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CLINICAL SCIENCE

Anticardiolipin and other antiphospholipid antibodies in critically ill COVID-19 positive and negative patients

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Handling editor Josef S Smolen

► Additional supplemental material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/annrheumdis-2021-220206).

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Received 20 February 2021
Revised 16 March 2021
Accepted 7 April 2021

To cite: Trahtemberg U, Rottapel R, Dos Santos CC, *et al*. *Ann Rheum Dis* Epub ahead of print: [*please include* Day Month Year]. doi:10.1136/annrheumdis-2021-220206

ABSTRACT

Background Reports of severe COVID-19 being associated with thrombosis, antiphospholipid antibodies (APLA), and antiphospholipid syndrome have yielded disparate conclusions. Studies comparing patients with COVID-19 with contemporaneous controls of similar severity are lacking.

Methods 22 COVID-19$^+$ and 20 COVID-19$^-$ patients with respiratory failure admitted to intensive care were studied longitudinally. Demographic and clinical data were obtained from the day of admission. APLA testing included anticardiolipin (aCL), anti-β2glycoprotien 1 (β2GP1), antidomain 1 β2GP1 and antiphosphatidyl serine/prothrombin complex. Antinuclear antibodies (ANAs) were detected by immunofluorescence and antibodies to cytokines by a commercially available multiplexed array. Analysis of variance was used for continuous variables and Fisher's exact test was used for categorical variables with α=0.05 and the false discovery rate at q=0.05.

Results APLAs were predominantly IgG aCL (48%), followed by IgM (21%) in all patients, with a tendency towards higher frequency among the COVID-19$^+$. aCL was not associated with surrogate markers of thrombosis but IgG aCL was strongly associated with worse disease severity and higher ANA titres regardless of COVID-19 status. An association between aCL and anticytokine autoantibodies tended to be higher among the COVID-19$^+$.

Conclusions Positive APLA serology was associated with more severe disease regardless of COVID-19 status.

Trial registration number NCT04747782

Key messages

What is already known about this subject?

- COVID-19 is associated with coagulopathy and high morbidity and mortality.
- COVID-19 shares some of these clinical features with antiphospholipid syndrome.
- Reports of an association of antiphospholipid antibodies with high-risk COVID-19 have yielded disparate conclusions, but they lacked longitudinal follow-up and control groups of similar severity.

What does this study add?

- Antiphospholipid syndrome serology assessed longitudinally was predominantly anticardiolipin IgG autoantibodies, in 48% of patients.
- Anticardiolipin serology was associated with worse disease severity in both COVID-19 positive and negative patients.

How might this impact on clinical practice or future developments?

- The use of antiphospholipid antibodies tests in the COVID-19 clinical setting needs to be taken in context; whereas they are associated with more serve disease, they do not discriminate between COVID-19 positive and negative patients.

INTRODUCTION

Antiphospholipid antibodies (APLAs) are biomarkers of a spectrum of clinical features observed in antiphospholipid syndrome (APS).[1] Features of APS include venous and arterial thrombosis involving multiple organs and having various presentations.[1] APLAs that are components of APS criteria include IgG and/or IgM anticardiolipin (aCL), anti-β2-glycoprotein1 (anti-β2GP1) and the 'lupus anticoagulant' (LAC).[2] Other non-criteria APLA such as antiphosphatidylserine/prothrombin (PS/PT) complex, anti-PT and antidomain 1 of β2-GP1 have also found a diagnostic niche in APS.[3 4]

One of the salient features of COVID-19 is the development of thrombotic events associated with severe morbidity and mortality.[5–8] In the context of systemic inflammation and dysregulated immunity,[9] some reports have linked APLA to these thromboses,[10 11] severe COVID-19[6 12] and release of neutrophil extracellular traps.[6] However, APLAs are also described in a variety of other infectious diseases[13] and critically ill patients have high rates of thromboembolism that were not linked to APS or APLA[14] (critically reviewed in ref. 15). Therefore, the association of COVID-19 with APLA and their potential pathogenic role[16] has not been clearly demonstrated due to the lack of contemporaneous, COVID-19 negative controls. Here, we compare the prevalence and clinical correlations of APLA in patients with severe COVID-19 as compared with contemporaneous non-COVID19 patients with similar clinical characteristics.

METHODS

Informed consent was obtained from all patients or their legal surrogates. Inclusion criteria were age ≥18 years, admission to intensive care unit (ICU)

Table 1 Patient demographics, clinical and autoantibody status

	Cohort	All	COVID$^+$	COVID$^-$
	N	42	22	20
Age	Mean (CI)	58.2 (62.7 to 54.1)	60.9 (66.6 to 55.3)	55.7 (62 to 48.7)
Sex	N male (%)	29/42 (69)	17/22 (77)	12/20 (60)
Censored?	N (%)	5/42 (12)	4/22 (18)	1/20 (5)
No of days before censoring	Mean (CI)	39.4 (59.4 to 19.4)	44.3 (66.2 to 22.3)	20 (NA)
Days from symptom onset to ICU	Mean (CI)	6 (8.3 to 3.7)	7.5 (9.9 to 5.2)	4.2 (8.5 to 0)
APACHE II on ICU admission	Mean (CI)	25.3 (27.6 to 22.9)	23.7 (27 to 20.4)	27 (30.5 to 23.5)
Mean of SOFA score for first 3 days	Mean (CI)	9.6 (10.7 to 8.5)	9.3 (11 to 7.7)	9.9 (11.6 to 8.3)
Mean of SOFA score for first 7 days	Mean (CI)	8.9 (10.1 to 7.8)	9.1 (11 to 7.3)	8.7 (10.3 to 7.2)
ICU days (censored)	Mean (CI)	14.1 (17.3 to 10.8)	14.2 (20.5 to 7.8)	14 (16.9 to 11.1)
Death in ICU	N (%)	13/42 (31)	7/22 (32)	6/20 (30)
Mechanical ventilation days (censored)	Mean (CI)	14.4 (18.9 to 10)	16.8 (25.1 to 8.6)	11.8 (14.9 to 8.7)
Total days of ventilation rescue measures	Mean (CI)	2.9 (4.3 to 1.4)	4.4 (7 to 1.8)	1.2 (2 to 0.4)
Therapeutic anticoagulation used	N (%)	8/42 (19)	3/22 (14)	5/20 (25)
Mean platelet count	Mean (CI)	239 (269 to 209)	264 (313 to 214)	212 (245 to 179)
Mean platelet to neutrophil ratio	Mean (CI)	35.2 (42 to 28.4)	38.7 (48.4 to 29)	31.4 (41.6 to 21.2)
aCL IgG	N (%)	20/42 (48)	13/22 (59)	7/20 (35)
aCL IgM	N (%)	9/42 (21)	7/22 (32)	2/20 (10)
Anti-β2GPI IgG	N (%)	0	0	0
Anti-β2GPI IgM	N (%)	0	0	0
Anti-domain 1 β2GP1 IgG	N (%)	0	0	0
Anti-PS/PT IgG	N (%)	0	0	0
Anti-PS/PT IgM	N (%)	1/42 (2)	1/22 (5)	0

The data were censored on 31 May 2020. Days from symptom onset were self-reported by the patients or their representatives. The SOFA score was performed daily for all patients; the average was calculated for the first 3 and 7 days in the ICU for each patient, and the mean of those averages are reported. For patients who underwent tracheostomy, mechanical ventilation days are counted until successfully weaned from ventilatory support for 24 hours. Rescue measures included use of paralytics, proning and inhaled NO (counted additively if more than one intervention used in the same day). The clinical outcomes were measured for up to 3 months. All the serologies were tested longitudinally and are reported for the first 10 days from admission to the ICU (for standardisation among patients). There was no statistically significant difference between COVID$^+$ and COVID$^-$ patients for all variables, using ANOVA for continuous variables and Fisher's exact test for categorical variables at α=0.05, followed by the false discovery rate at q=0.05.

aCL, anticardiolipin; ANOVA, analysis of variance; APACHE, Acute Physiology and Chronic Health Evaluation (score); β2GP1, beta two glycoprotein I; ICU, intensive care unit; PS/PT, phosphatidyl serine/prothrombin complex; SOFA, sequential organ failure assessment (score).

with acute respiratory failure. Exclusion criteria were inability to ascertain the primary outcome or obtain a baseline blood sample, and SARS-CoV2 infection in the 4 weeks prior to admission. COVID-19 status was determined with PCR of nasopharyngeal swabs and/or endotracheal aspirates. Follow-up was 3 months post-ICU admission or hospital discharge. Primary outcome was death in the ICU. Secondary outcomes were in hospital-death, ICU utilisation metrics, organ dysfunction measures and severity scores. Clinical data and serum samples were collected longitudinally at days 0, 1, 3, 5, 7 and 10; after day 10 or ICU discharge. aCL, anti-β2GP1 and anti-PS/PT were tested for IgG and IgM, as well as IgG anti-domain 1 β2-GP1; all by ELISA or chemiluminescence (Inova Diagnostics, San Diego, California, USA). Analysis of variance was used for continuous variables and Fisher's exact test was used for categorical variables at α=0.05, followed by a false discovery rate adjustment at q=0.05. Detailed methods are available (online supplemental file), including methods for detection of anti-nuclear autoantibodies (ANA) by HEp-2 immunofluorescence assay (IFA) (Inova Diagnostics) and antigen-specific autoantibodies (TheraDiag, Paris, France) and anticytokine autoantibodies (Millipore, Oakville, Ontario, Canada) using addressable laser bead immunoassays.

RESULTS

The demographic and clinical parameters of 22 COVID-19 positive (COVID$^+$) and 20 COVID-19 negative (COVID$^-$) patients (table 1) included an average of 14.1-day stays in ICU and 31% mortality, but no statistically significant differences between the two cohorts, including the lack of significant differences in the number of thrombotic events requiring therapeutic anticoagulation, platelet counts or platelet counts normalised to the neutrophil counts (to index for severity) (table 1). None of the patients had a history of antecedent APS, systemic lupus erythematosus (SLE) or other conditions associated with APS, nor were there significant differences in other past medical history between COVID$^+$ and COVID$^-$ patients (online supplemental table 1).

Frequency, development and distribution of aCL

Forty-eight per cent of all the ICU cohort had a positive IgG aCL test (table 1); interestingly, fewer patients had elevated titres of IgM aCL (n=9, 21%), with only two patients having IgM without IgG. Although more COVID-19$^+$ had aCL antibodies, the difference was not statistically significant (table 1); aCL titres were slightly higher among the COVID-19$^+$ (not statistically significant, (online supplemental table 2) and online supplemental figure 1). Longitudinally testing for anti-β2-GP1 and anti-PS/PT for IgG and IgM, as well as domain 1 anti-β2-GP1 IgG revealed only one patient (COVID-19$^+$) with positive serology for any of these autoantibodies. This patient seroconverted to IgM anti-PS/PT at days 5–7 of ICU hospitalisation. Table 2 shows the temporal development of the aCL IgG and IgM antibodies stratified by COVID-19 status. Late appearing (beyond 10 days after admission) aCL antibodies were not included in the statistical

Table 2 Development of ACL IgG and IgM over time

Cohort		aCL detected on admission	aCL developed within 10 days	Late appearing aCL (after 10 days)
aCL IgG positive	COVID$^+$	4	9	2
	COVID$^-$	3	4	0
aCL IgM positive	COVID$^+$	1	6	2
	COVID$^-$	1	1	1

Late aCL was not included in the statistical analyses to avoid survival and availability bias, and is shown here for qualitative assessment.
aCL, anticardiolipin antibodies.;

analyses to avoid survival and availability bias. Anti-CL were not associated with age or sex (not shown).

aCL versus disease severity, platelet counts and need for anticoagulation

Patients positive for aCL IgG demonstrated a consistent trend for worse outcomes in all the measures tested but this did not reach statistical significance after adjusting for multiple comparisons (table 3). These trends remained when analysed separately for COVID$^+$ and COVID$^-$ (not shown). aaCL IgG positive patients showed no significant differences in platelet counts, platelet to neutrophil ratio or the need for therapeutic anticoagulation (table 3).

aCL association with ANA, antigen-specific autoantibodies and anti-cytokine autoantibodies

We tested a broad range of non-APS autoantibodies to understand the autoimmune context of these patients and their potential relationship to APS autoantibodies. Although aCL IgG positivity was not associated with the presence of HEp-2 IFA ANA at a dilution of 1:160, it was significantly associated with higher ANA titres (online supplemental figure 2), p=0.03). This trend remained when analysing the COVID$^+$ and COVID$^-$ patients separately (data not shown). IgG aCL positivity was also significantly associated with anticytokine autoantibodies, both when analysed for positive or high-positive anticytokine titres (p=0.003 for both, adjusted for multiple comparisons); this was not related to any particular anticytokine autoantibody, although anti-interferon-γ, anti-IL10 and anti-IL-17f were the most prevalent (online supplemental table 3). When analysing the aCL IgG positive according to their COVID-19 status, the COVID$^+$ had significantly higher levels of anticytokine autoantibodies than the COVID$^-$ (online supplemental table 4). aCL IgG was not associated with antigen-specific autoantibodies, including SLE and myositis-related autoantibodies (not shown).

DISCUSSION

In the year since the onset of the SARS-CoV2 pandemic, there has been a remarkable surge in publications about one disease, COVID-19, chronicling the clinical onset and outcomes, and a host of biomarkers purported to have related pathophysiological significance (reviewed in references 17 18). The key observation of this study is that patients with positive IgG aCL showed a trend towards more severe disease regardless of whether they were COVID$^+$ and COVID$^-$. That is, while COVID$^+$ patients showed non-significant trends towards worse respiratory outcomes when compared with COVID$^-$, aCL status had an independent association with disease severity, and did not modulate the outcomes differentially based on COVID status. The pathological significance of aCL seropositivity is unclear since there were no major differences in platelet counts or thrombotic events in the two cohorts. Others have reported a high prevalence of aCL autoantibodies among COVID$^+$ patients, but these studies lacked contemporaneous COVID$^-$ control groups of similar disease severity.[6 15 19 20]

Although aCL tended to associate with COVID-19$^+$, they did not associate with the presence of other antigen-specific autoantibodies, although they had a strong association with certain anticytokine autoantibodies, which are reported to neutralise corresponding type I IFNs ability to block SARS-CoV-2 infection in vitro.[21] Interestingly, some patients had positive IgG aCL serology on ICU admission (table 2) in the absence of another relevant comorbidity such as APS or SLE (online supplemental table 1). These observations suggest that aCL positivity in the setting of acute severe respiratory illness may be a marker of a unique phenotype with variable temporal expression of aCL and anticytokine antibodies. The temporal dynamic is evidenced by the relatively long time frame from symptom onset to ICU

Table 3 Association between ACL IgG and disease severity, platelet counts and need for anticoagulation

	Cohort	All	aCL IgG positive	aCL IgG negative
	N	42	20	22
Age	Mean (CI)	58.2 (62.7 to 54.1)	55.9 (62.9 to 49)	60.7 (66.4 to 55)
Sex	N male (%)	29/42 (69)	13/20 (65)	16/22 (73)
Days from symptom onset to ICU	Mean (CI)	6 (8.3 to 3.7)	8.7 (12.8 to 4.6)	3.4 (5.4 to 1.5)
APACHE II on ICU admission	Mean (CI)	25.3 (27.6 to 22.9)	25.7 (28.5 to 22.9)	24.9 (28.8 to 20.9)
Mean of SOFA score for first 3 days	Mean (CI)	9.6 (10.7 to 8.5)	10.6 (12.2 to 9.1)	8.7 (10.3 to 7)
Mean of SOFA score for first 7 days	Mean (CI)	8.9 (10.1 to 7.8)	10 (11.7 to 8.4)	8 (9.5 to 6.4)
ICU days (censored)	Mean (CI)	14.1 (17.3 to 10.8)	16.6 (21.9 to 11.3)	12.1 (16.5 to 7.6)
Death in ICU	N (%)	13/42 (31)	8/20 (40)	5/22 (23)
Mechanical ventilation days (censored)	Mean (CI)	14.4 (18.9 to 10)	18.2 (25.5 to 10.8)	11.1 (16.4 to 5.7)
Total days of ventilation rescue measures	Mean (CI)	2.9 (4.3 to 1.4)	3.6 (5.6 to 1.5)	2.3 (4.4 to 0.1)
Therapeutic anticoagulation used	N (%)	8	4/20 (20)	4/22 (18)
Mean platelet count	Mean (CI)	239 (269 to 209)	268 (321 to 216)	212 (246 to 179)
Mean platelet to neutrophil ratio	Mean (CI)	35.2 (42 to 28.4)	34.8 (45.2 to 24.3)	35.6 (45.4 to 28.9)

See table 1 for details on the variables shown. There were no statistically significant differences between aCL IgG positive and aCL IgG negative patients for all variables, using ANOVA for continuous variables and Fisher's exact test for categorical variables at α=0.05, followed by the false discovery rate at q=0.05.
aCL, anticardiolipin; ANOVA, analysis of variance; APACHE, Acute Physiology and Chronic Health Evaluation (score); ICU, intensive care unit; SOFA, sequential organ failure assessment (score).

admission to the development of IgG aCL (table 3). Our findings highlight the importance of longitudinal monitoring of acutely ill patients. It seems plausible that disparate conclusions in the literature with respect to the significance of APLAs in COVID-19 may relate to arbitrary sampling times and lack of longitudinal follow-up in the setting of dynamic inflammatory diseases.

While some reports have included LAC in their analyses, we did not because LAC is known to be an unreliable biomarker in severe illnesses where C reactive protein, anticoagulant use and other factors confound its detection.[22 23] In this study, we used the anti-PS/PT test regarded by some as a surrogate for LAC (reviewed in reference 3). However, only one patient developed anti-PS/PT 5–7 days after admission. Further, our observation that no patient had antibodies to β2-GP1 (an APS criteria antibody) or to domain 1 β2-GPI (reportedly higher specificity for APS) argues against the presence of APS in our cohort. In addition, aCL in isolation and/or the depletion of β2-GPI reactivity has been associated with the loss of pathogenic thrombosis formation (reviewed in reference 3). In a study of 37 COVID+ acute respiratory disease vs 31 prepandemic (not contemporaneous) acute respiratory disease controls using a sample collected within 48 hours of admission, Frapard *et al* reported that 37 patients with COVID-19 exhibited more thrombotic events as compared with 31 prepandemic controls but the occurrence of APLA in the two groups was similar.[24] Using APLA assays similar to ours, Borghi *et al* reported a low prevalence of APLA in COVID$^+$ sera, where the most common target was IgG β2-GP1 (15.6%).[20] In addition, the primary β2GP1 antibody targets were in domains 2–4 which are less specific for APS.[20] In agreement with our study, Bertin *et al*[12] and Borghi *et al*[20] concluded that APLA were not associated with major thrombotic events.

The main limitation of our study is the small sample size, although studies using somewhat larger COVID-19 cohorts have reached similar conclusions.[12 20] The strengths of our study include its prospective, contemporaneous COVID$^-$ cohort with similar severity of disease. Importantly, we tested a broad APLA serological panel longitudinally, providing a more robust assessment of its true prevalence and incidence than in other reported studies; this is particularly relevant for such acutely ill patients with dynamic clinical courses. Finally, our use of an extensive serological panel allowed us to better characterise the broad phenotype associated with aCL.

Acknowledgements The authors acknowledge the technical assistance of Haiyan Hou, Meifeng Zhang and Emily Walker in the MitogenDx Laboratory at the University of Calgary. We thank Marlene Santos, Gyan Sadhu, Imrana Khalid and Sebastian Duncan, the research coordinators at St Michael's Hospital Critical Care Research Unit. We are grateful to patients and families that have generously consented to the study.

Contributors This report is part of the COLOBILI study (Coronavirus longitudinal biomarkers in lung injury). AB and CDS are principal investigators; MJF, RR and AS are collaborators/coinvestigators and UT is the research lead. RR, MJF, UT and CDS conceived of the study; MJF, UT and RR wrote the manuscript drafts; AS, AB and CDS provided critique and technical guidance; UT performed the data analysis and creation of the figures. All authors edited the manuscript, through to the final version, read and approved the final submission.

Funding St Michael's Hospital Foundation, internal competitive grant to AB and CDS. Autoantibody testing was provided as a gift in kind by MitogenDx (Calgary, AB, Canada).

Competing interests MJF is the Director of MitogenDx. MJF is a consultant for and received speaking honoraria from Inova Diagnostics Inc (San Diego, California, USA) and Werfen International (Barcelona, Spain). All the other authors have no disclosures to declare.

Patient and public involvement statement Patients and public were not involved in the design of the study. During the initial phases of the study, we obtained feedback from the patients and their substitute decision makers. Their concerns, questions and preferences were incorporated into improved processes for consent and collection of biological samples. The consent forms have checkboxes with optional aspects of the study, to accommodate different patient preferences. The results of the study will be disseminated in lay versions by St. Michael's Hospital public relations and communications departments for the benefit of the public.

Patient consent for publication Not required.

Ethics approval This is an observational cohort study of patients admitted to St. Michael's Hospital (Toronto, ON, Canada), as approved by the Research Ethics Board (REB# 20–078).

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement All data relevant to the study are included in the article or uploaded as online supplemental information. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Online materials and methods

Study design. This report is part of the COLOBILI study – Coronavirus Longitudinal Biomarkers in Lung Injury, being conducted at St. Michael's Hospital (Toronto, ON, Canada). This is an observational cohort study that includes analysis of biological samples. The study was approved by the Research Ethics Board of St. Michael's Hospital (REB# 20-078). The inclusion criteria were all patients above age 18 years admitted to the Medical-Surgical or Trauma-Neuro intensive care units (ICU) with acute respiratory distress, suspected to have COVID-19. COVID-19 status was determined according to diagnostic PCR of nasopharyngeal swabs and/or endotracheal aspirates as described in detail below. The exclusion criteria were refusal to participate, inability to ascertain mortality status during the first 2 weeks of the study, failure to obtain a blood sample on either day 0 or 1, or individuals known to have had COVID-19 in the 4 weeks prior to admission in any setting. Patients were followed for up to 3 months in hospital or hospital discharge, whichever occurred first. The primary outcome was death in the ICU; secondary outcomes included death outside the ICU, ICU utilization metrics, and organ dysfunction measures and scores. Clinical data and blood samples were collected longitudinally immediately upon admission, as available, defined as day 0, and on the morning of days 1, 3, 5, 7 and 10; after day 10 or ICU discharge, they were sampled every 2 weeks. The study started on March 26th, 2020, and the first patient was recruited on March 29^{th,} 2020. The study is ongoing; the last patient from the cohort presented in this manuscript was recruited on May 17th, 2020, and the data was censored for analysis on May 31st, 2020. No COVID-19 treatments were given to the patients beyond the standard of care since at the time there was no evidence of efficacy for any such treatments. Informed consent was obtained from the patients or their legal representatives; in case that was not possible, the patients were enrolled using a deferred consent model and kept in the study until they regained capacity, or a surrogate decision maker was identified.

Data and sample collection. Demographics, clinical data and clinical laboratory were collected from the patients' paper and electronic medical records, with auditing performed reciprocally by research coordination team members and curated by UT. To standardize handling and processing, blood samples were collected in EDTA tubes between 8:00 and 12:00 AM and kept on ice for up to 60 minutes until their processing in a dedicated translational research station located inside the ICU. They were then immediately frozen at -20 ^{0}C on site, and transferred to -80 ^{0}C for storage within 48 hrs. All procedures were performed by dedicated research

personnel. Nasopharyngeal samples were obtained from all patients by bedside nurses and analyzed by the clinical laboratory using either the Altona RealStar SARS-CoV-2 RT-PCR Kit 1.0 or Cepheid GeneXpert Xpert Xpress SARS-CoV-2 assay. Endotracheal tube aspirates were analyzed using the Seegene Allplex 2019-CoV Assay. All patients had a nasopharyngeal PCR performed; intubated patients had an endotracheal aspirate sent as well. Further PCR tests were repeated by the clinical and infection control teams at their discretion if there was suspicion of a false negative result based on clinical observations or to confirm negativity. All patients in the PCR negative cohort had at least two negative tests performed acutely, except one patient who had only one test done acutely. To analyze longitudinal trends, only patients with 3 or more longitudinal sampling times were included in the study. To mitigate bias, five patients with shorter ICU admissions were included; 2 had early deaths and 3 had early discharges.

Experimental procedures. Plasma samples were stored and managed under a standard operating procedure which included shipping on dry ice and storage at -80C until assay performance by Mitogen Diagnostics Laboratory (MitogenDx, Calgary, AB, Canada). Anti-cardiolipin, anti-β2-GP1 and anti-PS/PT complex were tested by ELISA for IgG and IgM antibodies and for IgG anti-domain 1 β2-GP1 by chemiluminescence immunoassay (Inova Diagnostics, San Diego, CA USA). For all of the anti-phospholipid antibodies listed above, the manufacturer's cutoffs were utilized and previously validated for routine diagnostic testing (MitogenDx: https:// https://mitogendx.com/); 20 Units (U) for anti-cardiolipin, anti-β2-GP1 and anti-PS/PT complex and 20 chemiluminescence units (CU) for the anti-domain 1 β2-GP1 immunoassay. The anti-PS/PT assay is approved for use in serum and EDTA plasma, as it includes calcium protein stabilizers and calcium to overcome any chelating effect of EDTA. A HEp-2 indirect immunofluorescence assay (IFA) was used to detect anti-cellular antibodies (also referred to as anti-nuclear antibodies (ANA) – see "nomenclature" below)[1] (NOVA Lite HEp-2, Inova Diagnostics, San Diego, CA) at a serum dilution of 1:80 and read on an automated instrument (Nova View, Inova Diagnostics) which interpolates fluorescence intensity to an end point titer[2]. IFA staining patterns were classified according to the International Consensus on Autoantibody Patterns (ICAP, https://anapatterns.org/index.php)[3], and considered positive at a dilution ≥1:160. All samples were also tested for systemic autoimmune disease-related autoantibodies by a FIDIS Connective13 addressable laser bead immunoassay (ALBIA) (TheraDiag, Paris, France) detecting antibodies to Sm/U2-U6 ribonucleoprotein (RNP), U1-RNP, SSA/Ro60, SSB/La, Ro52/Tripartite Motif Protein 21 (TRIM21), histones, and ribosomal P, read on a Luminex 200

system using the MLX-Booster software. A cut-off of >40 units was considered positive. Anti-dsDNA positivity and titers were detected by a chemiluminescence test (Inova Diagnostics, San Diego, USA). A cut-off of <27 chemiluminescence units was considered within normal range, 27-35 was indeterminate, and >35 was positive. All samples were also tested for autoantibodies associated with autoimmune inflammatory myopathies using a multiplexed solid phase line immunoassay: Ro-52/TRIM21, OJ, EJ, PL-12, PL-7, SRP, Jo-1, PM-75, PM-100, Ku, SAE1, NXP2, MDA5, TIF1γ, Mi-2α, Mi-2β (Euroimmun AG, Luebeck, Germany), and anti-NT5c1A by ALBIA[4]. The following anti-cytokine antibodies were assayed using an ALBIA (Millipore, Oakville, ON, Canada; HCYTAAB-17K-15) read on a Luminex 200 system: BAFF, GMCSF, IFN-β, IFN-γ, IL-1a, IL-6, IL-8, IL-10, IL-12p40, IL-15, IL-17a, IL-17f, IL-18, IL-22 and TNF-α. The manufacturer's thresholds were 500 for positive and 1000 for high-positive (arbitrary units). All tests were performed according to the manufacturer's instructions.

Nomenclature. There is considerable heterogeneity in the nomenclature of autoimmune assays in the literature and clinical practice; therefore, we used the most contemporary nomenclature. Autoantibodies is a general term that encompasses the autoimmune humoral responses assayed. The HEp-2 IFA, although including anti-cytoplasmic and anti-mitotic cell antibodies, are commonly referred as anti-nuclear antibodies (ANA) [1], and we have adopted that usage for clarity. The AAB test results that identified specific, named antigens (see details above), were called collectively antigen-specific autoantibodies. We have further separated them into myositis-related and non-myositis-related AAB. Anti-cytokine autoantibodies are referred to directly.

Data analysis. All the data was organized and analyzed by UT. The data was censored on May 31st, 2020; only 5 patients had censored data for the primary outcome, death in the ICU within 3 months. Given the elapsed time until censoring, the risk of right-censoring bias is low. ANOVA was used for continuous variables and Fisher's exact test was used for categorical variables at $\alpha=0.05$, adjusted for multiple comparisons as indicated in the text using the false discovery rate at $q=0.05$. All statistical and graphical analyses were performed on JMP Pro (version 15.2.1; SAS Institute Inc, Cary, NC, USA).

References

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Anti-cardiolipin and other anti-phospholipid antibodies in critically ill COVID-19 positive and negative patients

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Supplemental Table 1: Premorbid Clinical Characteristics and Therapeutics

	All (N)	COVID+ (N)	COVID- (N)
	42	22	20
Respiratory PMH	18	8	10
Cardiovascular PMH	19	11	8
Renal PMH	7	6	1
Type 2 Diabetes	20	12	8
Hypertension	24	14	10
Other comorbidities	37	18	19
Premorbid steroid used	3	1	2
Premorbid immunomodulatory medication use	2	1	1
Premorbid ACEi/ARB use	15	10	5

Abbreviations: ACEi, Angiotensin-converting enzyme inhibitors; ARB, angiotensin receptor blocker; PMH, past medical history.

Legend: "Other comorbidities" include autoimmune diseases: Myasthenia gravis among COVID+, and autoimmune hemolytic anemia, rheumatoid arthritis and multiple sclerosis among the COVID-. No statistically significant difference between COVID+ and COVID- patients for all variables were detected using ANOVA for continuous variables and Fisher's exact test for categorical variables at $\alpha=0.05$

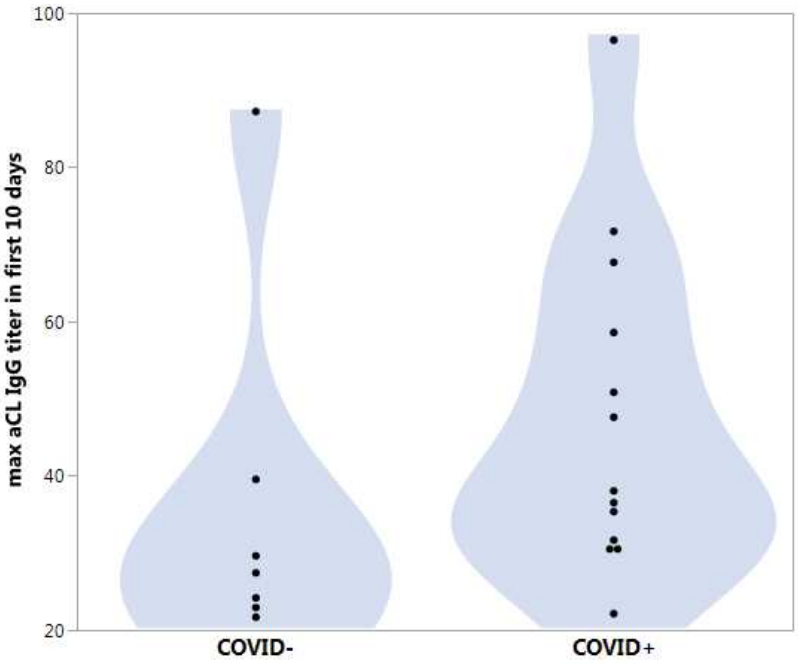
Supplemental Table 2: distribution of aCL titers among the aCL positive

	N	Mean	SD	Median	Range	IQR
COVID^{+}	13	47.5	21.2	38.1	22.1 – 96.5	31.2 – 63.1 (31.9)
COVID^{-}	7	36.1	23.3	27.4	21.7 – 87.3	22.9 – 39.6 (16.7)

Abbreviations: aCL, anti-cardiolipin antibodies; IQR, interquartile range (25%-75%); SD, standard deviation.

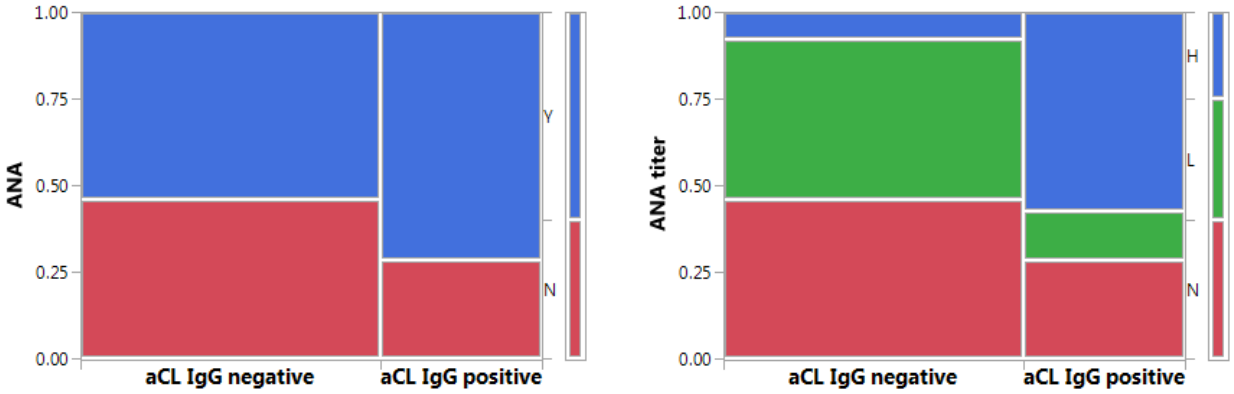
Legend: Descriptive statistics of aCL titers in acL positive patients were stratified into COVID^{+} and COVID^{-}. *N* is the number of patients included in each group. These differences were not statistically significant (ANOVA, significance threshold at α=0.05).

Supplemental Figure 1: aCL titers according to COVID status



Legend: This is a graphical representation of the data shown in supplemental Table 2, where aCL absorbance units (AU) are displayed according to COVID-19 status.

Supplemental Figure 2: ANA positivity and titers in aCL positive and aCL negative patients



Abbreviations: aCL, anti-cardiolipin antibodies; ANA, anti-nuclear antibodies.

Legend:

Left panel: ANA positivity vs aCL antibodies. The Y axis is the proportion of ANA positivity (red are negative (N), blue are positive (Y)). These differences are not statistically significantly (Fisher's exact test, significance threshold at α=0.05).

Right Panel: Same as left panel, but ANA positive patients are subdivided into high and low titers. The Y axis is the proportion of ANA titers: red are negative, green are low (1:160-1:320), and blue are high (>1:320) titers. These differences are statistically significantly (Fisher's exact test, p=0.03).

Supplemental Table 3: Association between aCL IgG and high-titer anti-cytokine autoantibodies

	Cohort	**All**	**aCL positive**	**aCL negative**
	N	42	20	22
ALL	*N (%)*	16/42 (38%)	13/20 (65%)	3/22 (14%)
anti-GMCSF	*N (%)*	1/42 (2%)	1/20 (5%)	0/22 (0%)
anti-IFN-γ	*N (%)*	7/42 (17%)	6/20 (30%)	1/22 (5%)
anti-IL-1a	*N (%)*	1/42 (2%)	0/20 (0%)	1/22 (5%)
anti-IL-6	*N (%)*	5/42 (12%)	3/20 (15%)	2/22 (9%)
anti-IL-10	*N (%)*	5/42 (12%)	5/20 (25%)	0/22 (0%)
anti-IL-12p40	*N (%)*	2/42 (5%)	2/20 (10%)	0/22 (0%)
anti-IL-17a	*N (%)*	2/42 (5%)	2/20 (10%)	0/22 (0%)
anti-IL-17f	*N (%)*	4/42 (10%)	4/20 (20%)	0/22 (0%)
anti-IL-22	*N (%)*	1/42 (2%)	0/22 (0%)	1/22 (5%)

Abbreviations: aCL, anticardiolipin; GMCSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin.

Legend: The table reports the number of patients with high titers of anti-cytokine antibodies at any time during the first 10 days of ICU admission. “ALL” represents all patient having a high titer of anti-cytokine antibody of any type during that period. The numbers of the specific anti-cytokine antibodies sum to more than “ALL” since some patients had more than one high titer anti-cytokine antibody. Once adjusted for multiple comparisons, there were no statistically significant differences between aCL positive and aC negative- for any of the results (Fisher’s exact test at $\alpha=0.05$ followed by the false discovery rate at $q=0.05$). The following anti-cytokine AAB did not show high levels in any of the patients: anti-BAFF, anti-IFN-β, anti-TNF-α, anti-IL8, anti-IL-15 and anti-IL-18.

Supplemental Table 4: Association between aCL IgG and anti-cytokine autoantibodies per COVID-19 status

			Anti-cytokine autoantibody titers	
			Positive	**High-positive**
COVID^{+}	aCL IgG positive	*N, %*	12/13, 92%*	9/13, 69%
COVID^{-}			4/7, 57%	4/7, 57%

Abbreviations: aCL, anti-cardiolipin antibodies.

Legend: The asterisk (*) represents a significant association between aCL IgG and anti-cytokine autoantibodies (Fisher's exact test, p=0.006, adjusted for multiple comparisons).