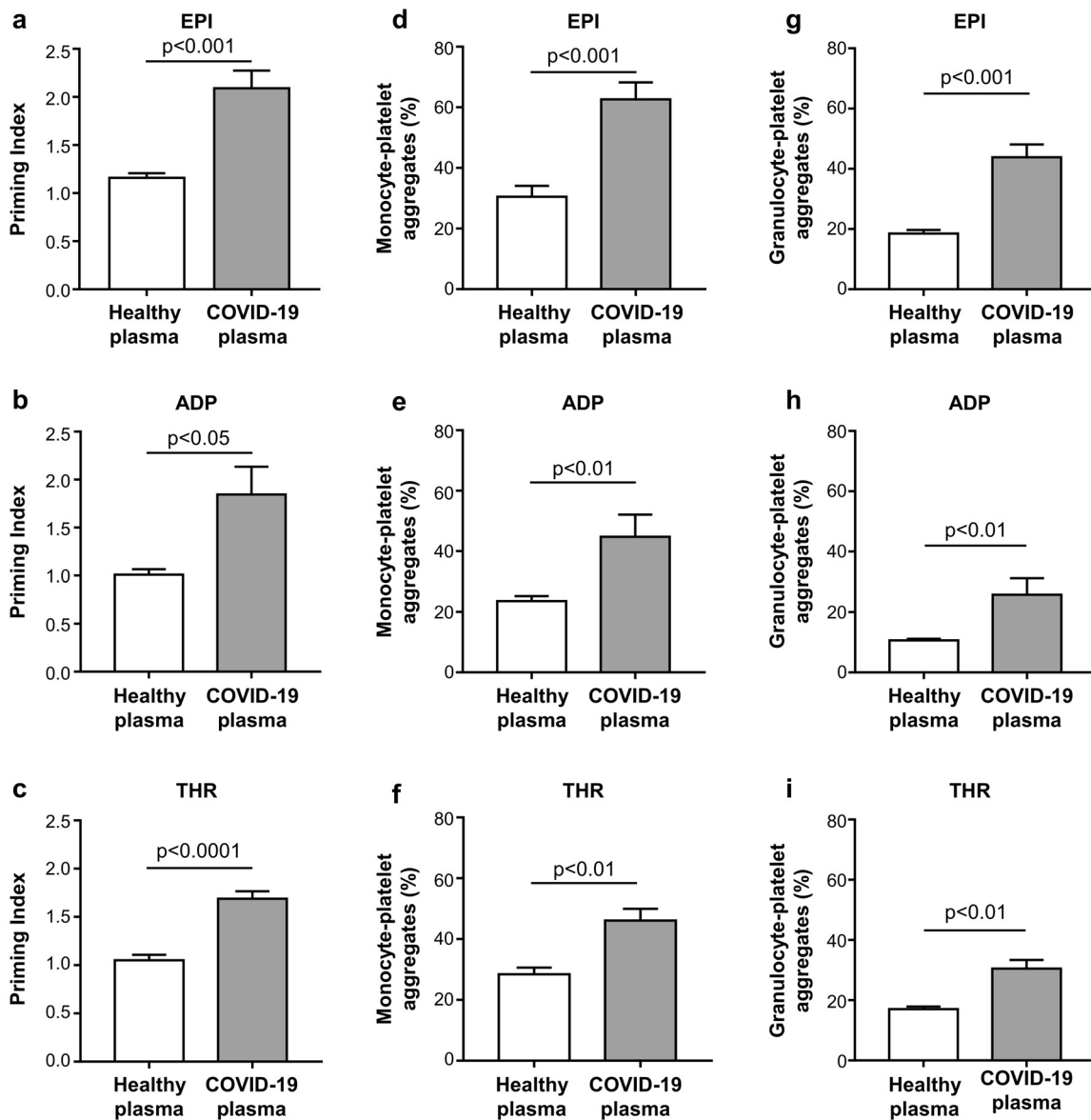
Analogously, pre-treatment of PRP with Ab  $\alpha$ THPOR decreased the priming effect exerted by plasma from COVID-19 patients on platelet aggregation in PRP induced by EPI (Figure 6d), ADP (Figure 6e), and THR (Figure 6f), whereas it did not modify the



**Figure 3.** Decision curves for THPO and IL-6 for the diagnosis of COVID-19 (a) and for the risk of severe COVID-19 development (b).



**Figure 4.** Effect of tocilizumab (TCZ) administration on THPO and IL-6 plasma levels in six COVID-19 patients. Scatter plots of THPO (a) and IL-6 (b) plasma levels measured pre- and post-TCZ administration in COVID-19 patients. Data were analyzed by using Wilcoxon matched-pairs signed rank test.



**Figure 5.** Effect of plasma from healthy subjects and COVID-19 patients on *in vitro* platelet aggregation in platelet-rich plasma (PRP).

Bar graph showing the *in vitro* priming activity induced by plasma from healthy subjects and COVID-19 patients on platelet aggregation in PRP induced by epinephrine (EPI) (a), adenosine-diphosphate (ADP) (b), and thrombin (THR) (c).

Bar graph showing the *in vitro* effect of plasma from healthy subjects and COVID-19 patients on monocyte-platelet aggregation induced in whole blood by epinephrine (EPI) (d), adenosine-diphosphate (ADP) (e), and thrombin (THR) (f).

Bar graph showing the *in vitro* effect of plasma from healthy subjects and COVID-19 patients on granulocyte-platelet aggregation induced in whole blood by epinephrine (EPI) (g), adenosine-diphosphate (ADP) (h), and thrombin (THR) (i).

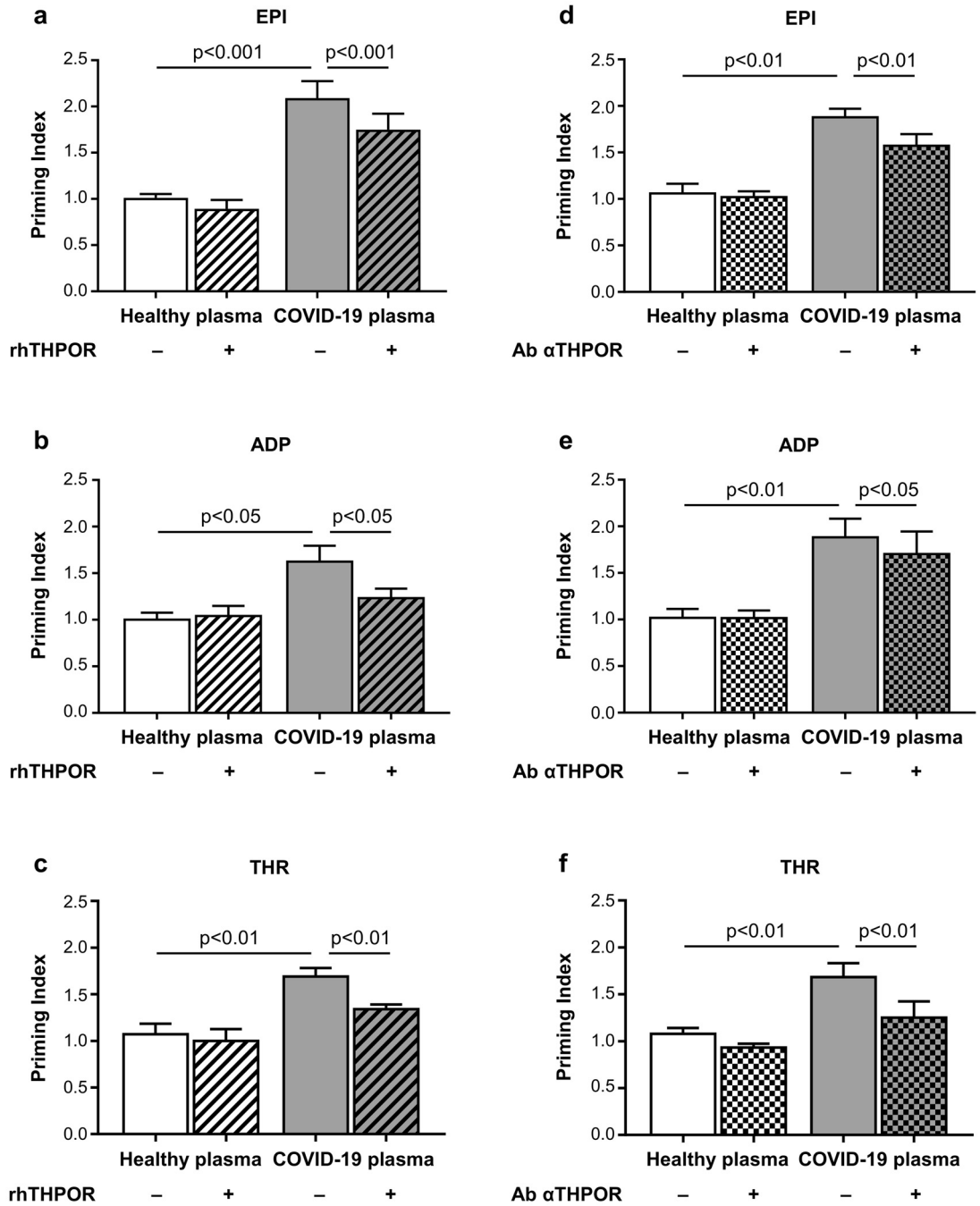
A minimum of five experiments for each experimental conditions was performed. Data represent means  $\pm$  SE, and were analyzed by using Student's t-test.

effect of plasma samples from healthy subjects (Figure 6, panels d-f).

Representative aggregation traces are shown in Supplementary Figure 6.

THPO concentrations measured *in vivo* also correlated with the *in vitro* priming activity of COVID-19

plasma samples on leukocyte-platelet adhesion in whole blood, as evaluated by flow cytometry analysis. Plasma THPO levels, indeed, correlated with the increase in monocyte-platelet aggregates induced in whole blood by EPI ( $r = 0.7626$ ;  $p = 0.0022$ ), ADP ( $r = 0.7857$ ;  $p = 0.0008$ ), and THR ( $r = 0.6455$ ;  $p = 0.0368$ ), as well as



**Figure 6.** Inhibitory effect of rhTHPOR and Ab αTHPOR on *in vitro* platelet aggregation in platelet-rich plasma (PRP).

Bar graph showing the effects exerted by plasma of COVID-19 patients pre-incubated or not with rhTHPOR on platelet aggregation induced in PRP by EPI (a), ADP (b), and THR (c).

Bar graph showing the effects exerted by plasma of COVID-19 patients, after pre-incubation of PRP or not with Ab αTHPOR, on platelet aggregation induced in PRP by EPI (d), ADP (e), and THR (f).

A minimum of five experiments for each experimental conditions was performed. Data were analyzed by using paired Student's t-test.

with the increase in granulocyte-platelet aggregates induced in whole blood by EPI ( $r = 0.7855$ ,  $p = 0.0013$ ) and ADP ( $r = 0.8322$ ;  $p = 0.0013$ ), but not by THR ( $r = 0.4455$ ;  $p = 0.1730$ ).

Moreover, the pre-treatment of plasma from COVID-19 patients with rhTHPOR decreased monocyte-platelet binding induced in whole blood by EPI, ADP, and THR (Figure 7, panels a-c), as well as granulocyte-platelet binding induced by EPI, ADP, and THR (Figure 8, panels a-c). On the contrary, pre-incubation of plasma samples from healthy subjects with rhTHPOR did not modify the effects observed after stimulation with EPI, ADP and THR on both monocyte-platelet aggregation (Figure 7, panels a-c) and granulocyte-platelet aggregation (Figure 8, panels a-c).

In the same way, pre-treatment of whole blood samples from healthy subjects with Ab  $\alpha$ THPOR reduced the increase induced by plasma from COVID-19 patients in monocyte-platelet binding after stimulation with EPI (Figure 7d), ADP (Figure 7e), and THR (Figure 7f), as well as in granulocyte-platelet binding (Figure 8, panels d, e, and f for EPI, ADP, and THR stimulation, respectively). On the contrary, the pre-treatment of whole blood samples with Ab  $\alpha$ THPOR did not modify the effects observed with plasma samples from healthy subjects either on monocyte-platelet binding (Figure 7, panels d-f) or granulocyte-platelet binding (Figure 8, panels d-f).

Finally, we evaluated the effect of plasma samples obtained from COVID-19 patients who were treated with tocilizumab as compared to plasma samples obtained from the same patients before tocilizumab administration. *In vivo* blocking of IL-6 biological activity markedly reduced the priming index induced in PRP (Figure 9). These results further suggest that the elevated concentrations of THPO in plasma samples from COVID-19 patients are involved in priming platelet activation in COVID-19.

## Discussion

Our study is the first evaluating THPO in patients with COVID-19 at the time of diagnosis and first evaluation in the ED, and the potential involvement of THPO in enhancing platelet activation in COVID-19 patients.

Key findings of the present study are that THPO concentrations were higher both in COVID-19 compared to Non-COVID-19 patients, and in patients developing severe COVID-19 compared to patients with persistently mild COVID-19. These results suggest that increased THPO concentrations are specific of COVID-19 pathogenesis, and that THPO may be proposed as potential diagnostic and prognostic biomarker in the early phase of COVID-19. This represents incremental knowledge over previous evidence showing increased circulating THPO levels in severe COVID-19 patients compared to healthy controls.<sup>52</sup>

Although several previous studies had shown that THPO is elevated in patients affected by several critical diseases,<sup>40-46</sup> very little data was available until now on the levels of THPO in patients with COVID-19, and from a different clinical setting.<sup>52</sup>

On the basis of the ability of THPO to enhance platelet activation in several diseases,<sup>36</sup> including unstable angina<sup>40</sup> and burn injury, especially after sepsis development,<sup>44</sup> as well as in cigarette smokers,<sup>61</sup> we sought to evaluate whether THPO may be involved in sustaining platelet activation also in COVID-19 patients.

The presence of THPO in the circulation of COVID-19 patients precludes the evaluation of its role on platelet aggregation directly on blood samples obtained from patients. In addition, we were not allowed, in our laboratory, to work directly on blood and plasma samples or cells obtained from COVID-19 patients, which represents an important limit of our study. Therefore, in order to reproduce the potential effect of increased THPO concentrations in COVID-19, we choose to study *in vitro* the effects of patient plasma samples both on platelet aggregation in PRP and on leukocyte-platelet adhesion in whole blood from healthy subjects, and to indirectly evaluate the role of THPO by inhibiting its biological activity by using two different inhibitors.

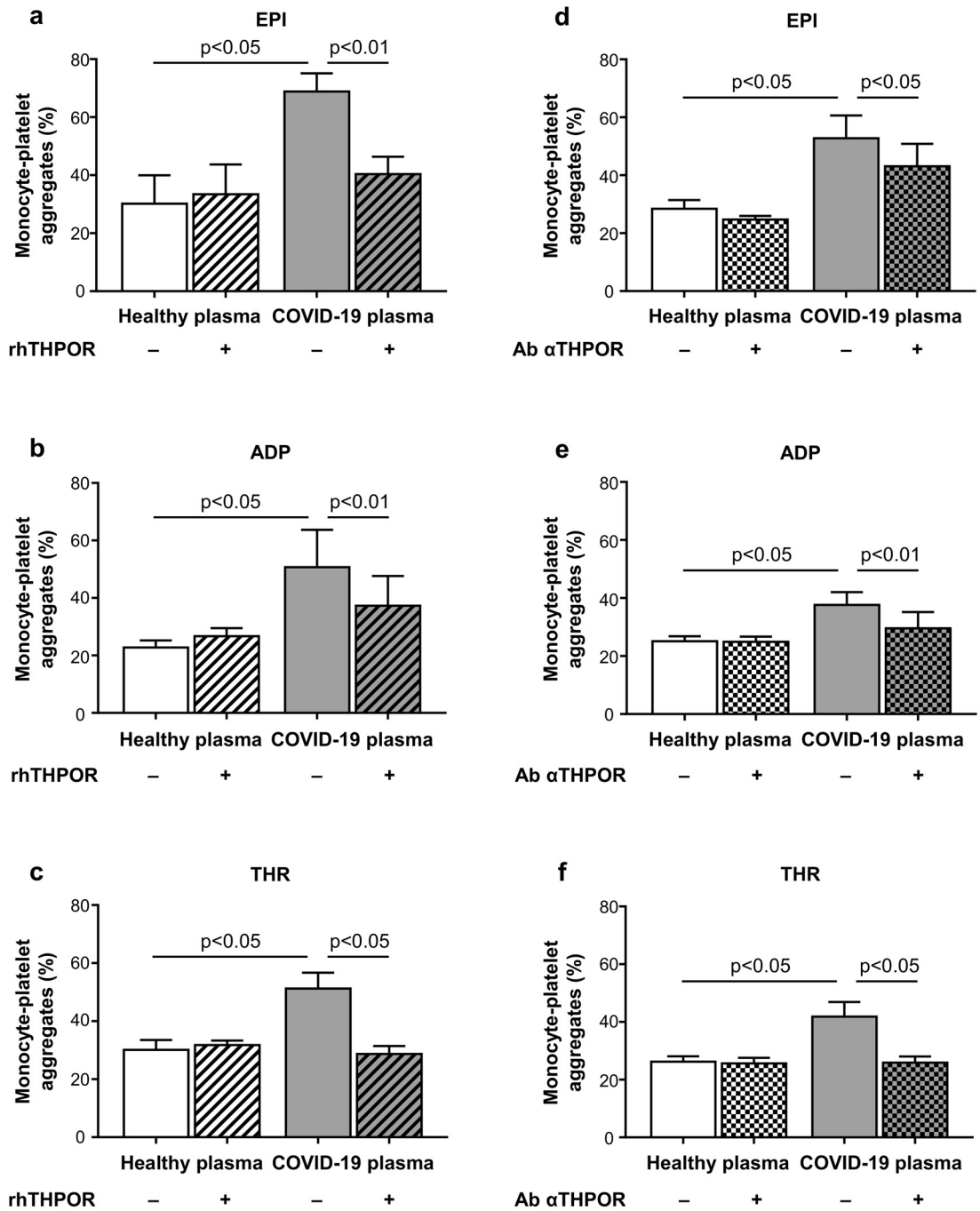
In preliminary experiments, we verified that both inhibitors, the rhTHPOR and the neutralizing antibody against hTHPOR, are able to block the biological activity of rhTHPO *in vitro*, and that they have no effect when added to plasma samples from healthy donors neither on platelet aggregation in PRP nor on leukocyte-platelet binding in whole blood.

In these experimental conditions, plasma from COVID-19 patients did not stimulate *per se* platelet aggregation in PRP as well as monocyte- and neutrophil-platelet binding in blood from healthy donors, but it was able to enhance the effect of different agonists (EPI, ADP, and THR) on these biological events.

The contribution of THPO to this priming effect is suggested by: (1) the correlation analysis showing that THPO levels and both EPI-, ADP- and THR-induced priming index in PRP consensually increased; (2) the correlation analysis between THPO levels and leukocyte-platelet adhesion in whole blood, and (3) the inhibitory effect of rhTHPOR and Ab  $\alpha$ THPOR on these phenomena.

Taken together, our *in vitro* data support the hypothesis that THPO present in the circulation of COVID-19 patients may be implicated in COVID-19-related thromboinflammation by sensitizing circulating platelets to the action of other agonists, thus concurring to precipitate the occurrence of microvascular thrombosis and the clinical onset of respiratory failure and/or organ damage.

Although we observed a significant reduction of the priming effect exerted by plasma samples from COVID-19 patients by inhibiting THPO biological activity, this

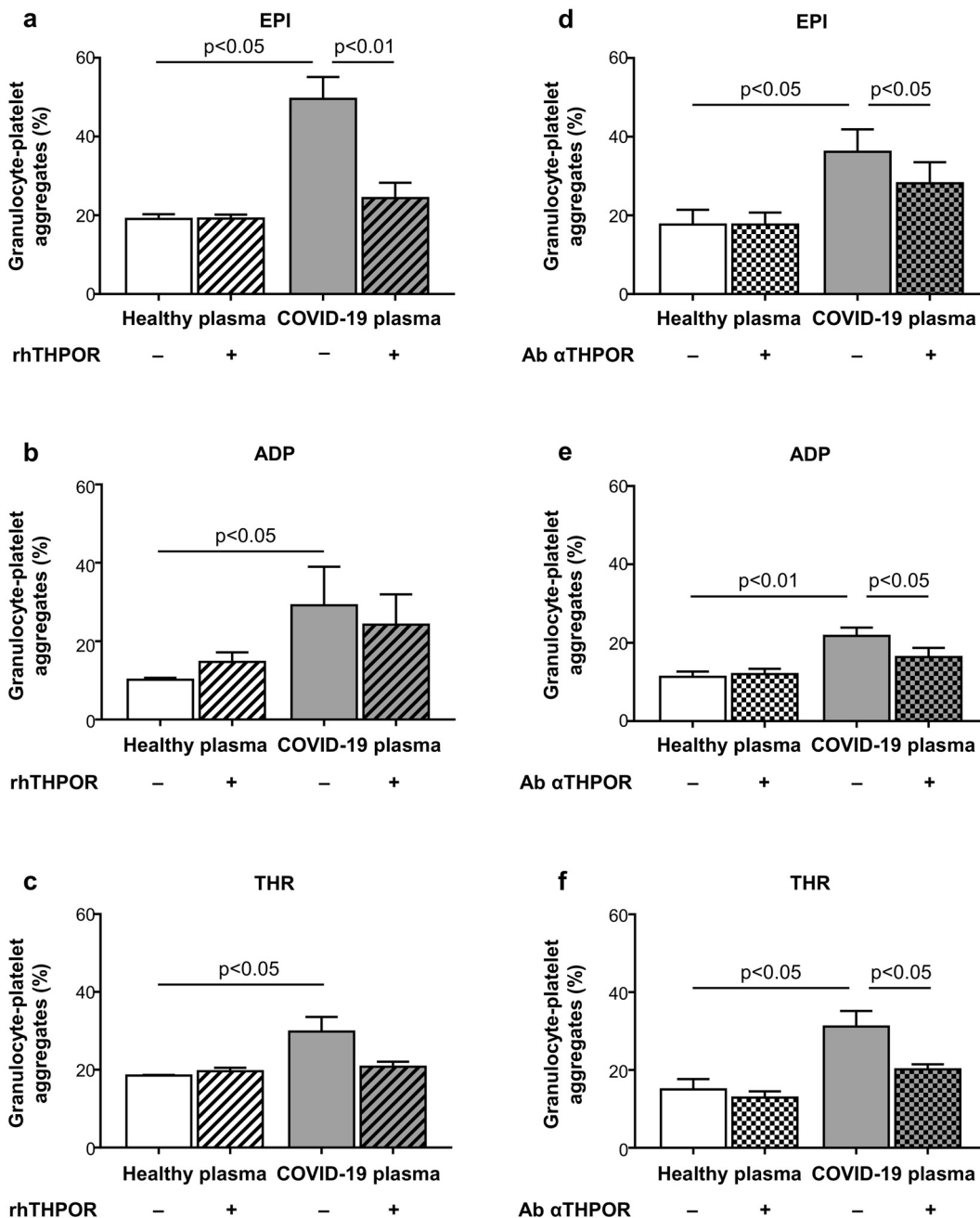


**Figure 7.** Inhibitory effect of rhTHPOR and Ab  $\alpha$ THPOR on *in vitro* monocyte-platelet aggregation in whole blood.

Bar graph showing the effects exerted by plasma of COVID-19 patients pre-incubated or not with rhTHPOR on monocyte-platelet aggregation induced in whole blood by EPI (a), ADP (b), and THR (c).

Bar graph showing the effects exerted by plasma of COVID-19 patients, after pre-incubation of whole blood or not with Ab  $\alpha$ THPOR, on monocyte-platelet aggregation induced in whole blood by EPI (d), ADP (e), and THR (f).

A minimum of five experiments for each experimental conditions was performed. Data were analyzed by using paired Student's t-test.



**Figure 8.** Inhibitory effect of rhTHPOR and Ab  $\alpha$ THPOR on *in vitro* granuloocyte-platelet aggregation in whole blood.

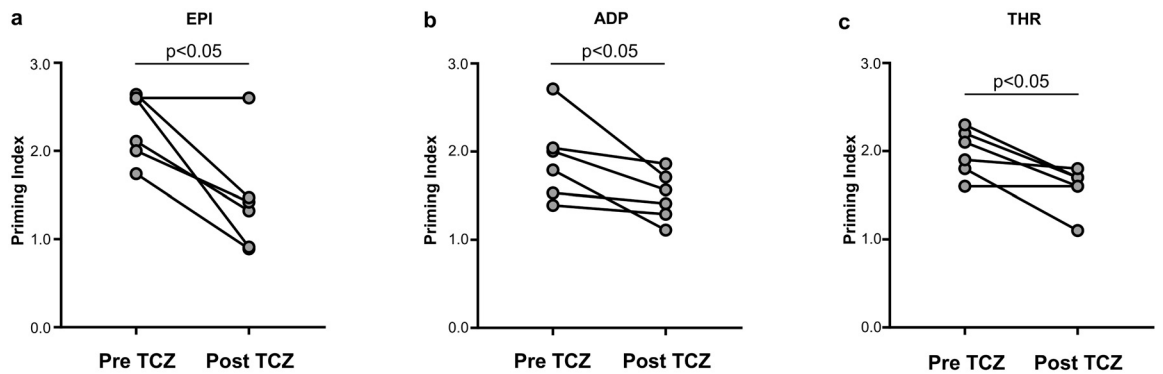
Bar graph showing the effects exerted by plasma of COVID-19 patients pre-incubated or not with rhTHPOR on granuloocyte-platelet aggregation induced in whole blood by EPI (a), ADP (b), and THR (c).

Bar graph showing the effects exerted by plasma of COVID-19 patients, after pre-incubation of whole blood or not with Ab  $\alpha$ THPOR, on granuloocyte-platelet aggregation induced in whole blood by EPI (d), ADP (e), and THR (f).

A minimum of five experiments for each experimental conditions was performed. Data were analyzed by using paired Student's t-test.

inhibitory effect was only partial, accounting for about 30% of the priming effect observed. This observation suggests that other mediators, in addition to THPO,

also participate in inducing the priming effect exerted by plasma from COVID-19 patients on platelet activation. In our experimental model, no priming effect on



**Figure 9.** Effect of plasma obtained from six COVID-19 patients before and after tocilizumab (TCZ) administration on *in vitro* platelet aggregation in platelet-rich plasma (PRP).

Scatter plots showing the *in vitro* priming activity induced by plasma obtained from COVID-19 patients before and after TCZ administration on platelet aggregation in PRP induced by epinephrine (EPI) (a), adenosine-diphosphate (ADP) (b), and thrombin (THR) (c).

Data were analyzed by using Wilcoxon matched-pairs signed rank test.

platelet aggregation in both PRP and whole blood was previously seen using TNF- $\alpha$ , IL-1, IL-6, IL-3, GCSF, or GM-CSF,<sup>44</sup> as also shown by other studies.<sup>62</sup> However, we can hypothesize that other inflammatory mediators or activated coagulation factors themselves may cooperate in inducing this effect.

A central open question coming from our and other studies is whether plasma factors directly mediate platelet hyperactivity or make platelets intrinsically hyperactive. Since, in our laboratory, we are not allowed to manipulate platelets isolated from COVID-19 patients, we cannot provide evidence directly exploring this phenomenon *in vivo* in COVID-19 patients. Moreover, the presence of plasma factors in the patient circulation makes extremely difficult to assess their role on platelet function *in vivo*.

Another hypothesis that can be proposed is that sustained or prolonged signaling triggered by THPO receptor stimulation *in vivo* would be at the basis of platelet hyper-reactivity in COVID-19. Unfortunately, we cannot directly address this research question, which is beyond the scope of the present study.

An observation that could argue against the role of THPO in platelet activation in COVID-19 is the down-regulation of platelet THPO receptor demonstrated by transcriptomic analysis,<sup>28</sup> or the decrease of surface expression of THPO receptor following internalization upon binding with its ligand THPO.<sup>40,63,64</sup> Whereas it is reasonable to hypothesize that the down-regulated expression of THPO receptor could make platelets less responsive to THPO, it is possible that the stimulation of platelets by THPO, in association with other soluble mediators, may induce a phenotypic switch in circulating platelets that makes the pro-aggregatory phenotype stable for longer time. In accordance with this hypothesis, previous experiments from our group had shown

that the stimulation of platelets *in vitro* with THPO has long-term effect, lasting up to 24 h.<sup>37</sup>

In addition to its actions on platelet functions, THPO also has other biological activities on different cell types that may be important in the pathophysiology of inflammatory reactions and thrombotic complications during COVID-19.<sup>36</sup> For instance, THPO stimulates IL-8 and reactive oxygen species release from neutrophils,<sup>65</sup> promotes angiogenesis,<sup>66</sup> and modulates the apoptotic processes in different mature cells.<sup>64,67</sup>

Interesting insights on the origin of the rise in THPO levels observed in COVID-19 patients came from the study of circulating IL-6 levels. High levels of IL-6 in COVID-19 patients have been described in several studies, and correlate with disease severity,<sup>13</sup> leading to propose the use of neutralizing antibodies against IL-6 as a therapeutic option, especially in the most critically ill COVID-19 patients.<sup>60</sup> In our study, IL-6 levels did not significantly differ between COVID-19 patients and Non-COVID-19 patients, whereas they resulted more elevated in severe compared to mild COVID-19 patients. Interestingly though, the concentrations of IL-6 significantly correlated with those of circulating THPO, suggesting a possible relationship between the increased concentrations of these two cytokines. Finally, when we measured the levels of IL-6 and THPO in COVID-19 patients treated with tocilizumab, we found that IL-6 levels increased, as already reported in the literature,<sup>59,68</sup> while THPO concentrations markedly decreased. These results, in accordance with previous studies showing increased hepatic synthesis stimulated by IL-6 in hepatocytes,<sup>47</sup> suggest that IL-6-driven THPO synthesis in the liver has a prominent role in COVID-19 patients in sustaining elevated THPO levels, which, in turn, may activate circulating platelets.



Another hypothesis that may be evoked to explain the rise in THPO levels is based on data indicating platelet mass as the primary regulator of circulating THPO levels.<sup>35</sup> In our study, we found an inverse correlation between THPO level and platelet count in COVID-19 patients. In contrast, we did not find any correlation between THPO and indexes of increased platelet turnover, such as MPV and PDW, a result that does not support a causative role of higher platelet turnover in sustaining the rise of THPO levels. Nonetheless, others studies have previously shown a trend for increased MPV in COVID-19 patients, a result that may be coherent with the hypothesis of an increase in THPO concentrations secondary to reduced platelet number and mass.<sup>16,69</sup> Ideally, it would have been very interesting to study other parameters of increased platelet turnover, such as immature platelet number (IPN) and immature platelet fraction (IPF), but the measurement of these parameters was not possible in our clinical laboratory.

Finally, we cannot exclude that the increase in circulating THPO were, at least partially, due to its release from activated platelets themselves,<sup>70</sup> and therefore be the consequence, rather than the cause, of increased platelet activation determined by other mechanisms. However, the results of the experiments on COVID-19 patients treated with tocilizumab seem to exclude this hypothesis in favor of the prominent role of IL-6-driven increased hepatic synthesis.

Our study has some major limitations: it is a single-center investigation, and it enrolled a small number of patients. Therefore, our results need to be confirmed in larger multi-institutional randomized trials. As already anticipated above, another major limit of our study is represented by the impossibility of directly working with blood and plasma samples or cells obtained from COVID-19 patients. In addition, due to the limited number of patients enrolled and of those who developed severe infection, although we have considered performing a multivariate analysis, this was not possible due to the reduced degrees of freedom and the high risk of overfitting. Finally, we cannot exclude that other confounding factors, which were not explored in our study, may also have had a role in determining the results obtained.

In conclusion, our results suggest that elevated levels of THPO, probably driven by IL-6-stimulated increased hepatic synthesis, may contribute to enhance platelet activation and leukocyte-platelet interaction in COVID-19 patients, thus potentially participating in the pathogenesis of thromboinflammation and organ damage development in this disease. In addition, our data provides the first evidence for the potential of circulating THPO as early diagnostic and prognostic biomarker useful to timely identify patients affected by COVID-19 in the ED.

## Contributors

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## Data sharing statement

Deidentified participant data will be made available on a collaborative basis upon reasonable request with publication.

## Declaration of interests

Authors declare that no conflict of interest exists.

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## Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:[10.1016/j.ebiom.2022.104305](https://doi.org/10.1016/j.ebiom.2022.104305).

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