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Maria Orietta Borghi, Asmaa Beltagy, Emirena Garrafa, Daniele Curreli ...+23 more authors

**Institutions:** University of Milan, Alexandria University, University of Brescia, Saint Louis University

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# Anti-Phospholipid Antibodies in COVID-19 Are Different From Those Detectable in the Anti-Phospholipid Syndrome

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### \*Correspondence:

Nicola Pozzi  
nicola.pozzi@health.slu.edu  
Pier Luigi Meroni  
pierluigi.meroni@unimi.it

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Maria Orietta Borghi<sup>1,2</sup>, Asmaa Beltagy<sup>1,3</sup>, Emirena Garrafa<sup>4,5</sup>, Daniele Curreli<sup>1</sup>, Germana Cecchini<sup>6</sup>, Caterina Bodio<sup>1</sup>, Claudia Grossi<sup>1</sup>, Simonetta Blengino<sup>7</sup>, Angela Tincani<sup>8</sup>, Franco Franceschini<sup>8</sup>, Laura Andreoli<sup>8</sup>, Maria Grazia Lazzaroni<sup>8</sup>, Silvia Piantoni<sup>8</sup>, Stefania Masneri<sup>8</sup>, Francesca Crisafulli<sup>8</sup>, Duilio Brugnoli<sup>8</sup>, Maria Lorenza Muiesan<sup>9</sup>, Massimo Salvetti<sup>9</sup>, Gianfranco Parati<sup>7</sup>, Erminio Torresani<sup>6</sup>, Michael Mahler<sup>10</sup>, Francesca Heilbron<sup>7</sup>, Francesca Pregnotato<sup>1</sup>, Martino Pengo<sup>7</sup>, Francesco Tedesco<sup>1</sup>, Nicola Pozzi<sup>11\*</sup> and Pier Luigi Meroni<sup>1\*</sup>

<sup>1</sup> Immunorheumatology Research Laboratory, Istituto Auxologico Italiano, Istituto di Ricovero Cura a Carattere Scientifico (IRCCS), Milan, Italy, <sup>2</sup> Department of Clinical Sciences and Community Health, University of Milan, Milan, Italy, <sup>3</sup> Rheumatology and Clinical Immunology Department, Faculty of Medicine, Alexandria University, Alexandria, Egypt, <sup>4</sup> Department of Molecular and Translational Medicine, University of Brescia, Brescia, Italy, <sup>5</sup> Department of Laboratory Diagnostics, Azienda Socio-Sanitaria Territoriale (ASST) Spedali Civili, Brescia, Italy, <sup>6</sup> Department of Chemical Chemistry, Istituto Auxologico Italiano, Istituto di Ricovero Cura a Carattere Scientifico (IRCCS), Milan, Italy, <sup>7</sup> Department of Cardiovascular, Neural and Metabolic Sciences, Istituto Auxologico Italiano, Istituto di Ricovero Cura a Carattere Scientifico (IRCCS), San Luca Hospital, Milan, Italy, <sup>8</sup> Rheumatology and Clinical Immunology Unit, Department of Clinical and Experimental Sciences, Azienda Socio-Sanitaria Territoriale (ASST) Spedali Civili and University of Brescia, Brescia, Italy, <sup>9</sup> Unità Operativa Complessa (UOC) 2° Medicina, Department of Clinical and Experimental Sciences, Azienda Socio-Sanitaria Territoriale (ASST) Spedali Civili and University of Brescia, Brescia, Italy, <sup>10</sup> Research and Development, Inova Diagnostics, Inc., San Diego, CA, United States, <sup>11</sup> Department of Biochemistry and Molecular Biology, Saint Louis University School of Medicine, St. Louis, MO, United States

**Background:** Critically ill patients with coronavirus disease 2019 (COVID-19) have a profound hypercoagulable state and often develop coagulopathy which leads to organ failure and death. Because of a prolonged activated partial-thromboplastin time (aPTT), a relationship with anti-phospholipid antibodies (aPLs) has been proposed, but results are controversial. Functional assays for aPL (i.e., lupus anticoagulant) can be influenced by concomitant anticoagulation and/or high levels of C reactive protein. The presence of anti-cardiolipin (aCL), anti-beta2-glycoprotein I (anti- $\beta_2$ GPI), and anti-phosphatidylserine/prothrombin (aPS/PT) antibodies was not investigated systematically. Epitope specificity of anti- $\beta_2$ GPI antibodies was not reported.

**Objective:** To evaluate the prevalence and the clinical association of aPL in a large cohort of COVID-19 patients, and to characterize the epitope specificity of anti- $\beta_2$ GPI antibodies.

**Methods:** ELISA and chemiluminescence assays were used to test 122 sera of patients suffering from severe COVID-19. Of them, 16 displayed major thrombotic events.

**Results:** Anti- $\beta_2$ GPI IgG/IgA/IgM was the most frequent in 15.6/6.6/9.0% of patients, while aCL IgG/IgM was detected in 5.7/6.6% by ELISA. Comparable values were found by

chemiluminescence. aPS/PT IgG/IgM were detectable in 2.5 and 9.8% by ELISA. No association between thrombosis and aPL was found. Reactivity against domain 1 and 4-5 of  $\beta_2$ GPI was limited to 3/58 (5.2%) tested sera for each domain and did not correlate with aCL/anti- $\beta_2$ GPI nor with thrombosis.

**Conclusions:** aPL show a low prevalence in COVID-19 patients and are not associated with major thrombotic events. aPL in COVID-19 patients are mainly directed against  $\beta_2$ GPI but display an epitope specificity different from antibodies in antiphospholipid syndrome.

**Keywords:** anti-phospholipid antibodies,  $\beta_2$ -glycoprotein I, prothrombin, autoimmunity, COVID-19, thrombosis

## INTRODUCTION

Critically ill patients with coronavirus disease 2019 (COVID-19) have a profound hypercoagulable state and often develop thrombosis in veins, arteries and in the microcirculation (1, 2). A recent analysis showed several coagulation abnormalities in these patients, including prominent elevation of fibrin/fibrinogen degradation products (i.e., D-dimer) and a prolonged activated partial-thromboplastin time (aPTT). While high levels of D-dimer are consistent with sustained activation of the clotting and fibrinolytic cascades, the combination of prolonged aPTT and both arterial and venous thrombosis was, however, surprising, and it is reminiscent of a clinical scenario known as antiphospholipid syndrome (APS) (3).

Looking at the causes of aPTT prolongation, recent studies have shown that lupus anticoagulant (LA) can be detected in a significant percentage of COVID-19 samples (4–6). Since LA is often caused by anti-phospholipid antibody (aPL), these findings support the idea that aPL may play a role in COVID-19 (7). However, it is important to point out that LA is a very sensitive assay and its outcome can be influenced by several factors, most notably heparin administration (8) and a profound inflammatory state characterized by high levels of C reactive protein (CRP) (9, 10). Both of them are present in COVID-19 patients (11).

Another method to detect aPL that is in principle insensitive to anticoagulation and other confounding agents relies on the detection and quantification of autoantibodies using solid-phase assays (3). Using this method, the presence of aPL was recently reported in a handful of case reports and small cohorts of patients (4, 6, 7, 12, 13). While encouraging, this data is limited and its interpretation remains controversial, with some investigators proposing an important role of aPL in COVID-19 patients (7) while others suggesting a very poor correlation between aPL and thrombotic events (14). There is no information on the antigen specificity of COVID-19 aPL in comparison with APS antibodies. Such information and a larger study, possibly multicenter, may be instrumental to clarify the real clinical value of these autoantibodies.

## MATERIALS AND METHODS

### Patients

A total of 122 patients were enrolled from two COVID-19 referral centers in Lombardia. All patients tested positive to

SARS-CoV-2, and classified as severe or critical COVID-19 (11). The mean age was 68.5 ( $\pm$  SD 16.4) years; 77 were men and 45 women. No diagnosis of previous autoimmune diseases was made; six patients had a thrombotic event (three arterial and three venous) in the past clinical history. The presence of antinuclear antibodies (ANAs) was investigated in 58 patients at Istituto Auxologico Italiano by HEp2-IIF and solid phase CTD screening following the guidelines described in Agmon-Levin et al. (15). Of the 58 samples, none was positive for ANA.

Eighty-seven patients suffering from APS were also tested for anti-cardiolipin (aCL) and anti- $\beta_2$ GPI IgG/IgM (16). The study was approved by the Ethics Committees (Istituto Auxologico Italiano 3-04-2020 - Milan and ASST Spedali Civili NP4187 - Brescia).

### Detection of aPL

aCL and anti- $\beta_2$ GPI IgG/IgA/IgM were detected by chemiluminescence immunoassay (CIA; Quanta Flash, Inova, San Diego, CA, US) and a home-made ELISA as described (16, 17). Anti- $\beta_2$ GPI domain 1 IgG (anti-D1) were detected by CIA (16, 17), IgG anti-D4-5 by a home-made ELISA, as described (16, 17). Detailed methods are reported in the **Supplementary Material**. Anti-phosphatidylserine/prothrombin (aPS/PT) IgG/IgM were detected by a commercial ELISA as reported (18). Blood samples were collected in the first week after hospital admission.

### Statistical Analysis

Data were analyzed using R v3.4.0. Descriptive statistics was used to summarize data. Associations and differences between categorical or continuous variables were tested by Fisher's exact test and non-parametric Mann-Whitney test, respectively. A p-value < 0.05 was considered statistically significant.

## RESULTS

### Patients

**Table 1** reports the median with minimum and maximum values for different coagulation and inflammation parameters in 122 severe or critical COVID-19 patients. In particular, prolonged aPTT (>30 s) was found in 57.6% while PT INR values were above the cut-off in 24.8% of the cases. Most of the patients (120/122) were on anticoagulation with low molecular weight heparin (70% on therapeutic and the remaining on prophylactic dosage).

**TABLE 1** | Coagulation and inflammation parameters expressed as median with minimum and maximum in severe or critical COVID-19 patients.

	D-dimer μg/L	CRP mg/dl	Ferritin μg/L	IL-6 ng/L	White cells n/μl	Neutrophils n/μl	Plateletsn × 10 <sup>3</sup> /μl	PTratio	aPTTs	Fibrinogen mg/dl
COVID-19	984.47	126.99	1,024	25.1	8000	6600	350	1.196	30.13	521
	200–40,234	0.1–470.3	55–9,002	3–496	2,500–12,900	1,560–12,510	60–800	0.9–6.9	21–75.4	202–840
Normal range	<500	0.00–0.05	30–400	<10	4,300–10,500	1,800–8,100	140–450	≤1.2	<30 s	200–400

Despite anticoagulation, we observed sixteen thrombotic events (13.1%, 8 in veins and 8 in arteries). These statistics are in agreement with previous reports (2, 19–24) and document a systemic inflammation and a coagulopathy in our patients.

## Anti-Cardiolipin and Anti-β<sub>2</sub>GPI Antibody Testing

In the APS field, testing for LA is not recommended when patients are on heparin, since the presence of heparin, even if neutralized, may lead to false-positive results (8). Likewise, high levels CRP, such as those found in our cohort of patients, have

been shown to prolong aPTT independently from the presence of aPL (9, 10). On these bases, the presence of aPL was researched using solid-phase assays, and not LA. First, we investigated the presence of aCL and anti-β<sub>2</sub>GPI, two APS classification criteria (3). Testing was independently performed in Milan and Brescia, using harmonized methodologies (25). The prevalence of COVID-19 patients positive for aCL and anti-β<sub>2</sub>GPI IgG/IgA/IgM detected by ELISA and CIA is summarized in **Table 2**. The ELISA raw data are shown in **Figure 1**. We found IgG/IgM aCL in 5.7/6.6% of patients, whereas anti-β<sub>2</sub>GPI IgG/IgA/IgM were found in 15.6/6.6/9.0% of patients. Similar values were obtained for aCL antibodies using CIA (**Table 2**), whereas a slightly lower sensitivity was obtained for anti-β<sub>2</sub>GPI antibodies (26). The positivity for aCL and anti-β<sub>2</sub>GPI antibodies was at medium/low titer in contrast with the medium/high titers found in the control group of primary APS (**Figure 1**). There is no association between aPL positivity and thrombotic events.

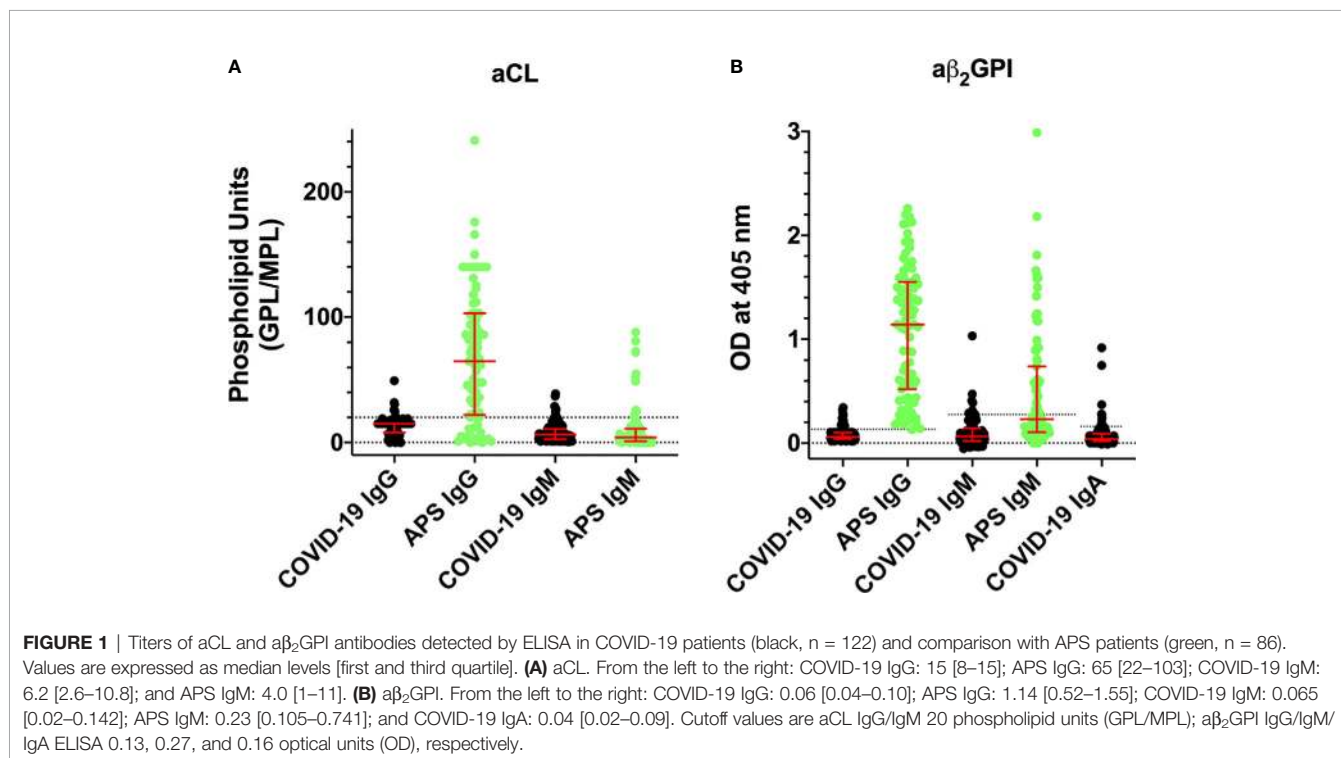
**TABLE 2** | Prevalence of COVID-19 patients positive for aPL.

	ELISA		CIA	
	aCL	aβ <sub>2</sub> GPI	aCL CIA	aβ <sub>2</sub> GPI CIA
IgG	5.7 (7)*	15.6 (19)	9.8 (12)	5.0 (6)
IgM	6.6 (8)	9.0 (11)	6.6 (8)	5.0 (6)
IgA	nd	6.6 (8)	2.5 (3)	0.8 (1)

\*Values are expressed as percentage (n) of positive patients. aCL, anti-cardiolipin antibodies; aβ<sub>2</sub>GPI, anti-β<sub>2</sub> glycoprotein I antibodies; ELISA, enzyme linked immunosorbent assay; CIA, chemiluminescence immunoassay; nd, not done.

## Epitope Characterization of Anti-β<sub>2</sub>GPI Antibodies

Fifty-eight sera were also tested with D1 and D4-5-coated plates in order to characterize their epitope specificity. **Figure 2B** shows that three out of 58 samples reacted with D1, while in **Figure 2C**,



three samples tested positive for D4-5. None of the sera was positive for both domains and all displayed a weak reactivity with no association with thrombosis.

## Anti-Phosphatidylserine/Prothrombin Antibody Testing

Prolonged aPTT (>30 s) was found in 57.6% of the patients. Although aPS/PT are not included in the APS classification laboratory tests, they can be associated with a prolonged aPTT and with the presence of LA (18). Consequently, we looked at the presence of aPS/PT antibodies in our cohort and we found fifteen out of 122 sera positive for aPS/PT (12.3%), mostly of the IgM isotype (12 out 15) and at a low titer (**Figure 3**). There was no association between prolonged aPTT and the presence of aPS/PT antibodies nor with thrombotic events in our COVID-19 cohort.

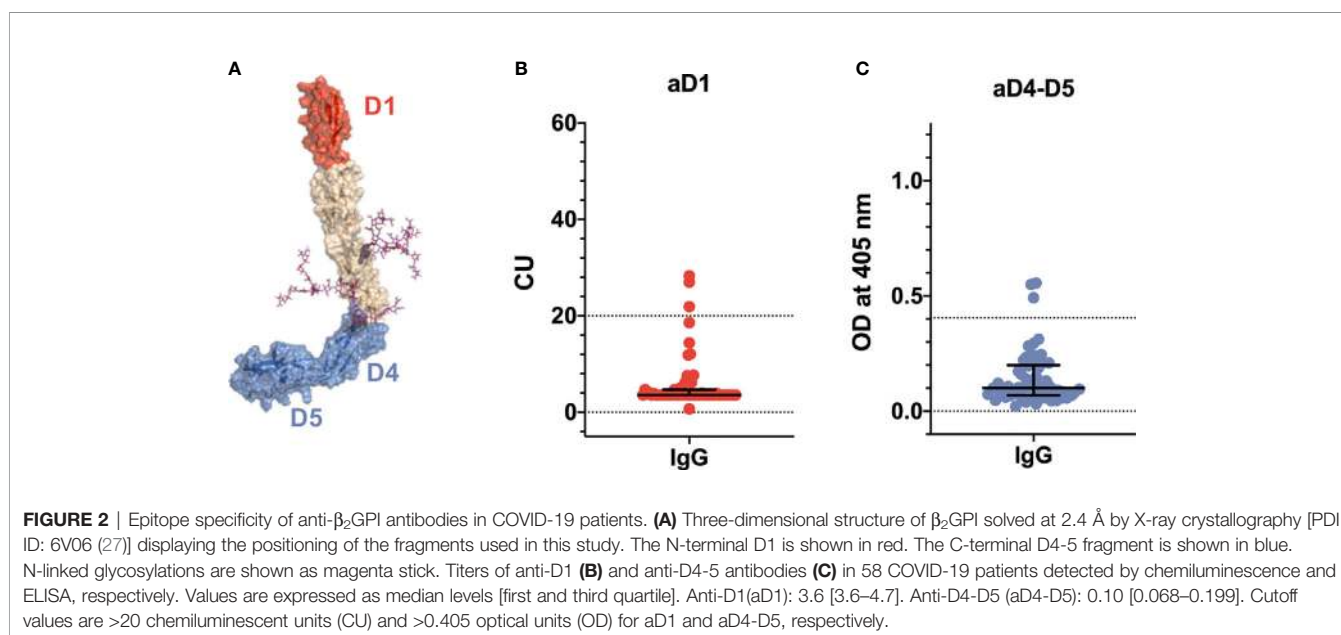
## DISCUSSION

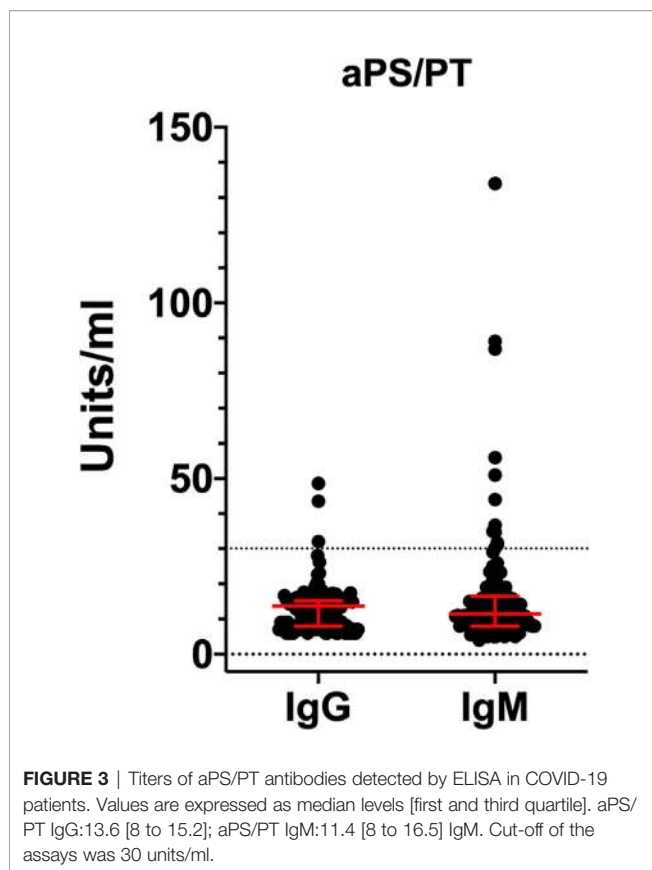
Taken together, our data show a low prevalence of classification criteria aPL in COVID-19 patients. In this regard, our study confirms recent studies obtained with smaller cohorts of patients (4, 14, 24). Importantly, our data also shows that aPL are slightly more reactive towards  $\beta_2$ GPI-coated plates as compared to CL-coated ones and that, regardless of the nature of aPL, there is no association between aPL positivity and thrombotic events ( $p = 1$ ).

A striking difference between the autoantibody profile in COVID-19 patients as compared to the one in APS concerned the titers of aPL. Medium/low aPL titers were consistently found in patients with COVID-19. By contrast, medium/high titers are usually found in APS patients (**Figure 1**). This difference suggests that aPL found in COVID-19 may be different from aPL found in APS and led us to further investigate the epitope specificity of anti- $\beta_2$ GPI antibodies. We focused on autoantibodies directed against the N-terminal domain 1 (anti-D1) or the C-terminal domains 4-5

(anti-D4-5) of the molecule (17) (**Figure 2A**). This is because anti-D1 antibodies are associated with an increased risk of thrombosis and pregnancy complications in APS (16, 17, 28). By contrast, anti D4-5 antibodies are associated neither with vascular nor obstetric APS manifestations (16, 29). Furthermore, anti D4-5 antibodies are also reported at high levels in the so called asymptomatic aPL carriers and are frequently found in non-APS (e.g., patients with leprosy, atopic dermatitis, atherosclerosis, and in children born to mothers with systemic autoimmune diseases) (29). We found that three out of 58 samples reacted with D1, and three samples tested positive for D4-5. None of the sera was positive for both domains and all displayed a weak reactivity. Although the number of the investigated sera is relatively small, this finding is quite different from the results found in APS in which almost all the sera positive for the whole  $\beta_2$ GPI molecule also reacted with domain D1 at high titer (16, 28). Furthermore, at variance with APS patients, none of the anti-D1 positive patients displayed thrombotic events (28).

Approximately, 57% of COVID-19 patients have prolonged aPTT. Yet, only a small proportion of COVID-19 patients carry aCL and anti- $\beta_2$ GPI antibodies. This suggests that other factors must be responsible for the prolonged aPTT phenomenon and likely for the LA activity. LA may be affected by the concomitant heparin treatment and the high CRP levels. Although more sensitive and specific diagnostic algorithms have been suggested (30), we followed the ISTH guidelines available at the beginning of the study (31). Since aPS/PT can be associated with a prolonged aPTT and with the presence of LA (18), we tested our cohort for aPS/PT antibodies. We found a small percentage (12.3%) of positive sera, mostly of the IgM isotype (12 out 15) and at a low titer. Again, there was no association between prolonged aPTT and the presence of aPS/PT antibodies nor with thrombotic events in our COVID-19 cohort. This indicates that aPS/PT are not responsible for the prolongation of aPTT nor are predictors of adverse clinical outcomes. Furthermore, in contrast to what we would have expected in





APS (32), we found no associations between the presence of aPS/PT, aCL, and anti- $\beta_2$ GPI antibodies. This data is in line with the unusual epitope specificity of anti- $\beta_2$ GPI antibodies documented in **Figure 2**, supporting the hypothesis that aPL found in COVID-19 patients are different from aPL found in APS patients. Whether COVID-19 aPL are similar to the ones found in other infectious diseases such as HCV, HBV and HIV (33) remains to be determined.

Despite heparin treatment, 13.1% of our patients displayed thrombotic events. Although we cannot exclude that treatment could be protective, the prevalence of vascular events was in line with that reported by other studies as recently reviewed (34).

In conclusion, while the medium/high aPL titers with D1 specificity are associated with vascular events in APS, low antibody titers with reactivity against  $\beta_2$ GPI epitope(s) different from D1 or D4,5 can be found in COVID-19. This may explain the lack of association with thrombotic events in COVID-19. In addition, our data do not support the hypothesis that aPL can be the main cause of prolonged aPTT in these patients. Although low titer aPL are not predictive of vascular events in the APS, it is important to keep in mind that COVID-19 patients suffer from an acute form of systemic inflammation with complement activation (35), which may be responsible for endothelial perturbation. In this context, since  $\beta_2$ GPI can accumulate on the activated endothelium at high density, even low titers of aPL may become pathogenic thus potentiating or

even triggering thrombus formation, especially when anticoagulation is suspended. A comparable condition in which low titers of aPL can cause substantial damage is seen in obstetric APS, where high levels of  $\beta_2$ GPI can be found in the placenta (36). Hence, while transitory aPL are likely to be clinically irrelevant in COVID-19 patients as in other infections (33), detection of aPL may be useful for identifying patients potentially at risk of thrombosis after the hospital discharge. Accordingly, anticoagulant prophylaxis or therapies affecting cell signaling involved in inflammatory and coagulation responses could be justified before a confirmatory assay (3, 37).

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Istituto Auxologico Italiano 3-04-2020 - Milan and ASST Spedali Civili NP4187 - Brescia. The ethics committee waived the requirement of written informed consent for participation.

## AUTHOR CONTRIBUTIONS

MB, MP, AT, FF, FT, NP, and PM designed the study. SB, GP, FH, MP, ML, MLM, and MS collected clinical samples. EG, DC, GC, CB, CG, SP, SM, FC, DB, ET, MM, and LA performed research. MB, AB, FP, FT, NP, and PM analyzed data. MB, FT, NP, and PM wrote the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.584241/full#supplementary-material>

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**Conflict of Interest:** MM was employed by Inova Diagnostics, Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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