

Increased von Willebrand Factor Platelet-Binding Capacity Is Related to Poor Prognosis in COVID-19 Patients

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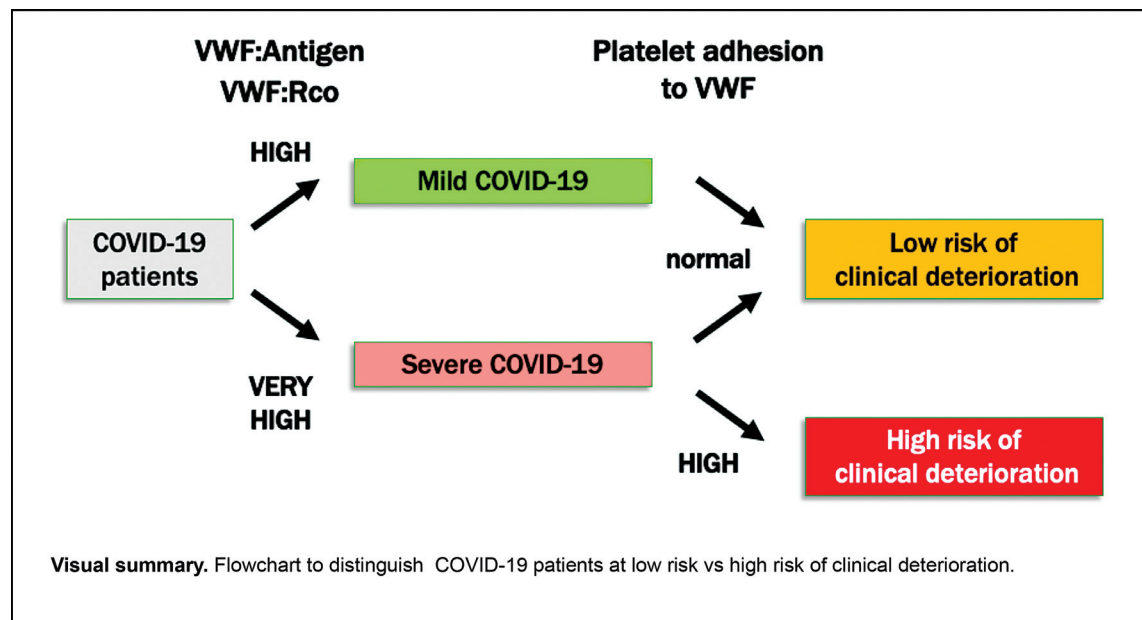
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Thromb Haemost 2023;123:118–122.



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received
 April 7, 2022
 accepted after revision
 August 31, 2022
 accepted manuscript online
 October 17, 2022

DOI <https://doi.org/10.1055/a-1962-5447>.
 ISSN 0340-6245.

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Coronavirus disease 2019 (COVID-19) is still a global health emergency. Its causative agent, the novel coronavirus SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2), has infected more than 300 million people and caused more than 5 million deaths worldwide.

SARS-CoV-2 infection increases the risk of thrombotic complications and patients with pre-existing cardiovascular disease comorbidities have a higher risk of experiencing severe symptoms and death. The prothrombotic state observed in severe patients is not just an epiphenomenon that occurs in patients experiencing a cytokine storm and virus-driven endothelial damage, but also contributes to the rapid worsening of the disease. In the pulmonary microcirculation, microthrombi^{1,2} impair pulmonary gas exchange and aggravate the acute respiratory distress syndrome.³ Systemically, platelet-mediated thrombotic microangiopathy and consumptive coagulopathy may lead to multiple organ failure.⁴

Nevertheless, the mechanisms responsible for the formation of thrombi in COVID-19 patients have yet to be fully elucidated. One of the key features of COVID-19 is endothelial injury⁵ that results in a dramatic increase of the circulating levels of von Willebrand factor (VWF).⁶ The imbalance between VWF and ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) increases the hypercoagulable state promoted by COVID-19 disease⁷ and it is associated with disease severity^{8,9} and short-term mortality.⁴ In a previous study, our research network provided evidence that critically ill COVID-19 patients experience increased VWF-induced platelet agglutination.¹⁰ To assess if the ability of platelets to bind VWF is different between severe and mild COVID-19 patients, we performed a prospective study in which we evaluated VWF antigen levels (VWF:Ag; ASSERACHROME VWF:AG by Stago), VWF ristocetin cofactor activity (VWF:RCo; BCS XP instrument by Siemens), and ristocetin-induced platelet agglutination (RIPA) in COVID-19 patients admitted at the Umberto I Hospital from April to December 2020 ($n = 49$) in relation to disease severity and to the short-term mortality. The RIPA assay was performed by adding scalar doses of ristocetin (0.75, 1, 1.25, 1.5, and 2 mg/mL) to platelet-rich plasma. If the response to 0.75 mg/mL ristocetin was higher than 40%, ristocetin was also tested at a lower dose (0.5 mg/mL). SARS-CoV-2-positive patients (age >18 years, at least one positive swab for COVID-19) were stratified based on their required respiratory support at the time of blood sampling, among severe (on assisted or controlled mechanical ventilation and $\text{PaO}_2/\text{FiO}_2 < 200$; $n = 35$) and mild (on nasal cannula or face mask; $n = 14$). Severe patients were further stratified based on short-term mortality ($n = 21$ of 35), defined as mortality occurring less than 20 days after presentation to the hospital. For comparison, we evaluated the same parameters in a group of healthy SARS-CoV-2-negative volunteers (HVs; $n = 20$). The clinical characteristics of patients and HVs upon enrollment are shown in ►Tables 1 and 2. The study was approved by the Ethics Committee of the Umberto I Hospital of Rome.

In agreement with previous reports,⁸ both VWF:Ag and VWF:RCo were significantly higher in severe compared

Table 1 Characteristics of COVID-19 patients (severe and mild) and healthy volunteers (HVs)

| Characteristic | COVID-19 severe, $n = 35$ | COVID-19 mild, $n = 14$ | HV, $n = 20$ |
|-----------------------------|---------------------------|-------------------------|--------------|
| Demographic characteristics | | | |
| Age, y | 67.9 ± 11.2 | 66.6 ± 19.9 | 36.3 ± 9 |
| Gender (F/M) | 19/16 | 11/11 | 9/11 |
| SAPS II | 42.1 ± 12.8 | 22.9 ± 10.6 | 0 |
| Medical history | | | |
| Hypertension | 74.2% | 36.4% | 14.0% |
| Diabetes | 32.2% | 18.2% | 0% |
| Smoke | 19.4% | 9.0% | 31.0% |
| Obesity | 19.3% | 4.5% | 10.0% |
| Symptoms at disease onset | | | |
| Fever | 100% | 63.6% | 0% |
| Dyspnea | 51.6% | 45.5% | 0% |
| Cough | 64.8% | 0% | 0% |

with mild patients (►Fig. 1A), and both patient populations displayed significantly higher levels compared with the normal reference values (50–160 IU/dL for VWF:Ag; 41–130 IU/dL for VWF:RCo). However, in the RIPA assay, mild patients required the same amount of ristocetin as HVs to achieve 20 or 40% platelet agglutination, while severe patients needed significantly less ristocetin to achieve the same level of agglutination (►Fig. 1B). This data suggested that platelets of severe COVID-19 patients bind more easily to plasmatic VWF, and that this does not depend solely on the VWF concentration. In addition, no

Table 2 Characteristics of severe COVID-19 patients divided for mortality and survival

| Characteristics | Mortality < 20 d, $n = 21$ | Survival > 20 d, $n = 14$ |
|-----------------------------|----------------------------|---------------------------|
| Demographic characteristics | | |
| Age, y | 68.2 ± 11.7 | 63.5 ± 11.2 |
| Gender (F/M) | 9/12 | 6/8 |
| SAPS II | 39.9 ± 11.9 | 45.3 ± 13.8 |
| Medical history | | |
| Hypertension | 76.1% | 71.1% |
| Diabetes | 33.3% | 27.3% |
| Smoke | 23.8% | 9.1% |
| Obesity | 19.1% | 18.1 |
| Symptoms at disease onset | | |
| Fever | 100% | 100% |
| Dyspnea | 52.4% | 45.4% |
| Cough | 66.7% | 62.5% |

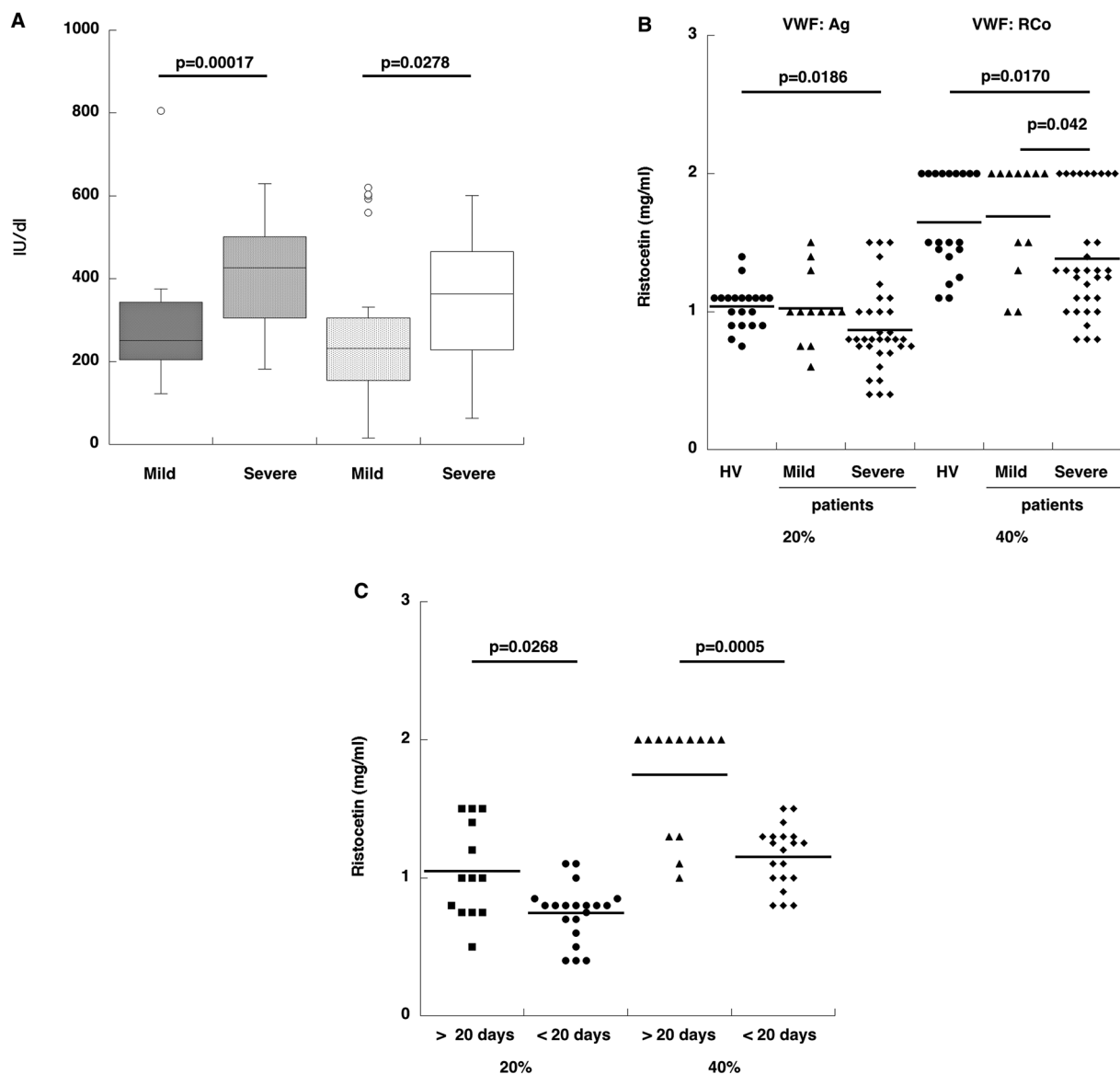


Fig. 1 (A) Box plot of the plasma concentration of von Willebrand factor antigen (VWF:Ag) and von Willebrand ristocetin cofactor (VWF:RCo) evaluated in severe and mild COVID-19 patients. Normal reference values were 50 to 160 IU/dL for VWF:Ag and 41 to 130 IU/dL for VWF:RCo. (B) Dot blot of ristocetin (0.75–2 mg/mL)-induced platelet agglutination in severe ($N=35$) and mild ($N=22$) COVID-19 patients versus healthy volunteers ($N=20$). The results are reported as the ristocetin concentrations that exceed 20 and 40% of agglutination. Statistical data were evaluated by the Wilcoxon–Mann–Whitney rank sum test for unpaired data. (C) Dot blot of ristocetin (0.75–2 mg/mL)-induced platelet agglutination in the subpopulation of severe COVID-19 patients between those whose survival was greater than 20 days (>20 days; $N=14$) and those who died within 20 days from enrollment (<20 days; $N=21$). The results are reported as the ristocetin concentrations that exceed 20 and 40% of agglutination. Statistical data were evaluated by the Wilcoxon–Mann–Whitney rank sum test for unpaired data.

correlation was found between ristocetin sensitivity and VWF concentration (data not shown). Interestingly, the minimal ristocetin concentration capable of inducing 20 or 40% platelet agglutination for patients with survival less than 20 days was significantly reduced compared with patients with survival greater than 20 days ($p=0.0268$ and 0.0005 , respectively), mild COVID-19 patients ($p=0.0117$ and $p=0.0010$, respectively), and HVs ($p=0.0004$ and $p<0.0001$, respectively) (→Fig. 1C). No differences were found between patients with survival greater 20 days compared with both mild patients and HVs. Thus, platelets from patients experiencing short-term mortality appear to

bind more readily to VWF compared with platelets from patients with survival greater 20 days who have comparable amounts of plasmatic VWF.

VWF binds to platelets via the glycoprotein (GP)Ib–IX receptor. Spontaneous or aberrant adhesion to VWF has been associated with thromboinflammation.¹¹ Thus, we speculate that platelet agglutination is facilitated by differences in VWF–GPIb interactions. However, we detect no differences in the levels of VWF:Ag and VWF:RCo among the two subpopulations studied (data not shown). Moreover, previous studies show that, even though ADAMTS13 level and activity are reduced in COVID-19, circulating levels of

ultra-large VWF multimers are not increased in severe patients.¹²

A potential explanation could be that the increased platelet agglutination is due to other components present in the blood of severe patients. A recent study demonstrates that COVID-19 severity correlates with the presence of high plasmatic concentrations of anti-SARS-CoV-2 spike immunoglobulin G (IgG) with aberrant glycosylation (hypofucosylated).^{13,14} Moreover, a new study by Bye and colleagues¹¹ went on to show that immune complexes formed of hypofucosylated anti-SARS-CoV-2 IgG and the spike protein increase thrombus formation on VWF in vitro by triggering FcγRIIA signaling. There are indeed reports suggesting that inside-out signaling can upregulate ristocetin-dependent platelet binding to VWF.¹⁵

Based on this evidence, we hypothesize that the increased ability of platelets to agglutinate that we observe in severe patients with short-term mortality is due to the presence of antibodies with aberrant glycosylation in the plasma of these patients. In our cohort we did not detect any difference in the levels of anti-spike IgG based on the short-term mortality (data not shown). Future studies in vivo are needed to confirm this hypothesis.

In other virus infections, IgG antibodies contribute to the worsening of the disease¹⁶ and greater adhesion of platelets with VWF has been reported.¹⁷ Collectively, these data suggest that COVID-19 thrombotic complications are not solely dependent on SARS-CoV-2 but are the result of the aberrant antibody response that amplifies the virus-induced endothelial injury and the VWF-induced platelet adhesion. As other viruses such as adenovirus, dengue, and Epstein-Barr virus can induce platelet activation and thrombosis,¹⁸ we could envisage that pathogens other than SARS-CoV-2 could impair the homeostatic balance with a similar mechanism.

A limitation of our work is that we did not measure collagen binding or ADAMTS13 activity as COVID-19 patients had a reduced activity of ADAMTS13.⁸

In conclusion, we report that platelet adhesion to VWF, observed using RIPA, correlates with the mortality of patients and can be a useful tool to identify patients with high risk of clinical deterioration. We also provide indirect supporting evidence that a plasmatic component, possibly immune complexes containing hypofucosylated IgG, could synergize with VWF to recruit platelets into microthrombi that precipitate the condition of severe COVID-19 patients. The growing understanding of SARS-CoV-2 infection pathogenesis and how it contributes to critical illness and its complications may help to improve risk stratification and develop targeted therapies to reduce the acute and long-term consequences of the disease.

Author Contributions

L.S. designed research, analyzed data, and wrote the article. F.R. designed research, analyzed data, selected patients, and collected clinical and epidemiological data. M.C. performed research and analyzed data. E.S. performed research and analyzed data. A.C. performed research and analyzed data. R.M. performed research and

analyzed data. M.V. performed research and analyzed data. G.C. analyzed data, selected patients, and collected clinical and epidemiological data. G.D. analyzed data, selected patients, and collected clinical and epidemiological data. C.S. performed research and analyzed data. O.G. performed research and analyzed data. E.F.M. selected patients and collected clinical and epidemiological data. V.Z. selected patients and collected clinical and epidemiological data. F.P. designed research, analyzed data, selected patients, and collected clinical and epidemiological data. F.M.P. designed research, performed research, analyzed data, and wrote the article.

Funding

This study was funded by Sapienza Università di Roma, University Funds to F.M.P.

Conflict of Interest

None declared.

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