Incidence of autoantibodies related to systemic autoimmunity in patients with severe COVID-19 admitted to the intensive care unit

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Abstract

Objective

To assess the prevalence of autoantibodies (AAbs) in mechanically ventilated COVID-19 patients and to investigate whether AAbs influence the clinical outcome.

Methods

Serum samples were drawn within the first 48 hours upon admission to the intensive care unit (ICU) from 217 consecutive patients, from January 1st, 2021, to May 10th, 2021, and investigated for the presence of AAbs using conventional techniques. Serum samples (n=117) of age- and sex-matched healthy individuals collected before COVID-19 pandemic were used as controls.

Results

COVID-19 patients in the ICU had more commonly AAbs compared to age- and sex-matched controls (174/217, 80.2% vs. 73/117, 62.4%, p<0.001). Patients expressed more frequently ANAs (48.4% vs. 21.4%, p<0.001), anti-dsDNA (5.1% vs. 0%, p=0.01), anti-CCP (8.3% vs. 1.7%, p=0.014) and anti-CL IgM AAbs (21.7% vs. 9.4%, p=0.005) than controls, respectively. Simultaneous reactivity against at least three autoantigens, occurred in 144 out of 174 (82.8%) patients. The two groups did not differ in terms of clinicoepidemiologic characteristics or the mortality ratio within the ICU. Patients who died compared to convalescents were older, had higher ferritin, D-dimers levels, APACHE II score, lower oxygen saturation, higher prevalence of comorbidities and cognitive dysfunction. However, AAbs were not found to correlate with the clinical outcome.

Conclusion

Patients with severe COVID-19 express AAbs more commonly compared to controls. No correlation was found between AAbs and disease outcome.

Key words

autoantibodies, COVID-19, outcome

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Received on May 6, 2022; accepted in revised form on July 18, 2022.

Funding: the experimental arm of the current study was financially supported by donation grants from SYN-ENOSIS (Athens, Greece) and Institute for Autoimmune Systemic and Neurologic Diseases (Athens, Greece).

Competing interests: A.G. Tzioufas has received research grants from Novartis, Pfizer, UCB, AbbVie and GSK pharmaceutical companies, through the National and Kapodistrian University of Athens, not relevant to the submitted work. The other authors have declared no competing interests.

Introduction

COVID-19 is potentially a multisystemic disorder associated with acute respiratory distress syndrome (ARDS), sepsis, coagulopathy (1-3), neurologic complications (4), and heightened inflammatory responses, including cytokine storm syndrome (5). Clinical features of COVID-19 compatible with autoimmunity include: (i) certain overlaps with immune-mediated disorders (6), (ii) new onset autoimmune phenomena such as Kawasaki-like syndrome (7), Guillain-Barre syndrome (8), immune thrombocytopenic purpura, haemolytic anaemia (9), systemic lupus erythematosus (SLE) (10), inflammatory arthritis, vasculitis (11), myopathy (12), chilblain-like lesions (13), and (iii) exacerbation of a pre-existing autoimmune disease (14).

The presence of autoantibodies (AAbs), detected frequently in the more severe cases, highlights the state of immune dysregulation in COVID-19. Theoretically, SARS-CoV-2 may break peripheral self-tolerance through a variety of well-described mechanisms including molecular mimicry, bystander activation, epitope spreading, viral persistence and formation of neutrophil extracellular traps (15). In the advent of the pandemic, we reported that more than a half of severely-ill hospitalised COVID-19 cases were found positive for an AAb related to systemic autoimmunity (16). Subsequent reports described a high frequency of AAbs in patients with poor prognosis, while other researchers identified that a significant subset of patients developed new-onset AAbs (17). Of relevant clinical importance is the association between the presence of antiphospholipid AAbs (aPLs) and coagulopathy in COVID-19 patients (18). Given that healthy elderly subjects have a high prevalence of AAbs (19), concerns have been raised regarding the clinical significance of AAbs in COVID-19, since most of the patients with severe COVID-19 are in the middle and third age. To address the unresolved clinical questions related to the presence of AAbs in critically-ill patients with COVID-19, we conducted a prospective study involving consecutive patients admitted to the intensive care unit (ICU) and investigated the: (i) clinical significance of AAbs in COVID-19, (ii) comparison with age- and sex-matched healthy individuals, (iii) association with anti-SARS-COV-2 antibody responses and (iv) proportion of newly generated AAbs.

Patients and methods

Patients and study design

Consecutive patients admitted to the ICU of Evaggelismos Hospital, Athens, Greece between January 1st, 2021 and May 30th, 2021 (n=217) were included in this study based on the following criteria: (a) RT-PCR diagnosed SARS-CoV-2 infection, (b) age >18 years old, and (c) critical COVID-19 illness based on the NIH classification criteria necessitating intubation and subsequent mechanical ventilation (20). Patients with a documented prior medical history of systemic autoimmune rheumatic disease were excluded. Serum samples for AAbs and anti-SARS-CoV-2 antibody testing were obtained upon admission of patients in the ICU (217 patients), as well as on day 15 of their hospitalisation (60 out of 217 patients). Baseline characteristics [age, gender, body mass index (BMI)], time duration from symptoms onset until hospital admission, adverse outcomes (myositis, thrombotic events, cognitive dysfunction), prognostic scores [APACHE II, Sequential Organ Failure Assessment (SOFA)], laboratory measurements (ferritin, D-dimers, C-reactive protein, creatinine, HbA1c), COVID-19 related treatment regimens and outcomes were monitored. Serum samples from anonymous, age- and sex-matched healthy controls (n=117) were obtained prior to the COVID-19 pandemic from the Laboratory of Clinical Immunology, Department of Pathophysiology, School of Medicine, National and Kapodistrian University of Athens (NKUA), Athens, Greece. The present study was approved by the Ethics Committee of School of Medicine, NKUA, Athens, Greece (leading partner; protocol no: 456) and was conducted according to the principles of the Helsinki Declaration and the GDPRs of the European Union. All patients or their legally authorised representatives, provided written informed consent.

Blood samples and serology testing

Sera were separated by centrifugation at 2500g for 10 min and stored at -20°C within 1 hour from blood collection at Evaggelismos Hospital. Serum samples were assessed for the presence of the AAbs and anti-SARS-CoV-2 antibodies in the Laboratory of Clinical Immunology, Department of Pathophysiology, School of Medicine, NKUA, Athens, Greece.

- Detection of antibodies against SARS-CoV-2

Anti-SARS-CoV-2 IgG antibodies against the S1 domain of SARS-CoV-2 spike protein were detected using an FDA-approved ELISA method (Euroimmun, Lübeck, Germany), according to manufacturer's instructions. The cut-off positive threshold was >1.1, after calculating the ratio of optical density (OD) of samples measured at 450 nm, divided by the OD value provided by the calibrator. The aforementioned ratio corresponds to anti-SARS-CoV-2 antibody titres throughout the current manuscript. For the assessment of neutralising activity of the anti-SARS-CoV-2 antibodies, a cPass ELISA SARS-CoV-2 Surrogate Virus Neutralisation Test Kit (GenScript Biotech B.V, USA), testing antibody-mediated inhibition of SARS-CoV-2 RBD binding to the human host receptor angiotensin-converting enzyme type 2, was used and a cut-off of >30% inhibitory concentration was considered as positive.

- Detection of autoantibodies

AAbs against nuclear antigens (ANA), neutrophil cytoplasmic antigens (ANCA), cyclic citrullinated peptides (anti-CCP), double stranded-DNA (anti-dsDNA), cardiolipin (anti-CL), β2-glycoprotein-I (anti-β2-GPI), thyroid peroxidase (anti-TPO), and thyroglobulin (anti-TG) were routinely tested in all patients and controls. Laboratory testing was performed blindly, without knowledge of patient's characteristics and status. ANA and ANCA AAbs were evaluated in serial serum dilutions (starting from 1/160 and 1/20, respectively) by standard indirect immunofluorescence on commercially available Hep-2 cells and ethanol-fixed neutrophils using the NOVA Lite HEp-2 ANA and NOVA Lite ANCA kits (Inova Diagnostics Inc, San Diego, CA, USA), respectively, according to the manufacturer's instructions, followed by evaluation of the staining pattern by fluorescence microscopy (by EKK and KB). Titres ≥1/160 and 1/20 for ANA and ANCA, respectively, were considered as positive. The levels of IgG and IgM AAbs against CL, β2-GPI and ds-DNA were determined by home-made ELISAs, as previously described (21, 22). Anti-CCP, anti-TPO and anti-TG were measured by commercially available ELISAs (QUANTA Lite CCP, QUANTA Lite TPO and QUANTA Lite Thyroid T ELISA kits, Inova Diagnostics Inc) according to manufacturer's instructions. Extractable nuclear antigens (ENA), including Ro52/SSA, Ro60/SSA, La/SSB, Sm, U1-nRNP, Jo-1, Scl70, and myositis-related AAbs were additionally evaluated in the patient group. ENAs were tested by immunoblotting using the Euroline Anti-ENA ProfilePlus1 (IgG) kit (Euroimmun, Lübeck, Germany) and myositis-specific autoantibodies by immunoblotting using Autoimmune Inflammatory Myopathies 16 Ag (Euroimmun Lübeck, Germany) according to manufacturer's instructions.

Statistical analysis

Data analyses and statistics were performed using SPSS-v. 26, Python-v. 3.7 and GraphPad-v. 9 packages. Univariable statistical analyses for categorical data were performed using the chi-square test or Fisher's exact test when cell counts were <5. For continuous variables, normality was tested with the Shapiro-Wilk test and Mann-Whitney U-test (Wilcoxon rank-sum test), or t-test were applied appropriately. Comparison among several groups was based on Kruskal-Wallis with *post-hoc* analysis, after testing the normality of the variables.

For multivariable analyses, a data-driven analysis was performed based on the combination of the Fast Correlation Based Feature (FCBF) selection method with the Logistic Regression (LR) algorithm (FCBF/LR). This combined model was applied on the unified dataset of all patients, in order to identify independent associated risk factors for death, followed by a conventional 10-fold cross-validation approach to evaluate the performance of the FCBF/LR model. Unlike the classical statistical analyses which hampers the identification of hidden patterns within the variables in the data based on a target outcome, data-driven analysis is suitable for this classification task, since it involves automated methods for the extraction of hidden patterns within the variables in the dataset, minimising potential selection bias, as previously described (23).

Results

Patients and controls

A total of 217 intubated patients with COVID-19 (66 women) were enrolled in the study (median age of 68 years; range 32–102). The demographic, biometrical and comorbidity characteristics of all patients are shown in Supplementary Table S1. The median time period (range) from the onset of COVID-19-related symptoms and hospital admission was 5 (1–15) days, while the median (range) of hospitalisation until the final outcome (discharge or death) was 20 (4–137) days. Upon admission in the ICU, the median (range) SOFA and APACHE II scores were 5 (2–11) and 15 (3–24), respectively. During their hospitalisation, all except one patient, manifested with bilateral pneumonia with a median (range) oxygen (O2) saturation of 56% (40.5–85) on room air. Eighteen (8.3%) patients had cognitive dysfunction, three (1.4%) myositis and two (0.9%) pulmonary embolisms. The COVID-19 related laboratory abnormalities during ICU admission including the values for C-reactive protein, D-dimers and ferritin levels are shown in Supplementary Table S2. Thirty-nine (21%) patients received azithromycin prior to hospital admission. All patients were treated with dexamethasone upon admission, while other COVID-19-related treatments, such as anti-cytokine biologics and fresh-frozen plasma, were applied in two individuals. One hundred and six patients (48.8%) died in a median (range) duration of 18 (4–120) days.

Table I. Comparison of COVID-19 patients and controls.

	Patients (n=217)	Controls (n=117)	*p*-value
Female gender, n (%)	66 (30.4)	42 (35.9)	0.307
Age, median (min-max)	68 (32–102)	65 (32–90)	0.057
Presence of autoantibodies, n (%)*	174 (80.2)	73 (62.4)	**<0.001**
ANA, n (%)	105 (48.4)	25 (21.4)	**<0.001**
Anti-dsDNA IgG, n (%)	11 (5.1)	0 (0)	**0.01**
ANCA, n (%)	30 (13.8)	14 (12)	0.632
C-ANCA, n (%)	17 (7.8)	2 (1.7)	**0.024**
P-ANCA, n (%)	13 (6)	12 (10.3)	0.158
Myositis related autoantibodies, n (%)	22/203* (10.8)	1 (0.9)	**<0.001**
Anti-CCP, n (%)	18 (8.3)	2 (1.7)	**0.015**
Titres of anti-CCP, median (min-max)	6 (0-4–468)	9 (2–25)	**0.014**
Anti-TPO, n (%)	57 26.3)	33 (28.2)	0.703
Titres of anti-TPO, median (min-max)	78 (57–4099)	79 (62–14129)	0.281
Anti-TG, n (%)	10 (4.6)	12 (10.3)	**0.047**
Anti-β2-GPI IgG, n (%)	27 (12.4)	17 (14.5)	0.590
Titres of anti-β2-GPI IgG, median (min-max)	54 (1–445)	52 (5–1862)	0.333
Anti-β2-GPI IgM, n (%)	24 (11.1)	8 (6.8)	0.211
Titres of anti-β2-GPI IgM, median (min-max)	30 (2–1114)	35 (5–238)	0.094
Anti-CL IgG, n (%)	54 (24.9)	23 (19.7)	0.279
Titres of anti-CL IgG, median (min-max)	57 (2–744)	72 (19–453)	**0.014**
Anti-CL IgM, n (%)	47 (21.7)	11 (9.4)	**0.005**
Titres of anti-CL IgM, median (min-max)	59 (2–664)	36 (4–557)	**<0.001**

*Calculation of autoantibodies without presence of myositis related autoantibodies and extractable nuclear antigens (ENAs)
ANA: antinuclear antibodies; Anti-dsDNA: anti-double stranded DNA (IgG) antibodies; ANCA: antineutrophil cytoplasmic antibodies; Anti-CCP: anti-citrullinated protein antibodies; Anti-TPO: anti-thyroid peroxidase antibodies; Anti-TG: anti-thyroglobulin antibodies; Anti-β2-GPI: anti-β2-glycoprotein I antibodies; Anti-CL: anti-cardiolipin antibodies.

Prevalence of serum autoantibodies in patients and controls

174 (80.2%) patients displayed at least one autoantibody; 68 of them (39.1%) had one autoantibody, 49 (28.2%) two, 27 (15.5%) three, 20 (11.5%) four and 10 (5.8%) more than five autoantibodies. In the control group, there were 73 (62.4%) cases with at least one AAb; 35 of them (47.9%) had one autoreactivity, 15 (20.5%) had two, 15 (20.5%) three, 5 (6.8%) four and 3 (4.1%) five autoreactivities. A detailed report of autoantibody specificities in both study and control group are shown in Table I and Supplementary Tables S4-6.

The frequency of any AAb in the COVID-19 group was significantly higher than that in the control group (80.2% *vs*. 62.4%; $p<0.001$). Individual autoantibody differences with statistical significance were as follows: positive ANA in 48.4% of the study group *vs*. 21.4% in the control group ($p<0.001$); cANCA in 7.8% *vs*. 1.7%, ($p=0.02$); anti-CCP in 8.3% *vs*. 1.7%, ($p=0.02$); anti-CL IgM in 21.7% *vs*. 9.4%, ($p=0.005$) and anti-dsDNA in 5.1% *vs*. 0%, ($p=0.01$). On the contrary, the prevalence of anti-TG AAbs was higher in the control group compared to patients (10.3% *vs*. 4.6%, $p=0.047$) (Table I). Patients had higher positive anti-CL IgG titres than controls, while controls had higher positive anti-β2-GPI IgG titres than patients (Fig. 1). At least one from the myositis-specific AAbs were detected in 16 (7.4%) patients; more specifically, the seropositivity rates among the different antigens were: U1-nRNP 3 (1.4%), Sm 1 (0.5%), Ro60/SS-A 1 (0.5%), Ro52/SS-A 6 (2.8%), La/SS-B 3 (1.4%), Scl70 3 (1.4%), Jo1 1 (0.5%). Regarding myositis specific ENAs, 22 out of 203 serums tested (10.8%) were found positive for at least one autoreactivity; Mi-2a 1 (0.5%), Mi-2β 0 (0%), TIF1γ 3 (1.5%), MDA5 1 (0.5%), NXP2 1 (0.5%), SAE1 0 (0%), Ku 5 (2.5%), PM-Scl100 2 (1%), PM-Scl75 4 (2%), Jo1 1 (0.5%), SRP 0 (0%), PL-7 2 (1%), PL-12 4 (2%), EJ 0 (0%), and OJ 3 (1.5%), these being statistically more common among patients than controls [22/203 (10.8%) *vs*. 1/117 (0.9%), $p<0.001$] (Suppl. Table S6). The titres of all autoantibodies tested ranged from low to high and were similar to those observed in autoimmune disorders (Suppl. Table S7).

Correlation of autoantibody positivity with clinicopathologic features and outcome

The comparison of patients with at least one serum AAb (n=174) and those without AAb (n=43) revealed that the AAb-positive subgroup had more frequently anti-SARS-CoV-2 antibodies and neutralising activity above cut-off [150 (86.2%) *vs*. 28 (65.1%), $p=0.001$ and 159 (91.4%) *vs*. 33 (76.7%), $p=0.007$], significantly lower O_2 saturation levels [55.2 (42-85) *vs*. 57.5 (40.5–75), $p=0.037$] and less often abnormal creatinine values [14 (8.1%) *vs*. 8 (18.6%), $p=0.041$] than the AAb-negative subgroup. Importantly, the AAb-positive subgroup did not have more severe prognosis (as defined by SOFA and APACHE II scores) and outcomes (as defined by death incidence) (Suppl. Table S2). The presence and/or the titre of the autoantibodies tested was not found to correlate with other clinical features, including thrombotic events). The subgroup with the adverse outcome was significantly older [73.5 (36–102) *vs*. 62 (32–85) years old, $p<0.001$], had more frequently underlying cardiovascular disease [21 (19.8%) *vs*. 9 (8.3%), $p=0.015$] and higher APACHE II scores [16 (7–24) *vs*. 14 (3-22), $p<0.001$], CRP values [12.7 (0.4–41.1) *vs*. 9.3 (0.3–30) mg/dl, $p=0.001$], D-dimer values [1.6 (0.3–17.6) *vs*. 1.17 (0.1–10), $p=0.007$], and ferritin levels [633.8 (70.9–25466) *vs*. 471.8 (38–14959), $p=0.008$] compared to the convalescent subgroup. Moreover, patients who ultimately died had higher incidence of cognitive dysfunction [15 (14.2%) *vs*. 3 (2.7%), $p=0.003$] and lower O2 saturation levels on room air [55 (40.5–77) *vs*. 58 (42–85), $p=0.002$].

Newly induced autoantibodies triggered by SARS-CoV-2 infection

To determine if AAbs were generated *de novo,* we analysed two consecutive samples from 60 patients, taken 2 to 4 weeks after ICU admission. Baseline characteristics of these patients are detailed in Supplementary Table S3. A heatmap of the dynamics of various AAbs is depicted in Figure 2. Forty-five out of 60 patients (75%) had at least one newly induced AAb. Among these

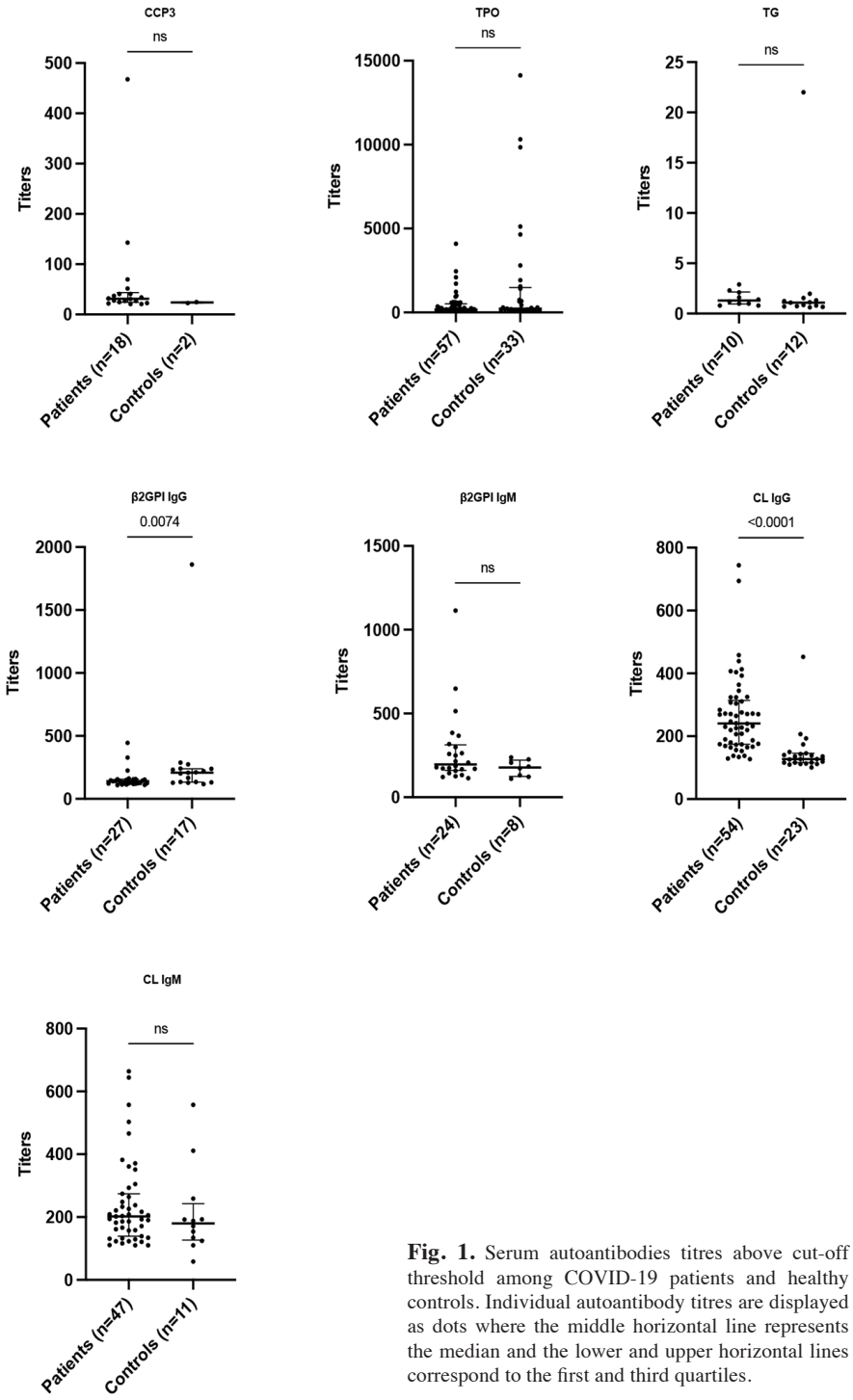

Fig. 1. Serum autoantibodies titres above cut-off threshold among COVID-19 patients and healthy controls. Individual autoantibody titres are displayed as dots where the middle horizontal line represents the median and the lower and upper horizontal lines correspond to the first and third quartiles.

45 patients, 17 (37.8%) had one new-onset autoreactivity, 12 (26.7%) had 2 new-onset autoreactivities, 7 (15.6%) had 3 new-onset autoreactivities, while 4–6 autoreactivities were identified in 9 (20%) patients. On the contrary, 28 of 60 patients (46.7%) lost at least one AAb; 20/28 (71.4%) lost one, 6/28 (21.4%) lost 2, and the rest 2/28 (7.1%) lost 4 AAb.

New-onset reactivity against ANAs was identified in 20 of 60 cases (33.3%); 13 regarding 1/160 dilution, 6 regarding 1/320 dilution and 1 regarding 1/640 dilution. IgG anti-CL and IgG anti-β2GPI new onset autoreactivities were detected in 21 (35%) and 11 (18.3%) cases, respectively. Quite the reverse, the most frequently detected losses of reactivity were in IgG anti-β2-GPI (7/60, 11.7%), IgM anti-CL (7/60, 11.7%), and IgG anti-CL (6/60, 10%). New onset reactivity against extractable nuclear antigens was detected in 3 of 60 (5%) patients, with 2 of them regarding Ro52. Myositis-related autoantibodies showed in 9 patients (15%) with Mi2a (4/9) and PL7 (3/9) being identified more frequently. Neither new-onset or loss of reactivity were found to correlate with any clinical or serological parameters.

Multivariable data driven logistic regression analysis for identification of independent risk factors associated with death, presence of any autoantibody and aPL antibodies

To address the predicting factors of death we constructed a working dataset that included 217 patients and 29 features (variables) which are described in Table III. The results of the FCBF-based multivariable logistic regression analysis, using death as an outcome are presented in Table III. The FCBF algorithm identified 4 potentially independent risk factors associated with death: age, APACHE II score, oxygen saturation on room air, and anti-β2-GPI (IgM). Logistic regression analysis disclosed only age, oxygen saturation on room air and APACHE II score as independent negative modifiers of adverse outcome. The performance of the data driven approach was quite favourable, yielding an average accuracy 0.70, sensitivity 0.69, specificity 0.72 and AUC 0.83. To study the effect of variable baseline and clinical characteristics of patients on "antibody formation", another FCBF/LR multivariable model was conducted, without identifying any feature as an independent factor influencing auto-antibody formation (data not shown).

Discussion

In accordance with the tendency of SARS-CoV-2 to trigger autoimmune phenomena in susceptible individuals, a high proportion of critically-ill COVID-19 patients developed AAbs, and their prevalence significantly compared to that of age- and sex-matched individuals, evaluated prior to the pandemic. Notably, some of the observed AAbs in severe COVID-19 patients including

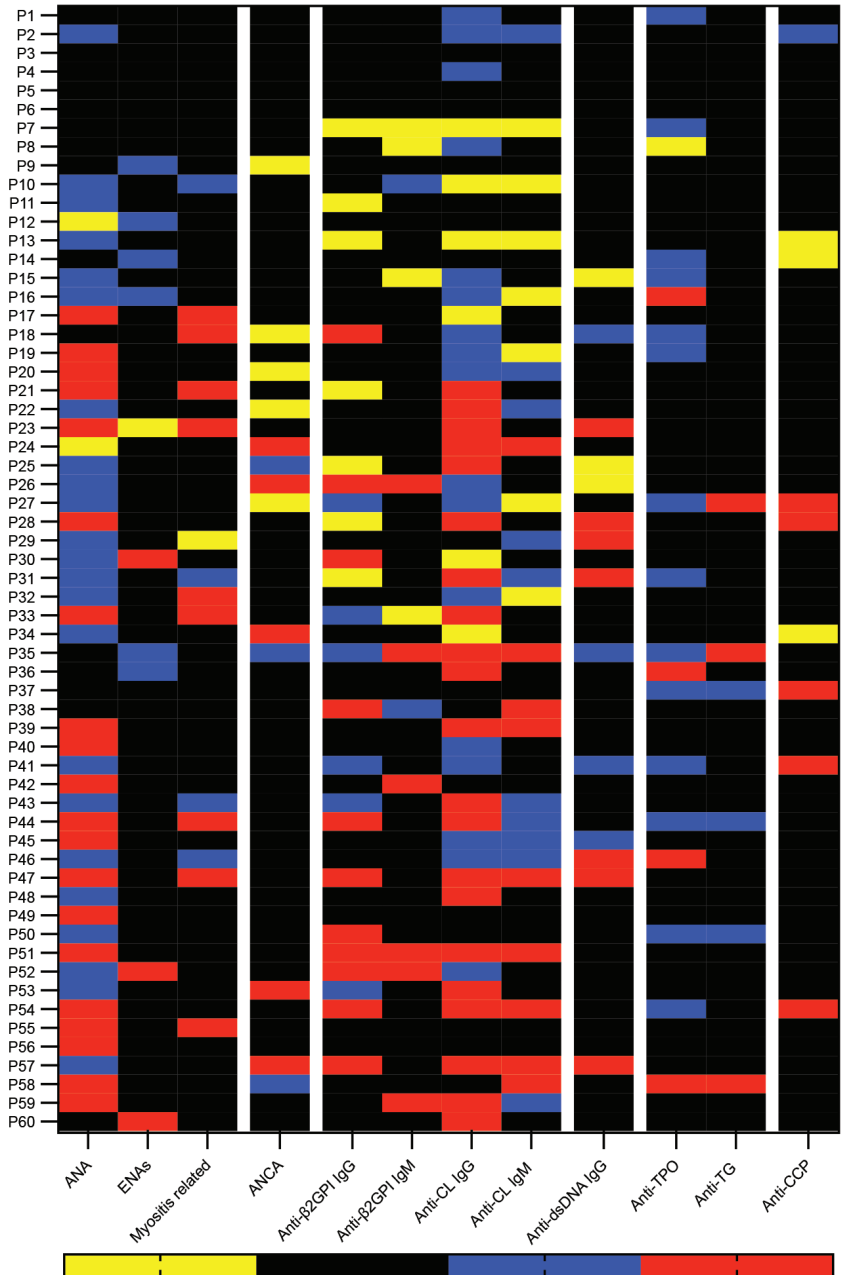

Fig. 2. A heatmap of SARS-CoV-2 antibodies and autoantibodies dynamics during hospitalisation. Black indicates unaltered absence of autoreactivity. Blue indicates persistently positive autoantibodies. Yellow indicates loss of reactivity. Red indicates new-onset induction of antibodies.

anti-CCP, anti-dsDNA and cANCA are specific for certain systemic autoimmune diseases (24). In addition, some of these were new-onset, generated during acute illness and hospitalisation. However, these were not found to correlate with any potential risk factors or clinical outcomes.

Interestingly, autoimmunity associated with severe COVID-19 infection lacks Bcl6+ germinal centres, suggesting a predominantly extrafollicular response, as was described in the lymph nodes of COVID-19 patients (25). B cells, of extrafollicular areas, known as double-negative B cells, lack IgD, CD27, CXCR5, and CD21. Thus, they are poised to become antibody secreting cells, tending to produce pathogenic autoantibodies (26).

Using well-characterised, clinically validated and widely applicable standard techniques for identifying autoantibodies correlated with classifiable autoimmune diseases, we observed that 80% of critically-ill and mechanically-ventilated COVID-19 patients had at least one circulating autoantibody as opposed to pre-pandemic non-acutely ill individuals who were tested positive in 60% of cases. Among other studies, composed mainly of non-critically COVID-19 cases, frequency of any autoantibody positivity ranged from 30% to 52% (17, 27); nevertheless, their frequency remained significantly higher than that in age- and sex-matched patients with fever and/or pneumonia with etiologies other than COVID-19 (27, 28). Although the most commonly detected autoantibodies identified were ANAs, there is a substantial diversity in their frequency (range 10–84%), intensity and staining patterns reported in the literature (6, 27-33). We observed, among others (29), that the reactive specimens against ANA demonstrate generally low positivity, at dilutions of 1/160 with speckled nuclear pattern (~60%) and the momentous majority (~95%) less than 1/640, while other investigators have found more intense reactivities (greater than 1/320 levels) primarily with nucleolar pattern) (6, 27). To better characterise ANA-related autoreactivity, patients' samples were further crisscrossed for ENAs, and myositis-specific autoantibodies and it was found that approximately 7% and 10% of patients reacted against at least one ENA or myositis-specific antigen, respectively. Still, only a few ANA-positive cases were shown to target specific antigenic targets (up to 20%) and the most common reactivities were against Ro52, Ku, PM-Scl75 and PL-12. Although our results are consistent with another study by Gazzaruso *et al*. who found similar ENA reactivities (~5% of cases referring to SS-A/Ro52) (30), other investigators have reported reactivities against highly specific autoantigens (*i.e*. MDA5 and RIG1) in up to 20% of COVID-19 patients which correlated with worse pulmonary involvement at lung computerised tomography scans (31). This high inter-individual variation among published studies in which autoantigens are targeted (most commonly detecting RNP) (17, 34), suggest that autoimmune responses in the context of COVID-19 are rather non-specific.

Table II. Comparison between convalescent COVID-19 patients and those who died.

	Total (n=217)	Convalescent (n=111)	Deaths (n=106)	*p*-value
Female gender, n (%)	66 (30.4)	36 (32.4)	30 (28.3)	0.509
Age, median (min-max)	68 (32–102)	62 (32–85)	73.5 (36–102)	**<0.001**
Covid-19 IgG antibodies, n (%)	178 (82)	96 (86.5)	82 (77.4)	0.080
Titres of Covid-19 IgG antibodies, median (min-max)	7.6 (0.1–11.4)	7.9 (0.1–11.4)	6.9 (0.1–11.1)	0.131
Activity of neutralising SARS-CoV-2 RBD antibodies, n (%)	192 (88.5)	99 (89.2)	93 (87.7)	0.737
Titres of neutralising SARS-CoV-2 RBD antibodies, median (min-max)	91 (4–100)	91 (4–99)	88 (6–100)	0.292
Presence of autoantibodies, n (%)**	174 (80,2)	91 (82)	83 (78.3)	0.497
ANA, n (%)	105 (48.4)	52 (46.8)	53 (50)	0.642
Anti-dsDNA IgG, n (%)	11 (5.1)	3 (2.7)	8 (7.5)	0.128
ANCA, n (%)	30 (13.8)	11 (9.9)	19 (17.9)	0.087
C-ANCA, n (%)	17 (7.8)	6 (5.4)	11 (10.4)	0.173
P-ANCA, n (%)	13 (6)	5 (4.5)	8 (7.5)	0.345
Anti-CCP, n (%)	18 (8.3)	10 (9)	8 (7.5)	0.696
Anti-TPO, n (%)	57 (26.3)	32 (28.8)	25 (23.6)	0.380
Anti-TG, n (%)	10 (4.6)	8 (7.2)	2 (1.9)	0.062
Anti-β2-GPI IgG, n (%)	27 (12.4)	16 (14.4)	11 (10.4)	0.368
Anti-β2-GPI IgM, n (%)	24 (11.1)	14 (12.6)	10 (9.4)	0.455
Anti-CL IgG, n (%)	54 (24.9)	27 (24.3)	27 (25.5)	0.845
Anti-CL IgM, n (%)	47 (21.7)	24 (21.6)	23 (21.7)	0.989
Clinical features				
Time duration between symptoms onset and hospital admission, median (min-max)	5 (1–15) / 186*	5 (1-13)	5 (1–15) / 102*	0.431
Time duration between hospital admission and outcome, median (min-max)	20 (4–137) / 186*	25 (6-37)	18 (4–120) / 102*	**0.005**
BMI 25-30, n (%)	148/186* (79.5)	63/84* (75)	85/102* (83.3)	0.161
BMI 30-35, n (%)	31/186* (16.7)	18/84* (21.4)	13/102* (12.7)	0.114
BMI >35, n (%)	8/186* (4.3)	4/84* (4.8)	4/102* (3.9)	0.779
Comorbidities				
Hypertension, n (%)	101/215* (47)	50/109* (45.9)	51 (48.1)	0.742
Dyslipidaemia, n (%)	55/215* (25.6)	32/109* (29.4)	23 (21.7)	0.198
COPD, n (%)	10/225* (4.7)	4/109* (3.7)	6 (5.7)	0.543
Neoplasia, n (%)	15/215* (7)	8/109* (7.3)	7 (6.6)	0.832
CD, n (%)	30/215* (14)	9/109* (8.3)	21 (19.8)	**0.015**
CKD, n (%)	9/215* (4,2)	4/109* (3.7)	5 (4.7)	0.701
DM, n (%)	28/215* (13)	11/109* (10.1)	17 (16)	0.195
Thyroid dysfunction, n (%)	6/215* (2,8)	4/109* (3.7)	2 (1.9)	0.683
ICU prognostic indicators				
SOFA score (>5), n (%)	83 (38.2)	43 (38.7)	40 (37.7)	0.879
SOFA score points, median (min-max)	5 (2–11)	5 (2–11)	5 (3–11)	0.574
APACHE II score (>14), n (%)	140 (64.5)	53 (47.7)	87 (82.1)	**<0.001**
APACHE II score points, median (min-max)	15 (3–24)	14 (3–22)	16 (7–24)	**<0.001**
Clinical characteristics				
pO2 on air (%), median (min-max)	56 (40.5-85) / 186*	58 (42-85) / 84*	55 (40.5-77) / 102*	**0.002**
Pulmonary embolism, n (%)	2 (0.9)	1 (0.9)	1 (0.9)	1
Myositis, n (%)	3 (1.4)	3 (2.7)	0 (0)	0.247
Cognitive dysfunction, n (%)	18 (8.3)	3 (2.7)	15 (14.2)	**0.003**
Laboratory findings				
Hyperferritinaemia (>250 μg/l), n (%)	154/191* (80.6)	72/91* (79.1)	82/100* (82.4)	0.715
Ferritin levels, median (min-max)	556.2 (38–25466)	471.8 (38–14959)	633.8 (70.9–25466)	**0.008**
D-dimers, n (%)	55/194* (28.4)	23/92* (25)	33/102* (31.4)	0.325
D-dimer levels, median (min-max)	1.38 (0.1–17.6)	1.17 (0.1–10)	1.6 (0.3–17.6)	**0.007**
CRP (>0.5 mg/dl), n (%)	208/214* (97.2)	106/110* (96.4)	102/104* (98.1)	0.684
CRP levels, median (min-max)	11.4 (0.3–41.1)	9.3 (0.3–30)	12.7 (0.4–41.1)	**0.001**
Creatinine (>1.4 mg/dl), n (%)	22/216* (10.2)	8/110 (7.3)	14 (13.2)	0.149
Creatinine levels, median (min-max)	0.9 (0.4–5.8)	0.9 (0.4–4.2)	1 (0.4–5.8)	**0.050**
HbA1c (>6.5), n (%)	34/150* (22.7)	16/75* (21.3)	18/75* (24)	0.697
HbA1c levels, median (min-max)	6 (4.5–13)	6 (4.5–13)	6 (4.7–10.4)	0.745
Treatment				
Azythromycin before hospitalisation, n (%)	39/186* (21)	18/84* (21.4)	21/102* (20.6)	0.889
Dexamethasone after intubation, n (%)	186/186* (100)	84/84* (100)	102/102* (100)	1

*Available data; **Calculation of autoantibodies without presence of myositis related autoantibodies and extractable nuclear antigens (ENAs).
ANA: antinuclear antibodies; Anti-dsDNA: anti-double stranded DNA (IgG) antibodies; ANCA: antineutrophil cytoplasmic antibodies; Anti-CCP: anti-citrullinated protein antibodies; Anti-TPO: anti-thyroid peroxidase antibodies; Anti-TG: anti-thyroglobulin antibodies; Anti-β2-GPI: anti-β2-glycoprotein I antibodies; Anti-CL: anti-cardiolipin antibodies; BMI: Body Mass Index; COPD: chronic obstructive pulmonary disease; CD: cardiovascular disease; CKD: chronic kidney disease; DM: diabetes melitus; SOFA: Sequential Organ Failure Assessment score; APACHE II: acute physiology and chronic health evaluation II; CRP: C-reactive protein; HbA1c: haemoglobin A1c

Table III. FCBF-based multivariable logistic regression analysis for risk factors associated with death among critically-ill COVID-19 patients in the ICU.

Prominent feature*	Regression coefficient	Odds ratio	*p*-value	CI low	CI upper
Age**	0.08	1.081	<0.001	1.050	1.113
Oxygen saturation on air**	-0.07	0.935	0.002	0.901	0.972
APACHE II score**	0.18	1.194	0.001	1.084	1.314
Anti-β2-GPI (IgM)	0.01	1.0	0.851	0.997	1.003

*The strongest potentially independent variables identified by the FCBF algorithm to construct the logistic regression model, after analysing initially the following features included in the dataset: Age, Oxygen saturation on air, APACHE II score, Anti-β2-GPI (IgM), ferritin levels, cardiopathy, BMI, CRP, SOFA score, anti-SARS-CoV-2 antibody titres, anti-dsDNA (IgG), at least one extractable nuclear antigen, D-dimers, (c+p) ANCA, creatinine, diabetes mellitus, SARS-CoV-2 neutralising activity, At least one myositis related autoantibody, dyslipidaemia before admission, anti-CCP, Anti-β2-GPI (IgG), COPD, azythromycin before admission, anti-CL (IgG), female gender, ANAs, hypertension before admission, anti-CL (IgM), dexamethasone after admission. **<0.05 (95% confidence interval): final independent risk factors associated with death.

Regarding ANCA autoantibodies, there was no difference in their prevalence between critically-ill COVID-19 patients and controls (14% *vs*. 12%) and although significantly more patients were found positive for cANCA autoantibodies (7.8% *vs*. 1.7%), no association with death was found. Other published studies also support that ANCA reactivity is not associated with acute COVID-19 (27, 30), even when compared to controls (28). The only exception is the study by Sacchi *et al*. who found 25% of patients positive for ANCA autoantibodies and worse outcomes among those with atypical presentation (33).

Similar to our findings among critically-ill COVID-19 patients, Chang *et al*. (32), found a higher prevalence of anti-CCP antibodies in more severe cases, while in the study by Lingel *et al*. (35), anti-CCP were more frequently identified in convalescent COVID-19 patients when compared to those with acute illness or healthy controls and remained long-term after recovery along with anti-tissue transglutaminase antibodies. Another significant difference found in the current study was that COVID-19 patients had more common anti-dsDNA autoantibodies in line with the findings by Gomes *et al*. (36). The investigators note that, despite the fact that anti-dsDNA AAbs were comparably increased in COVID-19 and malaria-infected patients, their presence correlated strongly with later development of severe disease.

Various published reports in the literature have shown an increased prevalence of aPL antibodies of various isotypes in patients with COVID-19 (27, 37, 38), and their presence was associated with thrombosis and severity of COVID-19 (18, 39-42). However, multiple other studies did not demonstrate either differences between COVID-19 and control populations or clinical associations (33, 34, 43-46). The significantly higher frequency of anti-CL IgM autoantibodies that we found in COVID-19 patients compared to controls was not associated with unfavorable outcomes as previously reported (29). The incidence of macro-thrombosis (explicitly occurring as pulmonary embolisms) was pretty low in our cohort, considering the patients' high risk for thrombosis in ICU settings. On the other hand, aPLs displayed a relatively high prevalence and analogous volatility in a 15-days period of hospitalisation (new-onset production up to 35% while loss up to 12%), as observed in other viral infections where autoantibodies production is transient and non-pathogenic. This discrepancy between the few thrombotic events and the high prevalence of aPL antibodies, especially of non-clinically significant IgM isotypes, argues in favour of a temporary breakdown of self-tolerance during COVID-19 and not a direct causal effect on thrombosis or adverse outcomes (3, 47). However, due to the absence of autopsies in our study, a possible association between high anti-CL-IgG titres (Fig. 1), and organ micro-thrombosis that is commonly described in COVID-19, cannot be ruled out (48, 49).

By comparing paired samples from two time−points during hospitalisation, similarly to other studies (17), it was found that: (i) some of the autoantibodies detected in COVID-19 patients predate the infection and remain constantly stable as in the case of autoantibodies against thyroid tissue and ANA which are relatively common in the general population (19), (ii) some other autoantibodies develop new-onset during acute COVID-19 possibly due intermolecular epitope spreading and molecular mimicry between SARS-CoV-2 and self-molecules (15, 50), and (iii) several autoreactivities are lost probably after a temporal disruption of immunity due to acute illness (29).

The limitations of this study are: (i) the study group included exclusively COVID-19 patients who were severely ill and mechanically ventilated in the ICU preventing us from examining the effect of COVID-19 severity on autoantibody production; (ii) the control group lacks acutely ill subjects and as such the effect of an infectious disease other than COVID-19 on autoantibody production could not be assessed; (iii) long-term follow-up of patients was not available therefore autoantibody longevity and its clinical significance could not be studied; (iv) intrahospital co-existing disorders (*e.g*. coinfections) and individualised treatments could have introduced bias.

In conclusion, patients who were severely ill with COVID-19 present in higher prevalence autoantibodies related to systemic autoimmune rheumatic diseases compared to age and sex matched controls. Sequential samples revealed that in a significant proportion the autoantibodies are formed after the infection. The clinical significance of these finding for future development of clinical or subclinical autoimmune disease will be addressed with future prospective studies.

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