Markers and mediators in sepsis: extracellular vesicles and beyond

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CHAPTER 1

General Introduction and thesis outline

Sepsis

Sepsis is a life-threatening systemic illness associated with a dysregulated host response to an infection caused by pathogen(s) such as bacteria, viruses, or fungi [1]. The global burden of sepsis is increasing, with approximately 49 million new cases and 11 million deaths annually [2]. It is more common than heart attack and claims more lives than any cancer [3]. Moreover, sepsis is a common disease in the elderly and its incidence increases exponentially with age [4]. It is the leading cause of death in hospitals and intensive care units, with mortality rates ranging from 10% to 50% depending on the severity of the infection and the patient's overall health status [5]. Furthermore, sepsis survivors are at substantial risk for poor quality of life, functional disability, and cognitive impairment [2,3].

Sepsis can occur when an infection in one part of the body, such as the lungs, urinary tract, or skin, spreads to the bloodstream and triggers a generalized immune response [6]. Clinical features include consumptive thrombocytopenia, coagulopathy, and multiorgan dysfunction syndrome. In the most severe forms of the disease, sepsis can lead to septic shock owing to circulatory and cellular metabolism abnormalities that are sufficient to increase mortality [7]. In addition, sepsis has a rapid onset and symptoms similar to those of other critical diseases [8].

Given that there is currently no gold standard diagnostic tests, early recognition and timely management are key to helping reduce patient prognosis and mortality while also reducing hospital stay, which is cost-effective and resource-conserving [3]. Furthermore, there is increasing awareness that biomarkers are essential for understanding the pathophysiology of sepsis and guiding therapeutic choices. The development of novel sepsis markers will likely lead to a better characterization of sepsis and may help for its early detection and for the evaluation of patient's clinical courses.

Extracellular vesicles

Extracellular vesicles (EVs) are nano-sized membrane vesicles that are secreted by a variety of cell types [9]. They play a key role in communication networks via their diverse array of biological cargo, including DNA, mRNA, microRNAs, proteins and bioactive lipids, carrying information to target cells in a paracrine fashion or via circulation [10,11]. There is evidence that EVs are involved in many pathophysiological processes, including cellular homeostasis [12], infection transmission [13], cancer development [14], and cardiovascular disease [15].

Over the past two decades, the multifaceted role of EVs in inflammation has entered the focus of sepsis research as inducers, mediators, and markers [16]. Both sepsis and EVs are very complex

research fields, and the existing evidence regarding their relationship is limited. The role of EVs as sepsis mediators was shown in experiments with septic-EV transfer. The transfer of EVs derived from septic mice in healthy mice led to the induction of a sepsis-like condition indicated by leukopenia, intrahepatic inflammation, and bone marrow hyperplasia [17]. A triggering event, such as injury or infection in the context of environmental and genetic factors, causes alterations in EV functions, and homeostasis is interrupted [18]. Septic patients have substantially higher concentrations of circulating EVs than matched controls, and EV number correlates with the severity of sepsis [19]. The pathophysiological changes of sepsis are dynamic, and EVs released by multiple activated cells can synergistically lead to specific pathophysiological changes, such as endothelial dysfunction, coagulation abnormalities, and circulatory and organ dysfunction [19–21].

With the development and application of omic technologies, more and more studies have shown that the expression profile of cargo carried by EVs in sepsis is dynamic, which helps correlate protein, RNA expression and metabolic alterations in EVs with specific clinical features [22]. EVs represent a hidden reservoir of immune modulators with cargo that dictates cell-to-cell and tissue-to-tissue communication that can, in certain clinical statuses, act deleteriously to keep the immune system in a chronically activated inflammatory, or suppressed state [23]. EVs can carry altered cargo, including damage-associated molecular patterns (DAMPs), cytokine and miRNA species [24]. The sequelae of altered EV signaling to cells and tissues may result in immune activation (or suppression) and enhance tissue damage, triggering a vicious cycle of immune dysfunction driven by further tissue damage and further release of altered EVs [21]. Furthermore, EVs possess surface markers that reflect their cell of origin, enabling them to serve as biomarkers with high fidelity [25–27]. EVs may also act as a “biopsy” for other tissues not routinely collected from patients [28,29]. Taken together, EVs are an attractive area of research for understanding and predicting disease-associated pathology and mortality in sepsis.

Sepsis in burn patients and extracellular vesicles

Sepsis is the leading cause of death in burn patients, involving up to 65% of burn patients, depending on age and burn severity [30,31]. Several differences exist between sepsis in the general population and sepsis occurred after burn injury [32]. Burn patients lose the first barrier to infection, their skin, and thus the risk of infection persists as long as that this barrier is absent [33]. The initial phase after burn injury is characterized by an enormous release of pro-inflammatory cytokines, leading to systemic inflammatory response syndrome (SIRS) [34,35]. Secondary complications typically occur

because of systemic immune dysfunction that develops in response to injury, which can lead to immunosuppression, coagulopathy, multiple organ failure, and unregulated inflammation [32].
The diagnosis and management of sepsis in burn wound infections remain challenging due to the many features unique to burn injuries. Often, burn patients were excluded from every sepsis study due to their heterogeneity. Therefore, due to the critical complications that characterize burn patients, the role of EVs in these patients has not been fully investigated.

COVID-19

In recent years, the COVID-19 pandemic has profoundly impacted healthcare systems and individual's health. By applying the Sepsis-3 definitions [1], it became obvious that almost 80% of hospitalized patients infected by severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) had viral sepsis [36]. Thus, not surprisingly, many features and parameters reported for bacterial sepsis were recovered in COVID-19 [37]. COVID-19 symptoms can range from flu-like symptoms to severe pneumonia, acute respiratory distress syndrome (ARDS), clotting disorder, multi-organ dysfunction syndrome (MODS), and death [38]. Clinically, the COVID-19 infection triggers a biphasic illness [39]. In the first phase, the virus infects the host and replicates, causing flu-like symptoms, and most patients recover in 5-6 days without further complications. However, some patients enter a second phase, which is characterized by hyper-inflammation causing pneumonia and/or clotting disorders. While reliable tests are available for rapid, early detection of SARS-CoV-2 infection, there are no reliable tests available that can anticipate this second phase of the illness.

Thrombopoietin

Thrombopoietin (THPO) is a growth factor recognized for its function as the primary regulator of megakaryocyte (MK) expansion and differentiation [40,41]. THPO is widely expressed with mRNA being present at the highest levels in the liver, but also found in the kidney, smooth muscle, and bone marrow stromal cells [40,42]. In normal physiologic conditions, plasma concentrations of THPO vary inversely with platelet count [43]. Platelets can absorb constitutively synthesized THPO and destroy it, thereby helping to maintain a physiologic balance of the cytokine [42]. Despite multiple mechanisms whereby THPO is autoregulated, many inflammatory states are associated with THPO levels well above the expected levels based on patient's platelet counts [43,44]. Significant elevation of THPO and secondary thrombocytosis, indeed, is observed in many clinical inflammatory conditions, including malignancy [45,46]. This elevation in THPO and platelet count is mediated by interleukin (IL)-6, which increases THPO production both in vitro and in vivo [42].

Increased plasma levels of THPO have been described in several critical diseases where platelet activation represents a crucial pathogenic mechanisms, including unstable angina [47], burn injury [48], acute pancreatitis [45], and severe acute respiratory syndrome [49]. Furthermore, disease severity is a major determinant of elevated THPO in patients with sepsis [50,51], suggesting that THPO may be proposed as a potential marker and mediator in this pathologic condition.

microRNAs

microRNAs (miRNAs) are small non-coding RNAs that play relevant roles in regulating gene expression by binding the 3' untranslated region of target RNAs [52]. Another important role played by miRNAs is intercellular signaling [53]. Even though most of the miRNAs are found inside the cell, there is a large proportion that migrates outside it and can be found in bodily fluids [53]. These are called circulating miRNAs, and they are discharged in blood, urine, saliva, and other fluids as a consequence of tissue damage, apoptosis, and necrosis, active passage in extracellular vesicles, or bonding to a protein [54]. In recent years, extraordinary progress has been made in terms of elucidating the origin and functions of miRNAs and their potential use in research and clinical practice [55,56]. Of note, several miRNAs are key regulators of inflammation-related mediators, being essential in some inflammatory diseases [57]. However, probably the most promising role of circulating miRNAs is that of potential biomarkers. Numerous authors have investigated this potential application in various medical fields [55,56,58]. Evidence suggests that miRNAs could have an essential role as biomarkers in heart failure [59] or, in case of infectious diseases [60], for the diagnosis of sepsis [61]. This novel class of molecules possesses several advantages that could turn them into ideal candidates as biomarkers in a variety of pathological conditions. The ideal biomarker needs to be easily accessible, a condition that applies to miRNAs that can easily be extracted through liquid biopsies from blood and other bodily fluids. Moreover, it also needs to possess high specificity for the tissue or cell type of provenance and to be sensitive in the way that it varies according to the disease progression. Finally, the technologies for the detection of nucleic acids already exist, and the development of new assays requires less time and lower costs in comparison to producing new antibodies for protein biomarkers.

Thesis outline

Considering the rising burden of sepsis, the improvement and development of novel markers for a better characterization of sepsis are in high demand. With the recent advances in molecular approaches, EVs and miRNAs have been functionally annotated to have several roles in sepsis onset and progression. Concomitantly, strategies to modulate their expression have also been emerging,

presenting EVs and miRNAs as versatile tools to alter gene expression and extend the druggable genome.

Recent evidence suggests that EVs are associated with several phases of sepsis development [62]. Furthermore, the biological potential of EVs as mediators of the mechanisms of sepsis is still only partially known. However, the rapid development of novel molecular platforms is contributing to envisioning and developing theragnostic applications of EVs in critical illness.

In this thesis, we aim to shed light on the complex biology of EVs and miRNAs and their potential as markers and mediators in sepsis.

Chapter 2 summarizes the current experimental evidence on the role of EVs in sepsis and COVID-19, with particular attention to their potential role in the pathophysiological mechanisms of thromboinflammation. The complex interrelationship and similarities between sepsis and COVID-19 have recently emerged with particular emphasis [63,64]. Based on the well-established pathogenic role of thromboinflammation in the development of organ damage in critically ill patients affected by sepsis, we discuss the experimental evidence on the role of EVs in thromboinflammation, both in bacterial sepsis and in COVID-19, aiming to explore potential targets useful to translate the knowledge into the discovery of novel potential diagnostic and therapeutic applications of EVs in these diseases.

Our mechanistic understanding of post-burn inflammation and of its systemic propagation is limited. Moreover, the relationship between sepsis in burn patients and EV release in this setting remains unexplored. In **chapter 3**, we characterize, for the first time, plasma-derived EVs released during septic shock in burn patients and performed multiplexed phenotyping aiming for a more detailed view of their origin. Moreover, we evaluated the effects of EVs from burn septic shock patients on *in vitro* platelet aggregation, identifying them as potential novel mediators of platelet activation and thromboinflammation during septic shock in burn patients.

In chapter 2, we introduced the concept that sepsis is the final common pathway to death from most infectious diseases worldwide, including viral infections such as SARS-CoV-2. COVID-19 may profoundly vary from mild to lethal forms and inflammation-associated thrombosis, named thromboinflammation, occurs commonly in a broad range of human disorders, including COVID-19, heavily affecting patient's prognosis. One of the most impelling clinical needs is the identification of reliable markers to early define patient severity and predict clinical prognosis at the time of first medical evaluation. On the other hand, a better understanding of pathophysiologic mechanisms

underlying the development of thromboinflammation in COVID-19 and other critical diseases is needed.

In **chapter 4**, we aimed to investigate the levels of THPO and IL-6 in COVID-19 patients at the time of first evaluation in the Emergency Department and to evaluate THPO as early biomarker for the diagnosis of COVID-19 patients. In addition, we studied the potential correlation of THPO with IL-6 levels, and the role of THPO in stimulating *in vitro* platelet priming.

In **chapter 5** we aimed to investigate miRNA profile and explore its potential utility as diagnostic and severity marker in patients with COVID-19 by matching *in vitro* and *in silico* approaches.

Finally, **Chapter 6** presents a discussion of the findings of this thesis in light of the current literature and proposes future research directions in the field.

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CHAPTER 2

Extracellular vesicles: new players in the mechanisms of sepsis- and COVID-19-related thromboinflammation

Extracellular vesicles: new players in the mechanisms of sepsis- and COVID-19-related thromboinflammation

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Abstract

Sepsis and COVID-19 patients often manifest an imbalance in inflammation and coagulation, a complex pathological mechanism also named thromboinflammation, which strongly affects patient prognosis. Extracellular vesicles (EVs) are nanoparticles released by cells into extracellular space that have a relevant role in cell-to-cell communication. Recently, EVs have been shown to act as important players in a variety of pathologies, including cancer and cardiovascular disease. The biological properties of EVs in the mechanisms of thromboinflammation during sepsis and COVID-19 are still only partially known. Herein, we summarize the current experimental evidence on the role of EVs in thromboinflammation, both in bacterial sepsis and in COVID-19. A better understanding of EV involvement in these processes could be useful in describing novel diagnostic and therapeutic applications of EVs in these diseases.

Keywords: extracellular vesicles; sepsis; COVID-19; thromboinflammation.

Introduction

Sepsis is defined as a life-threatening organ dysfunction due to a dysregulated host response to infection [1]. It is the final common pathway to death from most infectious diseases worldwide, including bacterial and viral infections such as Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) [2]. The World Health Organization has listed sepsis as one of the global health priorities for the next few years, [3] and this is the most common cause of emergency admission to intensive care units (ICUs) in Europe [4,5].

Evidence has grown that the relationship between inflammation and coagulation, described by the term “thromboinflammation”, is critical in the pathogenesis of sepsis [6].

The same pathogenic mechanism has also been indicated as the most crucial leading to elevated morbidity and mortality in patients affected by Coronavirus Disease-2019 (COVID-19), the complex clinical syndrome caused by the recently emerged Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) [7–9], which can lead to acute respiratory distress syndrome, sepsis, and multi-organ failure [10].

Although the precise mechanims are not fully understood, inflammation through complement activation and cytokine release, platelet hyperactivity and apoptosis (thrombocytopathy), as well as coagulation abnormalities (coagulopathy) play critical roles in this complex scenario [10,11].

Extracellular vesicles (EVs) are membrane-derived vesicles released into extracellular space by all cell types, and they emerged as novel mediators of cell-to-cell and organ-to-organ crosstalk in many physiological and pathological conditions [12,13].

The diversity of EVs lies in different mechanisms of biogenesis as well as in their different cellular origin [12]. EVs can be found in many biological fluids such as plasma [14], saliva [15] and urine [16]. In addition, their ability to modulate inflammation has suggested EVs as potential novel biomarkers and next-generation biological therapeutics. Finally, there is growing interest in their ability to exchange functional components between cells. EVs may, indeed, communicate with cells and affect their phenotype or function via surface molecule-triggered uptake and intracellular signalling via cargo content [13].

The biological potential of EVs in the mechanism of sepsis- and COVID-19-related thromboinflammation is still only partially known; however, the rapid development of novel molecular platforms is contributing to envisage and develop theranostic applications of EVs also in critical illness.

The aim of this review is to outline the involvement of EVs in thromboinflammation associated with sepsis and COVID-19. Based on the well-established pathogenic role of thromboinflammation in the development of organ damage in critically ill patients affected by these diseases, we will discuss the current experimental evidences on the role of EVs in thromboinflammation, both in bacterial sepsis and in COVID-19, aiming to explore potential targets in the coagulation processes useful to translate the knowledge into the discovery of novel potential diagnostic and therapeutic values of EVs in these diseases.

Thromboinflammation in sepsis and COVID-19: differences and overlaps

Thromboinflammation or immunothrombosis?

In 2004, Tanguay J-F et colleagues were the first using the term "*thromboinflammation*" to indicate the platelet-leukocyte interaction mediated by P-selectin and P-selectin glycoprotein ligand 1 (PSGL-1) implicated in stent restenosis [17]. Inflammation-associated thrombosis, now known as thromboinflammation, occurs commonly in a broad range of human disorders.

Thrombosis is well defined as an exaggerated hemostatic response, leading to the formation of an occlusive blood clot obstructing blood flow through the circulatory system. By comparison, inflammation is the term applied to the complex protective immune response to harmful stimuli, such as pathogens or damaged cells. Increasingly well-defined is the recognition that inflammation stimulates thrombosis, and in turn, thrombosis promotes inflammation [18].

In 2013, Engelmann and Massberg coined the term "immunothrombosis" to explain a tricky and mutual interaction, whereby, on the one hand, the activation of coagulation cascade triggers the immune system, cooperating with the identification, containment and destruction of pathogens [19], whereas, on the other hand, the innate immune cells promote the development of thrombi [20]. Today, thromboinflammation or immunothrombosis are considered interchangeable terms indicating dysregulation of the physiologic anti-thrombotic and anti-inflammatory functions of endothelial cells, which negatively influences hemostasis and favours thrombus deposition both in micro and macro-vasculature [18,21].

Cellular mechanisms of sepsis-related thrombosis

Sepsis is a life-threatening systemic illness associated with the invasion of the bloodstream by pathogens such as bacteria, viruses and fungi [1]. It is considered an extremely complex illness both on the cellular and on the molecular level, involving, among different pathophysiologic mechanisms, an imbalance in the inflammatory response, immune dysfunction, mitochondrial damage,

coagulopathy, and immune network abnormalities, ultimately leading to multi-organ failure (MOF) and death [22,23].

It is known that septic patients are at high risk of developing thrombotic complications ranging from widespread microvascular involvement, such as disseminated intravascular coagulation (DIC), to venous thromboembolism, arising as deep vein thrombosis (DVT) or pulmonary embolism (PE). The development of these potentially fatal complications is believed to be triggered by a common final cascade of events characterized by serious injury to the microvasculature of affected tissues and organs and by the excessive activation of the coagulation system resulting in increased thrombus formation [6,24,25].

At the beginning of the infectious process, components of the bacterial cell wall such as pathogen-associated molecular patterns (PAMPs) are recognized by pattern recognition receptors (PRRs) present on the surface of endothelial cells, platelets, and leukocytes [26]. PRRs transduce signals leading to the release of inflammatory chemokines and cytokine, and of other inflammatory mediators that increase the expression of leukocyte-adhesion molecules [27]. As a consequence, the natural anticoagulant system on endothelial cells is altered and tissue factor (TF) production by monocytes and endothelial cells is increased. The relevance of TF in promoting thromboinflammation in sepsis has been confirmed using pharmacological TF inhibitors or through the inhibition of TF expression in mice exposed to endotoxin, which leads to diminished coagulation and inflammation and improved survival [28].

The location and grade of thromboinflammatory lesions are largely determined by the severity of damage to the endothelial cells (ECs) lining vessels. Usually, endothelium expresses both anti-inflammatory and anti-thrombotic substances, which antagonize leukocyte adhesion to and platelet accumulation [29]. Upon vascular injury, platelets are recruited to the site of damage. Their activation and adherence to the vessel wall lead to the release of platelet agonists, such as adenosine diphosphate (ADP), which further induce paracrine platelet activation and platelet aggregation [30].

Another crucial point is the activation of neutrophils that release nuclear DNA and granular protein, known as neutrophil extracellular traps (NETs) [31], with a strong antibacterial activity. Furthermore, platelets attached on the surface of neutrophils increase the formation of NETs that damage microcirculation, promote immunothrombosis, and lead to diffuse intravascular coagulation, since they facilitate the formation of thrombus acting as a scaffold [32].

COVID-19-related thromboinflammation

SARS-CoV-2 is an enveloped, single-stranded RNA virus [33] that uses the angiotensin-converting enzyme 2 (ACE2) receptor for internalization in the host cells, aided by transmembrane serine protease 2 (TMPRSS2) receptor [34]. ACE2 receptor is highly expressed in many human cells, including nasal and oral epithelial cells, endothelial cells, and myocardial cells [35].

SARS-CoV-2 infection may cause diffuse lung alveolar and endothelial cell damage with severe inflammation and increased vascular permeability leading to acute respiratory distress syndrome (ARDS). In addition to systemic inflammation and severe respiratory disease, COVID-19 is frequently complicated by the development of a hypercoagulable state and by the appearance of thrombotic events, which represent a primary cause of mortality in these patients [7,8,11,36–38]. Histopathological findings, indeed, have reported pulmonary microthrombi in 57% of SARS-CoV-2 infection patients, as compared with 24% of H1N1 influenza patients, a subtype of influenza A virus [39], with increased angiogenesis and pulmonary microthrombosis, respectively, three and nine times more prevalent in COVID-19 patients [40].

Although the appearance of a pro-thrombotic profile is now a well-recognized hallmark of COVID-19 [36,40–42], the mechanisms underlying the development of thromboinflammation are not completely elucidated yet.

In the process of SARS-CoV-2 infection progressing to a systemic and severe disease, multiple proinflammatory cytokines, which include interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α, and chemokines, recruit more innate immune cells (such as macrophages, neutrophils and dendritic cells) to produce additional inflammatory cytokines, in a loop known as cytokine storm. This increases vascular dysfunction and thrombosis and favours the development of multiorgan failure [43,44]. Vascular dysfunction is primarily sustained by the abundant expression on endothelial cells of the ACE2 receptor, which leads to increased virus tropism in blood vessels [45]. In general, the vascular endothelium is considered the first responder of the host defence. Once homeostasis is disrupted by SARS-CoV-2 infection, endothelial cells lose their anti-thrombotic capacity as a consequence of glycocalyx damage [44]. In addition, they express on their surface procoagulants and proapoptotic factors, such as TF and phosphatidylserine (PS), thereby leading to the exposure of the basement membrane and the activation of the coagulation cascade [46,47]. SARS-CoV-2 also directly activates platelets and exacerbates the thromboinflammatory cascade by promoting platelet-neutrophil binding, which, in turn, increases the formation of NETs, activates additional inflammatory responses, and increases other prothrombotic pathways [48,49].

In this complex cellular and molecular interplay, our research group has recently demonstrated that thrombopoietin (THPO), a humoral growth factor involved in the proliferation and differentiation of megakaryocytes in the bone marrow, appears as an additional crucial candidate mediator of platelet hyper-activation and immunothrombosis/thromboinflammation in COVID-19 [50].

Are sepsis and COVID-19 overlapping?

The complex inter-relationship and similarities between sepsis and COVID-19 are topics that have recently emerged with particular emphasis [51–53].

Data obtained in hospitalized COVID-19 patients has revealed that serum cytokine and chemokine levels are high in these patients, at levels comparable to those found in patients with sepsis [54,55].

Some researchers have also pointed out that severe and critically ill COVID-19 patients meet the diagnostic criteria for sepsis and septic shock according to the Sepsis-3 International Consensus, and thus recommend using the term 'viral sepsis' instead of the terms severe or critical illness because more appropriate [56,57].

Patil et co-authors have suggested that SARS-CoV-2 itself likely causes sepsis as a consequence of various mechanisms, which include immune-dysregulation, respiratory dysfunction leading to hypoxemia, and metabolic acidosis due to circulatory dysfunction [58].

Multiorgan failure seen in COVID-19 could also be explained by hypoxia and circulatory disorders that occur as a consequence of microvascular dysfunction, anagolously to what described in sepsis [59].

Finally, other authors have suggested that microvascular dysfunction may also contribute to hypoxia and subsequent organ failure by interrupting the blood flow to the lungs due to disseminated intravascular coagulation and micro-embolism [60].

Extracellular Vesicles

Definition and classification

EVs are a heterogeneous population of membrane-bound particles released by cells that contain both distinct cargoes of proteins, lipids, metabolites, nucleic acids, which include small non-coding RNA such as microRNAs, long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs), and organelles that reflect the parental cells and modulate the functions of recipient cells [12].

EVs are released from all cell types, including those circulating in the blood such as platelets, monocytes, and granulocytes [61,62].

A univocal classification of EVs is still lacking, thus continuously evolving. With the aim of answering this need, in 2014, the International Society for Extracellular Vesicles (ISEV) enacted the Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines [63], which were then updated in 2018 [64].

The current ISEV guidelines settle that "EVs" remain a collective term describing a complex continuum of vesicles of different sizes and compositions, resulting from various mechanisms of formation and release [63,65].

Conventionally, EVs are classified into different subclasses based on size and biogenesis. The studies of the scientific community mostly focused on two classes, namely medium/large EVs or microvesicles (MVs) (>200 nm), which are produced through the outward budding of the cell membrane, and small EVs or exosomes (<200 nm), produced from the endosomal pathway and formed through inward invagination of the endosomal membrane to form the multi-vesicular body [12,65,66].

Analogously to the use of cell-specific surface markers for cell characterization, cluster of differentiation (CD) markers are widely used to characterize different EV subpopulations.

Small EVs are enriched of non-tissue specific markers, such as tetraspanins (CD63, CD9 and CD81), proteins involved in multi-vesicular body formation (ALIX and TSG101), membrane trafficking proteins (RAB proteins and annexins), and MHC class I (HLA-A/B/C) [65]. On the contrary, large vesicles, such as MVs, carry surface markers that derive from the composition of the cellular plasma membrane upon release, and also express cell/tissue-specific markers, such as CD45 (if derived from immune cells), CD41 and CD42a (from platelets), CD14 (from monocytes), or other cell-specific surface molecules [67].

Methods to study extracellular vesicles

Although ISEV has provided technical protocols and recommendations for EV isolation [64,68], a standardized procedure has not yet been established [69], especially for biofluids, such as blood, where viscosity, as well as fat and protein content, are highly variable. These factors may affect EV purity and yield and require the isolation protocols to be adjusted according to the biofluid of interest [70,71]. A standardization of pre-analytical steps is crucial in order to minimize the artefacts in EV analysis from biological fluids. On the contrary, the use of cell culture medium enables a more controlled environment for EV isolation [72].

Therefore, the choice of the EV isolation method largely depends on the sample analyzed, and on planned downstream analysis and applications.

Several researchers have compared different methodologies to improve the efficiency of the isolation and characterization of EVs [46–48]. The need for robust, standardized and reproducible EV-isolation methods is an essential requirement, especially for the translation in a clinical setting [74].

Some authors sustain that the combination of different methods is often the best option for EV isolation since the different origin, complex nature, and heterogeneity of EVs require sophisticated isolation approaches [75].

The main isolation techniques include ultracentrifugation (UC), immunoaffinity capture (IC), size-exclusion chromatography (SEC), ultrafiltration (UF) and precipitation [64,73,76]. Each one of these methods offers some advantages and disadvantages compared to the others. The most popular isolation method still in use is based on UC, which is surely cost-efficient and widely accepted, but also time-expensive and burdened with the undesired co-purification of non-EV-associated proteins [61]. Immunoaffinity capture methods are usually based on the coating with specific CD antibodies and theoretically allow to specifically isolate only one EV subpopulation [77]. Size-exclusion chromatography, ultrafiltration and precipitation are relatively widely-used methods [78]. In particular, SEC has been used as a method to enrich EVs whilst depleting protein contaminants, and is often used in EV-based omics analysis [79,80].

For more details on the discussion of the adequate isolation method of EVs, we adress the readers to several comprehensive reviews or position statements already published on this topic [61,81,82].

After isolation, EV populations need to be characterized for intended downstream applications, for which there is a variety of techniques available. These methods include dynamic light scattering/nanoparticle tracking analysis (NTA), which is the most popular quantitative method for EV particle analysis [83], flow cytometry, the most widely employed technique to study large extracellular vesicles [84], and transmission electron microscopy (TEM), a routine method that has been used with great success for the study of EVs [85].

EVs in cell-to-cell communication

EVs are pivotal elements in cell-to-cell communication, in both physiological and pathological states, via the transfer of selective biomolecular cargos to recipient cells in an autocrine, paracrine, or endocrine manner to regulate cell function [86].

Although the uptake of EVs by different cell types is a well-described phenomenon, the processes directing EV entry into recipient cells remain poorly understood.

The initial meeting with the recipient cell is based on a variety of receptors and/or molecules located on the plasma membrane. This binding is recognized and drives intercellular signalling events, finally establishing the process of cell entry, as well as other downstream effects [87].

An attractive paradigm of EV-mediated cell-cell communication depends on the existence of an "address system" where the EV membrane composition provides a high degree of selectivity in terms of targeting specific recipient cell types [12].

Various evidence *in vivo* sustain that EVs with distinct composition are able to target specific organs to elicit microenvironment change in a remarkably selective way [88].

Due to their stability in the circulation, low immunogenicity, biocompatibility and biodegradation, besides their ability to deliver proteins, lipids, and nucleotides from one cell to another, and specifically to selected cell types, EVs have started to attract attention for their potential applications as therapeutic delivery systems in various medical fields [89], including immunology [90], cancer research [91], cardiovascular [92], inflammatory diseases [93], and infection [94].

Considering the very preliminary nature of the evidence on therapeutic applications of EVs in sepsis, in the following paragraphs, we specifically focus on highlighting the role of EVs as contributors to the pathological mechanisms described above.

EV role in sepsis-related thromboinflammation

In sepsis, increased circulating levels of proinflammatory and procoagulant EVs are well documented and may contribute to coagulation disorders likely involved in the pathophysiology of DIC [62,95–98].

EVs may exhibit direct procoagulant properties, in part due to PS expressed on their surfaces, a cell membrane phospholipid that supports the assembly of coagulation enzymes and TF [99]. Septic patients have increase circulating PS-positive EVs that are mainly released by endothelial cells, monocytes and platelets [100–102].

Zhang et al. [102] reported that endothelial cells treated with EV-containing serum obtained from septic patients exhibited more exposed PS than those treated with serum from healthy controls. These data support the concept that procoagulant properties of effector cells can be transmitted to target cells by EVs.

Tripisciano et al. [103] investigated the thrombogenicity of platelet-derived EVs and compared the contributions of PS and TF exposure on thrombin generation. They demonstrated that annexin V (which binds and marks PS), but not anti-TF antibodies, efficiently inhibit this effect, indicating that thrombin generation is primarily due to the exposure of PS on platelet-derived EVs [103].

In addition to PS exposure, EVs can carry TF, whose activity contributes to their pro-coagulant activity in various diseases, including cancer, acute coronary syndromes, and sepsis itself [104–106]. Higher amounts of TF-positive EVs are associated with increased occurrence of DIC in sepsis [101]. However, the role of TF-positive EVs in septic thromboinflammation is still considered controversial. For more detailed information on this specific topic, we address the readers to some interesting reviews exploring it [107,108].

New data support the notion that EVs from different cell types have distinct characteristics and play different pathogenic roles in thromboinflammation during sepsis.

Platelet-derived EVs are considered dominant in DIC, but those derived from other cell types, including leukocytes and endothelial cells, are believed to also contribute to procoagulant mechanisms in sepsis [107,109–113].

Platelet-derived EVs adhere to platelets, leukocytes, and endothelial cells and induce pro-inflammatory and pro-thrombotic functions in these cells [109,114,115] since they contain a distinctive subset of proteins and microRNAs involved in hemostasis and thrombosis [109]. Recently, it was seen that platelet-derived exosomes promote excessive NET formation in sepsis, through modulating the Akt/mTOR-related autophagy pathway partly by the transfer of selected microRNAs [110]. This suggests that exosomes released from activated platelets are essential mechanisms in sepsis-induced thromboinflammation [109,110,114,115].

Leukocyte-derived EVs, originating from neutrophils, monocytes/macrophages and, lymphocytes, may also contribute to disrupt vascular homeostasis via their cytoplasmatic contents, for instance through reactive oxygen species (ROS). Mortaza and coll. have shown, indeed, that EVs isolated from septic rats reproduce hemodynamic, inflammatory and oxidative stress patterns of sepsis in healthy rats [111]. These effects were mediated by increased superoxide anion production, NF-kB activation, NO synthase (NOS)-2 expression, and NO overproduction [111]. Similarly, Mastronardi et al. found that EVs isolated from septic patients induce tissue-selective expression of pro-inflammatory proteins in rats, whereas EVs derived from healthy subjects did not [112]. These results further support the possibility of distant dissemination of EVs and their involvement in the pathogenesis of MOF in sepsis.

Other evidences suggest that activated ECs increase their procoagulant activity during sepsis by enhancing the production of EVs that bind to neutrophils. Endothelial-derived EVs stimulate the oxidative activity with increased binding to leukocytes in patients with severe systemic inflammatory response syndrome [113].

Profiling exosome cargos, including proteins, mRNAs, lncRNAs and miRNAs, is crucial in order to identify molecular markers of sepsis-related thromboinflammation, and thus elucidate the functions and the related regulatory mechanisms of EVs in this process, potentially leading to the development of new tailored therapeutic approaches in sepsis. Only one experimental study until now addresses this topic using a multi-omics approach, showing that serum exosomes from septic mice may exert a therapeutic effect through cytokine storm suppression, inhibition of complement and coagulation system activation, endothelial cell junction stabilization, and vascular permeability reduction [116]. Further studies on the regulation of gene expression mediated by miRNAs contained in EVs and on their relation to the thromboinflammatory mechanisms present in sepsis would certainly be needed.

Finally, recent evidence suggests that EV-mediated transfer of mitochondrial content may alter metabolic and inflammatory responses [117], similar to what is described in neuroinflammation [118]. Understanding the potential role of mitochondrial in sepsis-related thromboinflammation and deepening this aspect associated with EVs could also reveal yet unexplored mechanisms.

The main concepts of this paragraph are schematically depictedin Figure 1.

EV role in COVID-19-related thromboinflammation

In support of the role of EVs in promoting thrombosis in COVID-19, a systematic analysis of surface antigen expression of EVs circulating in COVID-19 patients showed 37 antigens involved in inflammation, platelet activation, coagulation processes, and endothelial dysfunction [119]. The cytokine storm occurring during COVID-19 might influence the cell release of substantial amounts of procoagulant EVs that may act as clotting initiation agents. In particular, the elevated levels of TNF-α in COVID-19 patients serum strongly correlate with TF (CD142) expression onto the surface of EVs [119], as well as with its procoagulant activity [120]. Several studies [121–124] have reported a significant increase in circulating TF-expressing EVs that correlated with D-dimer levels, vWF, circulating leukocytes, and inflammatory markers, assuming the contribution of TF-EVs in disease determining severity and thrombosis in COVID-19 patients. However, the identification of the exact cellular origin of TF-exposing EVs, as well as of the underlying pathogenic mechanism remains challenging and requires additional study.

Controversial data have been reported on PS exposure from EVs isolated from COVID-19 patients: some studies have reported an increase [124,125], others a decrease [126]. These differences may be related to the heterogeneity of patient cohorts.

EVs are released by activated platelets, leukocytes, and endothelial cells [127,128]. In many cardiovascular diseases, EVs have been shown to be able to propagate inflammation and coagulation, contributing to tissue injury and thrombosis [129]. These mechanisms are likely contributing to exacerbate vascular conditions also in COVID-19 [130]. For instance, platelet-derived EVs were shown to promote the formation of NETs during SARS-CoV-2 infection through toll-like receptor (TLR)-2-dependent mechanisms [131]. Moreover, neutrophils-derived EVs synergize with NETs in neutrophil activation and endothelial adhesion through a high-mobility group box protein 1 (HMGB1)/TLR pathway [132].

Proteomic analysis of EVs has been used in different pathological conditions, including sepsis [133] and cancer [134], with the aim of elucidating those metabolic pathways that are modulated by the action of EVs. This same methodologic approach has shown that circulating EVs in plasma from COVID-19 patients exhibit a distinct procoagulant profile. Especially in the most severe patients, this is reflected by a profound proteomic change, mainly related to coagulation activation, complement cascade, and platelet degranulation [135].

In addition, microRNA cargo of EVs could contribute to the pathogenesis of thrombotic complications in COVID-19 patients by downregulating two specific miRNAs (miR-145 and miR-885) that promote higher levels of TF and von Willebrand Factor, thus determining a prothrombotic state [136].

The accumulated evidence on the pathophysiological role of EVs in COVID-19-related-thromboinflammation has enhanced our understanding of the relationship between viruses and coagulation and may help formulate effective targeted strategies.

The main concepts of this paragraph are schematically depictedin Figure 1.

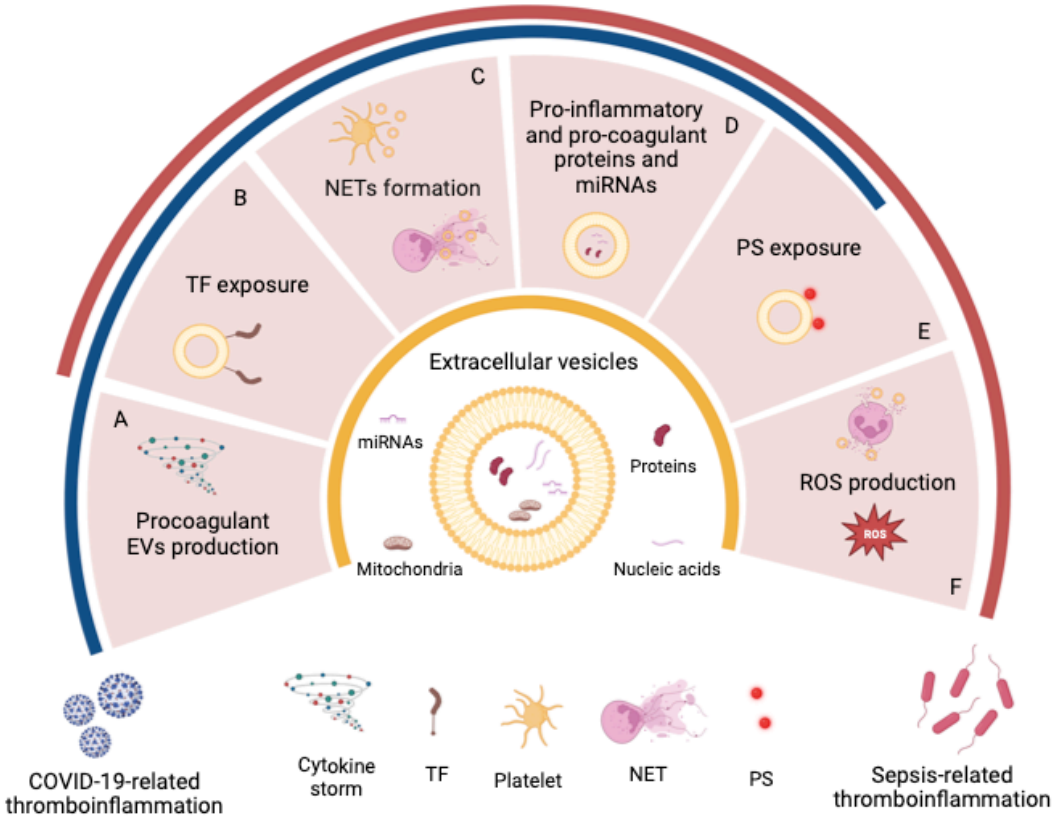

Figure 1. Extracellular vesicles as new players in sepsis- and COVID-19- thromboinflammation. Schematic summary of some of the main roles of EVs in sepsis- (red line) and COVID-19- (blue line) related-thromboinflammation. **A)** The cytokine storm occurring during sepsis and COVID-19 might influence the cell release of substantial amounts of procoagulant EVs involved in in thromboinflammation development. **B)** Higher amounts of TF-positive EVs are associated with increased occurrence of both DIC in sepsis and thrombosis in COVID-19. **C)** Activated platelets release EVs that promote excessive NET formation both in sepsis and COVID-19. **D)** Protein and miRNA cargo in EVs could contribute to the pathogenesis of thrombotic complications in sepsis and COVID-19 patients. **E)** EVs exhibit direct procoagulant properties due to PS expressed on their surfaces in sepsis and (probably) in COVID-19 patients. **F)** Leukocyte-derived EVs contribute to thromboinflammation via excessive ROS production. TF, tissue factor; NETs, neutrophil extracellular traps; PS, phosphatidylserine; ROS, reactive oxygen species. Created with BioRender.com.

Challenges and Future Directions

The emerging interest in the role of EVs in critical illness is supported by their early involvement in relevant pathobiological functions of vital organs exposed to severe stressors. Although EVs offer innovative theragnostic possibilities in detecting, monitoring, and modulating the onset of tissue dysfunction before the development of overt organ failure, technical challenges based on isolation, characterization methods, and standardization of clinically suitable EV collections represent a significant issue.

The development of EV-based biomarkers is promising, particularly for diseases such as cancer and neurological conditions [137,138].

In the last years, EVs have gained progressive importance in sepsis and, more recently, in COVID-19, two similar but heterogenous critical diseases.

EVs represent novel players involved in the mechanisms of thromboinflammation in these diseases since they concur to promote a switch toward a procoagulant phenotype, as already observed in cardiovascular disorders [92] and cancer [91]. However, a complete picture of EV active functions in sepsis and COVID-19 is still lacking. Therefore, a better understanding of the role of EVs in thromboinflammation is needed, in order to improve our knowledge about the mechanisms underlying these diseases, to be subsequently translated into clinical practice, for effective and personalized management of septic and COVID-19 patients. Further investigations are required in these critical illnesses, which will take advantage also of the next-generation approaches using novel imaging techniques, omic-based methodologies, and organ-on-chip systems to reveal new features of EVs. There are still a significant number of questions to be answered in order to better clarify the involvement of EVs in this scenario. More one discovers, more questions arise.

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CHAPTER 3

Characterization of plasma-derived extracellular vesicles in patients with burn injury complicated by septic shock reveals CD42-EVs as potential marker and mediator of septic shock

Characterization of plasma-derived extracellular vesicles in patients with burn injury complicated by septic shock reveals CD42-EVs as a potential marker and mediator of septic shock

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[in preparation]

Abstract

Septic shock is the leading cause of death in severe burn patients. Although significant efforts have been made, precisely timed detection of sepsis is a difficult challenge in burn patients. Extracellular vesicles (EVs) have emerged as novel cell-to-cell mediators and promising biomarkers that may aid in the diagnosis of septic shock. The present study aims to characterize the release of EVs in patients with burn injury complicated by septic shock and explore their role in platelet aggregation. We enrolled twenty-nine burn patients, including 23 patients with (BSP) and 6 without septic shock (BnSP). Ten healthy subjects (HS) were used as controls. Plasma-derived EVs were isolated by ultracentrifugation and characterized by nanoparticle tracking analysis, transmission electron microscopy, and flow cytometry. EV surface antigens were investigated by bead-based multiplex flow cytometry. In *vitro*, we reproduced the effects of EVs on platelet function by adding EVs from BSP or HS to platelet-rich plasma from healthy donors. BSP had increased plasma-derived EV concentrations compared to BnSP and HS. BSP-EVs phenotyping revealed a pattern of platelet-cell surface markers. Of note, we found a significant increase of CD42a in BSP compared to BnSP- and HS-derived EVs. In *vitro* experiments showed that BSP-EVs increase platelet aggregation and that this priming effect was inhibited by a CD42b neutralizing antibody. The increased release of CD42a-EVs might be suggested as a marker and mediator of septic shock in burn patients, reflecting a possible augmented pro-thrombotic cellular activity.

Keyword: septic shock, burn patients, extracellular vesicles, CD42.

Introduction

Severe burns are one of the most life-threatening injuries, associated with substantial morbidity and mortality [1]. After burns, patients leave skin and other tissue vulnerable to bacterial infections that increase the risk of sepsis [1]. This is defined as organ dysfunction caused by a dysregulated host response to infection that can lead to severe disease or septic shock [2]. Sepsis is still the major cause of death after the first 24 hours of burn, representing the final cause of death in 50-60% of non-survivors [3,4]. Although significant efforts have been made in the past and multiple studies have expanded knowledge in the field of sepsis, precisely timed detection of sepsis is a difficult challenge due to its diverse, non-specific clinical signs and its incompletely understood pathophysiology. In addition, the diagnosis of sepsis is even more difficult in the burned patients, who are possibly even at an even higher risk than the general critical-care patients [4–6]. Under physiological conditions, there is a delicate balance between procoagulant and anticoagulant mediators within the vasculature [7]. In sepsis, several proinflammatory cytokines promote the generation and release of procoagulant factors that disrupt vascular homeostasis and result in a procoagulant state [8]. Thrombosis and vascular leaking are implicated in hypoperfusion of multiple organs and subsequent multiple organ dysfunction syndrome, which is the initiating event of septic shock that impairs tissue oxygenation and aggravates organ dysfunction [8].

Extracellular vesicles (EVs) are particles released by all cell types and covered by a lipid bilayer [9]. The increasing interest in EVs is based on their bioactive cargo components and mediators, such as cytokines, enzymes, proteins and microRNAs to target cells in a paracrine fashion or via circulation [10,11]. EVs play a key role in cell-to-cell communication, cross-organ signaling, and tissue homeostasis [12,13]. Recent studies have demonstrated that circulating EV populations and their content can change during sepsis [14–17], and can be associated with the severity of organ failure becoming predictive of mortality in critically ill patients with sepsis [18]. In addition, the possible involvement of EVs as markers and mediators of thromboinflammation in sepsis has been recently described by our group and others [19,20]. It has been also shown that EV surface antigens reflect their cellular origin and could help to understand cellular communication mechanisms in several diseases [21,22], including trauma [23].

Here, we investigated plasma-derived EVs released during septic shock in burn patients, and performed multiplexed phenotyping, aiming for a more detailed view of their origin. Moreover, we evaluated the effects of EVs from burned patients with septic shock on *in vitro* platelet aggregation, hypothesizing their potential role in thromboinflammation during septic shock.

Methods

Patients

The study population consisted of 29 burn patients with ≥ 10% Total Body Surface Area (TBSA) admitted to the Burn Center of the "Città della Salute e della Scienza di Torino – CTO Site" – University Hospital, Turin, Italy, from November 2020 to December 2022. We enrolled 23 patients with septic shock (burn septic patients - BSP) and 6 with no evidence of infection (burn non-septic patients - BnSP) until 72 hours after the enrolment. Ten healthy subjects (HS) with comparable age and sex were enrolled as control group.

The diagnosis of septic shock was for the International Guidelines for Management of sepsis and septic shock: 2016 [2]. According to this, the Sequential (Sepsis-related) Organ Failure Assessment (SOFA) score, which includes respiration, coagulation, liver cardiovascular, central nervous system and renal score, should be ≥ 2 and calculated within 24 hours of blood collection. The presence of inhalational injury was recorded and incorporated as specified into the Revise Baux Score [24]. Systemic treatment of burn patients, diagnosis and treatment of septic shock was managed by a multidisciplinary team based on guidelines in place at the time [2,25,26].

Exclusion criteria were: age < 18 years; TBSA <10%; cancer (active or recent history); autoimmune or chronic inflammatory disease; SARS-CoV-2 infection; presence of other diseases that affect blood coagulation.

Plasma EV isolation

Blood was collected into EDTA-containing tubes using a central venous catheter or, for healthy subjects, by clean venipuncture using a 21-gauge butterfly infusion set.

Within 1 hour from blood collection, blood was centrifuged for 10 min at 1600 *g* at 4°C, to eliminate cellular components; then, one centrifuge was performed to further purify the plasma, eliminating apoptotic bodies and platelets (5000 *g*, 30 min at 4°C). Plasma samples were aliquoted and stored at -80°C or, in preliminary experiment, immediately processed to compare fresh versus stored samples. Pre-analytical factors for sample handling and storage complied with the recommendations of the International Society for Extracellular Vesicles [27].

In preliminary experiment, 10 mL of plasma from healthy subjects were immediately aliquoted into fresh or frozen and stored at -80°C and then thawed 30 days later.

EVs were isolated from 1-10 mL of plasma by ultracentrifugation at 100,000 *g* for 2 hours at 4°C (L8-80M Ultracentrifuge, Beckham Coulter, Brea, CA). After discarding the supernatant, EV pellets were resuspended, washed with PBS, and again ultracentrifugated at 100,000 *g* for 2 hours at 4°C. Finally,

EVs were resuspended in 100-200 µl of particle-free PBS with 1% DMSO, aliquoted and stored at -80°C.

In selected experiments, 1 ml of plasma underwent the precipitation procedure for EV isolation, according to the following procedure. Plasma samples were submitted to two centrifugations at 3000 *g* for 20 min at 4°C. The protamine (Sigma, St. Louis, MO, USA)/Polyethylene glycol (35,000; Merck KGaA, Darmstandt, Germany) precipitation solution (0.2 g PEG 35,000 and 1 mg protamine chloride/mL) was added to the plasma samples (1 volume precipitation solution:4 volume sample) that were processed as previously described [28]. After overnight incubation at 4°C, the mixture was centrifuged at 1500 *g* for 30 min and the pellet was re-suspended in 150 µl of particle-free PBS.

Nanoparticle tracking analysis (NTA)

Nanoparticle concentration and diameter were measured by NanoSight LM10 (Malvern Instruments, Malvern, UK) equipped with a 405 nm laser and NTA 3.0 software. EVs were diluted 1:1000 in particle-free PBS and three consequent videos of 30 seconds each were acquired. The minimum expected particle size, minimum track length, and biomedical light unit setting were set to automatic, and the detection threshold was set to 4 to reveal all particles. The particle concentration and the distribution graph of the particle size were determined per sample by averaging the results from the analysis of the 3 independent videos. For data analysis, Nanoparticle Tracking Analysis NTA 2.3 software was used (Malvern Panalytical, Malvern, UK).

Transmission electron microscopy (TEM)

TEM was performed on EVs isolated by ultracentrifugation and placed on 200 mesh nickel formvar carbon-coated grids (Electron Microscopy Science, Hatfield, PA, USA). After an adhesion step (20 minutes), the grids were incubated with 2.5% glutaraldehyde containing 2% sucrose and extensively washed in distilled water. Finally, the EVs were negatively stained with NanoVan (Nanoprobes, Yaphank, NK, USA) and acquired with a Jeol JEM 1010 electron microscope (Jeol, Tokyo, Japan).

Flow cytometry analysis

Forward scatter (FSC), side scatter (SSC) and fluorescence data were obtained with the settings in the logarithmic scale, as previously described [29] by Attune Nxt Acoustic Focusing Flow Cytometer system (ThermoFisher Scientific, MA, US). To enable side scatter (SSC) resolution at the scale required to visualize EVs, the Attune NxT Small Particle Side-Scatter Filter (488/10) (Invitrogen, ThermoFisher Scientific) was installed. The acquisition was performed at the lowest flow rate (150 µl/min). Gates were established using a mix of different spherical fluorescent beads with a nominal diameter of 0.2, 0.5 and 1.0 µm obtained from the flow cytometry sub-micro particle size reference

kit (Thermofisher Scientific). EVs were stained for 30 min in the dark at 4°C with anti-CD63-PE-Cy7 and anti-CD9-PE (Thermofisher Scientific). The data was analyzed with Attune NxT 3.2 Software.

MACSPlex exosome assay and flow cytometry analysis

To phenotype EVs isolated from patients and HS, the MACSPlex Exosome Kit (Miltenyi Biotec GmbH, Bergish Gladbach, Germany) was used. Briefly, it is based on 4.8 µm diameter polystyrene beads, labelled with different amounts of 2 dyes (phycoerythrin and fluorescein isothiocyanate), in order to generate 39 different bead subsets discriminable by flow cytometry analysis by size. Each bead subset is conjugated with a different capture antibody that recognizes the EVs carrying the respective antigen (37 EV surface epitopes plus 2 isotype controls). After beads and sample overnight incubation, EVs bound to the beads are detected by allophycocyanin-conjugated anti-CD9, anti-CD63, and anti-CD81 antibodies. EVs (3×10^9 particle/ml) diluted in buffed solution were analyzed with the MACSQuant Analyzer-10 flow cytometer (Miltenyi). Triggers for the SSC and the FSC were selected to confine the measurement on the multiplex beads. A blank control of MACSPlex Buffer only incubated with beads and detection antibodies was used to measure the background signal. This approach allows semi-quantitative analysis of differential surface epitopes. Each EV marker's median fluorescence intensity (MFI) was normalized to the mean MFI for specific EV markers (CD9, CD63, and CD81) obtaining normalized MFI (nMFI).

Platelet aggregation

Platelet aggregation was evaluated in 3.8% trisodium citrate-anticoagulated platelet-rich plasma (PRP) as previously described [30]. PRP was prepared by centrifugation (15 min at 180 *g*) of anticoagulated blood obtained from healthy donors by clean venipuncture using a 19-gauge butterfly infusion set, without venous stasis. PRP was incubated with 5µl, 10µl and 20µl of EV concentrate at 37°C for 10 minutes. When evaluating priming activity, epinephrine (EPI) or adenosine-diphosphate (ADP) (Helena Laboratories, Beaumont, TX) was added as agonist. For each experiment, the agonist concentration that induced the minimum measurable aggregation was determined: EPI and ADP dose ranges were 0.05-0.2 µm/L and 0.1-1.0 µm/L, respectively. The priming index (PI) was calculated as the response to plasma and agonist together, divided by the sum of individual responses elicited by EVs and agonists.

In separate experiments, designed to inhibit the biological effects of CD42b, test EVs were incubated with 20 µg/ml of antibody anti-CD42b (anti-GPIbα antibody, Clone 6B4, courtesy of Prof. SF De Meyer, University of KU Leuven Kulak, Belgium) for 10 min at 37°C. The mixture of EV sample and

anti-CD42b antibody was then added to PRP and further incubated for 10 min at 37°C. Finally, the agonist (EPI or ADP) was added and platelet aggregation was evaluated.

Data analysis

Data are presented as median (range) or mean ± standard error of mean (SEM), according to data distribution as asses by the Shapiro-Wilk test. Comparison between groups was carried out by Mann-Whitney rank sum test or Kruskal-Wallis one-way analysis of variance on ranks followed by Dunn's multiple comparison tests, Wilcoxon matched-pairs signed rank test or unpaired or paired Student's t-test, as appropriate. A *p*-value < 0.05 was considered significant. All statistical analyses have been performed using GraphPad Prism 9.0 software for Windows and Macintosh (GraphPad Software, La Jolla, CA, USA).

Study approval

This study was conducted according to the principles of the Declaration of Helsinki and approved by the Institutional Ethical Committee of A.O.U. Città della Salute e della Scienza di Torino, Molinette Hospital, Turin, Italy (n. CS2/815). Informed consent to the proposed treatments, retrospective review of the medical notes, and analysis of the collected data were obtained from the patients or substitute decision-makers.

Results

Characteristics of patients

Twenty-nine patients fulfilled the inclusion criteria. Clinical characteristics were similar between patients with (BSP) and without septic shock (BnSP) except for the higher median %TBSA in BSP compared to BnSP, (p=0.003) and the increased Revised Baux Index (p=0.04).

Eight BSP died in hospital while 15 survived and the median (range) SOFA score in BSP was 11 (4-16) (**Table 1**). None of the BnSP died (**Table 1**).

White blood cell counts as well as the concentrations of several biomarkers (procalcitonin, NT-pro-BNP, copeptin, troponin I, and myoglobin) were higher in BSP than in BnSP (**Table 1**). On the contrary, hemoglobin was lower in BSP than in BnSP. All clinical characteristics and laboratory findings are listed in **Table 1**.

Characterization of EVs

In light of the lack of standard procedures, mounting evidence suggests an impact of storage on plasma [31]; to determine whether this was true, we have preliminarily carried out TEM analysis, particle concentration and size distribution on plasma samples to compare EVs obtained from fresh

or stored plasma at -80°C for 30 days. No significant difference was found in plasma-derived EVs concentration and size distribution in stored compared to fresh plasma samples (**Supplementary S1**).

The presence and purity of EVs in the plasma isolated from healthy subjects (HS), burn non-septic patients (BnSP) and burn septic shock patients (BSP) were verified using TEM, which showed the typical round shape morphology with a diameter of approximately 200 nm, confirming the size measurements by NTA (**S2a-c**). The expression of markers of EVs, CD9 and CD63, were confirmed to be present by flow cytometer analysis (**S2d, e**).

Circulating EVs distinguish patients with septic shock from those with burn injury

The number of EVs present in plasma from BSP was increased compared to plasma from BnSP ($p<0.05$) and from HS ($p<0.0001$), whereas there was no difference between plasma from BnSP and from HS (**Fig. 1a**). Particles ranging in size from 200 to 300 nm did not show significant differences across the groups (**Fig. 1b**). Since NTA analysis of plasma EVs is based on light scatter, we confirmed using this technique that EVs detected in patient's plasma samples were membrane vesicles and not protein aggregates. As expected, CD9, CD63, and CD81, alone and together, were also increased in EVs derived from plasma samples from BSP compared to those from HS (CD9: $p=0.004$; CD63: $p=0.0005$; CD81: $p=0.04$; CD9-CD63-CD81: $p<0.0001$). Concurrently, CD63 was also increased in BSP compared to BnSP ($p=0.02$) (**Fig. 1c**).

EV surface antigen profiling reveals specific septic shock-related markers

In order to evaluate the surface epitopes and the cellular origin of plasma-EVs isolated from BSP, we performed the multiplex assay MACSPlex, analyzing the signal of 37 surface proteins simultaneously. The average MFI for CD9-CD63-CD81 was used as an internal normalizer of fluorescence levels for all 37 markers to enable comparison between different samples and experiments and to exclude non-specific binging due to small debris. The levels of EV surface epitopes in HS, BnSP and BSP are shown in **Fig. 2a-c.** In each group, the most abundantly expressed EV surface epitopes were CD42a (platelet/megakaryocytes membrane glycoprotein), CD41b (platelet/megakaryocytes membrane glycoprotein II-b), CD62p (p-selectin, vascular endothelial cells and platelet membrane protein), CD29 (platelet B1-integrin), and CD31 (monocytes, platelet, endothelial cell adhesion molecule-1), which were increased in BSP *vs.* HS (**Fig. 2**). In particular, CD42a was increased in BSP compared with BnSP ($p=0.03$) and HS ($p<0.0001$) (**Fig. 3a**), and CD41 was increased in BSP compared with HS ($p=0.02$) (**Fig. 3b**).

Receiver-operating characteristic curve (ROC) analysis showed that CD42a had a good value for the diagnosis of BSP, (**Fig. 3d**), with an AUC of 0.947 [(95% CI, 0.855-1.000), p=0.003], and a specificity of 100% and a sensitivity of 82% based on a threshold value of 3.340.

CD42a-EVs correlates with SOFA score severity in burn patients with septic shock

We stratified BSP based on (i) the severity of %TBSA <50% and ≥ 50% (p<0.001) and (ii) SOFA score < 10 points and ≥ 10 points (p<0.0001). Patients with %TBSA<50% were significantly older than patients with %TBSA ≥50 (p=0.007). %TBSA of burn was not significantly associated with mortality (**Table 2**), and no significant correlation existed between %TBSA and SOFA score (data no shown). Other detailed characteristics and laboratory findings are listed in **Table 2**.

When we analyzed the number of EVs in these two patient groups, we found a trend towards an increased amount of EVs in BSP with higher compared to those with lower SOFA score, based on CD9, CD63 and CD81 marker expression. In particular, a significant increase in CD63-EV expression (p=0.02) was shown in patients with high compared to those with low SOFA score (**Fig. 4 a, b**). On the contrary, the number of circulating EVs and the expression of CD9, CD63 and CD81 markers were not significantly affected by the increase of %TBSA (**Fig. 4 c, d**).

In addition, CD42a-EVs were more expressed in BSP with a higher SOFA score (p=0.03) (**Fig. 5**), whereas did not differ in BSP with higher %TBSA (**Fig. 5f**). ROC curve analysis displayed that CD42a-EV expression had a good value in discriminating those patients with higher septic shock severity, with an AUC of 0.769 (95% CI: 0.4569-0.969, p=0.035) and a specificity of 61.5% and a sensitivity of 77.8% for a cut-off value of 4.180 (**Fig. 6**).

***In vitro* effect of EVs isolated from BSP on platelet aggregation**

To determine the response of naïve platelets to EVs from BSP, we tested *in vitro* the effect of EVs isolated from BSP or HS on platelet aggregation in PRP from healthy donors by lumi-aggregometry. In preliminary experiments, we investigated the performance of EVs isolated by ultracentrifugation (UC) and precipitation (PEG) protocols in functional experiments on platelet aggregation. The different protocols used for EV isolation from HS and BSP did not change their effect on platelet aggregation (**S3a**). Moreover, we tested three different BSP-EV concentrations to establish the lowest concentration able to show an effect on platelet aggregation (**S3b**). Finally, when we studied the effect of BSP-EVs isolated from BSP on platelet aggregation, we demonstrated that the incubation of EVs from BSP with PRP significantly enhanced the aggregatory response induced when secondary agonists, EPI or ADP, were added (**Fig. 7 a, b)**. On the contrary, EVs isolated from HS did not show any effect on platelet aggregation in PRP from healthy donors (**Fig. 7 a, b).**

Role of CD42-EVs in priming activity exerted by EVs isolated from BSP on platelet aggregation

In order to investigate whether elevated circulating levels of CD42a-enriched EVs may contribute to enhanced platelet aggregation in BSP, we studied *in vitro* the effects of a neutralizing antibody directed against CD42b on the priming effect exerted by EVs isolated from BSP on platelet aggregation.

In preliminary experiments, we verified that the blocking antibody CD42b does not affect *per se* platelet aggregation in PRP (**S3c**).

Pre-incubation of EVs isolated from BSP with the anti-CD42b-antibody reduced the priming effect exerted on platelet aggregation in PRP. Conversely, the pre-incubation of EVs isolated from HS with the anti-CD42b-antibody did not show any effect (**Fig. 7 c, d**).

Discussion

Patients with burn injury complicated by septic shock represents one of the most critical categories of patients in terms of diagnosis and treatment [1,32], largely accounting for the dramatically high mortality in this group of patients. Therefore, considerable research efforts are directed toward searching for new diagnostic parameters that could reflect injury patterns, and provide reliable biomarkers to predict septic shock severity in burn patients, and guide therapeutic choices.

Although previous studies have investigated the role of EVs in septic patients [13–15,18], little is known about the presence, characteristics and role of EVs in the complex scenario of septic shock in burn patients. A few preliminary studies focused on EV characterization only in the first phases of burn injury [33], others did not consider the insurgence of septic shock although evaluating the length of hospital stay [34].

In this traumatic injury population, which induces rapid and persistent systemic inflammatory response syndrome in effectively all patients with severe injury, we demonstrated that plasma EV concentration is higher in burn patients with septic shock, suggesting an enhanced shedding of cellular membranes closely related to sepsis insult and not to the presence of burn trauma *per se*. Supporting this view, EV concentrations in burn patients with septic shock increase in parallel with SOFA score. Our data are in line with several clinical studies that demonstrated that EV quantification could be used to differentiate early septic shock from infection [35], or patients with septic shock from critically ill patients without infection [36].

The surface expression of tetraspanins such as CD63, CD9 and CD81 are the leading markers in EVs characterization [27]. Moreover, they are known to participate in a wide spectrum of physiological and pathological processes of the immune response to infections and inflammation [37,38]. For

instance, growing evidence suggests that CD63-EVs are involved in multiple processes of the inflammatory response to infection [39,40]. Additionally, the EVs expression of CD63 was related to platelet activation and its interaction with leukocyte and endothelial cells that promoted platelet consumption and coagulopathy and to overproduced proinflammatory cytokines, finally leading to apoptosis of cells and multiorgan failure [41,42]. Here we have shown that CD63-EVs are increased in burn patients with septic shock. These results are in line with previous studies that showed CD63's association with the severity of organ dysfunction and prediction of death in critically ill patients with sepsis [18].

A wide area of research is devoted to identify signature biomarkers of septic shock useful for diagnostic and prognostic purposes. EVs characterization, comprising both the study of specific surface markers and the miRNAs present in the EVs, is an area that has been receiving interest in this contest. Several EV subtypes have been already associated with various aspects of sepsis pathobiology from modulators of inflammation, lymphocyte apoptosis, and coagulation to organ dysfunction [43]. In this study, we have conducted a comprehensive characterization of plasma EVs using a standardized multiplex flow cytometric assay [44] aiming to identify a molecular signature diagnostic of septic shock in burn patients. We found that circulating EVs released during septic shock are mainly derived from platelets, endothelial cells and leucocytes, probably due to shear stress caused by increased blood flow on the walls of blood vessels or as a consequence of the hyper-coagulopathy and cardiovascular profile of the recipient cells [45]. When we evaluated separately different EVs-surface epitopes, two platelet-associated markers, CD42a and CD41b, resulted increased in burn patients with septic shock, but only CD42a was able to discriminate burn patients with septic shock from those without infection. Notably, when we looked at the potential diagnostic performance by ROC curve analysis, CD42a showed a good diagnostic performance. While previous studies on EV markers in septic shock patients focused on single markers [18], or a few cell type-specific markers [46], here we characterized, for the first time, a comprehensive panel of markers, which allowed us to identify those markers that achieved the highest diagnostic accuracy in burn-septic shock patients. EV biomarker profiling is emerging as an important general approach for precision medicine and personalized treatment [47]. This approach could be particularly valuable in critically ill patient and is probably a promising strategy to deconstruct and reassemble our knowledge about sepsis.

EVs are receiving increased attention not only as biomarkers, but also as mediators of cell communication and effectors of disease in sepsis [48,49]. Hypercoagulability is an important

component of the pathogenic cascade involved in severe burns as well as in sepsis and septic shock that can lead to disseminated intravascular coagulation (DIC), that is characterized by microvascular thrombosis and consumption of clotting factors, platelets and strongly affect patient morbidity and mortality [50]. Previous studies reported a higher level of platelet-derived EVs (PEVs) in HIV-infected patients [51]. Moreover, viral or bacterial infections stimulate platelets to secrete more PEVs [52]. In our study, we aimed to investigate whether EVs isolated from burn patients with septic shock may stimulate platelet aggregation, thus participating in maintaining the hypercoagulable state described in these patients. The results we obtained demonstrated that EVs isolated from burn patients with septic shock did not stimulate *per se* platelet aggregation in PRP from healthy donors, but were able to enhance the effect of different agonists (EPI and ADP) on platelet activation. EVs are small in size and could act as a bridge for cell-to-cell interaction leading to thrombus formation [53]. In light of our and others results [54–56], EVs should be considered among the mediators involved in platelet activation, thus contributing to cell-to-cell interaction, thrombus formation and, thromboinflammation in septic shock. In a preliminary study, EVs exert coagulation properties in septic patients with DIC through tissue factor-EV activity [57].

EVs isolated from burn patients with septic shock showed enrichment of adhesion molecules, such as CD62p (p-selectin) and CD142 (tissue factor), on their surface, analogously to what had been described for parent cells [58] that mediated procoagulant effects. In our study, in addition to CD63p and CD41b, CD42a resulted one the most abundantly expressed epitopes expressed on EV surface, which also was increased in BSP compared with BnSP and HS. In addition, CD42a was significantly more expressed in EVs isolated from burn septic shock patients with the higher, compared to those with lower SOFA score. This suggests a potential use of CD42a expressing EVs as biomarker of disease severity and prognosis in this category of patients.

Until now, nothing is known about the pathophysiologic functions of CD42-EVs in patients with sepsis/septic shock. GPIbα (CD42a), together with GPIbβ and GPIX (CD42b), forms the glycoprotein Ib-IX complex that is a platelet receptor fundamental for platelet recruitment during haemostasis [59]. When vasculature is exposed to abnormally high shear stress, platelet and endothelial cells secrete von-Willebrand Factor (vWF); next, platelet GPIbα receptors spontaneously associate with circulating plasma vWF, causing platelet activation and aggregation thereby inducing the formation of platelet thrombi that may have severe pathological consequences [60]. Given the dual role of vWF-GPIba in both thrombosis and inflammation [61], the vWF-GPIba axis has received attention in particular in the setting of ischemic stroke [62], leading to hypothesize that blocking the GPIb-vWF

axis could be an interesting novel treatment strategy in stroke. Our results suggest that the high concentration of CD42-expressing EVs in burn patients with septic shock may have a pathogenic role in this condition by facilitating platelet aggregation. At the same time, others demonstrated that GpIba from platelet-derived EVs supports monocyte recruitment in systemic inflammation [63], defining novel thrombo-inflammatory pathways in which EVs transfer a platelet adhesion receptor to monocytes and are likely to be pathogenic.

Our study had some limits. First, we studied a relatively low number of subjects, especially in the BnSP groups. Moreover, the study was conducted in a single referral center, which may limit the generalizability of our data. Future studies would also need to include patients with sepsis but without septic shock or enrolled at different time points of their hospital stay and may be compare the patient categories we studied with patients affected by other critical diseases.

Founding details

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Table 1. Patients and HS characteristics.

Characteristics	Healthy subjects (n=10)	Burn non-septic patients (n=6)	Burn-septic shock patients (n=23)	*p*-value
Age, year	56.38 (± 20.19)	58.46 (± 18.66)	57.97 (± 16.74)	0.971
Gender ratio (M/F)	5/5	3/3	16/7	0.466
COMORBIDITIES				
Diabetes Mellitus	-	-	3	>0.999
Hypertension	-	1	7	0.134
% TBSA	-	10% (10-25)	30% (10-90)	**0.003**
Prognostic scoring systems				
Revised Baux index	-	0.19 (± 0.18)	0.48 (± 0.28)	**0.038**
SOFA score	-	-	11 (4-16)	na
Dialysis, n (%)	-	-	5	0.553
MAP		78.8 (± 22.10)	81.5 (33-100)	
P/F	-	337.5 (315.0-360.0)	277.0 (112.0-470.0)	0.600
Norepinephrine	-	-	0.31 (± 0.24)	
In hospital death, n (%)	-	-	8 (39.13%)	0.138
LABORATORY FINDINGS				
White Blood Cell 10^9 /L	5.51 (4.6-6.04)*	6.31 (4.8-16.26)	12.24 (7.96-46.0)	**0.011**
Hemoglobin g/dL	13.5 (± 3.50)	13.54 (± 2.00) †	9.44 (± 1.34)	**<0.05**
Platelets 10^9 /L	253.0 (223.0-278.0)	179.0 (± 43.41)	235.3 (± 160.8)	0.541
Creatinine mg/dL	-	0.72 (0.68-1.01)	0.97 (0.41-3.47)	0.130
Procalcitonin ng/ml	-	0.38 (0.27-1.02)	2.74 (0.15-99.67)	**0.010**
Lactate mmol/L	-	0.70 (0.40-1.90)	1.4 (0.6-3.9)	0.060
Bilirubin mg/dL	-	0.80 (0.30-1.70)	1.20 (0.40-7.40)	0.370
NT-pro-BNP ng/L	-	157 (18.0-736.0)	1295 (106.0-44552.0)	**0.004**
Copeptin pmol/L	-	7.3 (5.1-16.30)	44.5 (3.0-167.3)	**0.002**
Troponin I ng/L	-	2.9 (2.9-6.0)	22.0 (6.0-303.0)	**<0.001**
Myoglobin µg/L	-	36.0 (26.0-135.0)	156.0 (15.0-14265.0)	**0.019**

% TBSA, Total Body Surface Area; SOFA, Sequential Organ Failure Assessment; MAP, mean arterial pressure; P/F, PaO2/FiO2; mean (± SD) or median (range) as appropriate. * BSP vs HS, † BSP vs BnSP.

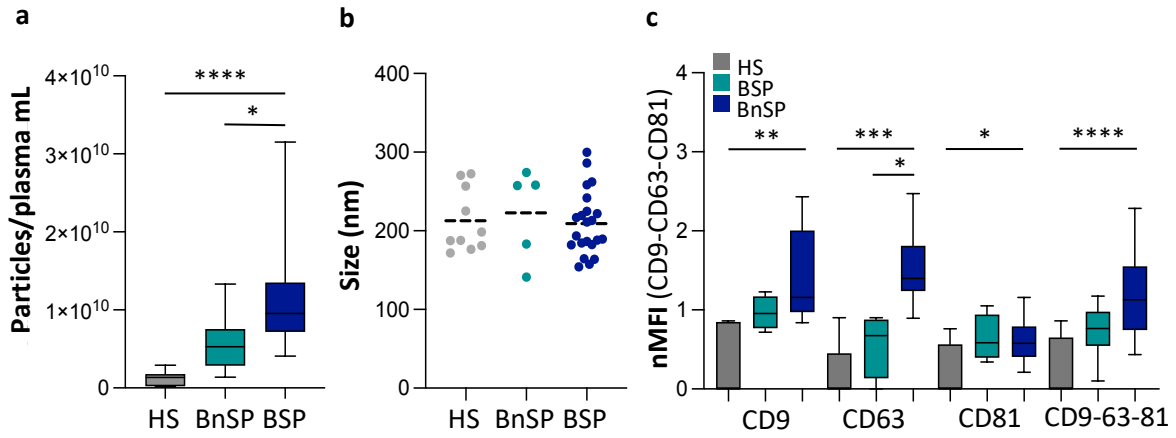


Figure 1 | Analysis for concentration (**a**) and size distribution (**b**) of plasma EVs in the three groups of study HS, BnSP and BSP by NTA. nMFI (normalized median fluorescence intensity) of CD9, CD63, CD81 and CD9/CD63/CD81 by bead-based flow cytometric analysis (**c**). Boxplots show median and range, bars show minimum and maximum values. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

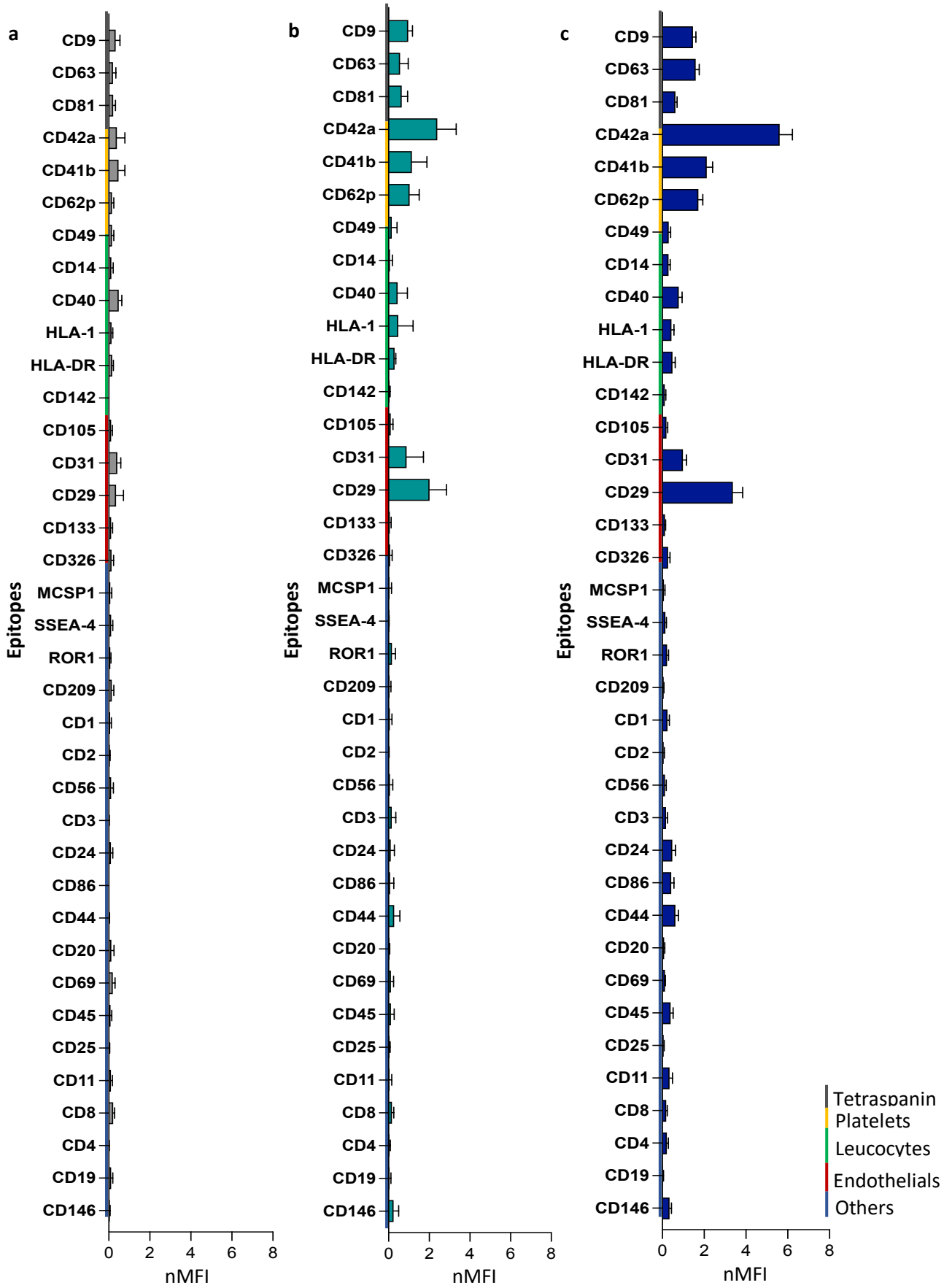


Figure 2 | Multiplex flow cytometric analysis of 37 EV surface epitopes in HS (**a**), BnSP (**b**) and BSP (**c**). Normalized median fluorescence intensity (nMFI).

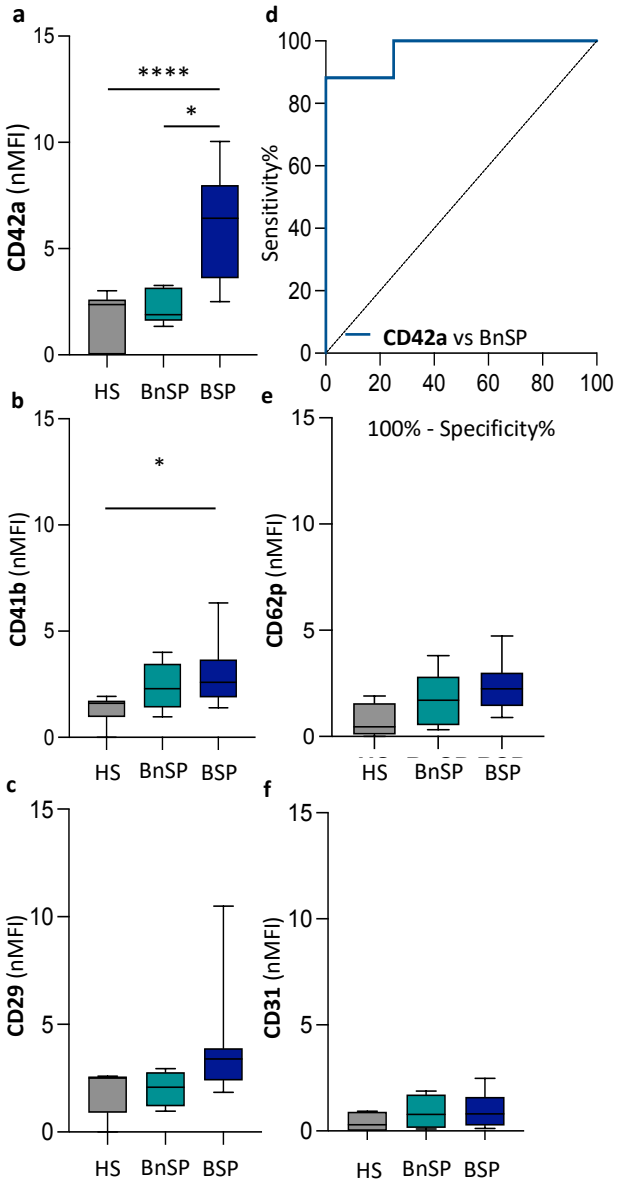


Figure 3| Expression of CD42a (**a**), CD41b (**b**), CD29 (**c**), CD62p (**e**), and CD31 (**f**) in HS. BnSP and BSP by multiplex flow cytometric analysis. ROC curve of CD42a-EVs (**d**) in the discrimination of BSP vs BnSP. *p<0.05; ****p<0.0001.

Table 2. Burn septic shock patient chracteristics.

	Burn-septic shock patients					
Characteristics	**%TBSA <50%** (n=17)	**%TBSA ≥50%** (n=6)	*p*	**SOFA score <10** (n=10)	**SOFA score ≥10** (n=13)	*p*
Age, year	63.3 (± 14.5)	42.9 (± 18.8)	**0.007**	59.2 (± 13.9)	57.1 (± 19.1)	0.773
Gender ratio (M/F)	13/4	3/3	0.318	6/4	10/3	0.650
COMORBIDITIES						
Diabetes Mellitus	3	-	0.539	1	2	>0.999
Hypertension	6	1	0.621	3	4	>0.999
% TBSA	25.7% (± 10.12)	67.5% (± 17.8)	**<0.001**	32.5% (15-60)	30% (8-90)	0.703
Prognostic scoring systems						
Revised Baux index	0.39 (± 0.25)	0.74 (± 0.18)	**0.006**	0.5 (± 0.3)	0.5 (± 0.3)	0.749
SOFA	10.8 (± 2.8)	8.3 (± 2.9)	0.082	7.5 (4.0-9.0)	12.0 (10.0-16.0)	**<0.0001**
Dialysis, n (%)	4	1	>0.999	1	4	0.339
MAP	80.0 (33.0-100.0)	84.5 (80.0-96.0)	0.360	83 (80-100)	83 (33-100)	0.288
P/F	258.6 (± 95.5)	371.5 (± 89.3)		367 (± 74.2)	227.4 (± 82.9)	**0.0004**
Norepinephrine	0.3 (± 0.2)	0.2 (± 0.2)	0.135	0.2 (± 0.2)	0.4 (± 0.2)	0.059
In hospital death, n (%)	7 (41.2%)	1 (16.7%)	0.369	2 (20%)	6 (46.1%)	0.379
LABORATORY FINDINGS						
White Blood Cell 10^9 /L	12.2 (8-46)	13.7 (10.7-31-4)	0.760	13.1 (11-31-4)	12.1 (8-46)	0.376
Hemoglobin g/dL	9.1 (7.5-13-7)	8.8 (8.3-10.7)	0.694	9.4 (7.5-11.2)	8.9 (8.3-13.7)	0.999
Platelets 10^9 /L	210.8 (± 117.4)	304.7 (± 248.8)	0.227	295.4 (± 195)	189.1 (± 116.5)	0.118
Creatinine mg/dL	1.3 (0.5-3.5)	0.7 (0.4-2.2)	**0.04**	0.8 (0.4-2.1)	1.8 (0.8-3.5)	**0.02**
Procalcitonin ng/ml	2.7 (0.1-99.7)	2.7 (0.5-11-6)	0.770	2.2 (0.1-24.0)	3.1 (0.3-99.7)	0.214
Lactate mmol/L	1.3 (0.6-3.9)	1.5 (1.3-2.5)	0.262	1.4 (± 0.4)	1.8 (± 1.0)	0.244
Bilirubin mg/dL	1.3 (0.5-7.4)	0.7 (0.4-3.9)	0.550	0.9 (0.4-2.1)	1.6 (0.5-7.4)	0.110
NT-pro-BNP ng/L	1685 (143-44552)	472 (10.9-167.3)	**0.04**	634 (106-35425)	2272 (143-44552)	**0.042**
Copeptin pmol/L	44.5 (10.9-167.3)	48.1 (3-122.8)	0.759	39.8 (3-167.3)	44.6 (15.5-141-4)	0.376
Troponin I ng/L	29.0 (6.0-303.0)	8.5 (3-13)	**0.01**	12 (3-95)	43 (3-303)	0.236
Myoglobin µg/L	207.0 (41.0-14265)	54.5 (15-276)	**0.02**	199.5 (41-690)	156 (15-14265)	0.636

% TBSA, Total Body Surface Area; SOFA, Sequential Organ Failure Assessment; MAP, mean arterial pressure; P/F, PaO2/FiO2; mean (± SD) or median (range) as appropriate.

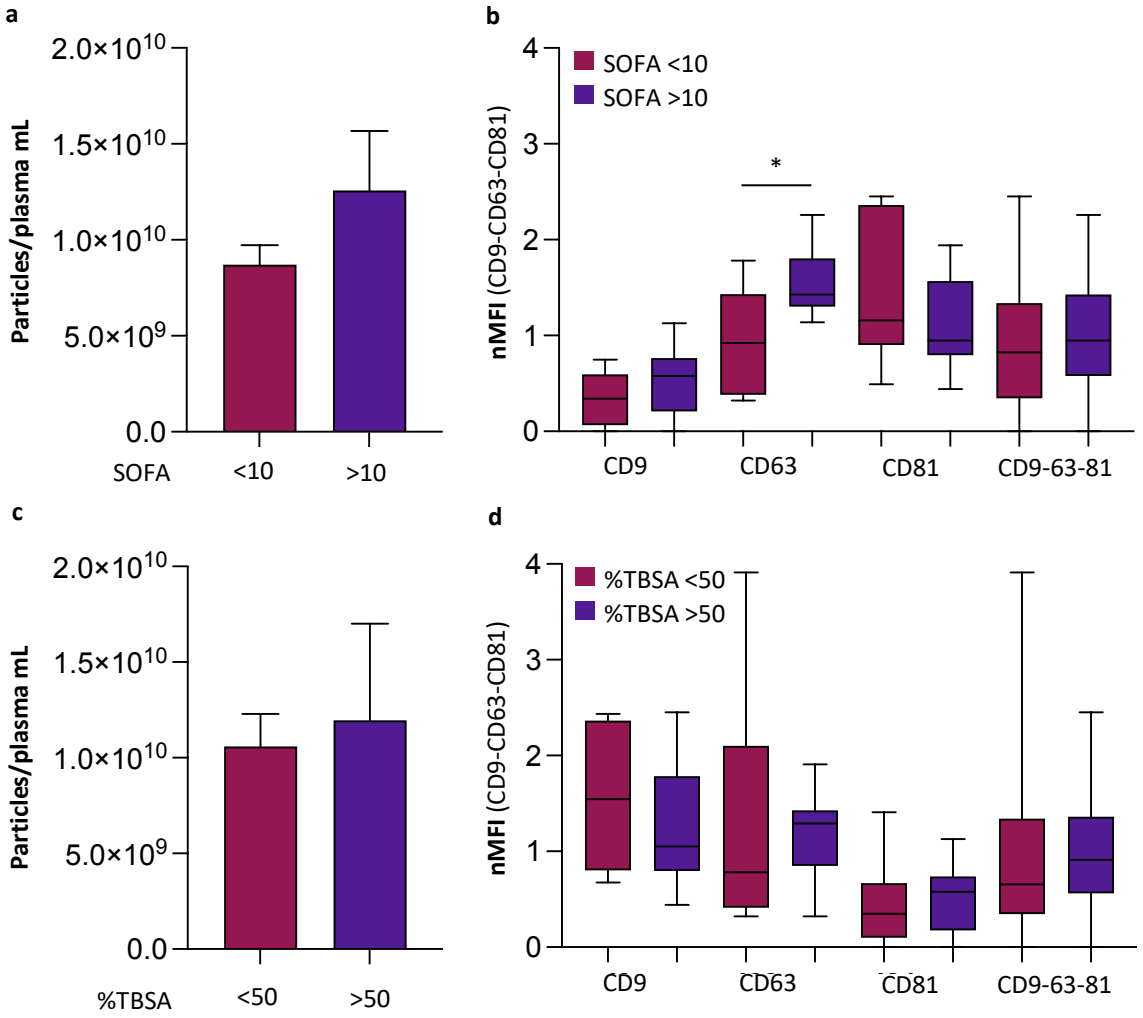


Figure 4 | EV concentration analysis in BSP with different SOFA score (**a**) and different %TBSA (**c**) by NTA. nMFI (normalized median fluorescence intensity) of CD9, CD63, CD81 and CD9/CD63/CD81 by bead-based flow cytometric analysis (**b, d**). Bar graphs show mean (± SEM). Boxplots show median and range, bars show minimum and maximum values. *p<0.05.

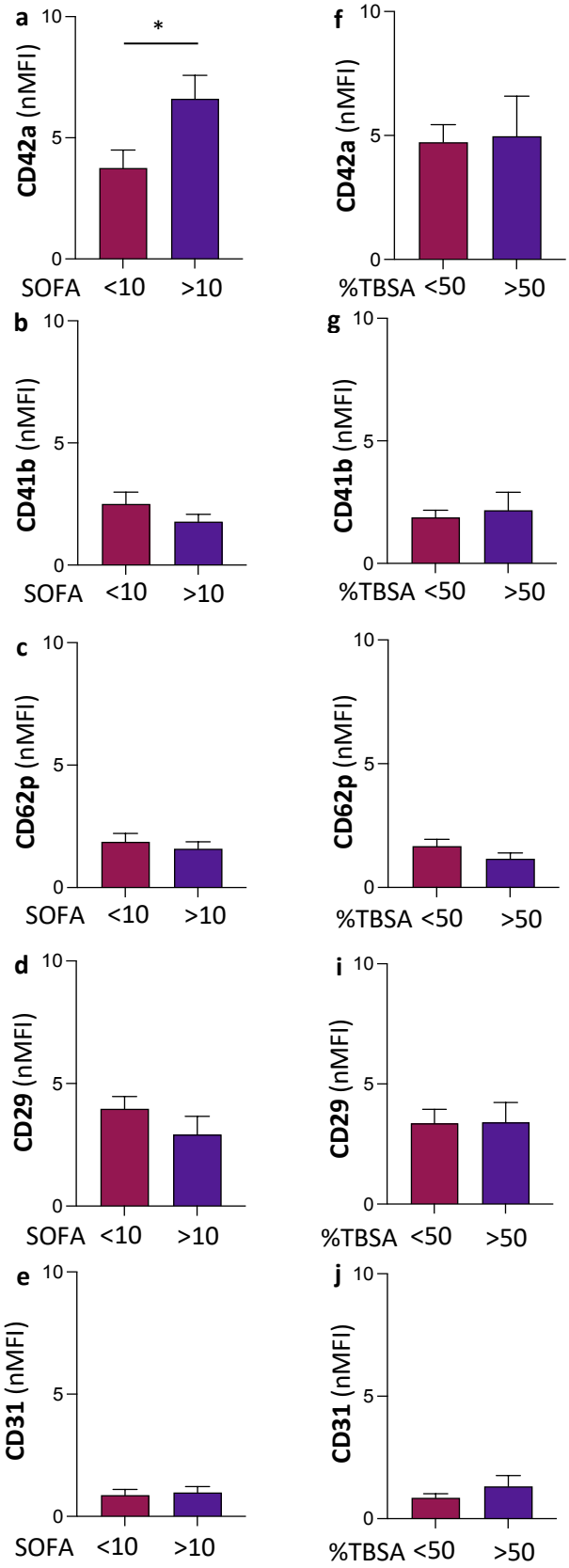


Figure 5 | EVs surface epitopes by flow cytometry analysis in BSP with different SOFA score and %TBSA. CD42a (**a**), CD41b (**b**), CD62p (**c**), CD29 (**d**) and CD31 (**e**). Bar graphs show mean (± SEM). *p<0.05.

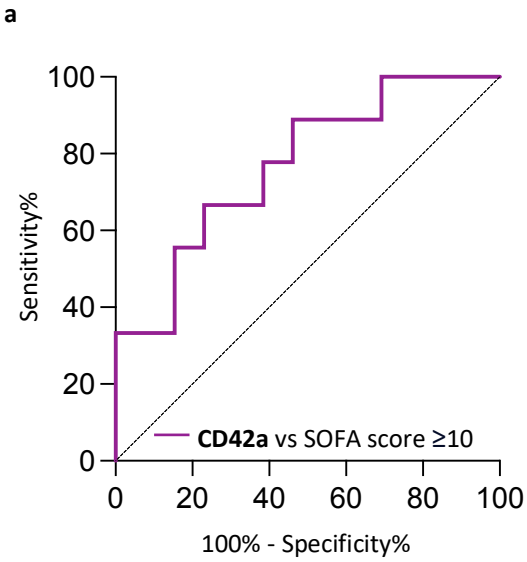


Figure 6| Performance of CD42a-EVs in the discrimination of BSP with different SOFA score (<10 points *vs.* ≥ 10 points) by ROC curve analysis.

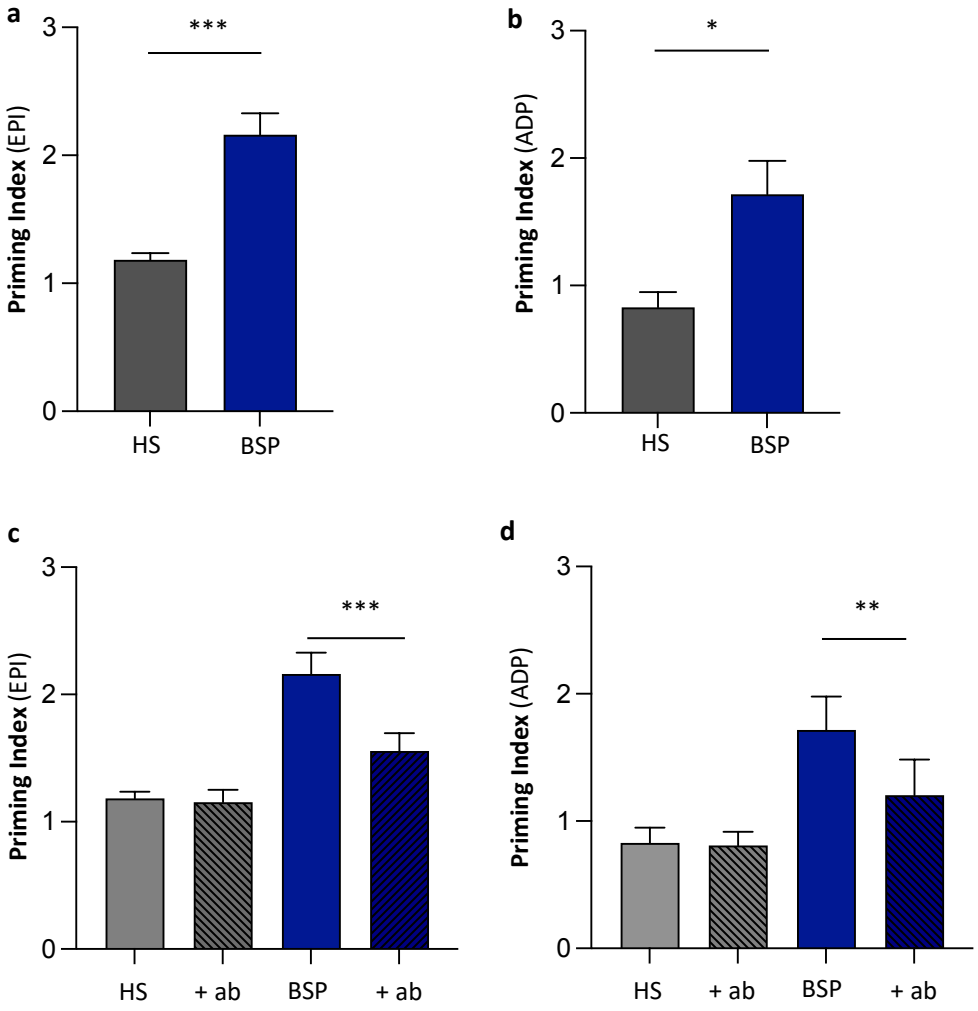
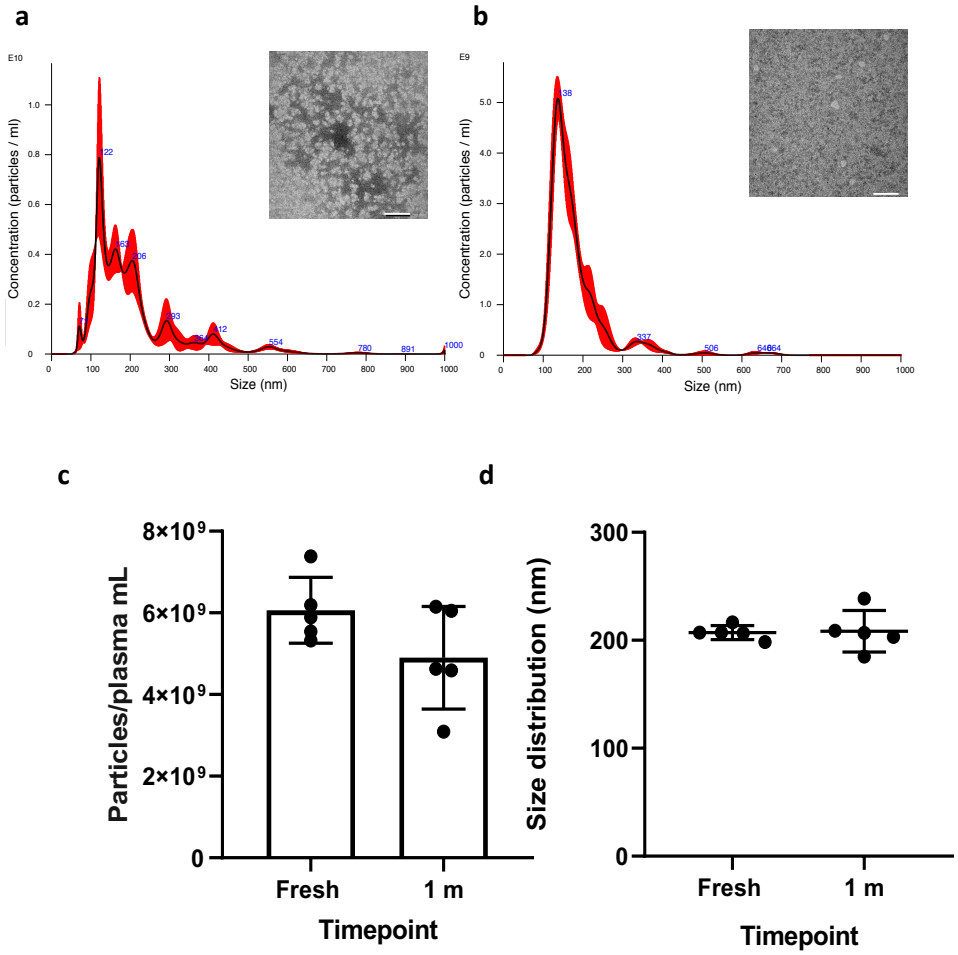
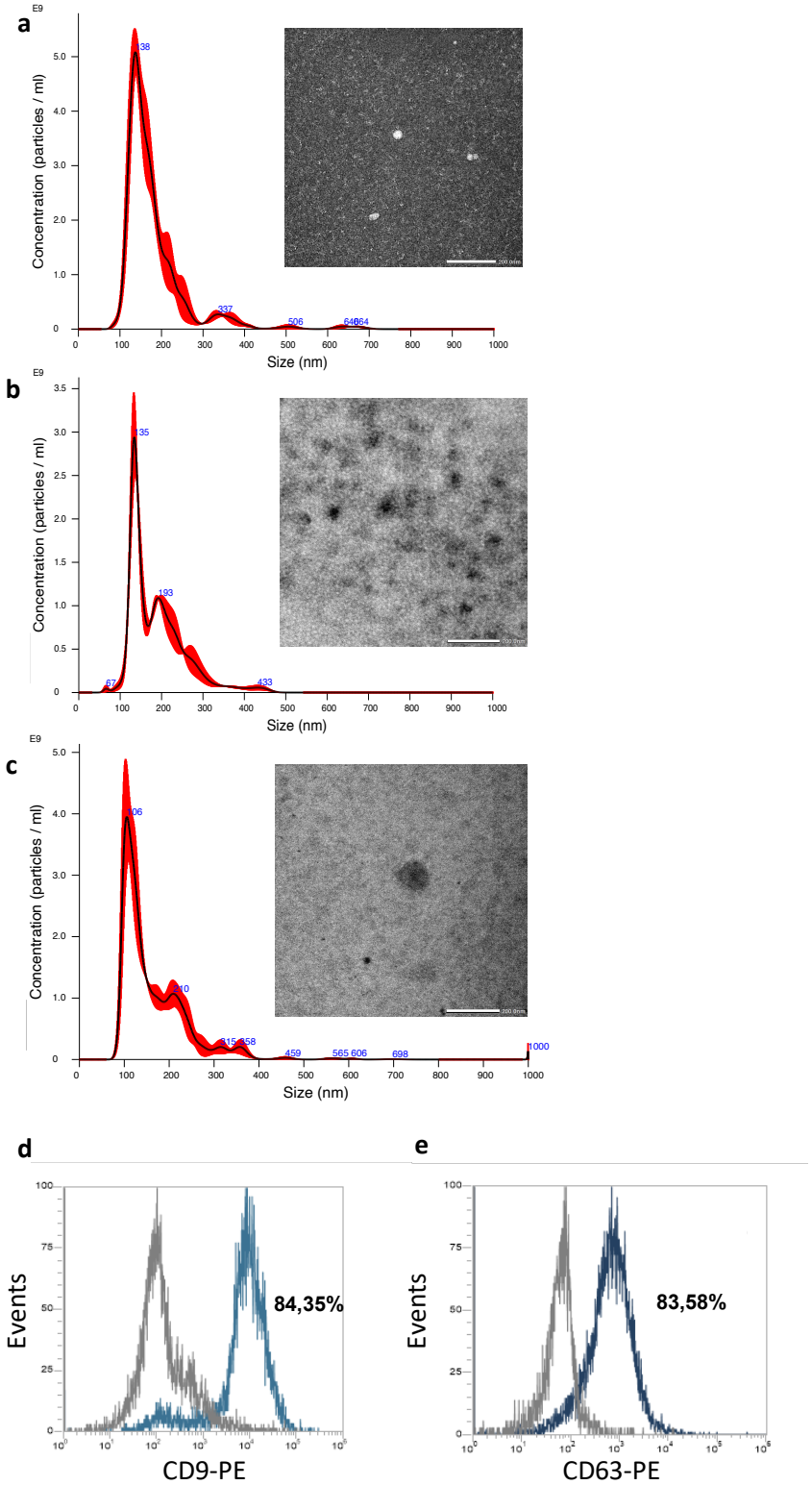


Figure 7 | *In vitro* effects of EVs on platelet activity. EVs isolated from BSP or HS were added to platelet-rich plasma (PRP) from healthy donors as described in the Methods section. When evaluating priming activity, epinephrine (EPI) or adenosine-diphosphate (ADP) was added as secondary agonist. EVs from BSP significantly increase platelet aggregation with both agonists (**a, b**) compared to HS. The pre-incubation of BSP-EVs with anti-CD42b antibody (+ab) (**c, d**) reduced platelet aggregation. Bar graphs show mean (± SEM). *p<0.05; **p<0.01; ***p<0.001.

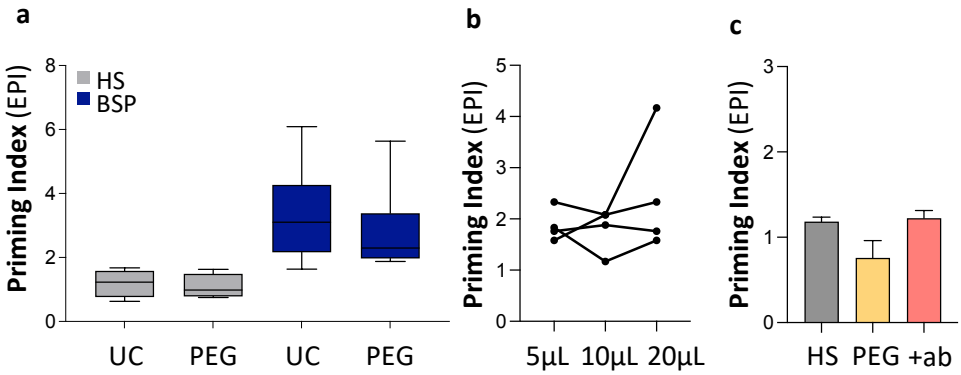
SUPPLEMENTARY RESULTS



Supplementary S1| Representative NTA and TEM images of EVs isolated from fresh (**a**) and stored (**b**) plasma samples. Concentration (**c**) and size distribution (**d**) of EVs isolated from in fresh and stored plasma samples from HS.



Supplementary S2| Representative NTA and TEM images of EVs isolated from HS (**a**), BnSP (**b**) and BSP (**c**). Representative flow cytometric analysis of tetraspanins CD9 (**d**) and CD63 (**e**) of HS-EVs compared with their appropriate isotype control (grey). Fluorescence (CD9-PE, CD63-PE; x-axis) *vs.* number of events (events; y-axis). The percentage expresses the overtone between isotype and antibody (**d**-**e**).



Supplementary S3 | EVs isolated from HS or BSP by ultracentrifugation or PEG-based precipitation method were added to platelet-rich plasma (PRP) from healthy donors, as described in the Methods section (**a**). When evaluating priming activity, epinephrine (EPI) was added as a secondary agonist. Evaluation of three different concentrations (5 µl, 10 µl, 20 µl) of EVs from BSP on platelet activity (**b**). Pre-incubation of EVs from HS, PEG precipitation buffer without EVs and anti-CD42b antibody without EVs, on PRP from healthy donor (**c**).

CHAPTER 4

Thrombopoietin participates in platelet activation in COVID-19 patients

Thrombopoietin participates in platelet activation in COVID-19 patients

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Abstract

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 megakariocyte 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 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 in priming *in vitro* platelet activation and leukocyte-platelet interaction in plasma samples obtained from COVID-19 patients.

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 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.

THPO concentrations correlated with those of IL-6 (r=0.2963; p=0.043), and decreased 24 hours 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 prevented 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 hyper-inflammation, 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 abolished by inhibiting the activity of thrombopoietin, thus suggesting that thrombopoietin participates in platelet activation in COVID-19.

Implications of all 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 (1,2), which strongly affect patient's prognosis (1). Microvascular or frank pulmonary thrombosis have been described in both autoptic (3,4) and imaging studies (2,5–8).

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

This complex interaction between coagulation and inflammatory pathways, also called thromboinflammation or immunothrombosis (16,19–25), 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 (16,19–23).

Patients with COVID-19 usually have a moderate thrombocytopenia, which can aggravate in the most severe cases, generally related to platelet-consumption (16,19,26,27). Several studies, however, have also provided evidence that circulating platelets in COVID-19 patients show features of hyper-activation and pro-coagulant state (19–21,28–32), which include changes in gene expression in pathways associated with protein ubiquitination, antigen presentation, and mitochondrial dysfunction (28). 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 (28), have increased basal expression of P-selectin, enhanced coagulation through Factor VIII, and increased release of pro-inflammatory cytokines and chemokines (28,30,32). Finally, COVID-19 patients also have higher circulating monocyte- and neutrophil-platelet aggregates (28,31,32). Platelet hyper-activation seems to correlate with the severity of COVID-19 (21).

Our and others research groups have previously shown that thrombopoietin (THPO), a growth factor essential to megakariocyte proliferation and differentiation in the bone marrow (33–35), is also able

to prime platelet aggregation and platelet-leukocyte adhesion in response to several stimuli (36–39).

Increased plasma concentrations of THPO have been described in several critical diseases where platelet activation represents a crucial pathogenic mechanism (36), including unstable angina (40), coronary artery disease (41), ischemic stroke (42), inflammatory bowel disease (43), burn injury (44), sepsis (45), and Severe Acute Respiratory Syndrome (SARS) (46). 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 (47). Furthermore, disease severity is a major determinant of elevated THPO levels in patients affected by sepsis (48) or acute pancreatitis (49), 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 (50), and that THPO cooperates with other cytokines, namely TNF-α and IL-1β, in mediating the depression of myocardial contractility induced by serum of patients with septic shock (51). 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 (52).

Based on available evidences, THPO appears as 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. The study was conducted according to the principles of Helsinki Declaration, and approved by our Institutional Ethical Committee (n. CS2/139).

Patients and Case Adjudication

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) during the study period. 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 (53), 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. 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-hours after the administration of tocilizumab (8 mg/kg BW *i.v.*, repeated after 12 hours), a blocking antibody directed against IL-6 receptor (54).

To obtain plasma samples, EDTA-anticoagulated tubes were centrifuged at 1,600 g for 15 minutes at 4°C within 2 hours 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 sqidlit system (SQI Diagnostics Systems Ltd., Toronto, Canada) with a time-to-result of 50 minutes. 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 forIL-6.

In Vitro Platelet aggregation

Platelet aggregation in 3.8% trisodium citrate-anticoagulated platelet-rich plasma (PRP) was evaluated as previously described (37,40). 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 (37,40).

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 (40). 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 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 (40).

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 whole blood with the Ab αTHPOR (3 µg/ml), and leukocyte-platelet adhesion was then evaluated as described above.

Cell Proliferation assay

The ability of rhTHPOR and Ab αTHPOR to block rhTHPO biological activity *in vitro* was tested by using the 5-bromo-2'-deoxyuridine (BrdU) Cell Proliferation Assay Kit (Abcam) on the megakaryoblast cell line M-07 (50,55), following manufacturer's instructions. Briefly, $2x10^4$ 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 rhTHPOR (2.5 µg/ml) for 1 hour, or M-07 cells were pre-treated with the Ab αTHPOR (3 µg/ml) for 1 hour.

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 ± standard error (SE), according to data distribution. 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, Wilkoxon 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) and area under the curve (AUC) statistics to test THPO and IL-6 accuracy as diagnostic biomarkers (56,57).

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

A p-value of < 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) (30 women, 17 men; median age 52 years; 22 to 97 years), whereas the diagnosis of COVID-19 was excluded in the remaining 19 patients (Non-COVID-19 patients) (14 women, 5 men; median age 53 years; 31 to 87 years).

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 (11 women, 7 men, all Caucasian; median age 51 years; 29 to 93 years), used as controls.

Demographic and clinical characteristics of study patients 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.

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 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).

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 Emergency Department, 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.

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 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 hours after the administration of tocilizumab, a blocking antibody directed against IL-6 receptor (54). THPO levels were significantly decreased 24 hours 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 (59,60), 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 αTHPOR, 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 αTHPOR 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 modifythe 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 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 whole blood 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 αTHPOR 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 αTHPOR did not modify the effects observed with plasma samples from healthy subjects either on monocyte- (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 (52).

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

On the basis of the ability of THPO to enhance platelet activation in several diseases (36), including unstable angina (40) and burn injury, especially after sepsis development (44), as well as in cigarette

smokers (61), 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 αTHPOR 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 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

platelet aggregation in both PRP and whole blood was previously seen using TNF-α, IL-1, IL-6, IL-3, GCSF, or GMCSF (44), as also shown by other studies (62). 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 (28), or the decrease of surface expression of THPO receptor following internalization upon binding with its ligand THPO (40,63,64).

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 hours (37).

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 (36). For instance, THPO stimulates IL-8 and reactive oxygen species release from neutrophils (65), promotes angiogenesis (66), and modulates the apoptotic processes in different mature cells (64,67).

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 (13), leading to propose the use of neutralizing

antibodies against IL-6 as a therapeutic option, especially in the most critically ill COVID-19 patients (60). 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 (59,68), while THPO concentrations markedly decreased. These results, in accordance with previous studies showing increased hepatic synthesis stimulated by IL-6 in hepatocytes (47), 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 (35). 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 (16,69). 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 (70), 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 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.

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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: 10.106/j.ebiom.2022.104305.

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	Healthy subjects (n = 18)	Non-COVID-19 (*n* = 19)	COVID-19 (*n* = 47)	Mild COVID-19 (*n* = 36)	Severe COVID-19 (*n* = 11)
Age, median (range), yr	51 (29−93)	53 (31−87)	52 (22−97)	48.5 (22−93)	68 (43−97)§
Gender, N (%)					
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)
Ethnicity, N (%)					
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
Symptoms, N (%)					
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)*	7 (19.4)	2 (18.2)
Comorbidities, N (%)					
Hypertension	-	2 (10.5)	12 (25.5)	6 (16.7)	6 (54.5)§
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)§
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)**	15 (41.7)	0§§
Ward Admission	NA	3 (15.8)	26 (55.3)**	20 (55.6)	6 (54.5)
ICU/HDU Admission	NA	1 (5.3)	6 (12.8)	1 (2.8)	5 (45.4)§§
MV (NIV/IMV)	NA	1 (5.3)	5 (10.6)	0	5 (45.4)§§§
In-hospital Mortality	NA	1 (5.3)	5 (10.6)	0	5 (45.4)§§§

***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.

	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 (10^9/L)	5.4 (3.9−6.9)	7.8 (4.7−20.3)†††	5.6 (2.4−17.3)**	5.5 (2.4−10.1)	7 (5.5−17.3)§§
Neutrophil count (10^9/L)	3.3 (1.7−4.3)	5.5 (1.9−18.2)††	3.6 (0.8−16.6)	3.2 (0.8−9.1)	5.3 (2.2−16.6)§§
Lymphocyte count (10^9/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)§§
Neutrophil / Lymphocyte (ratio)	2.0 (1.0−4.3)	2.3 (0.8−23.4)	2.9 (0.5−49)†	2.2 (0.5−1620)	19.5 (0.5−1020)§§
Leukocyte/Lymphocyte (ratio)	3.3 (2.3−5.9)	3.6 (2.5−26)	3.9 (1.6−51)†	3.5 (1.8−28.6)	9.7 (1.6−51)§§
Platelet count (10^9/L)	209 (119−365)	271 (185−386)††	190 (105−401)***	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)§§
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)†††	8.7 (0.3−133)	66.6 (37.9−261)§§§

***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).

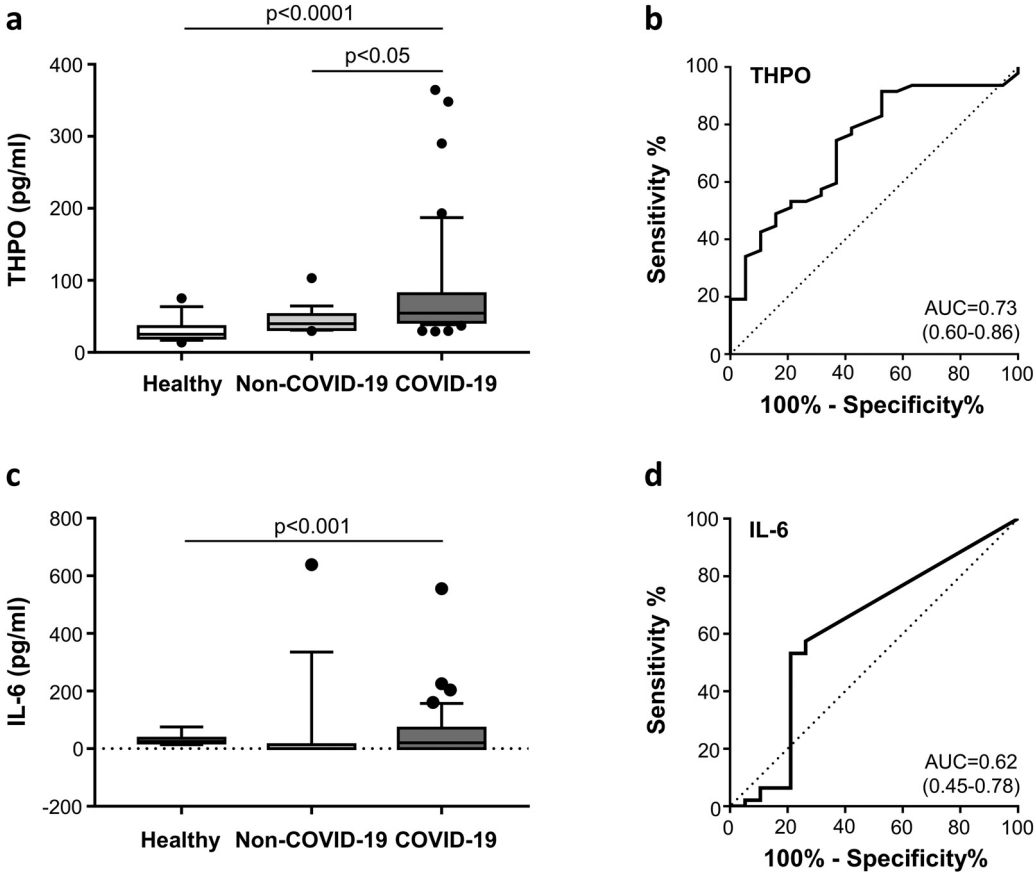

Figure 1| Comparison of plasma levels of THPO and IL-6 in healthy subjects, Non-COVID-19 patients, and COVID-19 patients and relative receiver-operating characteristics (ROC) curves .for the diagnosis of COVID-19. Box-and-whisker plots of THPO (**a**) and IL-6 (**c**) plasma concentrations in healthy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

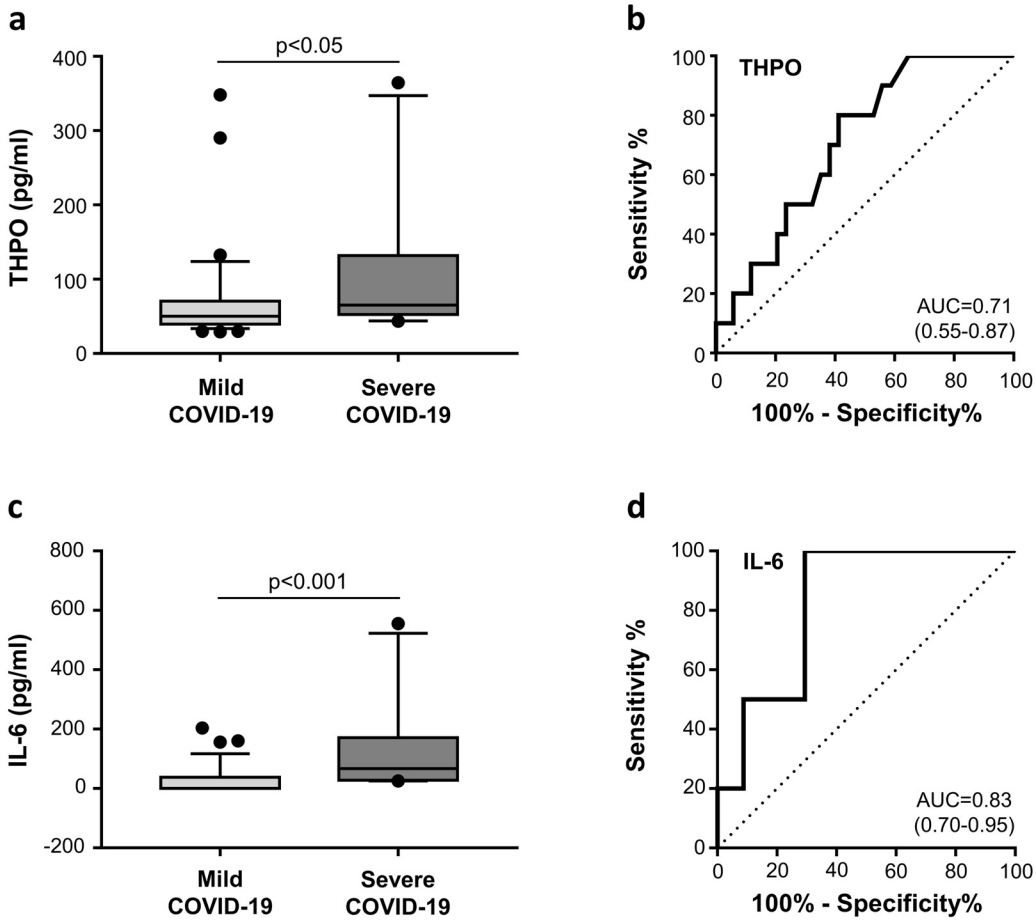

Figure 2| Comparison of THPO and IL-6 plasma levels in mild and severe COVID-19 patients and relative receiver-operating characteristics (ROC) curves for the development of severe COVID-19. Box-and-whisker plots of THPO (**a**) and IL-6 (**c**) plasma concentrations in mild (n=36) and severe (n=11) COVID-19 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.

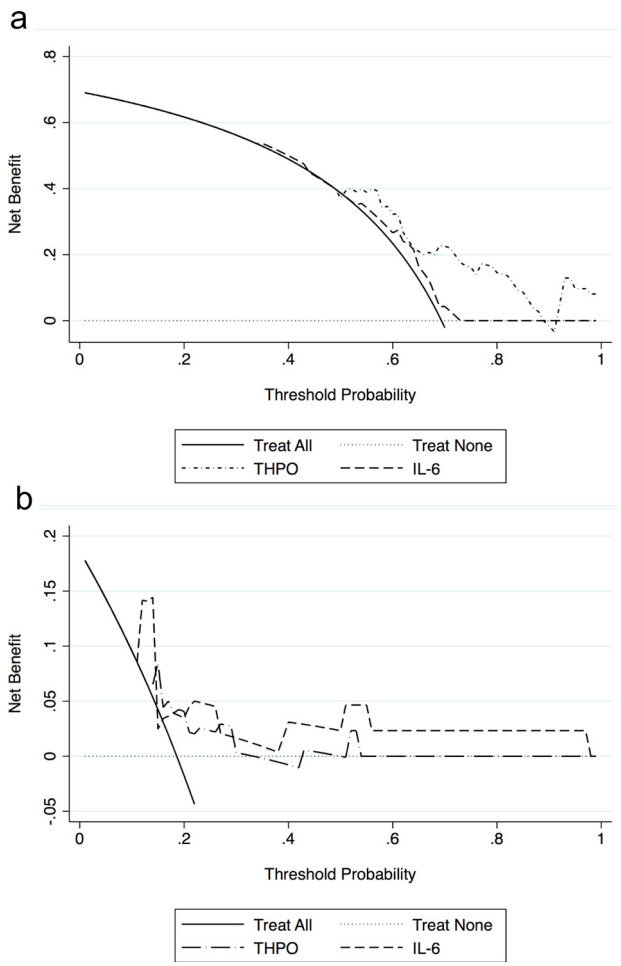

Figure 3| Decision curves for THPO and IL-6 for the diagnosis of COVID-19 (**a**) and for the risk of severe COVID-19 development (**b**).

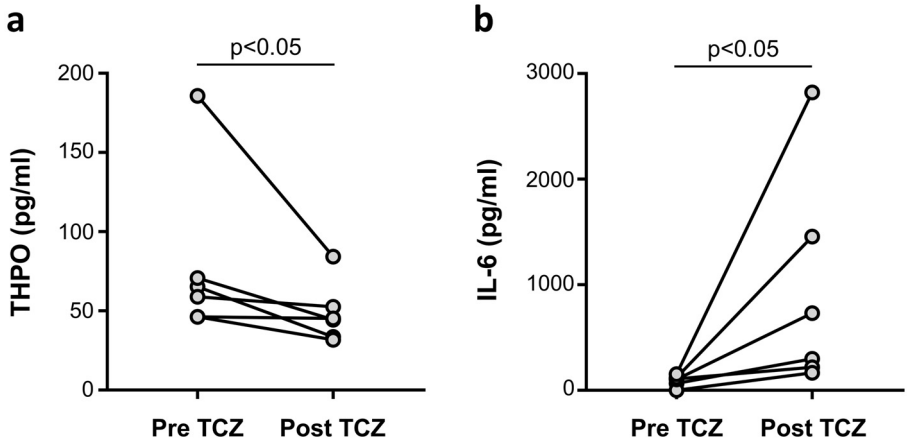

Figure 4 |Effect of tocilizumab (TCZ) administration on THPO and IL-6 plasma levels in six COVID-19 patients. Scatter plots of THPO (**a**) and IL-6 (**b**) plasma levels measured pre- and post-TCZ administration in COVID-19 patients. Data were analyzed by using Wilcoxon matched-pairs signed rank test.

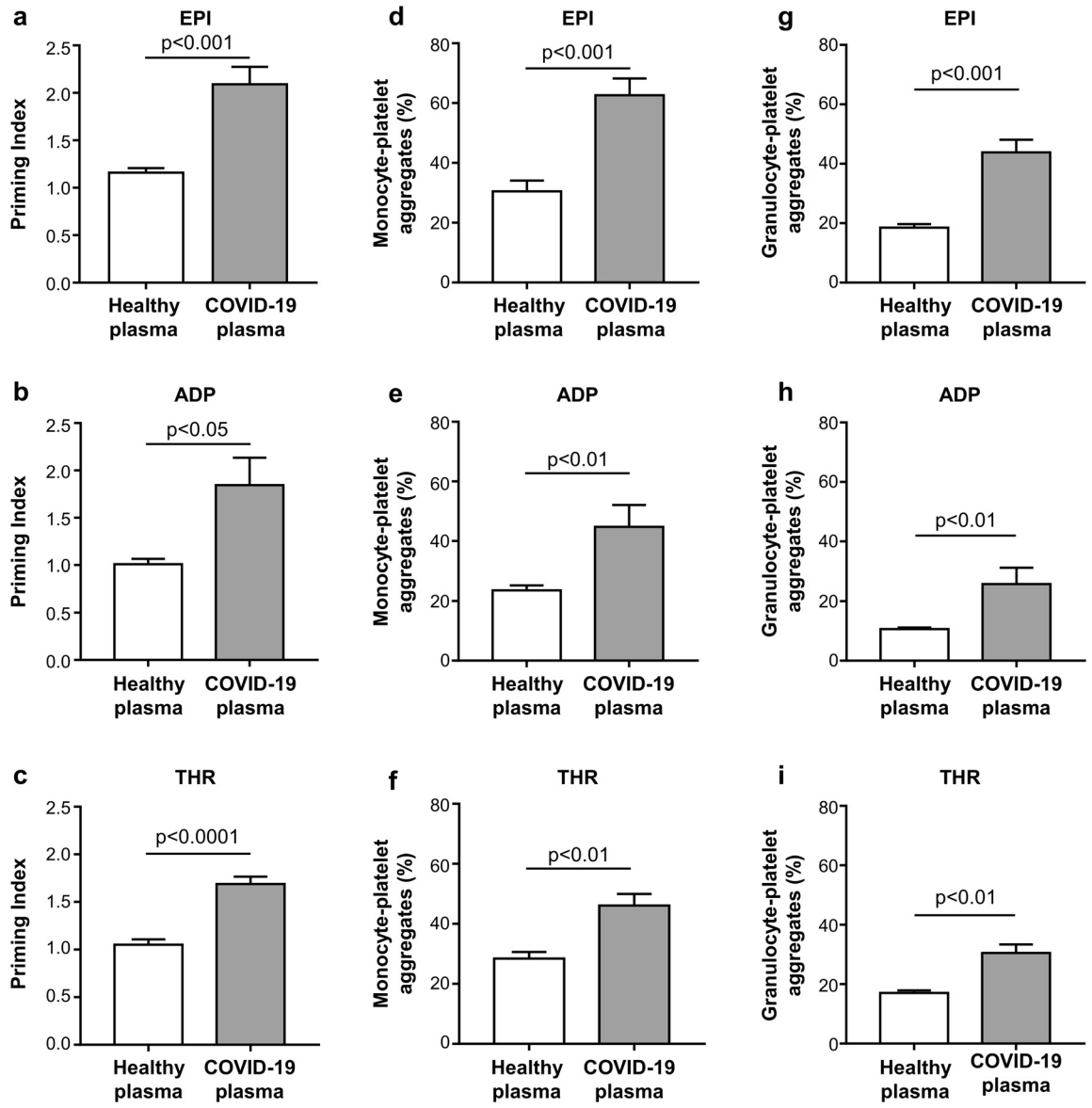

Figure 5| Effect of plasma from healthy subjects and COVID-19 patients on *in vitro* platelet aggregation in platelet-rich plasma (PRP). Bar graph showing the *in vitro* priming activity induced by plasma from healthy subjects and COVID-19 patients on platelet aggregation in PRP induced by epinephrine 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 were analyzed by using paired Student's t-test.

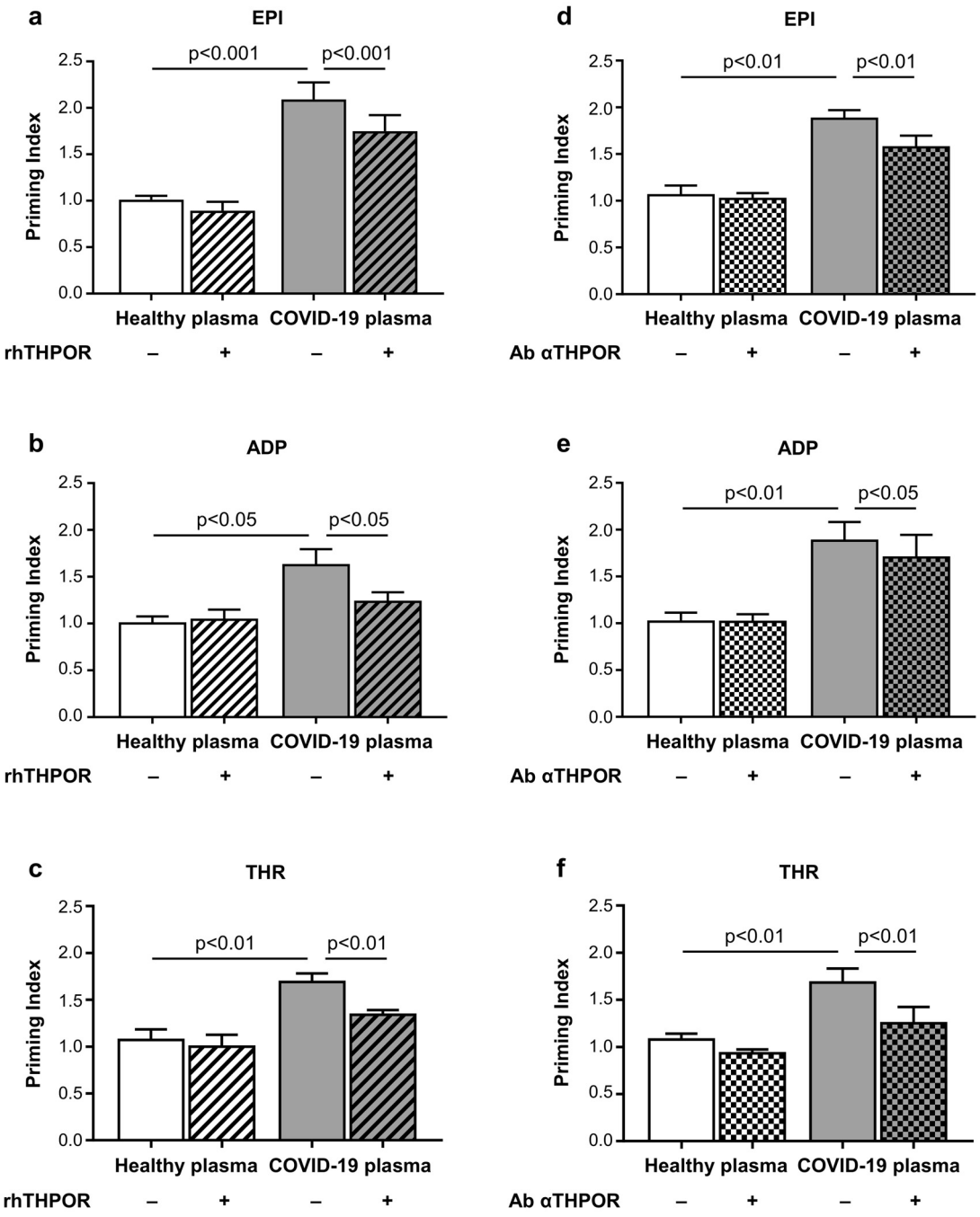

Figure 6| Inhibitory effect of rhTHPOR and Ab αTHPOR on *in vitro* platelet aggregation in platelet-rich plasma (PRP). Bar graph showing the effects exerted by plasma of COVID-19 patients pre- incubated or not with rhTHPOR on platelet aggregation induced in PRP by EPI (**a**), ADP (**b**), and THR (**c**). 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 condition was performed. Data were analyzed by using paired Student's t-test.

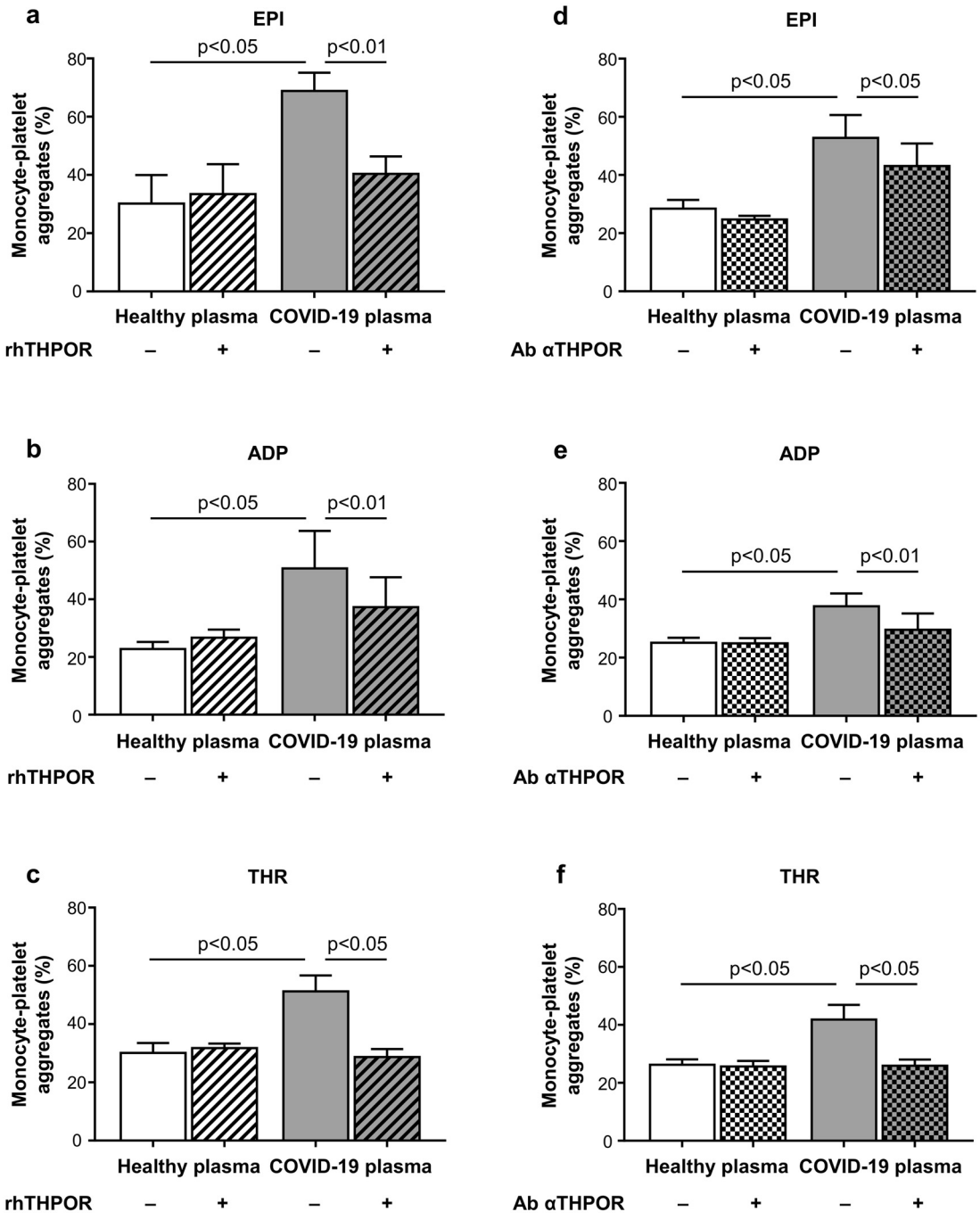

Figure 7| Inhibitory effect of rhTHPOR and Ab αTHPOR on *in vitro* monocyte-platelet aggregation in whole blood. Bar graph showing the effects exerted by plasma of COVID-19 patients pre- incubated or not with rhTHPOR on monocyte-platelet aggregation induced in whole blood by EPI (**a**), ADP (**b**), 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 αTHPOR, on monocyte-platelet aggregation induced in whole blood by EPI (**d**), ADP (**e**), and THR (**f**). A minimum of five experiments for each experimental condition was performed. Data were analyzed by using paired Student's t-test.

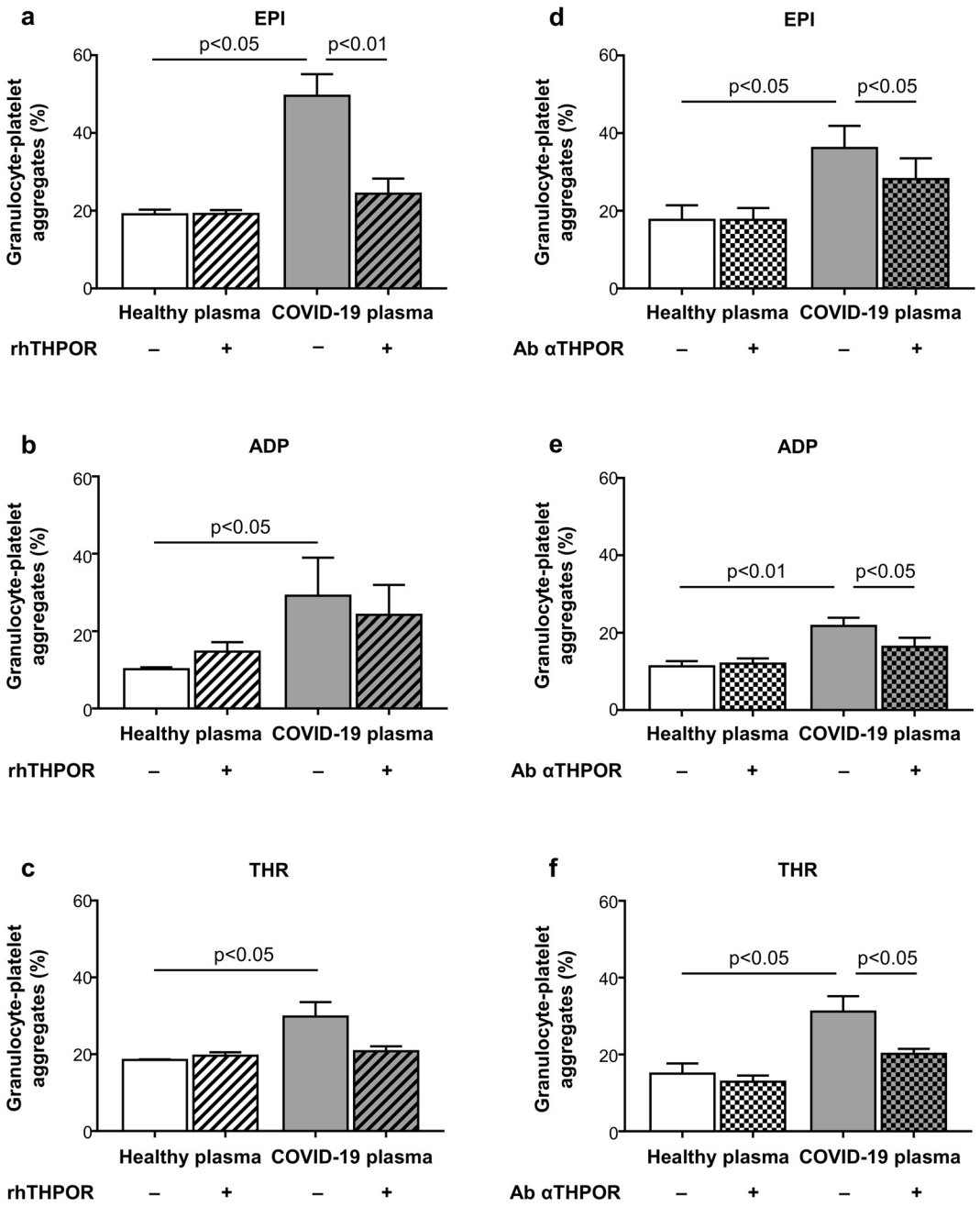

Figure 8 | Inhibitory effect of rhTHPOR and Ab αTHPOR on *in vitro* granulocyte-platelet aggregation in whole blood. Bar graph showing the effects exerted by plasma of COVID-19 patients pre- incubated or not with rhTHPOR on granulocyte-platelet aggregation induced in whole blood by EPI (**a**), ADP (**b**), 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 αTHPOR, on granulocyte-platelet aggregation induced in whole blood by EPI (**d**), ADP (**e**), and THR (**f**). A minimum of five experiments for each experimental condition was performed. Data were analyzed by using paired Student's t-test.

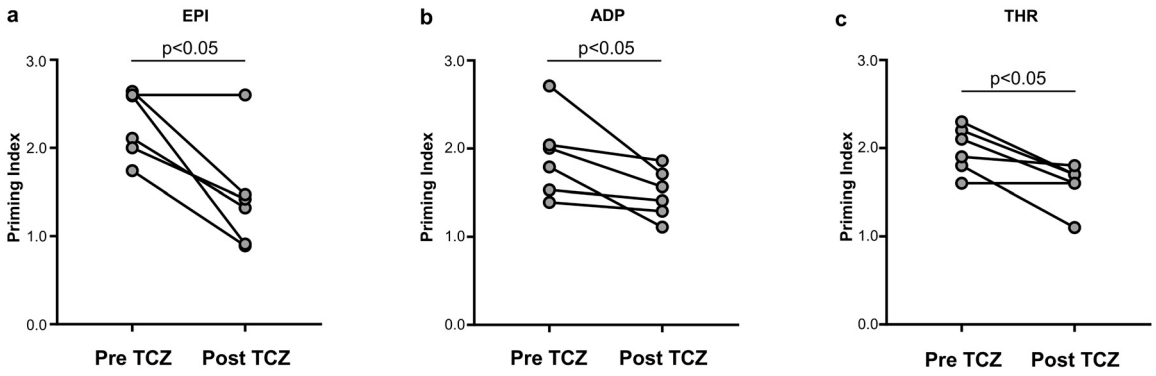

Figure 9: Effect of plasma obtained from six COVID-19 patients before and after tocilizumab (TCZ) administration on *in vitro* platelet aggregation in platelet-rich plasma (PRP). Scatter plots showing the *in vitro* priming activity induced by plasma obtained from COVID-19 patientsbefore and after TCZ 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.

CHAPTER 5

Utility of plasma microRNA profiling as diagnostic biomarker and early predictor of severity in COVID-19

Utility of plasma microRNA profiling as diagnostic biomarker and early predictor of severity in COVID-19

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[in preparation]

ABSTRACT

Background: The clinical course of coronavirus disease 2019 (COVID-19), which is caused by SARS-CoV-2 infection, may profoundly vary from mild to lethal forms according to the development of respiratory failure, target-organ damage, and thromboembolic events. MicroRNAs (miRNAs) are small non-coding RNAs that play pivotal roles in many biological processes. Several studies reveal the expression and involvement of miRNAs in several diseases, including viral respiratory infections. In this study, we aimed to investigate miRNA profiles and to explore miRNAs as potential diagnostic biomarkers and early predictors of severity in COVID-19 patients.

Methods: We enrolled, at the time of first medical evaluation in the Emergency Department, 40 patients with symptoms suggestive of COVID-19, in 30 of whom the diagnosis of COVID-9 was confirmed, whereas in 10 excluded (non-COVID-19 patients). Ten healthy subjects were also used as controls. In addition, 26 COVID-19 patients who required hospitalization were followed up and split in two groups (poor vs. good prognosis) according to the development of respiratory failure needing mechanical ventilation. Plasma miRNA profiling, single-tube validation, and miRNA annotations to target genes were performed by qRT-PCR and *in silico* analysis.

Results: We found that miR-133a-3p ($p=0.003$) and miR-199a-5p ($p<0.0001$) were higher in COVID-19 patients compared to healthy subjects. Moreover, miR-133a-3p ($p=0.004$) expression increased in COVID-19 compared to non-COVID-19 patients.

Moreover, miR-423-3p, miR-205a-5p, and miR-106b-5p expression levels were lower in patients with poor prognosis than in those with good prognosis. We also found miR-133a-3p, miR-199a-5p, miR-423-3p, miR-205a-5p, and miR-106b-5p as regulators in the platelet activity, coagulation and thrombosis pathways.

Conclusions: Plasma miRNA profiling may be useful to identify candidate biomarkers useful for the diagnosis of COVID-19 in patients with symptoms suggestive of this disease or for early prediction of severity.

Keywords: COVID-19, miRNA, profiling, biomarkers, bioinformatics.

Background

The clinical course of Coronavirus disease 2019 (COVID-19), which is caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infection, may profoundly vary from mild to lethal forms according to the development of respiratory failure, target-organ damage, and thromboembolic events [1,2]. Approximately 20-30% of hospitalized COVID-19 patients develop a severe phenotype of the disease that requires intensive care unit (ICU) admission [3] with a death rate reaching up to 50% [4].

One of the most impelling clinical needs is the identification of reliable markers to early define patient severity and predict clinical prognosis [5,6].Although risk modeling based on clinical characteristics and/or biomarkers has made remarkable progress, early prediction of vital status deterioration in COVID-19 patients remains a challenge. The search for alternative clinical indicators, including those provided by emerging omic technologies, may lead to develop new tools useful in COVID-19 patient management.

MicroRNAs (miRNAs) are small non-coding RNAs that play significant roles in many biological processes by binding to complementary regions of messenger RNA (mRNA), and leading to repression of its translation or even induction of its degradation [7]. Although many of the processes and mechanisms involved have not yet been fully elucidated, there is a strong association between miRNA expression and several diseases [8–10], including viral respiratory infections [11]. Moreover, the presence of many miRNAs has been discovered in various body fluids [12], including serum and plasma [13], suggesting the possible use of circulating miRNA analysis for monitoring critical patients. Several *ex vivo* studies documented an altered plasma miRNA profile in different COVID-19 patient cohorts [14–16]. Furthermore, by computational prediction studies, it has been shown that the different miRNA profiles could mirror distinct phases of the disease, from its onset to recovery [17,18].

In this study, we studied the expression of circulating miRNAs in patients with COVID-19 at the time of diagnosis. In addition, we investigated whether miRNA expression varies in those COVID-19 patients who later developed respiratory failure needing mechanical ventilation. Our evidence suggest that miRNA molecular phenotyping could represent a valuable, non-invasive diagnostic and prognostic early biomarker in COVID-19 patients.

Methods

Patient enrollment and study design

A prospective observational cohort study was carried out in 40 patients admitted to the Emergency Department (ED) of the "Città della Salute e della Scienza di Torino – Molinette Site", University Hospital, Turin, Italy, from February to May 2021.

We considered eligible all adult patients (age>18 years) who, at triage, screened positive for acute symptoms commonly associated with SARS-CoV-2 infection (*i.e.* fever, dyspnea, cough, sore throat, diarrhea, ageusia, anosmia). Patients already intubated at the time of ED arrival were excluded. Other exclusion criteria were as follows: death or orotracheal intubation (OTI) in the first 24 hours following ED admission, known hematological diseases affecting coagulation and/or platelet count, 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, and final case adjudication was performed by two expert physicians who independently assessed all patient's data.

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 between 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) were considered poor prognosis COVID-19 patients. Of note, none of these patients presented respiratory failure or organ damage at the time of first medical evaluation in the ED, when patients in both groups had comparable symptoms and clinical severity.

Finally, 10 healthy subjects (HS) were recruited among laboratory staff members, and used as an additional control group.

The experimental process was separated into three phases (screening, data analysis and validation) **(Figure 1).**

Blood collection and plasma preparation

Peripheral blood samples were collected by clean venipuncture using a 21-gauge infusion set in EDTA-vacutainers, and, after discharging the first mL, centrifuged for 10 min at 1,600 x *g* at 4°C

within 1 h from collection. The plasma obtained was further centrifuged at 13,300 x *g* for 10 min at 4°C, and stored at -80°C until use. No freeze-thaw cycles were performed during the experiments.

miRNA isolation

miRNAs were extracted from 200 µL of plasma by using the miRNeasy Serum/Plasma Advanced Kit (Qiagen, Hilden, Germany), as described by the manufacturer. A spike-in exogenous control, cel-miR-39-3p (478293_mir TaqMan Advanced Assay, Applied Biosystems, Thermo Fisher Scientific, MA, USA) from *Caenorhabditis elegans*, lacking sequence homology to human miRNAs, was added to each sample as an external reference miRNA (4.8 x 10^8 copies/sample). After silica-membrane-based purification on RNeasy UCP MinElute spin column, high-quality RNAs, primarily miRNAs and other small non-coding RNAs, were eluted into 20 μL of RNase-free water and stored at -80°C.

cDNA synthesis and pre-amplification

Since the overall amount of RNA present in plasma is low and the RNA concentration cannot be accurately determined, an equal amount (2 µL) of each miRNA extract was processed using the TaqMan Advanced miRNA cDNA synthesis kit (Applied Biosystems, Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, the cDNA templates were obtained through the following steps: poly-A-tailing, adapter ligation, reverse transcription (RT) and pre-amplification. The converted cDNAs were stored at -20°C.

miRNA relative quantification by TaqMan miRNA array

For the diagnostic and prognostic screening phase, four pooled samples composed of one pool obtained from HS (n=8) and three pools obtained from COVID-19 patients, each one from 8 patients, were processed by pooling a fixed volume of cDNAs using TaqMan™ Advanced miRNA Human Cards A and B (Applied Biosystems, Thermo Fisher Scientific) combined with 754 miRNA targets. Briefly, pooled cDNA was dispensed into each of the 8 loading ports per card. Plates were then centrifuged twice at 330 x *g* (1 min at room temperature) and sealed to ensure a uniform distribution across the plate. qRT-PCR reaction was performed under the following cycling conditions: 92°C (10 min), 40 cycles of 95°C (1 sec) and 60°C (20 sec) by QuantStudio 12K Flex Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific). Endogenous and exogenous miRNA controls for data normalization were included in the array. Data were analyzed by QuantStudio Design and Analysis Software v1.4.1 (Applied Biosystems, Thermo Fisher Scientific).

Data preprocessing

To ensure the optimal quality of the data, we first analyzed spike-in miRNA templates to monitor the uniformity of the RNA extraction procedure and the efficiency of qRT-PCRs. Then, the evaluation

of the difference in C_t values between miR-24a-3p and miR-451a was used to exclude hemolysis contamination that could affect the results of some miRNA expression; according to Blondal T et al. [13] samples with ΔCt (miR-24a-3p and miR-451a) above 5 were not analyzed. Then, miRNAs with a Ct > 33 were considered undetectable, and excluded from the analysis. Relative quantification (Rq) was performed using the $2^{-\Delta\Delta Ct}$ method, normalizing to cel-miR-39 expression levels.

Bioinformatics analysis

miRNA annotations to target genes were retrieved from miRbase, and a consensus analysis was applied to associate potential gene targets to each miRNA using R package multiMiR [19]. Specifically, the package provides both experimentally validated miRNA-mRNA targets from miRecords, miRTarBase and TarBase and predicted targets from Diana, ElMMo, MicroCosm, Miranda [20], MirDB [21], Pictar [22], Pita [23], and Targetscan [24]. We considered those target genes that were either annotated as experimentally validated in at least one database or predicted by at least three tools. We then functionally characterized the lists of selected miRNAs through a pathway enrichment analysis, collecting the annotations of the gene targets to 333 and 617 pathways from Kyoto Encyclopedia of Genes and Genomes (KEGG) [25] and WikiPathways [26] databases, respectively. Each gene-to-pathway annotation was converted into the corresponding miRNA-to-pathway association considering the consensus analysis described above. Fisher's Exact test was applied to each pathway, considering the number of annotated miRNAs and adjusting the p-values for multiple testing using the False Discovery Rate (FDR). We then mainly focused on those pathways that were significantly enriched and of interest for our study. From these pathways, we considered, among the miRNAs associated with the annotated genes, only those characterized by the highest/lowest fold-change values.

Experimental validation of selected miRNAs by single-tube miRNA assay

Validation of selected miRNAs was carried out by qRT-PCR on single-tube assays using the QuantStudio 1 Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific) for the following probes: hsa-miR-199a-5p (478231_mir), hsa-miR-200a-3p (478490_mir), hsa-miR-100-5p (478224_mir), hsa-miR-133a-3p (478511_mir), hsa-miR-28-5p (478000_mir), hsa-miR-139-5p (478312_mir), hsa-miR-374a-5p (478238_mir), hsa-miR-205-5p (477967_mir), hsa-miR-423-3p (478327_mir), hsa-miR-106b-5p (478412_mir), and cel-miR-39-3p (478293_mir) (Applied Biosystems, Thermo Fisher Scientific). We performed qRT-PCR for three technical replicates for all analyzed miRNAs, through the following steps: 95°C (10 min), followed by 40 cycles at 95°C (15 sec) and 60°C (1 min).

In silico evaluation of selected miRNAs and of their target genes

Among the diagnostic miRNAs selected by using the enrichment analysis, we aimed to identify those target genes that are preferentially involved both in COVID-19 pathophysiology and in blood coagulation, thrombosis, and platelet activation. Therefore, we downloaded two expression datasets from the Gene Expression Omnibus (GEO) database: 1) Human induced pluripotent stem cell-derived cardiomyocytes infected with SARS-CoV-2 vs control cultures (GSE150392); 2) Autopsies of SARS-CoV-2 infected tissues from which we selected datasets related to Lungs, Hearts and control samples (GSE150316). Differential expression analysis was applied to each dataset using DEseq2 R package [27]. Differentially expressed (DE) genes were selected considering p-values <0.05, adjusted by False Discovery Rate (FDR).

We then intersected the select genes with: 1) 98 genes associated with the term "thrombosis" from Disgenet database (https://www.disgenet.org/browser/0/1/0/C0040053/); 2) 67 genes associated with the term "thrombosis" from Malacards database (https://www.malacards.org/); 3) 222 genes related to "coagulation" (GO:0050817) and 123 related to "platelet activity" (GO:0030168) through Gene Ontology-based annotations, retrieved through GOfuncR R package (https://www.bioconductor.org/packages/release/bioc/html/GOfuncR.html).Also, in this case, in order to associate the genes to the identified miRNAs, we used multiMiR following the same approach described above.

Statistical analysis

Descriptive statistics were used to summarize the characteristics of the study population. All data are presented as median (range) or mean ± standard error (SEM), according to data distribution, evaluated through the Shapiro-Wilk test. All the other statistical analyses were applied by using Fisher's Exact Test for categorical variables, One-Way ANOVA followed by Tukey multiple comparison test and Student's t-test for normally distributed data or by Mann-Whitney test for skewed data. Receiver-operating-characteristic (ROC) and area under the curve (AUC) statistics were applied to test miRNA's accuracy as diagnostic biomarkers. The results were considered statistically significant when p was <0.05.

All statistical analyses were performed using GraphPad Prism 8 software (GraphPad Software, CA, USA).

Results

Patient characteristics

Forty patients with symptoms compatible with COVID-19 were included in the study. Of these, 30 patients were diagnosed with COVID-19 (COVID-19 patients), whereas the diagnosis of COVID-19 was excluded from the remaining 10 patients (non-COVID-19 patients). We also recruited 10 healthy subjects (HS), used as additional controls. Demographic and clinical characteristics of study subjects are detailed in **Tables 1-2**.

Whithin COVID-19 patients, 26, who, at the time of first clinical evaluation in the ED, did not present severe respiratory symptoms, were subsequently separated into two groups based on the development, during hospitalization, of respiratory failure requiring mechanical ventilation. Based on this criterion, 13 COVID-19 patients were classified as with poor prognosis and 13 with good prognosis.

Among non-COVID-19 patients, the final diagnosis was flu-like syndrome in 7 patients, bacterial pneumonia in 2, and acute gastroenteritis in 1 patient **(Table 1)**.

COVID-19 patients had lower lymphocyte and platelet counts than non-COVID-19 patients and HS, but they showed increased neutrophil/lymphocyte ratio, leukocyte/lymphocyte ratio, and C-reactive protein values **(Table 2).** No differences in demographic and clinical characteristics were found between good and poor prognosis COVID-19 patients (**Table 1-2**).

COVID-19 is characterized by distinct plasma miRNA signatures

In order to identify specific miRNA signatures in plasma samples of COVID-19 patients, we performed a high-throughput screening of 754 miRNAs using TaqMan Human microRNA Arrays. Out of 754 miRNAs screened, 219 were detectable in the pooled plasma samples below the cycle threshold (C_t) value of 33. Among these selected miRNAs, 8 were exclusively detected in COVID-19 patients, while 15 were only present in plasma samples of HS (data not shown). In addition, 38 miRNAs that in the three COVID-19 pools examined were not expressed in all of them or have different trend of expression vs HS, were excluded from the analysis.

Furthermore, among these 219 miRNAs we considered only those with an absolute fold change of 15. In the end, 43 miRNAs were found differentially expressed in COVID-19 patients vs healthy subjects and were further analysed by *in silico* analysis (**Table 3**).

To explore the pathways potentially affected by the panel of differentially expressed miRNAs (DEMs) in COVID-19 patients, we performed an enrichment analysis by using miRNA-pathway associations to the target genes. The 43 miRNAs selected were found enriched in 215 pathways **(Supplemental Table S1**). Since both KEGG and Wikipathways databases collect a wide variety of pathways that can fit different types of processes, we manually reviewed the lists of enriched

pathways, and we focused on those that were mainly related to COVID-19 pathophysiology at different levels: platelet activation, coronavirus disease, and chemokine, JAK-STAT, MAPK, NF-kappa B and IL-17 signaling pathways (**Table 4**). Next, by selecting those miRNAs that had higher FC expression and were associated with at least five of the most representative pathways in COVID-19, we selected the following six miRNAs: miR-139-5p, miR-28-5p, miR-133a-3p, miR-100-5p, miR-200a-3p, miR-199a-5p (**Fig. 2, panel A and table 4**).

The bubble plots displayed in Fig. 2 show the enrichment p-value of the six selected miRNAs on the listed enriched pathways involved in COVID-19 pathogenesis (bubble color), the number of genes that each miRNA targets (bubble size) and the gene ratio of each pathway. Since we wanted to be highly conservative in the results obtained from the enrichment analysis, all the selected pathways showed an adjusted FDR p-value <0.01. Out of the pathways we examined, platelet activation showed the highest ratio of genes targeted (28/124, 22.6%).

We next used qRT-PCR assay to confirm the differential expression of the six candidate diagnostic miRNAs in plasma samples from single patients. To do this, we studied 10 additional non-COVID-19 patients. With this approach, we identified two miRNAs, miR-133a-3p and miR-199a-5p, that confirmed their differential expression in COVID-19 vs. non-COVID-19 patients, while other miRNAs did not show a significant difference (data not shown). In particular, both miRNAs were more expressed in COVID-19 patients than in HS (p=0.0028 for miR-133a-3p, and p<0.0001 for miR-199a-5p) and, of note, miR-133a-3p was significantly higher in COVID-19 patients than non-COVID-19 patients (p=0.0041). The expression of both miRNAs did not differ significantly between non-COVID-19 patients and HS **(Figure 3a and b).**

Receiver operating characteristic (ROC) curves of miR-133a-3p [AUC=0.87 (95% CI, 0.76-0.99), p=0.0016] and miR-199a-5p [AUC=0.97 (95% CI, 0.92-1.03), p=0.0001] showed a high discriminatory power between COVID-19 patients and HS, with a specificity of 100% for both and a sensitivity of 75% and 96,3%, respectively, based on an optimal threshold value of 5.141 for miR-133a-3p and of 2.066 for miR-199a-5p **(Figure 3c)**. In addition, the threshold value for miR-133a-3p showing the best relationship between sensitivity (71.43%) and specificity (90%) to discriminate between COVID-19 and non-COVID-19 patients [AUC=0.86 (95% CI, 0.74-0.97), p=0.0009] was 5.791 **(Figure 3c).**

If combined, miR-133a-3p and miR-199a-5p yielded an AUC of 0.96 (95% CI, 0.90-1.02; p<0.0001) to discriminate COVID-19 patients from HS, and an AUC of 0.78 (95% CI, 0.63-0.92; p<0.0107) to discriminate COVID-19 from non-COVID-19 patients (**Figure 3d**). The cutoff values of 5.972 had the best sensitivity (89.29%) and specificity (100%) in discriminating COVID-19 from HS, whereas to

identify COVID-19 from non-COVID-19 patients the optimal cutoff was 22.19, with a sensitivity of 60.71 and a specificity of 100%.

miRNA profiles and integration bioinformatics analysis reveal specific circulating miRNAs as early predictors of COVID-19 severity

In order to identify circulating miRNAs potentially useful to early predict the worsening of clinical conditions in patients affected by COVID-19, 26 COVID-19 patients were divided into two groups based on the development of respiratory failure during hospitalization, as detailed in the Methods section. Of note, none of these patients presented respiratory failure or organ damage at the time of first medical evaluation in the ED and all presented comparable clinical status at the time of COVID-19 diagnosis.

Out of 754 miRNAs, 219 were detectable in the pooled plasma samples using the same criteria detailed in the previous section. Of these, 12 miRNAs were exclusively expressed in COVID-19 patients with poor prognosis, while 35 were specific for those with good prognosis (data not shown). After selecting only those miRNAs with an absolute fold change of 15, 47 different expressed miRNAs were considered for further analysis.

Using bioinformatic analysis (KEGG and wikipathway enrichment analysis), these 47 miRNAs differentially expressed in COVID-19 patients with poor vs good prognosis were found enriched in 254 pathways (**Supplemental Table S2).** After manual revision of the lists of enriched pathways, we selected those pathways associated with chemokine signaling, coronavirus disease, platelet activation, and MAPK signaling (**Table 5 and 6).**

By matching those miRNAs that had higher FC expression and were associated with the most representative pathways of COVID-19 pathogenesis, we identified seven miRNAs as potential predictors of clinical worsening: miR-205-5p, miR-100-5p, miR-423-3p, miR-106b-5p, miR-133a-3p, miR-374a-5p and miR-199a-5p. The bubble plot in **Figure 4** shows that, also in the evaluation of prognostic candidate miRNAs the higher gene ratio is associated with platelet activation.

Among these miRNAs, miR-423-3p, miR-205a-5p, and miR-106-5p resulted less expressed in COVID-19 patients with poor than in those with good prognosis (p=0.0473, p=0.0374, and p=0.0072, respectively) (**Figure 5 a-c**).

Using ROC analysis, these same miRNAs - [miR-423-3p (AUC= 0.75 (95% CI 0.54-0.97)], miR-205a-5p [AUC=0.76 (95% CI, 0.55-0.96)], and miR-106a-5p [AUC=0.81 (95% CI 0.63-0.98)] – showed a good performance in discriminating COVID-19 patients based on their clinical prognosis (p=0.0452, p=0.0364, and p=0.0083, respectively) (**Figure 5 d**).

Setting a cut-off value of 9.823 for miR-423-3p and of 6.646 for miR-205a-5p, both miRNAs had a specificity of 100% and a sensitivity of 54.55% and 58.33%, respectively; miR-106-5p performed even better by achieving a specificity of 92.31% and a sensitivity of 61.54 with a cut-off value of 3.583.

When we combined these three miRNAs, the ROC-curve analysis yielded a higher discriminatory power [AUC=0.76 (0.57-0.95), p=0.0225] with a specificity of 100% and a sensitivity of 54% at the cut-off of 20.62 (**Figure 5e**).

***In silico* evaluation on publicly available data and biological annotations**

We performed a first *in silico* validation to evaluate which genes targeted by the selected miRNAs are mainly associated with thrombosis, coagulation and platelet activation processes and, for the target genes of diagnostic miRNAs, which are associated with COVID-19, using publicly available data. **Supplementary Table S3** shows the 68 target genes found annotated to these biological processes. Among these, 17 genes resulted differentially expressed in the COVID-19-related GEO datasets: ACE2, PDE3A, IL6, TFPI, PTGS2, EGR1, KLF4, SERPINE1, PABPC4, JMJD1C, UBASH3B, C1QTNF1, SERPINE2, PLAUR, FAP, SLC4A1 and PDGFRA. Interestingly, these include several genes involved in the negative regulation of plasminogen activation (SERPINE1 and SERPINE2), platelet aggregation (UBASH3B, C1QTNF1 and SERPINE2) and blood coagulation (TFPI, PDGFRA, UBASH3B, C1QTNF1, SERPINE1 and SERPINE2). In addition, SERPINE1, SERPINE2 and PLAUR are involved in the dissolution of fibrin clot. Most of these genes are mainly targeted by hsa-miR-133a-3p (SERPINE1, SERPINE2, TFPI, PLAUR and UBASH3B).

Discussion

COVID-19 is an acute infectious respiratory disease caused by SARS-CoV-2. Patients with COVID-19 often worse rapidly, developing acute respiratory distress syndrome (ARDS), septic shock and coagulation dysfunctions, which are difficult to treat and heavily condition patient's survival [1]. While most patients with severe respiratory disorders successfully recover, a substantial number die of respiratory failure and/or systemic complications [3]. Determining which patients have the highest risk of encountering adverse outcomes, either by using clinically-identified risk factors and/or biomarkers for severe illness, would be ideal in order to optimize intensive medical management of COVID-19 patients. For this purpose, we investigated a cohort of COVID-19 patients enrolled at the time of hospital admission with the aim of discovering signatures of differentially expressed circulating miRNAs associated with COVID-19 onset. Our results identify plasma miRNA

profiles, namely miR-133a-3p and miR-199a-5p, which could discriminate between COVID-19, non-COVID-19 patients and HS at time as early as that of the first clinical evaluation in the ED.

After a battery of comprehensive screening and bioinformatic phases, six candidate diagnostic miRNAs, miR-139-5p, miR-28-5p, miR-133a-3p, miR-100-5p, miR-200a-3p, and miR-199a-5p, were carried out and validated by single qRT-PCR analysis in COVID-19 vs. HS. Furthermore, in order to better evaluate the diagnostic value of these miRNAs, an additional cohort of non-COVID-19 patients was considered. In our study, miR-133a-3p and miR-199a-5p resulted significantly higher in COVID-19 patients than in HS. In addition, miR-133a-3p demonstrated a high sensitivity and specificity to distinguish COVID-19 from non-COVID-19 patients. Therefore, the identification of miR-133a-3p and miR-199a-5p signature shows a higher diagnostic value in distinguishing COVID-19 patients from non-COVID-19 patients. Therefore, our results suggest that plasma microRNA profiling may be proposed as biomarker for the early diagnosis of COVID-19.

Previous studies [14,15,18,28,29] have already focused on investigating differentially expressed miRNAs in COVID-19 patients and attempted to evaluate their clinical significance for COVID-19. For instance, *Molinero et al*, analyzed miRNA profile of bronchial aspirate samples from COVID-19 and non-COVID-19 patients. They identified multiple miRNAs, including miR-133a-3p and miR-199a-5p, used as markers to distinguish between the two groups [12,28]. Moreover, in terms of functional analyses, it is known that miR-133a-3p and miR-199a-5p are implicated in fibrosis, coagulation, viral infections, immune responses and inflammation [12].

In addition, some data suggest an involvement of miR-133a and miR-199a-5p in affecting the clinical course of COVID-19 [12]. For instance, baseline levels of myocyte-derived miR-133a are associated with COVID-19 severity and 28-day ICU mortality [30]. Moreover, the up-regulation of miR-199a-5p above cited has been suggested to participate in the development of fibrotic lesions in the lung of post-COVID-19 patients [31].

These same two miRNAs we identified in our study as useful for the diagnosis of COVID-19 have been preciously implicated in the pathogenesis of other diseases. For instance, miR-133a was found up-regulated in patients with sepsis [32]. Similarly, circulating miR-133a levels are increased in patients with myocardial injury [33].

In our study, we also aimed to identify and evaluate plasma miRNA profiles that were able to discriminate those COVID-19 patients who, in the course of their disease, later develop respiratory failure and worst prognosis. In the past years, several risk factors, mainly based on clinical characteristics of patients, have been linked to a more severe course of COVID-19, such as age or

male gender [34]. However, the quest for additional biological factors determinant for affecting and able to predict the clinical course of COVID-19 patients remains essential.

One feature of our experimental design that is important to underline is that COVID-19 patients enrolled in our study had comparable moderate symptoms at the time of enrollment and evolve into different severity grades during the hospitalization that followed the first clinical evaluation in the ED.

In this section of our study, miR-106-5p, miR-205a-5p and miR-423-3p had lower expression in COVID-19 patients with poor than in those with good prognosis. ROC analysis demonstrated that all three miRNAs may be proposed as biomarkers for the development of respiratory failure in COVID-19 patients. Notably, the signature of the three-miRNAs together had a higher discriminatory power than miR-423-3p and miR-205a-5p alone, while miR-106a-5p alone presented the best prognostic performance in terms of sensibility and specificity.

Previous studies have implicated these miRNAs in the pathophysiology of other critical diseases [35,36]. For instance, in a recent study, miR-205 expression was increased in patients with sepsis-induced acute kidney injury who survived compared to those who did not survived [37]. Conversely, other studies found miR-106a-5p, together with other miRNAs, orchestrate the gene expression of IL-6, IL-10, IL-1b and IL-17 through STAT1 and STAT3 in tumor progression and in sepsis-induced acute kidney injury [38,39].

To elucidate the key pathways involved in determining COVID-19 severity is crucial in order to develop new therapeutic strategies that include additional innovative treatments devoted to reduce inflammatory signaling and damage, as well as to target molecular mediators of the maladaptive COVID-19 immune response [14]. In order to understand the molecular functions of the miRNAs we identified, we performed functional enrichment analysis by KEGG and GO. Interestingly, the *in silico* analysis shows that miRNAs we identified are known to be implicated in the regulation of the immune and/or inflammatory pathways at different levels: chemokine and cytokine signaling, platelet activation, coagulation, JAK-STAT and MAPK signaling, NF-kappa B and IL-17 signalling pathways.

However, more detailed mechanistic *in vitro* and *in vivo* studies are necessary to elucidate the extent to which the changes in the circulating miRNA profile we described are potentially translated into changes in the related biological pathways. It also remains unclear whether changes in circulating miRNA levels are mediators or consequences of the pathophysiological mechanisms implicated in COVID-19.

Finally, the identification of miRNA cellular origin in COVID-19 context is challenging: miRNAs appear in the bloodstream by active secretion or passive release during cell damage [28]. Dysregulated miRNAs have been described in a wide variety of tissues and the extracellular miRNA pattern does not necessary correlate with the intracellular miRNA expression pattern [28]. Although these points need clarification, they are not critical for the clinical application of the identified miRNAs as biomarkers. miR-106a-5p/miR-423-3p/miR-205a-5p signature has, indeed, a good prognostic power in discriminating COVID-19 patients who, starting from moderate symptoms, will move toward rapid improvement in clinical status from those with similar starting/initial conditions who will manifest critical complications requiring intensive care.

Our study has some limits. Although the study population seems to be representative of COVID-19 patients and prognosis, the number of patients enrolled is small. Further validation of our results in larger and international cohorts is encouraged and needed. Moreover, we assessed the biological and molecular functions by bioinformatics only. Further experimental studies should explore whether miRNAs participate mechanistically in the pathophysiology of COVID-19 complications that condition adverse prognosis.

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Ethics approval and consent to participate

The present study was conducted in compliance with the World Medical Association Declaration of Helsinki. All patients gave informed oral consent for the participation in the study (signed written consent form was waived due to safety protocol), and all data were immediately de-identified. The study was conducted according to the principles of the Helsinki Declaration, and approved by the Institutional Ethical Committee (A.O.U. Città della Salute e della Scienza di Torino, Molinette Hospital, Torino, Italy) (n. CS2/139).

Competing interests

The authors declare that they have no competing interests.

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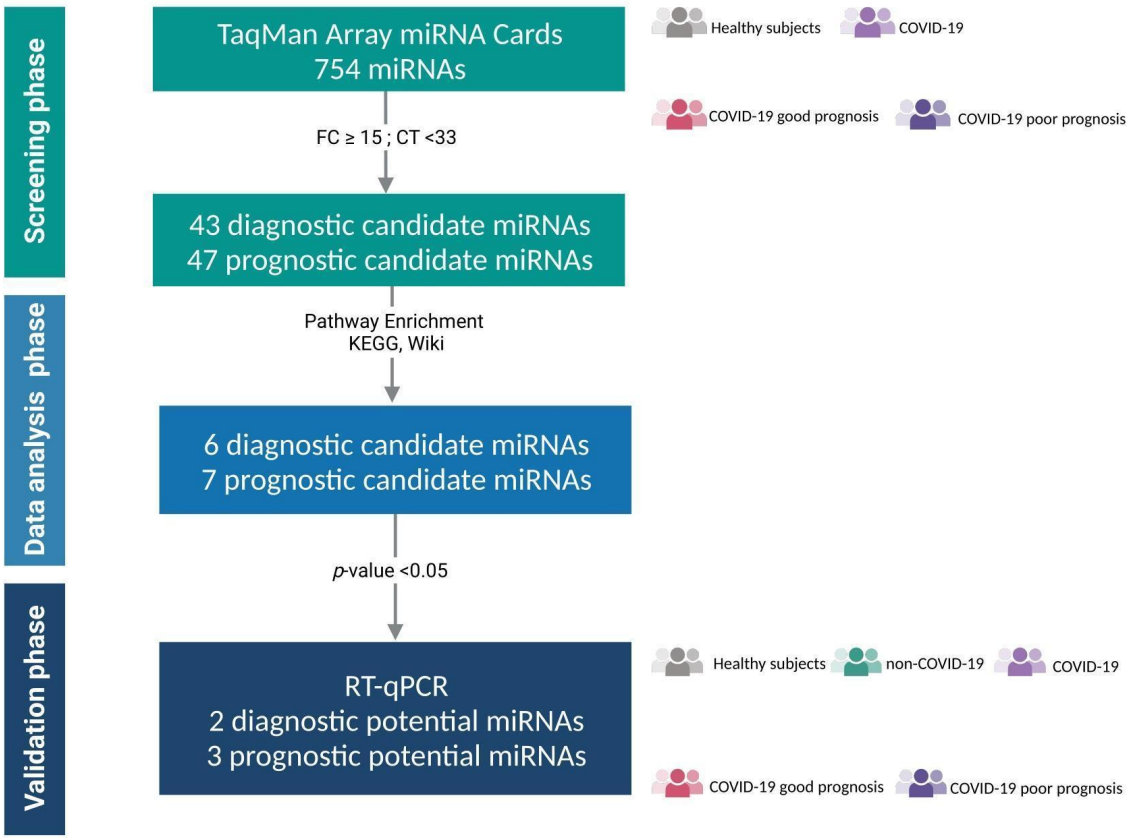

Figure 1 | Flow chart outlining the phases of the experiment design. Created with BioRender.com.

Table 1. Characteristics of patients at the moment of first medical evaluation in the ED.

	Healthy Subjects (n=10)	**non-COVID-19** (n=10)	**COVID-19** (n=30)	**COVID-19 Good Prognosis** (n=13)	**COVID-19 Poor Prognosis** (n=13)
Gender (M/F), N (%)	5/5 (50/50)	6/4 (60/40)	17/13 (56.7/43.3)	6/7 (46.1/53.8)	9/4 (69.2/38.8)
Age, mean (± SD), yr	64.11 (± 16.9)	51.8 (± 11.7)§§	69.1 (± 12.4)***	75.2 (± 10.7)	60.9 (± 9.6)°°
Symptoms, N (%)					
Fever	-	8 (80.0)	27 (90.0)	11 (84.6)	13 (100)
Cough	-	6 (60.0)	19 (63.3)	7 (53.8)	10 (76.9)
Shortness of breath	-	5 (50.0)	22 (73.3)	9 (69.2)	11 (84.6)
Sore throat	-	0	0	0	0
Diarrhea	-	3 (30.0)	11 (36.6)	4 (30.8)	6 (46.2)
Ageusia	-	1 (10.0)	7 (23.3)	2 (15.4)	4 (30.8)
Anosmia	-	0	3 (10.0)	0	3 (23.1)
Comorbidities, N (%)					
Hypertension	-	1 (10.0)	13 (43.3)	6 (46.2)	6 (46.2)
Diabetes Mellitus	-	0	5 (16.7)	2 (15.4)	3 (23.8)
CAD	-	0	1 (3.0)	1 (7.7)	0
Atrial fibrillation/flutter	-	1 (10.0)	4 (13.3)	3 (23.1)	0
Asthma/COPD	-	0	0	0	0
Cancer	-	0	0	0	0
Cerebrovascular disease	-	0	2 (6.7)	1 (7.7)	0
CKD	-	0	1 (3.0)	1 (7.7)	0
DVT/PE	-	0	0	0	0
Home discharge (%)	NA	10 (100)	0	0	0
Ward admission (%)	NA	0	30 (100)	13 (100)	13 (100)
ICU/HDU Admission (%)	NA	0	8 (26.7)	0	0
NIV (%)	NA	0	16 (53.3)	5 (38.5)	7 (53.8)
MIV (%)	NA	0	8 (26.7)	0	0
In-hospital Mortality (%)	NA	0	4 (13.3)	0	2 (15.4)
Day of hospitalization	NA	0	13.0 (± 9.4)	14.0 (± 10.9)	18.1 (± 11.0)

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. mean (± SEM) or median (range) as appropriate. ***p<0.0001 vs Non-COVID-19. §§ p<0.001 vs HS. °° p<0.001 *vs*. good prognosis.

Table 2. Laboratory findings.

	Healthy Subjects (n=10)	**Non-COVID-19** (n=10)	**COVID-19** (n=30)	**COVID-19 Good prognosis** (n=13)	**COVID-19 Poor prognosis** (n=13)
White blood cell count (10^9/L)	5.4 (± 0.9)	7.2 (± 1.6)§	7.6 (± 3.1)	7.1 (4.6-9.5)	6.8 (5.0-8.7)
Neutrophil count (10^9/L)	3.3 (± 0.8)	4.6 (± 1.5)	6.0 (± 3.1)§	5.6 (± 2.3)	5.6 (± 3.4)
Lymphocyte count (10^9/L)	1.6 (± 0.5)	2.0 (1.5-2.4)	0.9 (0.6-1.3)$^{**\ \S}$	1.0 (0.6-1.3)	0.8 (0.6-1.4)
Neutrophil/Lymphocyte (ratio)	2.3 (± 1.0)	2.1 (1.9-3.3)	5.2 (3.9-8.3)$^{**\ \S\S\S}$	5.2 (4.2-9.0)	5.1 (3.1-7.7)
Leukocyte/Lymphocyte (ratio)	3.7 (± 1.1)	3.5 (3.3-4.8)	6.7 (4.9-9.8)$^{**\ \S\S\S}$	6.5 (4.7-10.6)	6.5 (4.6-9.3)
Platelet count (10^9/L)	210.0 (± 75.3)	263.6 (± 44.1)	210.3 (± 72.8)*	191.0 (149.5-243.0)	190.0 (159.0-249.5)
Mean platelet volume (fL)	10.9 (± 0.74)	10.4 (10.0-11.1)	11.0 (10.1-11.6)	10.7 (10.0-11.3)	11 (9.9-11.9)
Platelet distribution width (fL)	13.4 (± 1.8)	12.4 (± 1.5)	12.7 (± 2.0)	12.4 (± 2.1)	12.8 (± 2.1)
C-Reactive Protein (mg/L)	1.9 (± 1.8)	3.9 (0.4-15.0)	76.9 (37.9-133.9)$^{***\ \S\S\S}$	62.8 (± 37.1)	87.2 (± 56.6)
P/F ratio	-	-	283.0 (± 62.3)	288.4 (± 45.7)	294.7 (± 53.5)

Mean (± SEM) or median (range) as appropriate. ***p<0.0001; **p<0.001; *p<0.05 vs Non-COVID-19. §§§ p<0.0001; §§ p<0.001; §p<0.05 vs HS.

Table 3. miRNAs fold change in plasma samples from COVID-19 patient *vs*. HS plasma.

miRNA	FC
hsa-miR-139-5p	0,03
hsa-miR-33a-5p	15,00
hsa-miR-328-3p	15,37
hsa-miR-30b-5p	16,07
hsa-miR-503-5p	16,17
hsa-miR-425-3p	16,37
hsa-miR-185-3p	16,50
hsa-miR-744-5p	16,62
hsa-miR-191-5p	17,17
hsa-miR-885-5p	17,19
hsa-miR-362-3p	17,65
hsa-miR-584-5p	17,95
hsa-miR-130b-3p	18,63
hsa-miR-874-3p	20,31
hsa-miR-151b	20,87
hsa-miR-494-3p	21,12
hsa-miR-340-5p	21,21
hsa-miR-127-3p	22,92
hsa-miR-1301-3p	24,79
hsa-miR-103a-2-5p	24,87
hsa-miR-655-3p	30,63
hsa-miR-21-3p	31,13
hsa-miR-653-5p	31,50
hsa-miR-183-5p	32,54
hsa-miR-182-5p	33,21
hsa-miR-495-3p	33,31
hsa-miR-326	40,62
hsa-miR-132-3p	47,97
hsa-miR-221-5p	55,63
hsa-miR-483-5p	57,56
hsa-miR-409-3p	60,24
hsa-miR-142-3p	65,33
hsa-miR-545-3p	66,60
hsa-miR-28-5p	72,71
hsa-miR-339-5p	102,50
hsa-miR-133a-3p	129,10
hsa-miR-100-5p	177,15
hsa-miR-190a-5p	206,41
hsa-miR-376c-3p	206,58
hsa-miR-1260a	229,70
hsa-miR-200a-3p	239,28
hsa-miR-627-5p	312,82
hsa-miR-199a-5p	1105,15

Table 4. Bioinformatics analysis of identified pathways associated to selected miRNAs.

Pathway	p value	FDR	SelemiRNA
Chemokine signaling pathway	1,37444E-06	0,0013057	hsa-miR-100-5p;hsa-miR-1301-3p;hsa-miR-130b-3p;hsa-miR-132-3p;hsa-miR-133a-3p;hsa-miR-139-5p;hsa-miR-142-3p;hsa-miR-151b;hsa-miR-182-5p;hsa-miR-183-5p;hsa-miR-185-3p;hsa-miR-199a-5p;hsa-miR-200a-3p;hsa-miR-21-3p;hsa-miR-28-5p;hsa-miR-30b-5p;hsa-miR-339-5p;hsa-miR-33a-5p;hsa-miR-340-5p;hsa-miR-376c-3p;hsa-miR-425-3p;hsa-miR-483-5p;hsa-miR-494-3p;hsa-miR-495-3p;hsa-miR-503-5p;hsa-miR-545-3p;hsa-miR-744-5p;hsa-miR-874-3p
Platelet activation	0,00064998	0,0106387	hsa-miR-1301-3p;hsa-miR-132-3p;hsa-miR-133a-3p;hsa-miR-139-5p;hsa-miR-142-3p;hsa-miR-151b;hsa-miR-182-5p;hsa-miR-183-5p;hsa-miR-185-3p;hsa-miR-200a-3p;hsa-miR-28-5p;hsa-miR-30b-5p;hsa-miR-326;hsa-miR-340-5p;hsa-miR-409-3p;hsa-miR-425-3p;hsa-miR-483-5p;hsa-miR-494-3p;hsa-miR-503-5p;hsa-miR-545-3p;hsa-miR-744-5p
Coronavirus disease - COVID-19	0,000995394	0,0124522	hsa-miR-100-5p;hsa-miR-130b-3p;hsa-miR-132-3p;hsa-miR-133a-3p;hsa-miR-139-5p;hsa-miR-142-3p;hsa-miR-182-5p;hsa-miR-185-3p;hsa-miR-199a-5p;hsa-miR-200a-3p;hsa-miR-21-3p;hsa-miR-221-5p;hsa-miR-28-5p;hsa-miR-30b-5p;hsa-miR-33a-5p;hsa-miR-409-3p;hsa-miR-425-3p;hsa-miR-494-3p;hsa-miR-495-3p;hsa-miR-503-5p;hsa-miR-545-3p;hsa-miR-744-5p;hsa-miR-874-3p
JAK-STAT signaling pathway	0,003064579	0,0232908	hsa-miR-100-5p;hsa-miR-103a-2-5p;hsa-miR-1301-3p;hsa-miR-130b-3p;hsa-miR-132-3p;hsa-miR-133a-3p;hsa-miR-139-5p;hsa-miR-151b;hsa-miR-182-5p;hsa-miR-183-5p;hsa-miR-191-5p;hsa-miR-199a-5p;hsa-miR-200a-3p;hsa-miR-21-3p;hsa-miR-28-5p;hsa-miR-30b-5p;hsa-miR-326;hsa-miR-33a-5p;hsa-miR-340-5p;hsa-miR-362-3p;hsa-miR-376c-3p;hsa-miR-494-3p;hsa-miR-495-3p;hsa-miR-503-5p;hsa-miR-545-3p;hsa-miR-874-3p
MAPK signaling pathway	0,007854022	0,0399001	hsa-miR-100-5p;hsa-miR-1260a;hsa-miR-130b-3p;hsa-miR-132-3p;hsa-miR-133a-3p;hsa-miR-139-5p;hsa-miR-142-3p;hsa-miR-151b;hsa-miR-182-5p;hsa-miR-183-5p;hsa-miR-185-3p;hsa-miR-191-5p;hsa-miR-199a-5p;hsa-miR-200a-3p;hsa-miR-21-3p;hsa-miR-28-5p;hsa-miR-30b-5p;hsa-miR-33a-5p;hsa-miR-340-5p;hsa-miR-362-3p;hsa-miR-376c-3p;hsa-miR-494-3p;hsa-miR-495-3p;hsa-miR-503-5p;hsa-miR-545-3p;hsa-miR-655-3p;hsa-miR-744-5p;hsa-miR-885-5p
NF-kappa B signaling pathway	0,00799177	0,0401703	hsa-miR-130b-3p;hsa-miR-132-3p;hsa-miR-133a-3p;hsa-miR-139-5p;hsa-miR-142-3p;hsa-miR-182-5p;hsa-miR-183-5p;hsa-miR-199a-5p;hsa-miR-30b-5p;hsa-miR-339-5p;hsa-miR-33a-5p;hsa-miR-362-3p;hsa-miR-425-3p;hsa-miR-503-5p;hsa-miR-545-3p;hsa-miR-744-5p;hsa-miR-874-3p
IL-17 signaling pathway	0,008215206	0,0409412	hsa-miR-100-5p;hsa-miR-132-3p;hsa-miR-139-5p;hsa-miR-142-3p;hsa-miR-182-5p;hsa-miR-183-5p;hsa-miR-185-3p;hsa-miR-191-5p;hsa-miR-199a-5p;hsa-miR-200a-3p;hsa-miR-28-5p;hsa-miR-30b-5p;hsa-miR-33a-5p;hsa-miR-494-3p;hsa-miR-495-3p;hsa-miR-503-5p;hsa-miR-545-3p;hsa-miR-744-5p

FDR, False Discovery Rate.

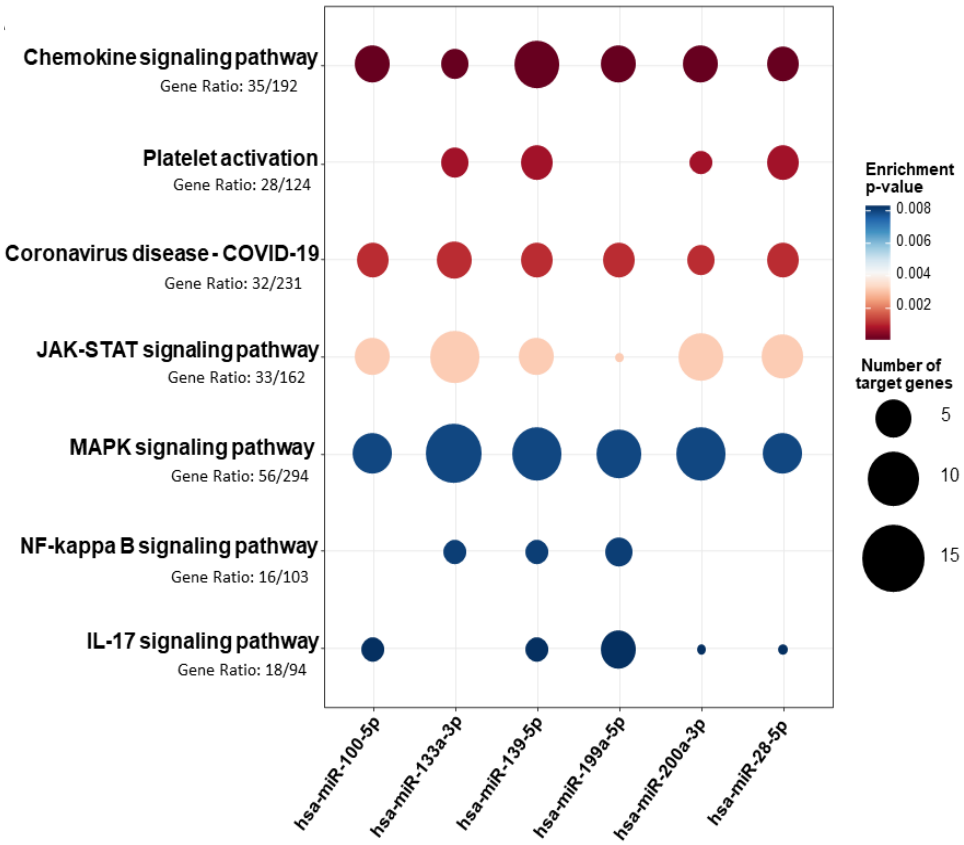

Figure 3 | Bubble plot of the enriched pathways mainly associated with the miRNAs identified in the study. Candidate diagnostic miRNAs most frequently annotated with the pre-selected pathways are displayed on the x-axis. The color scale represents the adjusted FDR p-values of the enrichment analysis performed through Fisher's Exact Test, considering the number of miRNAs annotated to each pathway (y-axis) after the detection of the target genes, whose ratio is included in the y-axis label. The size of the bubbles represents the number of target genes of each miRNA annotated to the corresponding pathway.

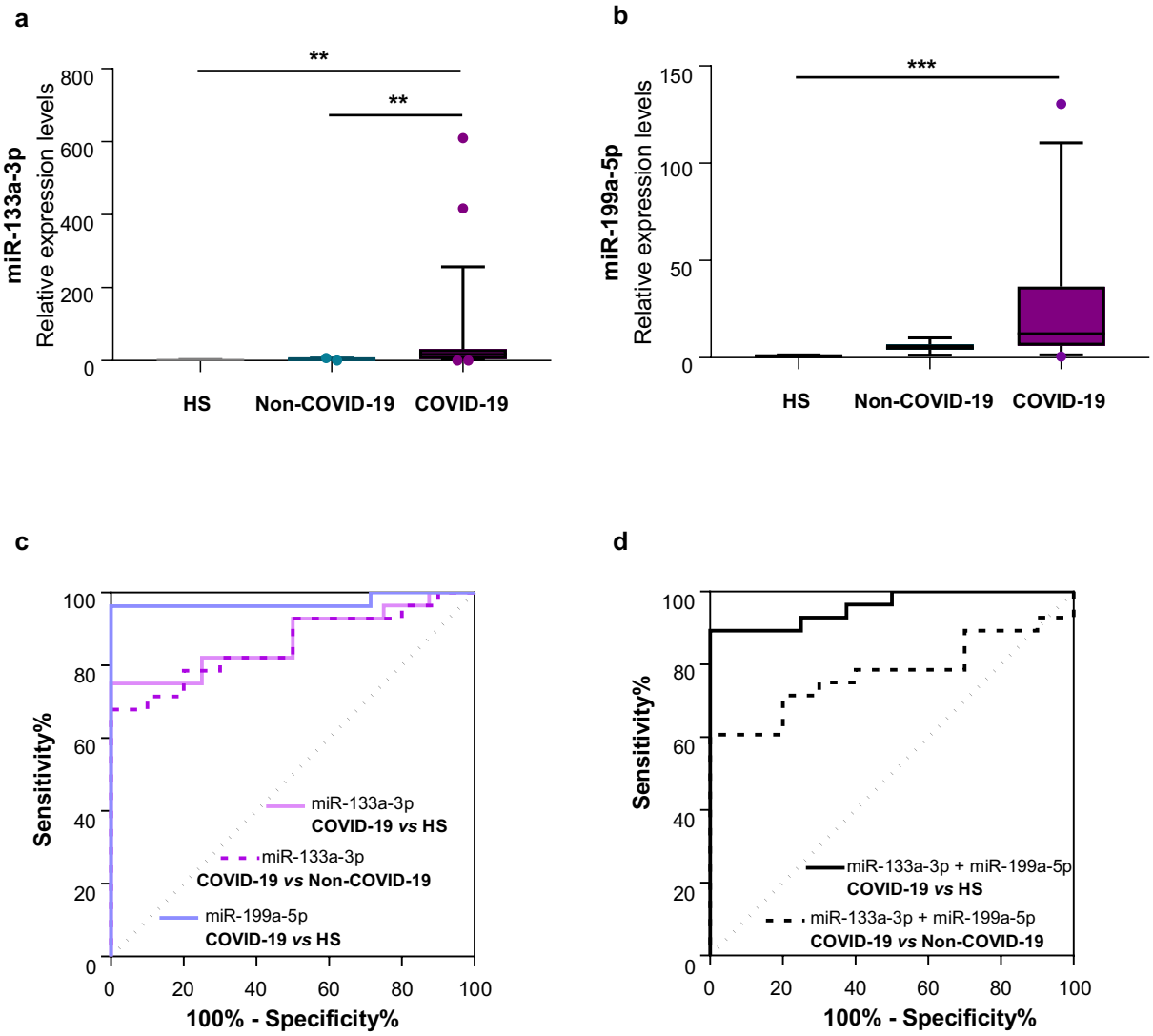

Figure 4 | Expression levels of miR-133a-3p (**a**) and miR-199a-5p (**b**) and ROC curves of selected miRNAs (**c-d**) in COVID-19 patients *vs.* non-COVID-19 patients and HS. The relative expression of miRNAs was quantified by qRT-PCR. * p<0.05, ** p<0.01, *** p<0.001 (Kruskal-Wallis test).

Table 5. miRNAs fold change in plasma samples from COVID-19 patients with poor *vs.* those with good prognosis.

miRNA	FC
hsa-miR-199a-5p	0,01
hsa-miR-627-5p	0,02
hsa-miR-374a-5p	0,02
hsa-miR-133a-3p	0,04
hsa-miR-223-5p	0,06
hsa-miR-590-5p	0,06
hsa-let-7b-5p	15,52
hsa-miR-19b-1-5p	15,81
hsa-miR-625-5p	16,89
hsa-miR-543	16,98
hsa-miR-331-3p	17,99
hsa-miR-205-5p	18,97
hsa-miR-219a-5p	18,97
hsa-miR-27b-3p	18,99
hsa-miR-200a-3p	19,60
hsa-miR-301a-3p	21,11
hsa-miR-431-5p	21,51
hsa-miR-421	21,59
hsa-let-7d-5p	22,19
hsa-miR-30d-5p	22,36
hsa-miR-548a-3p	23,26
hsa-miR-576-5p	25,07
hsa-miR-190a-5p	25,71
hsa-miR-340-5p	25,90
hsa-miR-654-5p	27,68
hsa-let-7a-5p	31,54
hsa-miR-487a-3p	32,58
hsa-miR-15b-3p	34,58
hsa-miR-103a-2-5p	40,90
hsa-miR-874-3p	41,16
hsa-miR-142-3p	42,81
hsa-miR-151b	43,08
hsa-miR-369-3p	50,21
hsa-miR-330-3p	51,88
hsa-miR-139-5p	60,05
hsa-miR-339-5p	62,73
hsa-miR-132-3p	84,86
hsa-miR-433-3p	92,48
hsa-miR-106b-5p	116,00
hsa-miR-574-3p	117,70
hsa-miR-758-3p	167,50
hsa-miR-423-3p	181,14
hsa-miR-154-3p	190,28
hsa-miR-524-3p	210,26
hsa-miR-100-5p	236,22
hsa-miR-374b-5p	1169,76
hsa-miR-376a-3p	4070,53

Table 5. Bioinformatics analysis of identified pathways associated to selected miRNAs.

Pathway	p value	FDR	Selected miRNA
Chemokine signaling pathway	6,32194E-05	0,011531434	hsa-let-7a-5p;hsa-let-7b-5p;hsa-let-7d-5p;hsa-miR-100-5p;hsa-miR-106b-5p;hsa-miR-132-3p;hsa-miR-133a-3p;hsa-miR-139-5p;hsa-miR-142-3p;hsa-miR-151b;hsa-miR-199a-5p;hsa-miR-200a-3p;hsa-miR-205-5p;hsa-miR-27b-3p;hsa-miR-301a-3p;hsa-miR-30d-5p;hsa-miR-330-3p;hsa-miR-331-3p;hsa-miR-339-5p;hsa-miR-340-5p;hsa-miR-369-3p;hsa-miR-374a-5p;hsa-miR-423-3p;hsa-miR-574-3p;hsa-miR-590-5p;hsa-miR-625-5p;hsa-miR-874-3p
Coronavirus disease - COVID-19	0,010832807	0,044587714	hsa-let-7a-5p;hsa-let-7b-5p;hsa-miR-100-5p;hsa-miR-106b-5p;hsa-miR-132-3p;hsa-miR-133a-3p;hsa-miR-139-5p;hsa-miR-142-3p;hsa-miR-199a-5p;hsa-miR-19b-1-5p;hsa-miR-200a-3p;hsa-miR-27b-3p;hsa-miR-301a-3p;hsa-miR-30d-5p;hsa-miR-331-3p;hsa-miR-374a-5p;hsa-miR-423-3p;hsa-miR-543;hsa-miR-548a-3p;hsa-miR-574-3p;hsa-miR-758-3p;hsa-miR-874-3p
Platelet activation	0,015994707	0,055059328	hsa-let-7a-5p;hsa-let-7b-5p;hsa-let-7d-5p;hsa-miR-106b-5p;hsa-miR-132-3p;hsa-miR-133a-3p;hsa-miR-139-5p;hsa-miR-142-3p;hsa-miR-151b;hsa-miR-19b-1-5p;hsa-miR-200a-3p;hsa-miR-205-5p;hsa-miR-301a-3p;hsa-miR-30d-5p;hsa-miR-331-3p;hsa-miR-340-5p;hsa-miR-374a-5p;hsa-miR-423-3p;hsa-miR-625-5p
MAPK signaling pathway	0,019371193	0,060336502	hsa-let-7a-5p;hsa-let-7b-5p;hsa-let-7d-5p;hsa-miR-100-5p;hsa-miR-106b-5p;hsa-miR-132-3p;hsa-miR-133a-3p;hsa-miR-139-5p;hsa-miR-142-3p;hsa-miR-151b;hsa-miR-199a-5p;hsa-miR-19b-1-5p;hsa-miR-200a-3p;hsa-miR-205-5p;hsa-miR-27b-3p;hsa-miR-301a-3p;hsa-miR-30d-5p;hsa-miR-330-3p;hsa-miR-331-3p;hsa-miR-340-5p;hsa-miR-369-3p;hsa-miR-374a-5p;hsa-miR-421;hsa-miR-423-3p;hsa-miR-433-3p;hsa-miR-543;hsa-miR-574-3p;hsa-miR-625-5p;hsa-miR-654-5p

FDR, False Discovery Rate.

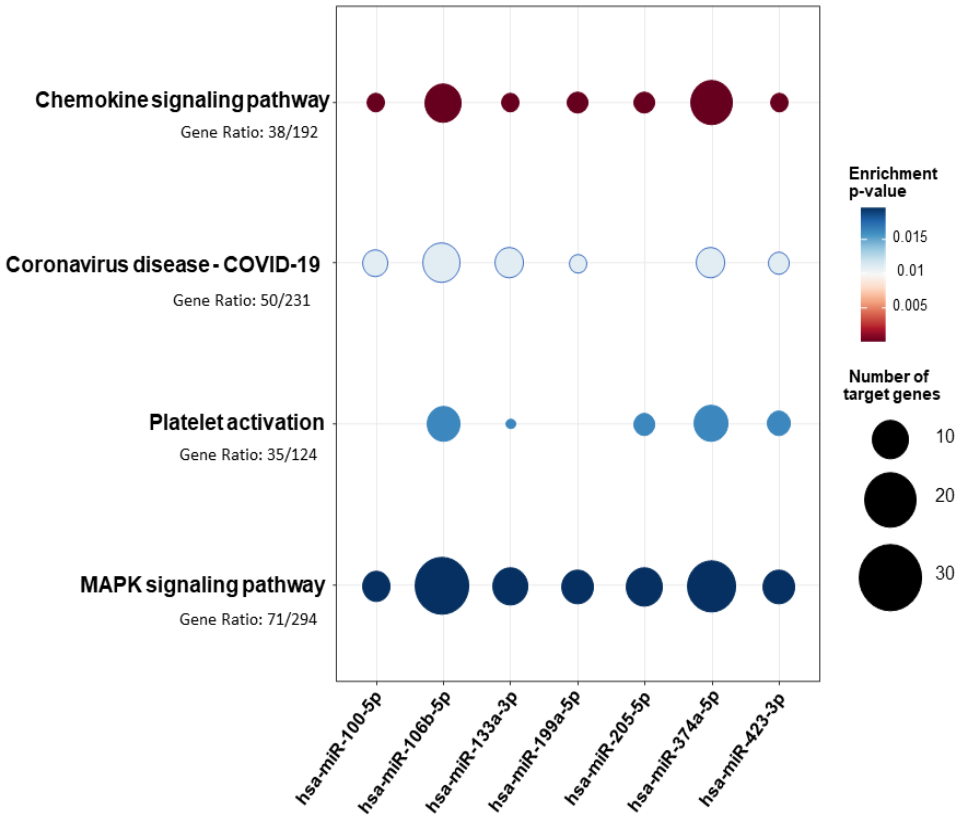

Figure 5 | Bubble plot of the enriched pathways mainly associated with the miRNAs identified in the study. Candidate prognostic miRNAs most frequently annotated with the pre-selected pathways are displayed on the x-axis. The color scale represents the adjusted FDR p-values of the enrichment analysis performed through Fisher's Exact Test, considering the number of miRNAs annotated to each pathway (y-axis) after the detection of the target genes, whose ratio is included in the y-axis label. The size of the bubbles represents the number of target genes of each miRNA annotated to the corresponding pathway.

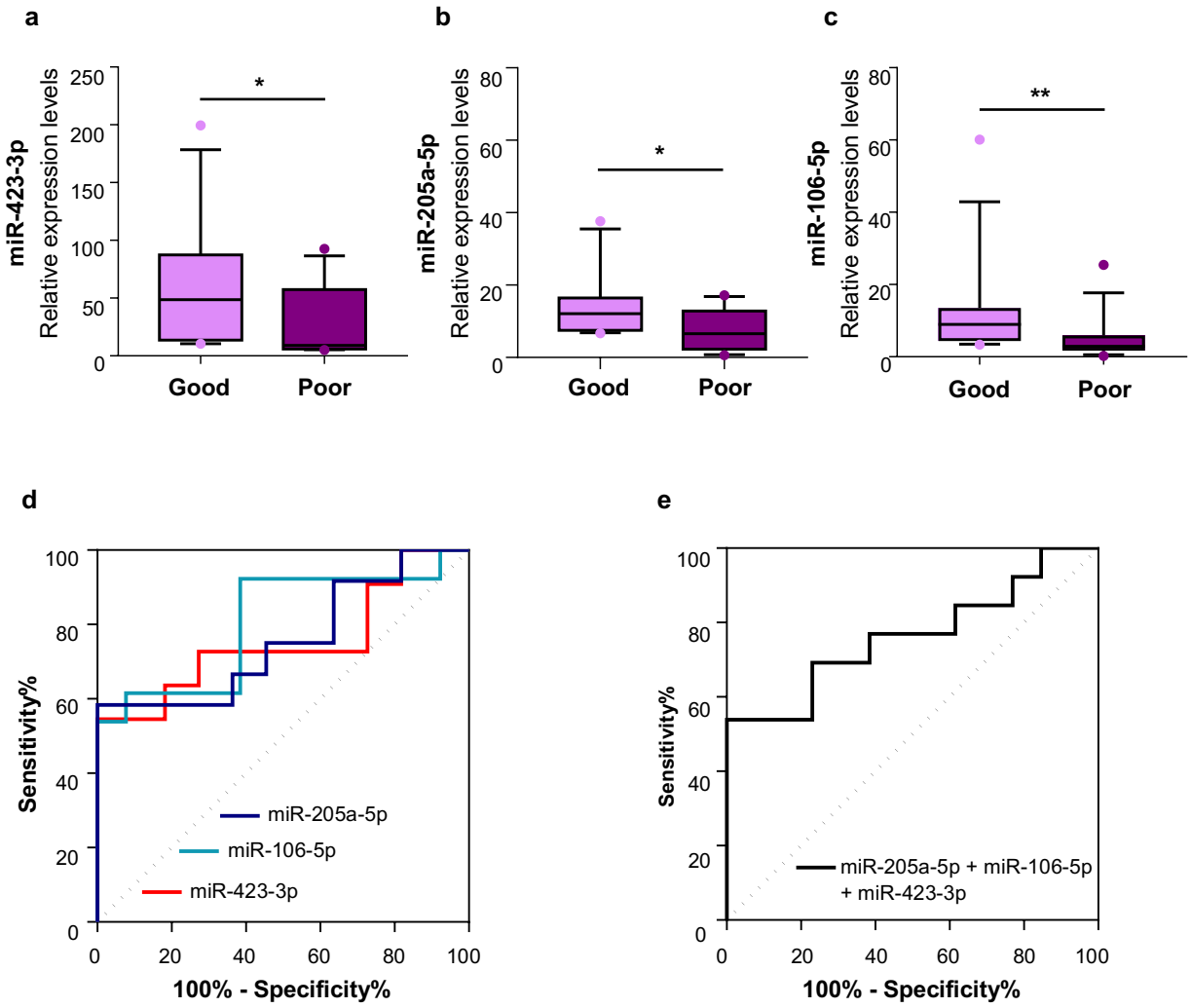

Figure 5 | Expressed levels (**a-c**) and ROC curves (**d-e**) of selected miRNAs in poor prognosis *vs.* good prognosis of COVID-19 patients. The relative expression of miRNAs was quantified by quantitative qRT-PCR. * p<0.05, ** p<0.01, *** p<0.001 (Mann-Whitney test).

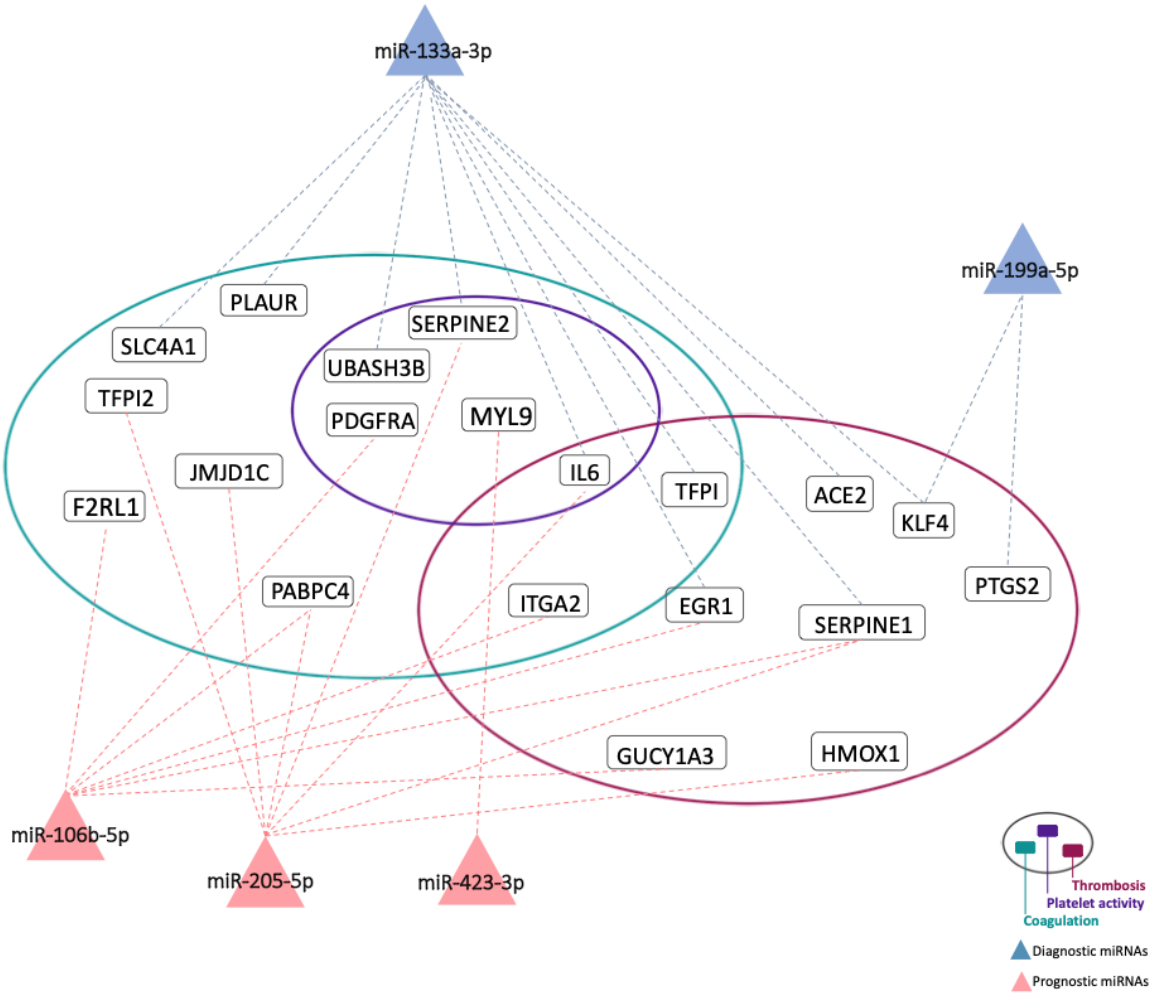

Figure 6 | miRNA-mRNA network. Associations of the 11 genes and 13 genes targeted by candidate diagnostic and prognostic miRNAs associated with COVID-19 and related to platelet activity, coagulation and thrombosis, respectively.

SUPPLEMENTAL MATERIAL

Table S1 Candidate diagnostic miRNAs annotated to the most interesting statistically enriched pathways. The first column reports the mean expression in COVID-19 samples.
https://docs.google.com/spreadsheets/d/1BfqLBpSbrIB7wmDwET6gWTh8-5IEMHR/edit#gid=270851285

Table S2 Candidate prognostic miRNAs annotated to the most interesting statistically enriched pathways. The first column reports the mean expression in COVID-19 samples. Ratio good prognosis vs poor prognosis in COVID-19 patients.
https://docs.google.com/spreadsheets/d/1Bfs4ZLlcGvdKlINSrUTqvrkgCke-5Xqv/edit#gid=1783676892

Table S3 List of target genes of the diagnostic miRNAs annotated to thrombosis, coagulation and platelet activation. In red, the genes found differentially expressed in the COVID-19 related GEO datasets.

MiRNA	Target Gene Symbol	Target Entrez ID	Sources	Biological Process
hsa-miR-199a-5p	SIRT1	23411	diana,elmmo,miranda,mirdb,mirtarbase,pita,targetscan	Thrombosis
hsa-miR-199a-5p	PODXL	5420	diana,elmmo,mirtarbase,pita,targetscan	Thrombosis
hsa-miR-139-5p	PDE3A	5139	elmmo,miranda,mirdb,pita,tarbase	Thrombosis
hsa-miR-133a-3p	VKORC1	79001	elmmo,mirdb,mirtarbase,tarbase	Thrombosis
hsa-miR-200a-3p	HGF	3082	diana,elmmo,miranda,mirtarbase	Thrombosis
hsa-miR-28-5p	MPL	4352	mirtarbase,tarbase	Thrombosis
hsa-miR-28-5p	VWF	7450	microcosm,tarbase	Thrombosis
hsa-miR-28-5p	PODXL	5420	tarbase	Thrombosis
hsa-miR-28-5p	PTPRJ	5795	mirtarbase	Thrombosis
hsa-miR-28-5p	VKORC1	79001	tarbase	Thrombosis
hsa-miR-100-5p	ACE2	59272	tarbase	Thrombosis
hsa-miR-100-5p	CYP4F2	8529	tarbase	Thrombosis
hsa-miR-100-5p	IL6	3569	tarbase	Thrombosis
hsa-miR-100-5p	PIK3CB	5291	tarbase	Thrombosis
hsa-miR-100-5p	TFPI	7035	tarbase	Thrombosis
hsa-miR-199a-5p	PLAU	5328	tarbase	Thrombosis
hsa-miR-199a-5p	PTGS2	5743	mirtarbase	Thrombosis
hsa-miR-199a-5p	VASP	7408	mirtarbase	Thrombosis
hsa-miR-139-5p	CYP3A5	1577	tarbase	Thrombosis
hsa-miR-133a-3p	ACE2	59272	tarbase	Thrombosis
hsa-miR-133a-3p	EGR1	1958	tarbase	Thrombosis
hsa-miR-133a-3p	IL6	3569	tarbase	Thrombosis
hsa-miR-133a-3p	KLF4	9314	tarbase	Thrombosis

hsa-miR-133a-3p	PTPRJ	5795	tarbase	Thrombosis
hsa-miR-133a-3p	SERPINE1	5054	tarbase	Thrombosis
hsa-miR-133a-3p	TFPI	7035	tarbase	Thrombosis
hsa-miR-200a-3p	EPRS1	2058	tarbase	Thrombosis
hsa-miR-200a-3p	VCAM1	7412	mirtarbase	Thrombosis
hsa-miR-133a-3p	TUBB1	81027	diana,elmmo,miranda	Thrombosis
hsa-miR-200a-3p	SIRT1	23411	diana,elmmo,miranda	Thrombosis
hsa-miR-133a-3p	MYH9	4627	diana,elmmo,miranda,mirdb,tarbase	Coagulation
hsa-miR-200a-3p	PABPC4	8761	elmmo,microcosm,miranda,pita,tarbase	Coagulation
hsa-miR-199a-5p	CAV1	857	elmmo,miranda,mirtarbase,tarbase	Coagulation
hsa-miR-199a-5p	FZD6	8323	diana,mirdb,mirtarbase,targetscan	Coagulation
hsa-miR-199a-5p	MYH9	4627	diana,elmmo,pita,tarbase	Coagulation
hsa-miR-133a-3p	VKORC1	79001	elmmo,mirdb,mirtarbase,tarbase	Coagulation
hsa-miR-200a-3p	FOXA2	3170	diana,elmmo,mirdb,mirtarbase	Coagulation
hsa-miR-200a-3p	JMJD1C	221037	diana,elmmo,miranda,tarbase	Coagulation
hsa-miR-200a-3p	MAPK14	1432	elmmo,mirtarbase,tarbase	Coagulation
hsa-miR-200a-3p	PSEN1	5663	diana,miranda,tarbase	Coagulation
hsa-miR-200a-3p	UBASH3B	84959	diana,elmmo,mirtarbase	Coagulation
hsa-miR-28-5p	MPL	4352	mirtarbase,tarbase	Coagulation
hsa-miR-28-5p	VWF	7450	microcosm,tarbase	Coagulation
hsa-miR-199a-5p	APOE	348	mirtarbase,tarbase	Coagulation
hsa-miR-139-5p	PIK3CA	5290	mirtarbase,tarbase	Coagulation
hsa-miR-133a-3p	ANXA2	302	mirtarbase,tarbase	Coagulation
hsa-miR-200a-3p	ANO6	196527	diana,tarbase	Coagulation
hsa-miR-200a-3p	HPS5	11234	diana,tarbase	Coagulation
hsa-miR-200a-3p	THBS1	7057	miranda,tarbase	Coagulation
hsa-miR-28-5p	ACTB	60	tarbase	Coagulation
hsa-miR-28-5p	C1QTNF1	114897	tarbase	Coagulation
hsa-miR-28-5p	GNA12	2768	tarbase	Coagulation
hsa-miR-28-5p	GNAS	2778	tarbase	Coagulation
hsa-miR-28-5p	HPS4	89781	tarbase	Coagulation
hsa-miR-28-5p	LNPK	80856	tarbase	Coagulation

hsa-miR-28-5p	SRF	6722	mirtarbase	Coagulation
hsa-miR-28-5p	VKORC1	79001	tarbase	Coagulation
hsa-miR-100-5p	ACTB	60	mirtarbase	Coagulation
hsa-miR-100-5p	CYP4F2	8529	tarbase	Coagulation
hsa-miR-100-5p	EDN1	1906	tarbase	Coagulation
hsa-miR-100-5p	IL6	3569	tarbase	Coagulation
hsa-miR-100-5p	PIK3CB	5291	tarbase	Coagulation
hsa-miR-100-5p	PPIA	5478	tarbase	Coagulation
hsa-miR-100-5p	SLC7A11	23657	tarbase	Coagulation
hsa-miR-100-5p	TFPI	7035	tarbase	Coagulation
hsa-miR-100-5p	THBS1	7057	tarbase	Coagulation
hsa-miR-199a-5p	CD59	966	tarbase	Coagulation
hsa-miR-199a-5p	EDN1	1906	mirtarbase	Coagulation
hsa-miR-199a-5p	FN1	2335	tarbase	Coagulation
hsa-miR-199a-5p	PLAU	5328	tarbase	Coagulation
hsa-miR-199a-5p	PRKCA	5578	tarbase	Coagulation
hsa-miR-199a-5p	SERPINE2	5270	tarbase	Coagulation
hsa-miR-199a-5p	THBS1	7057	tarbase	Coagulation
hsa-miR-199a-5p	VAV3	10451	mirtarbase	Coagulation
hsa-miR-139-5p	IL6ST	3572	tarbase	Coagulation
hsa-miR-139-5p	PPIA	5478	tarbase	Coagulation
hsa-miR-133a-3p	BLOC1S6	26258	tarbase	Coagulation
hsa-miR-133a-3p	CEACAM1	634	tarbase	Coagulation
hsa-miR-133a-3p	EDN1	1906	tarbase	Coagulation
hsa-miR-133a-3p	IL6	3569	tarbase	Coagulation
hsa-miR-133a-3p	PLAUR	5329	tarbase	Coagulation
hsa-miR-133a-3p	PLSCR1	5359	tarbase	Coagulation
hsa-miR-133a-3p	RAB27A	5873	tarbase	Coagulation
hsa-miR-133a-3p	SERPINE1	5054	tarbase	Coagulation
hsa-miR-133a-3p	SERPINE2	5270	tarbase	Coagulation
hsa-miR-133a-3p	STXBP1	6812	tarbase	Coagulation
hsa-miR-133a-3p	TFPI	7035	tarbase	Coagulation

hsa-miR-133a-3p	UBASH3B	84959	tarbase	Coagulation
hsa-miR-133a-3p	VAV3	10451	tarbase	Coagulation
hsa-miR-200a-3p	GNA12	2768	tarbase	Coagulation
hsa-miR-200a-3p	GNAQ	2776	tarbase	Coagulation
hsa-miR-200a-3p	IL6ST	3572	tarbase	Coagulation
hsa-miR-200a-3p	SRF	6722	mirtarbase	Coagulation
hsa-miR-200a-3p	VAV3	10451	tarbase	Coagulation
hsa-miR-200a-3p	VCL	7414	tarbase	Coagulation
hsa-miR-200a-3p	PLEK	5341	diana,elmmo,miranda,mirdb,targetscan	Coagulation
hsa-miR-200a-3p	FAP	2191	diana,elmmo,microcosm,miranda	Coagulation
hsa-miR-200a-3p	SRC	6714	diana,elmmo,miranda,mirdb	Coagulation
hsa-miR-199a-5p	PDPN	10630	diana,mirdb,targetscan	Coagulation
hsa-miR-139-5p	THBS1	7057	elmmo,miranda,pita	Coagulation
hsa-miR-133a-3p	IL6ST	3572	diana,elmmo,pita	Coagulation
hsa-miR-133a-3p	SLC4A1	6521	diana,elmmo,microcosm	Coagulation
hsa-miR-200a-3p	PDGFRA	5156	diana,elmmo,mirdb	Coagulation
hsa-miR-200a-3p	STXBP1	6812	elmmo,miranda,mirdb	Coagulation
hsa-miR-133a-3p	MYH9	4627	diana,elmmo,miranda,mirdb,tarbase	Platelet activity
hsa-miR-199a-5p	FZD6	8323	diana,mirdb,mirtarbase,targetscan	Platelet activity
hsa-miR-199a-5p	MYH9	4627	diana,elmmo,pita,tarbase	Platelet activity
hsa-miR-200a-3p	MAPK14	1432	elmmo,mirtarbase,tarbase	Platelet activity
hsa-miR-200a-3p	UBASH3B	84959	diana,elmmo,mirtarbase	Platelet activity
hsa-miR-28-5p	MPL	4352	mirtarbase,tarbase	Platelet activity
hsa-miR-28-5p	VWF	7450	microcosm,tarbase	Platelet activity
hsa-miR-199a-5p	APOE	348	mirtarbase,tarbase	Platelet activity
hsa-miR-139-5p	PIK3CA	5290	mirtarbase,tarbase	Platelet activity
hsa-miR-28-5p	ACTB	60	tarbase	Platelet activity
hsa-miR-28-5p	C1QTNF1	114897	tarbase	Platelet activity
hsa-miR-28-5p	GNAS	2778	tarbase	Platelet activity
hsa-miR-28-5p	SRF	6722	mirtarbase	Platelet activity
hsa-miR-100-5p	ACTB	60	mirtarbase	Platelet activity
hsa-miR-100-5p	IL6	3569	tarbase	Platelet activity

hsa-miR-100-5p	PIK3CB	5291	tarbase	Platelet activity
hsa-miR-100-5p	PPIA	5478	tarbase	Platelet activity
hsa-miR-100-5p	SLC7A11	23657	tarbase	Platelet activity
hsa-miR-199a-5p	FN1	2335	tarbase	Platelet activity
hsa-miR-199a-5p	PRKCA	5578	tarbase	Platelet activity
hsa-miR-199a-5p	SERPINE2	5270	tarbase	Platelet activity
hsa-miR-199a-5p	VAV3	10451	mirtarbase	Platelet activity
hsa-miR-139-5p	IL6ST	3572	tarbase	Platelet activity
hsa-miR-139-5p	PPIA	5478	tarbase	Platelet activity
hsa-miR-133a-3p	CEACAM1	634	tarbase	Platelet activity
hsa-miR-133a-3p	IL6	3569	tarbase	Platelet activity
hsa-miR-133a-3p	PLSCR1	5359	tarbase	Platelet activity
hsa-miR-133a-3p	SERPINE2	5270	tarbase	Platelet activity
hsa-miR-133a-3p	STXBP1	6812	tarbase	Platelet activity
hsa-miR-133a-3p	UBASH3B	84959	tarbase	Platelet activity
hsa-miR-133a-3p	VAV3	10451	tarbase	Platelet activity
hsa-miR-200a-3p	IL6ST	3572	tarbase	Platelet activity
hsa-miR-200a-3p	SRF	6722	mirtarbase	Platelet activity
hsa-miR-200a-3p	VAV3	10451	tarbase	Platelet activity
hsa-miR-200a-3p	VCL	7414	tarbase	Platelet activity
hsa-miR-200a-3p	PLEK	5341	diana,elmmo,miranda,mirdb,targetscan	Platelet activity
hsa-miR-200a-3p	SRC	6714	diana,elmmo,miranda,mirdb	Platelet activity
hsa-miR-199a-5p	PDPN	10630	diana,mirdb,targetscan	Platelet activity
hsa-miR-133a-3p	IL6ST	3572	diana,elmmo,pita	Platelet activity
hsa-miR-200a-3p	PDGFRA	5156	diana,elmmo,mirdb	Platelet activity
hsa-miR-200a-3p	STXBP1	6812	elmmo,miranda,mirdb	Platelet activity

Table S4 List of target genes of the prognostic miRNAs annotated to thrombosis, coagulation and platelet activity.

MiRNA	Target Gene Symbol	Target Entrez ID	Sources	Biological Process
hsa-miR-199a-5p	SIRT1	23411	diana,elmmo,miranda,mirdb,mirtarbase,pita,targetscan	Thrombosis
hsa-miR-106b-5p	F3	2152	diana,elmmo,miranda,mirdb,mirtarbase,tarbase,targetscan	Thrombosis
hsa-miR-205-5p	PTPRJ	5795	elmmo,miranda,mirdb,mirtarbase,pita,tarbase	Thrombosis
hsa-miR-199a-5p	PODXL	5420	diana,elmmo,mirtarbase,pita,targetscan	Thrombosis
hsa-miR-133a-3p	VKORC1	79001	elmmo,mirdb,mirtarbase,tarbase	Thrombosis
hsa-miR-106b-5p	F2R	2149	diana,mirtarbase,tarbase	Thrombosis
hsa-miR-106b-5p	GUCY1A1	2982	mirdb,tarbase,targetscan	Thrombosis
hsa-miR-374a-5p	CD55	1604	diana,elmmo,tarbase	Thrombosis
hsa-miR-374a-5p	ENTPD1	953	diana,elmmo,tarbase	Thrombosis
hsa-miR-374a-5p	TFPI	7035	diana,elmmo,tarbase	Thrombosis
hsa-miR-205-5p	PODXL	5420	diana,tarbase	Thrombosis
hsa-miR-106b-5p	EGR1	1958	pita,tarbase	Thrombosis
hsa-miR-106b-5p	VASP	7408	elmmo,tarbase	Thrombosis
hsa-miR-106b-5p	VHL	7428	diana,tarbase	Thrombosis
hsa-miR-374a-5p	ANXA5	308	miranda,tarbase	Thrombosis
hsa-miR-374a-5p	PTGS2	5743	diana,tarbase	Thrombosis
hsa-miR-100-5p	ACE2	59272	tarbase	Thrombosis
hsa-miR-100-5p	CYP4F2	8529	tarbase	Thrombosis
hsa-miR-100-5p	IL6	3569	tarbase	Thrombosis
hsa-miR-100-5p	PIK3CB	5291	tarbase	Thrombosis
hsa-miR-100-5p	TFPI	7035	tarbase	Thrombosis
hsa-miR-199a-5p	PLAU	5328	tarbase	Thrombosis
hsa-miR-199a-5p	PTGS2	5743	mirtarbase	Thrombosis
hsa-miR-199a-5p	VASP	7408	mirtarbase	Thrombosis
hsa-miR-205-5p	CYP3A5	1577	tarbase	Thrombosis
hsa-miR-205-5p	EPRS1	2058	tarbase	Thrombosis
hsa-miR-205-5p	F2RL2	2151	mirtarbase	Thrombosis
hsa-miR-205-5p	F5	2153	tarbase	Thrombosis
hsa-miR-205-5p	HMOX1	3162	tarbase	Thrombosis

hsa-miR-205-5p	IL6	3569	tarbase	Thrombosis
hsa-miR-205-5p	NOS3	4846	tarbase	Thrombosis
hsa-miR-205-5p	SERPINA3	12	tarbase	Thrombosis
hsa-miR-205-5p	SERPIND1	3053	tarbase	Thrombosis
hsa-miR-205-5p	SERPINE1	5054	tarbase	Thrombosis
hsa-miR-133a-3p	ACE2	59272	tarbase	Thrombosis
hsa-miR-133a-3p	EGR1	1958	tarbase	Thrombosis
hsa-miR-133a-3p	IL6	3569	tarbase	Thrombosis
hsa-miR-133a-3p	KLF4	9314	tarbase	Thrombosis
hsa-miR-133a-3p	PTPRJ	5795	tarbase	Thrombosis
hsa-miR-133a-3p	SERPINE1	5054	tarbase	Thrombosis
hsa-miR-133a-3p	TFPI	7035	tarbase	Thrombosis
hsa-miR-106b-5p	APOH	350	mirtarbase	Thrombosis
hsa-miR-106b-5p	C1QBP	708	tarbase	Thrombosis
hsa-miR-106b-5p	EPRS1	2058	tarbase	Thrombosis
hsa-miR-106b-5p	F13A1	2162	tarbase	Thrombosis
hsa-miR-106b-5p	ITGA2	3673	mirtarbase	Thrombosis
hsa-miR-374a-5p	BDKRB2	624	tarbase	Thrombosis
hsa-miR-374a-5p	F2R	2149	tarbase	Thrombosis
hsa-miR-374a-5p	F8	2157	tarbase	Thrombosis
hsa-miR-374a-5p	HMOX1	3162	tarbase	Thrombosis
hsa-miR-374a-5p	LEP	3952	tarbase	Thrombosis
hsa-miR-374a-5p	PIK3CB	5291	tarbase	Thrombosis
hsa-miR-374a-5p	PLAT	5327	tarbase	Thrombosis
hsa-miR-374a-5p	PLAU	5328	tarbase	Thrombosis
hsa-miR-374a-5p	PODXL	5420	tarbase	Thrombosis
hsa-miR-374a-5p	SCUBE2	57758	tarbase	Thrombosis
hsa-miR-374a-5p	SERPINA5	5104	tarbase	Thrombosis
hsa-miR-374a-5p	TBXAS1	6916	tarbase	Thrombosis
hsa-miR-423-3p	C1QBP	708	tarbase	Thrombosis
hsa-miR-423-3p	F2	2147	mirtarbase	Thrombosis
hsa-miR-423-3p	MTHFR	4524	mirtarbase	Thrombosis

hsa-miR-423-3p	SIRT1	23411	tarbase	Thrombosis
hsa-miR-133a-3p	TUBB1	81027	diana,elmmo,miranda	Thrombosis
hsa-miR-106b-5p	PTGER3	5733	diana,microcosm,pictar	Thrombosis
hsa-miR-374a-5p	CD40LG	959	diana,elmmo,miranda	Thrombosis
hsa-miR-374a-5p	ITGA2	3673	diana,elmmo,mirdb	Thrombosis
hsa-miR-374a-5p	LEPR	3953	diana,elmmo,miranda	Thrombosis
hsa-miR-106b-5p	F3	2152	diana,elmmo,miranda,mirdb,mirtarbase,tarbase,targetscan	Coagulation
hsa-miR-205-5p	ENPP4	22875	diana,elmmo,miranda,mirdb,mirtarbase,targetscan	Coagulation
hsa-miR-205-5p	LYN	4067	diana,miranda,mirtarbase,tarbase,targetscan	Coagulation
hsa-miR-205-5p	RAP2B	5912	diana,elmmo,mirtarbase,pita,targetscan	Coagulation
hsa-miR-133a-3p	MYH9	4627	diana,elmmo,miranda,mirdb,tarbase	Coagulation
hsa-miR-106b-5p	ANO6	196527	elmmo,mirdb,pictar,pita,tarbase	Coagulation
hsa-miR-374a-5p	ABAT	18	diana,elmmo,miranda,mirdb,tarbase	Coagulation
hsa-miR-199a-5p	CAV1	857	elmmo,miranda,mirtarbase,tarbase	Coagulation
hsa-miR-199a-5p	FZD6	8323	diana,mirdb,mirtarbase,targetscan	Coagulation
hsa-miR-199a-5p	MYH9	4627	diana,elmmo,pita,tarbase	Coagulation
hsa-miR-133a-3p	VKORC1	79001	elmmo,mirdb,mirtarbase,tarbase	Coagulation
hsa-miR-106b-5p	IL6ST	3572	diana,elmmo,mirdb,tarbase	Coagulation
hsa-miR-205-5p	THBS1	7057	diana,elmmo,tarbase	Coagulation
hsa-miR-106b-5p	CAV1	857	elmmo,mirtarbase,tarbase	Coagulation
hsa-miR-106b-5p	F2R	2149	diana,mirtarbase,tarbase	Coagulation
hsa-miR-106b-5p	NFE2L2	4780	elmmo,pita,tarbase	Coagulation
hsa-miR-106b-5p	SLC7A11	23657	diana,mirtarbase,tarbase	Coagulation
hsa-miR-106b-5p	VCL	7414	diana,elmmo,tarbase	Coagulation
hsa-miR-374a-5p	ENTPD1	953	diana,elmmo,tarbase	Coagulation
hsa-miR-374a-5p	JMJD1C	221037	diana,elmmo,mirtarbase	Coagulation
hsa-miR-374a-5p	LNPK	80856	mirdb,mirtarbase,tarbase	Coagulation
hsa-miR-374a-5p	NFE2L2	4780	diana,mirtarbase,tarbase	Coagulation
hsa-miR-374a-5p	PRKCD	5580	elmmo,miranda,mirtarbase	Coagulation
hsa-miR-374a-5p	SLC7A11	23657	diana,elmmo,tarbase	Coagulation
hsa-miR-374a-5p	TFPI	7035	diana,elmmo,tarbase	Coagulation

hsa-miR-199a-5p	APOE	348	mirtarbase,tarbase	Coagulation
hsa-miR-205-5p	FZD6	8323	diana,tarbase	Coagulation
hsa-miR-205-5p	GNAS	2778	mirtarbase,pita	Coagulation
hsa-miR-205-5p	JMJD1C	221037	diana,mirtarbase	Coagulation
hsa-miR-205-5p	SERPINA1	5265	elmmo,tarbase	Coagulation
hsa-miR-205-5p	SRC	6714	mirtarbase,tarbase	Coagulation
hsa-miR-133a-3p	ANXA2	302	mirtarbase,tarbase	Coagulation
hsa-miR-106b-5p	CD34	947	mirtarbase,tarbase	Coagulation
hsa-miR-106b-5p	F2RL3	9002	diana,mirtarbase	Coagulation
hsa-miR-106b-5p	FZD6	8323	pita,tarbase	Coagulation
hsa-miR-106b-5p	PDGFB	5155	elmmo,mirtarbase	Coagulation
hsa-miR-106b-5p	SERPINE2	5270	pita,tarbase	Coagulation
hsa-miR-106b-5p	TLN1	7094	microcosm,tarbase	Coagulation
hsa-miR-374a-5p	ANO6	196527	diana,tarbase	Coagulation
hsa-miR-374a-5p	ANXA5	308	miranda,tarbase	Coagulation
hsa-miR-374a-5p	BLOC1S6	26258	diana,tarbase	Coagulation
hsa-miR-374a-5p	CYP4F11	57834	mirtarbase,tarbase	Coagulation
hsa-miR-374a-5p	FZD6	8323	mirtarbase,tarbase	Coagulation
hsa-miR-374a-5p	IL6ST	3572	diana,tarbase	Coagulation
hsa-miR-374a-5p	LMAN1	3998	diana,tarbase	Coagulation
hsa-miR-374a-5p	METAP1	23173	diana,tarbase	Coagulation
hsa-miR-374a-5p	VAV3	10451	diana,tarbase	Coagulation
hsa-miR-423-3p	GNAS	2778	pita,tarbase	Coagulation
hsa-miR-100-5p	ACTB	60	mirtarbase	Coagulation
hsa-miR-100-5p	CYP4F2	8529	tarbase	Coagulation
hsa-miR-100-5p	EDN1	1906	tarbase	Coagulation
hsa-miR-100-5p	IL6	3569	tarbase	Coagulation
hsa-miR-100-5p	PIK3CB	5291	tarbase	Coagulation
hsa-miR-100-5p	PPIA	5478	tarbase	Coagulation
hsa-miR-100-5p	SLC7A11	23657	tarbase	Coagulation
hsa-miR-100-5p	TFPI	7035	tarbase	Coagulation
hsa-miR-100-5p	THBS1	7057	tarbase	Coagulation

hsa-miR-199a-5p	CD59	966	tarbase	Coagulation
hsa-miR-199a-5p	EDN1	1906	mirtarbase	Coagulation
hsa-miR-199a-5p	FN1	2335	tarbase	Coagulation
hsa-miR-199a-5p	PLAU	5328	tarbase	Coagulation
hsa-miR-199a-5p	PRKCA	5578	tarbase	Coagulation
hsa-miR-199a-5p	SERPINE2	5270	tarbase	Coagulation
hsa-miR-199a-5p	THBS1	7057	tarbase	Coagulation
hsa-miR-199a-5p	VAV3	10451	mirtarbase	Coagulation
hsa-miR-205-5p	AXL	558	tarbase	Coagulation
hsa-miR-205-5p	F2RL1	2150	tarbase	Coagulation
hsa-miR-205-5p	F2RL2	2151	mirtarbase	Coagulation
hsa-miR-205-5p	F5	2153	tarbase	Coagulation
hsa-miR-205-5p	IL6	3569	tarbase	Coagulation
hsa-miR-205-5p	ITPK1	3705	tarbase	Coagulation
hsa-miR-205-5p	MAPK14	1432	mirtarbase	Coagulation
hsa-miR-205-5p	METAP1	23173	tarbase	Coagulation
hsa-miR-205-5p	NOS3	4846	tarbase	Coagulation
hsa-miR-205-5p	PABPC4	8761	tarbase	Coagulation
hsa-miR-205-5p	PRDX2	7001	mirtarbase	Coagulation
hsa-miR-205-5p	SERPIND1	3053	tarbase	Coagulation
hsa-miR-205-5p	SERPINE1	5054	tarbase	Coagulation
hsa-miR-205-5p	SERPINE2	5270	tarbase	Coagulation
hsa-miR-205-5p	SLC7A11	23657	tarbase	Coagulation
hsa-miR-205-5p	STXBP3	6814	tarbase	Coagulation
hsa-miR-205-5p	TFPI2	7980	tarbase	Coagulation
hsa-miR-133a-3p	BLOC1S6	26258	tarbase	Coagulation
hsa-miR-133a-3p	CEACAM1	634	tarbase	Coagulation
hsa-miR-133a-3p	EDN1	1906	tarbase	Coagulation
hsa-miR-133a-3p	IL6	3569	tarbase	Coagulation
hsa-miR-133a-3p	PLAUR	5329	tarbase	Coagulation
hsa-miR-133a-3p	PLSCR1	5359	tarbase	Coagulation
hsa-miR-133a-3p	RAB27A	5873	tarbase	Coagulation

hsa-miR-133a-3p	SERPINE1	5054	tarbase	Coagulation
hsa-miR-133a-3p	SERPINE2	5270	tarbase	Coagulation
hsa-miR-133a-3p	STXBP1	6812	tarbase	Coagulation
hsa-miR-133a-3p	TFPI	7035	tarbase	Coagulation
hsa-miR-133a-3p	UBASH3B	84959	tarbase	Coagulation
hsa-miR-133a-3p	VAV3	10451	tarbase	Coagulation
hsa-miR-106b-5p	ABAT	18	tarbase	Coagulation
hsa-miR-106b-5p	AK3	50808	tarbase	Coagulation
hsa-miR-106b-5p	APOH	350	mirtarbase	Coagulation
hsa-miR-106b-5p	BLOC1S3	388552	mirtarbase	Coagulation
hsa-miR-106b-5p	DGKD	8527	mirtarbase	Coagulation
hsa-miR-106b-5p	DGKD	8527	mirtarbase	Coagulation
hsa-miR-106b-5p	DGKH	160851	tarbase	Coagulation
hsa-miR-106b-5p	ENPP4	22875	tarbase	Coagulation
hsa-miR-106b-5p	F11R	50848	tarbase	Coagulation
hsa-miR-106b-5p	F13A1	2162	tarbase	Coagulation
hsa-miR-106b-5p	F2RL1	2150	mirtarbase	Coagulation
hsa-miR-106b-5p	GNAS	2778	mirtarbase	Coagulation
hsa-miR-106b-5p	ITGA2	3673	mirtarbase	Coagulation
hsa-miR-106b-5p	LYN	4067	tarbase	Coagulation
hsa-miR-106b-5p	MYH9	4627	mirtarbase	Coagulation
hsa-miR-106b-5p	PABPC4	8761	tarbase	Coagulation
hsa-miR-106b-5p	PPIA	5478	mirtarbase	Coagulation
hsa-miR-106b-5p	TXK	7294	mirtarbase	Coagulation
hsa-miR-374a-5p	AK3	50808	tarbase	Coagulation
hsa-miR-374a-5p	ANXA2	302	tarbase	Coagulation
hsa-miR-374a-5p	AXL	558	tarbase	Coagulation
hsa-miR-374a-5p	C1QTNF1	114897	tarbase	Coagulation
hsa-miR-374a-5p	CAV1	857	tarbase	Coagulation
hsa-miR-374a-5p	CD36	948	tarbase	Coagulation
hsa-miR-374a-5p	CD59	966	tarbase	Coagulation
hsa-miR-374a-5p	CSRP1	1465	tarbase	Coagulation

hsa-miR-374a-5p	DGKE	8526	tarbase	Coagulation
hsa-miR-374a-5p	DGKG	1608	tarbase	Coagulation
hsa-miR-374a-5p	EDN1	1906	tarbase	Coagulation
hsa-miR-374a-5p	ENPP4	22875	tarbase	Coagulation
hsa-miR-374a-5p	F11R	50848	tarbase	Coagulation
hsa-miR-374a-5p	F2R	2149	tarbase	Coagulation
hsa-miR-374a-5p	F8	2157	tarbase	Coagulation
hsa-miR-374a-5p	FN1	2335	tarbase	Coagulation
hsa-miR-374a-5p	GNAQ	2776	tarbase	Coagulation
hsa-miR-374a-5p	LYN	4067	tarbase	Coagulation
hsa-miR-374a-5p	MYH9	4627	tarbase	Coagulation
hsa-miR-374a-5p	PDGFRA	5156	tarbase	Coagulation
hsa-miR-374a-5p	PIK3CB	5291	tarbase	Coagulation
hsa-miR-374a-5p	PLAT	5327	tarbase	Coagulation
hsa-miR-374a-5p	PLAU	5328	tarbase	Coagulation
hsa-miR-374a-5p	PLAUR	5329	tarbase	Coagulation
hsa-miR-374a-5p	PLCG2	5336	tarbase	Coagulation
hsa-miR-374a-5p	PTPN6	5777	tarbase	Coagulation
hsa-miR-374a-5p	SERPINA1	5265	tarbase	Coagulation
hsa-miR-374a-5p	SERPINE2	5270	tarbase	Coagulation
hsa-miR-374a-5p	SH2B3	10019	tarbase	Coagulation
hsa-miR-374a-5p	SHH	6469	tarbase	Coagulation
hsa-miR-374a-5p	SRF	6722	tarbase	Coagulation
hsa-miR-374a-5p	SYK	6850	tarbase	Coagulation
hsa-miR-374a-5p	TFPI2	7980	tarbase	Coagulation
hsa-miR-374a-5p	THBS1	7057	tarbase	Coagulation
hsa-miR-374a-5p	VCL	7414	tarbase	Coagulation
hsa-miR-423-3p	ACTB	60	tarbase	Coagulation
hsa-miR-423-3p	DGKE	8526	tarbase	Coagulation
hsa-miR-423-3p	F2	2147	mirtarbase	Coagulation
hsa-miR-423-3p	LYN	4067	tarbase	Coagulation
hsa-miR-423-3p	MYL9	10398	tarbase	Coagulation

hsa-miR-106b-5p	HPS5	11234	diana,elmmo,miranda,mirdb,targetscan	Coagulation
hsa-miR-106b-5p	HS3ST5	222537	diana,elmmo,miranda,mirdb,targetscan	Coagulation
hsa-miR-106b-5p	METAP1	23173	diana,elmmo,microcosm,miranda,pita	Coagulation
hsa-miR-106b-5p	PDGFRA	5156	diana,elmmo,miranda,mirdb	Coagulation
hsa-miR-199a-5p	PDPN	10630	diana,mirdb,targetscan	Coagulation
hsa-miR-133a-3p	IL6ST	3572	diana,elmmo,pita	Coagulation
hsa-miR-133a-3p	SLC4A1	6521	diana,elmmo,microcosm	Coagulation
hsa-miR-374a-5p	CD40LG	959	diana,elmmo,miranda	Coagulation
hsa-miR-374a-5p	ITGA2	3673	diana,elmmo,mirdb	Coagulation
hsa-miR-205-5p	LYN	4067	diana,miranda,mirtarbase,tarbase,targetscan	Platelet activity
hsa-miR-205-5p	RAP2B	5912	diana,elmmo,mirtarbase,pita,targetscan	Platelet activity
hsa-miR-133a-3p	MYH9	4627	diana,elmmo,miranda,mirdb,tarbase	Platelet activity
hsa-miR-374a-5p	ABAT	18	diana,elmmo,miranda,mirdb,tarbase	Platelet activity
hsa-miR-199a-5p	FZD6	8323	diana,mirdb,mirtarbase,targetscan	Platelet activity
hsa-miR-199a-5p	MYH9	4627	diana,elmmo,pita,tarbase	Platelet activity
hsa-miR-106b-5p	IL6ST	3572	diana,elmmo,mirdb,tarbase	Platelet activity
hsa-miR-106b-5p	F2R	2149	diana,mirtarbase,tarbase	Platelet activity
hsa-miR-106b-5p	SLC7A11	23657	diana,mirtarbase,tarbase	Platelet activity
hsa-miR-106b-5p	VCL	7414	diana,elmmo,tarbase	Platelet activity
hsa-miR-374a-5p	PRKCD	5580	elmmo,miranda,mirtarbase	Platelet activity
hsa-miR-374a-5p	SLC7A11	23657	diana,elmmo,tarbase	Platelet activity
hsa-miR-199a-5p	APOE	348	mirtarbase,tarbase	Platelet activity
hsa-miR-205-5p	FZD6	8323	diana,tarbase	Platelet activity
hsa-miR-205-5p	GNAS	2778	mirtarbase,pita	Platelet activity
hsa-miR-205-5p	SRC	6714	mirtarbase,tarbase	Platelet activity
hsa-miR-106b-5p	F2RL3	9002	diana,mirtarbase	Platelet activity
hsa-miR-106b-5p	FZD6	8323	pita,tarbase	Platelet activity
hsa-miR-106b-5p	PDGFB	5155	elmmo,mirtarbase	Platelet activity
hsa-miR-106b-5p	SERPINE2	5270	pita,tarbase	Platelet activity
hsa-miR-106b-5p	TLN1	7094	microcosm,tarbase	Platelet activity
hsa-miR-374a-5p	FZD6	8323	mirtarbase,tarbase	Platelet activity
hsa-miR-374a-5p	IL6ST	3572	diana,tarbase	Platelet activity

hsa-miR-374a-5p	METAP1	23173	diana,tarbase	Platelet activity
hsa-miR-374a-5p	VAV3	10451	diana,tarbase	Platelet activity
hsa-miR-423-3p	GNAS	2778	pita,tarbase	Platelet activity
hsa-miR-100-5p	ACTB	60	mirtarbase	Platelet activity
hsa-miR-100-5p	IL6	3569	tarbase	Platelet activity
hsa-miR-100-5p	PIK3CB	5291	tarbase	Platelet activity
hsa-miR-100-5p	PPIA	5478	tarbase	Platelet activity
hsa-miR-100-5p	SLC7A11	23657	tarbase	Platelet activity
hsa-miR-199a-5p	FN1	2335	tarbase	Platelet activity
hsa-miR-199a-5p	PRKCA	5578	tarbase	Platelet activity
hsa-miR-199a-5p	SERPINE2	5270	tarbase	Platelet activity
hsa-miR-199a-5p	VAV3	10451	mirtarbase	Platelet activity
hsa-miR-205-5p	AXL	558	tarbase	Platelet activity
hsa-miR-205-5p	F2RL2	2151	mirtarbase	Platelet activity
hsa-miR-205-5p	IL6	3569	tarbase	Platelet activity
hsa-miR-205-5p	MAPK14	1432	mirtarbase	Platelet activity
hsa-miR-205-5p	METAP1	23173	tarbase	Platelet activity
hsa-miR-205-5p	NOS3	4846	tarbase	Platelet activity
hsa-miR-205-5p	SERPINE2	5270	tarbase	Platelet activity
hsa-miR-205-5p	SLC7A11	23657	tarbase	Platelet activity
hsa-miR-205-5p	STXBP3	6814	tarbase	Platelet activity
hsa-miR-133a-3p	CEACAM1	634	tarbase	Platelet activity
hsa-miR-133a-3p	IL6	3569	tarbase	Platelet activity
hsa-miR-133a-3p	PLSCR1	5359	tarbase	Platelet activity
hsa-miR-133a-3p	SERPINE2	5270	tarbase	Platelet activity
hsa-miR-133a-3p	STXBP1	6812	tarbase	Platelet activity
hsa-miR-133a-3p	UBASH3B	84959	tarbase	Platelet activity
hsa-miR-133a-3p	VAV3	10451	tarbase	Platelet activity
hsa-miR-106b-5p	ABAT	18	tarbase	Platelet activity
hsa-miR-106b-5p	BLOC1S3	388552	mirtarbase	Platelet activity
hsa-miR-106b-5p	DGKD	8527	mirtarbase	Platelet activity
hsa-miR-106b-5p	DGKD	8527	mirtarbase	Platelet activity

hsa-miR-106b-5p	DGKH	160851	tarbase	Platelet activity
hsa-miR-106b-5p	F11R	50848	tarbase	Platelet activity
hsa-miR-106b-5p	GNAS	2778	mirtarbase	Platelet activity
hsa-miR-106b-5p	LYN	4067	tarbase	Platelet activity
hsa-miR-106b-5p	MYH9	4627	mirtarbase	Platelet activity
hsa-miR-106b-5p	PPIA	5478	mirtarbase	Platelet activity
hsa-miR-106b-5p	TXK	7294	mirtarbase	Platelet activity
hsa-miR-374a-5p	AXL	558	tarbase	Platelet activity
hsa-miR-374a-5p	C1QTNF1	114897	tarbase	Platelet activity
hsa-miR-374a-5p	CSRP1	1465	tarbase	Platelet activity
hsa-miR-374a-5p	DGKE	8526	tarbase	Platelet activity
hsa-miR-374a-5p	DGKG	1608	tarbase	Platelet activity
hsa-miR-374a-5p	F11R	50848	tarbase	Platelet activity
hsa-miR-374a-5p	F2R	2149	tarbase	Platelet activity
hsa-miR-374a-5p	FN1	2335	tarbase	Platelet activity
hsa-miR-374a-5p	LYN	4067	tarbase	Platelet activity
hsa-miR-374a-5p	MYH9	4627	tarbase	Platelet activity
hsa-miR-374a-5p	PDGFRA	5156	tarbase	Platelet activity
hsa-miR-374a-5p	PIK3CB	5291	tarbase	Platelet activity
hsa-miR-374a-5p	PLCG2	5336	tarbase	Platelet activity
hsa-miR-374a-5p	PTPN6	5777	tarbase	Platelet activity
hsa-miR-374a-5p	SERPINE2	5270	tarbase	Platelet activity
hsa-miR-374a-5p	SH2B3	10019	tarbase	Platelet activity
hsa-miR-374a-5p	SRF	6722	tarbase	Platelet activity
hsa-miR-374a-5p	SYK	6850	tarbase	Platelet activity
hsa-miR-374a-5p	VCL	7414	tarbase	Platelet activity
hsa-miR-423-3p	ACTB	60	tarbase	Platelet activity
hsa-miR-423-3p	DGKE	8526	tarbase	Platelet activity
hsa-miR-423-3p	F2	2147	mirtarbase	Platelet activity
hsa-miR-423-3p	LYN	4067	tarbase	Platelet activity
hsa-miR-423-3p	MYL9	10398	tarbase	Platelet activity
hsa-miR-106b-5p	METAP1	23173	diana,elmmo,microcosm,miranda,pita	Platelet activity

hsa-miR-106b-5p	PDGFRA	5156	diana,elmmo,miranda,mirdb	Platelet activity
hsa-miR-199a-5p	PDPN	10630	diana,mirdb,targetscan	Platelet activity
hsa-miR-133a-3p	IL6ST	3572	diana,elmmo,pita	Platelet activity
hsa-miR-374a-5p	CD40LG	959	diana,elmmo,miranda	Platelet activity

CHAPTER 6

Summary and general discussion

Summary and general discussion

Sepsis remains a clinical condition characterized by high heterogeneity [1]. This is evident on different levels, including the infection source, the individual patient's heterogeneity, and the plethora of pathways involving several processes (*e.g.*, inflammation and coagulation). Despite decades of research, the morbidity and mortality associated with sepsis and septic shock have not changed dramatically [2,3]. Early detection, suitable categorization and timely treatment administered during the incipient period of septic shock are vital for decreasing the death rate. Biological and molecular information assays allow us to reveal the molecular mechanisms of illness onset and progression, offering a new and valid method to acquire the underlying diagnosis markers and treatment targets that can help to prevent and treat septic shock [4]. Several strategies have been proposed over the years to improve our knowledge of the mechanisms involved in the pathophysiology of sepsis. To date, there are no direct molecular biomarkers to indicate tissue vulnerability to septic insult or to predict subsequent organ dysfunction [5]. It is not possible to use a single conventional biomarker in the personalized assessment of patients with sepsis since the available biomarkers do not directly reflect the inflammatory cellular response and do not predict the magnitude of early immune cellular activation.

Extracellular vesicles in sepsis

The emerging data on EVs suggests that they are potential players in critical diseases due to their early involvement in relevant pathobiological functions of vital organs exposed to serious stressors [6]. In **Chapter 2** we performed a narrative review that highlighted the recent advances in EV knowledge in sepsis and COVID-19, and their involvement in the mechanisms of thromboinflammation. According to our point of view, the investigation of EVs in sepsis is still at the beginning compared to other diseases such as cancer [7–9], neurodegenerative [10] and cardiovascular diseases [11]. Understanding the involvement of EVs in the pathogenesis of sepsis may offer the possibility of identifying new pathogenic insights or therapeutic targets to suppress inflammation, coagulopathy and bacterial growth, which may help to prevent end-organ damage. Therefore, some optimistic speculations about the promising role of EVs in sepsis are justified. There is a wide range of potential clinical applications for EVs, ranging from use as biomarkers to therapeutic targets [12–14]. On the other hand, standardized EV isolation methods and complete quality control as required by the guidelines in this field, are often lacking [15]. This represents a common pitfall in EV research which often impedes drawing unambiguous conclusions across studies.

Circulating EVs possess valuable potential in disease diagnosis, stratification, and prognosis definition. This is evident from a large portion of EV studies focusing on their role as biomarkers in several pathological conditions. For instance, increased levels of EVs have been reported in sepsis [16,17], suggesting that EVs mediate critical mechanisms of induction in sepsis pathophysiology and disease development [18–21]. Next to the role of different EV-releasing cell types, it is a matter of debate during which phase of sepsis or septic shock EVs could have the biggest influence [14]. Our data adds new knowledge to this area. In **Chapter 3**, we focused on characterizing EVs in burn patients with septic shock, a heterogeneous subgroup of sepsis patients, to identify potential novel markers and mediators of septic shock. Compared to other studies [22–25] that investigate burn severity not associated with septic shock, ours is unique because we provide a comprehensive, in-depth analysis of circulating EVs by using a standardized multiplex flow cytometric assay to define a molecular signature diagnostic for septic shock. To the best of our knowledge, this is the first screening of plasma EV concentration and epitope profile after burn injury that includes septic shock patients. Our results have shown that EV concentrations and specific cells-derived EVs are higher and closely related to septic shock and not burn trauma *per se*, suggesting the potential utility of measuring plasma EVs in the dynamic evaluation of patient's status. The increase in EV concentrations not only reflects the severity of septic shock but also might contribute to amplifying systemic inflammation [26]. We have shown, indeed, that EVs isolated from burn patients with septic shock may prime platelet activation and exacerbate coagulopathy. With this in mind and in the light of our results, EVs should be considered among those pathogenic mechanisms that, acting on platelets, may contribute to cell-to-cell interaction, thrombus formation, and thromboinflammation in septic shock. Further in-depth analysis of EVs may lead to the development of novel diagnostic tools or to the identification of new therapeutic targets for treating septic shock in burn patients.

Thrombopoietin and miRNAs in COVID-19

Although several previous studies had shown that THPO is elevated in patients affected by several critical disease [27–30], little is known about the levels of THPO in COVID-19 patients. In **chapter 4**, we suggest that elevated levels of THPO 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. On top of that, our data provides evidence for the potential of circulating THPO as early diagnostic and

prognostic biomarker useful to timely identify patients affected by COVID-19 and to recognizes those who will have a less favorable clinical course.

Emerging omic technologies are providing innovative tools for medical decision-making [31]. Current literature indicates that, among transcriptomic biomarkers, miRNAs are the most promising as diagnostic biomarker hotspots, indicators of disease severity, predictors of mortality, and therapeutic targets [32]. miRNAs may be useful in the diagnosis, prognosis, monitoring, and clinical management of COVID-19 and of its complications [33–36]. With this in mind, in **chapter 5**, we verify the utility of miRNA profiling as a diagnostic and prognostic early marker in patients affected by COVID-19. In this study, we provided a suitable panel of candidate miRNAs as useful biomarkers for the diagnosis of COVID-19. miRNA profiles could also inform molecular pathways implicated in determining disease severity, suggesting potential targets for therapeutic guidance [33]. Furthermore, we explored bioinformatics databases to retrieve a genetic network (mRNA-miRNAs) related to our *in vitro* results. Of note, the inclusion, in our study, of patients without COVID-19 infection but comparable symptoms enhanced the specificity of the miRNAs we identified as potential markers. Interestingly, we also aimed to identify and evaluate plasma miRNA profiling that was able to discriminate those COVID-19 patients who, in the course of their disease, later develop respiratory failure and the worst prognosis. One feature of our experimental design that is important to underline is that COVID-19 patients enrolled in our study had comparable moderate symptoms at the time of enrollment and evolved into different severity grades during the hospitalization that followed the first clinical evaluation in the ED.

Concluding remarks and future directions

The future of sepsis management relies on our ability to discover predictors of worse prognosis to phenotype and risk-stratify individual patients. Since the initial identification of EVs as natural carriers of biomolecules and mediators of intracellular communication, research in this field has been growing exponentially with the aim of better understanding EV biology and devising strategies to enhance EV targeting and bioactivity with the end to translate their use into clinical practice. EVs, as well as miRNAs, offer innovative theragnostic possibilities in detecting, monitoring and modulating the onset of tissue dysfunction before the development of organ failure. Given that the translation into clinical practice is still in progress, several areas could be improved to ensure their successful and timely translation into clinical practice. Before the routine use of EVs and miRNAs in clinical practice could be hypothesized, some issues will require further investigations, such as the improvement in techniques for EVs isolation and reproducibility between laboratories for circulating

miRNA quantification. An accurate understanding of this critical issue will improve the effective use of EVs and miRNAs for early diagnosis and management of critical illnesses within the framework of patient-centered management plans.

Future directions should include conducting large prospective studies to investigate the application of the different recently discovered EV biomarkers in sepsis and in predicting the clinical response to therapy.

In addition, since, in their mediator-like character, EVs are capable of conveying a multitude of information to distant tissues, this ability of mediation appears to be especially relevant in diseases involving endothelial activation and microvascular dysfunction (such as sepsis), given that they are accompanied by a robust release of EVs. Therefore, future applications of EVs as information mediators or therapeutic vectors in sepsis may also be envisaged.

One of the most pressing questions that arises in the management of patients affected by sepsis or COVID-19 is how we can contribute to designing and planning a personalized and host-directed treatment. The results we obtained on EVs, THPO and miRNAs may expand our knowledge in this direction and contribute to design novel therapeutic approaches. For instance, in preclinical models, EVs have demonstrated an ability to dampen inflammation and reduce proliferation caused by SARS-CoV-2, establishing the rationale for clinical trials [37]. In light of this several EV-based therapies have entered clinical trials to assess safety, efficacy, administration route, and optimal dosing in various respiratory conditions [38]. In the same direction, THPO silencing has also been shown to safely reduce platelet count and the rate of thrombotic events in different inflammatory diseases [39]. Finally, since miRNAs are known to interact with multiple pathways and reduce inflammatory biomarkers, thrombi formation, and tissue damage [40], some phase II clinical trials are currently testing the efficacy of miRNA-based drug able to downregulate viral RNA levels in patients with HCV infection and COVID-19 [41,42].

In conclusion, our results significantly contribute to the advancement of the scientific knowledge in the pathophysiological mechanisms involved in sepsis and COVID-19, potentially indicating new targets useful, in the future, to design innovative and personalized therapeutic approaches to these diseases.

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APPENDIX

Biography

Publications

Presentations arising from this thesis

Biography

Martina Schiavello was born on April 16, 1993 in Turin, Italy. In 2016, she received her Bachelor degree in Biological Science from the University of Turin. Then, she continued her education at the University of Turin, where she received her Master Degree in Medical Biotechnology, in 2018. In 2019, she was awarded an individual PhD fellowship from the University of Turin in PhD program in the Experimental Medicine and Therapy to fund her PhD project under the supervision of Prof. dr. Giuseppe Montrucchio and Prof. dr. Enrico Lupia. During the last year of PhD, she spent six months at the National Heart and Lung Institute of Imperial College London (Prof. Costanza Emanueli). During her PhD, she has been focusing on the study of extracellular vesicles and miRNAs for biomarkers discovery in sepsis and COVID-19. During her period abroad, she focused on the use of extracellular vesicles for cardiac therapy, exploring engineering strategies to enhance bioactivity and targeting the heart.

Publications

- **Schiavello, M**., Vizio, B., Bosco, O., Pivetta, E., Mariano, F., Montrucchio, G., & Lupia, E. (2023). Extracellular Vesicles: New Players in the Mechanisms of Sepsis- and COVID-19-Related Thromboinflammation. *International journal of molecular sciences*, *24*(3), 1920. https://doi.org/10.3390/ijms24031920
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- Schiavello M., Vizio B., Mariano F., Bruno S., Bosco O., Pensa A., Risso D., Camussi G., Montrucchio G., Lupia E. **Identification and characterization of plasma-derived extracellular vesilces in burn-septic shock patients**. 1st MOVE symposium, Malaga, Spain, 24-27 October 2023.
- Schiavello M., Vizio B., Mariano F., Bruno S., Bosco O., Pensa A., Risso D., Camussi G., Montrucchio G., Lupia E. **Extracellular vesilces isolated from human plasma as a potential diagnostic ana prognostic tool in burn septic shock patients**. 3rd Symposium EVITA, Urbino, Italy, 13-15 September 2023. DOI: 10.20517/evcna.2023.57.
- Schiavello M., Vizio B., Mariano F., Pensa A., Bosco O., Stella M., Lupia E., Montrucchio G. **Investigation of Novel Circulating Biomarkers in Burn Septic Shock Patients.** 19th European Burns Association Congress, Turin, Italy, 7-10 November 2022. https://doi.org/10.3390/ebj3040046.