Increased protease-activated receptor 1 autoantibodies are associated with severe COVID-19

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To the Editor:

Immune perturbation is a hallmark of coronavirus disease 2019 (COVID-19), with ambiguous roles of various immune cell compartments. Plasma cells, responsible for antibody production, have a two-pronged response while mounting an immune defence with 1) physiological immune response producing neutralising antibodies against protein structures of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and 2) potentially deleterious autoantibody generation. Growing evidence hints towards broad activation of plasma cells and the presence of pathological autoantibodies (abs) that mediate immune perturbation in acute COVID-19 [1]. Recently, a systematic screening for abs confirmed induction of diverse functional abs in SARS-CoV-2 infection, targeting several immunomodulatory proteins, including cytokines/chemokines and their respective G-protein coupled receptors (GPCR) [1]. Abs against GPCR act as agonistic and allosteric receptor modulators and are linked to chronic inflammatory diseases [2] and, as we demonstrated recently, disease severity in acute COVID-19 [3].

Immune-mediated thrombosis is a key pathogenic mechanism in COVID-19 linked to morbidity and mortality [4]. Peripheral blood megakaryocytes are potential biomarkers of severe COVID-19 [5], displaying prothrombogenic metabolic programmes and type I interferon signatures. Activated megakaryocytes and sequestering platelets might contribute to immune-mediated microthrombosis in COVID-19 [6]. Thrombin is another key factor in plasmatic coagulation, but it also induces platelet aggregation *via* GPCR protease-activated receptor 1 (PAR1), expressed on the plasma membrane of megakaryocytes, platelets and endothelial cells. Thrombin activation is linked to acute respiratory distress syndrome and fatal outcomes of COVID-19 [7], but it is (pre-)treatment with platelet inhibitors, not high-dose heparin therapy that reduces mortality and occurrence of thrombotic events in COVID-19 [8, 9]. Therefore, tackling platelet activation/PAR1-mediated coagulation could be a therapeutic target preventing microthrombotic complications in severe COVID-19 [10]. Additionally, thrombin mediates endothelial dysfunction in severe COVID-19 through PAR1 signalling [11]. Given these considerations, we hypothesised that anti-PAR1 abs are altered in COVID-19 and skew the coagulation system towards pro-thrombogenic states.

To investigate this, blood samples from 74 patients who tested positive for SARS-CoV-2 infection using reverse transcriptase-PCR detection (S gene) on nasopharyngeal swabs were collected after informed consent and ethical board review of the respective source studies from three hospitals (University Hospitals Schleswig-Holstein Kiel (identifier D466/20) and Lübeck (identifier 13-003) and Medical Clinic Research-Center-Borstel (identifier EK HL AZ 14-225)). 29 patients required intensive care unit (ICU) treatment and 14 died during hospital stay. Time of first sampling was within 48 h of hospital or ICU admission, while follow-up samples from 18 individuals were collected at random time points. In total, we collected 111 patient serum samples. Occurrence of thrombotic events within the COVID-19-related hospital stay was ascertained depending on the clinically suspected location of thrombosis by magnetic resonance imaging/computed tomography scanning with contrasting agents or duplex sonography. The most common manifestations in our cohort were lung embolism, stroke and intestinal embolic ischaemia. 29 single time point samples from healthy controls age-matched to the patients admitted to ICU were collected in Lübeck (identifier AZ16-199). All serum samples were subjected to duplicate quantification of anti-PAR1 abs by IgG-specific indirect sandwich ELISA (CellTrend, Luckenwalde, Germany) as described previously [12]. We use linear mixed models (LMMs) to evaluate differences between anti-PAR1 abs,

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In patients with severe #COVID19, increased levels of autoantibodies against PAR1 were found. These might serve as allosteric agonists of PAR1 on endothelial cells and platelets, and thus might contribute to the pathogenesis of microthrombosis in COVID-19. https://bit.ly/3pqM9Vv

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disease severity and outcome (survival and thrombotic events), accounting for repeated measures *via* inclusion of patient-specific random intercepts. Lab analytes (including anti-PAR1 abs) were natural log transformed and residual plots visually inspected for deviations from normality or homoscedasticity. LMMs are reported with p-values (obtained using Satterthwaite's method) and 95% confidence intervals for log-transformed coefficient estimates. We assessed the power of anti-PAR1 abs in the prediction of survival and thrombotic events relative to established markers alone and in combination (D-dimers, C-reactive protein (CRP) and interleukin (IL)-6) using logistic regression, and evaluated the resultant models using receiver operating characteristic (ROC) analysis. Analyses were run in R (version 4.2.1; R

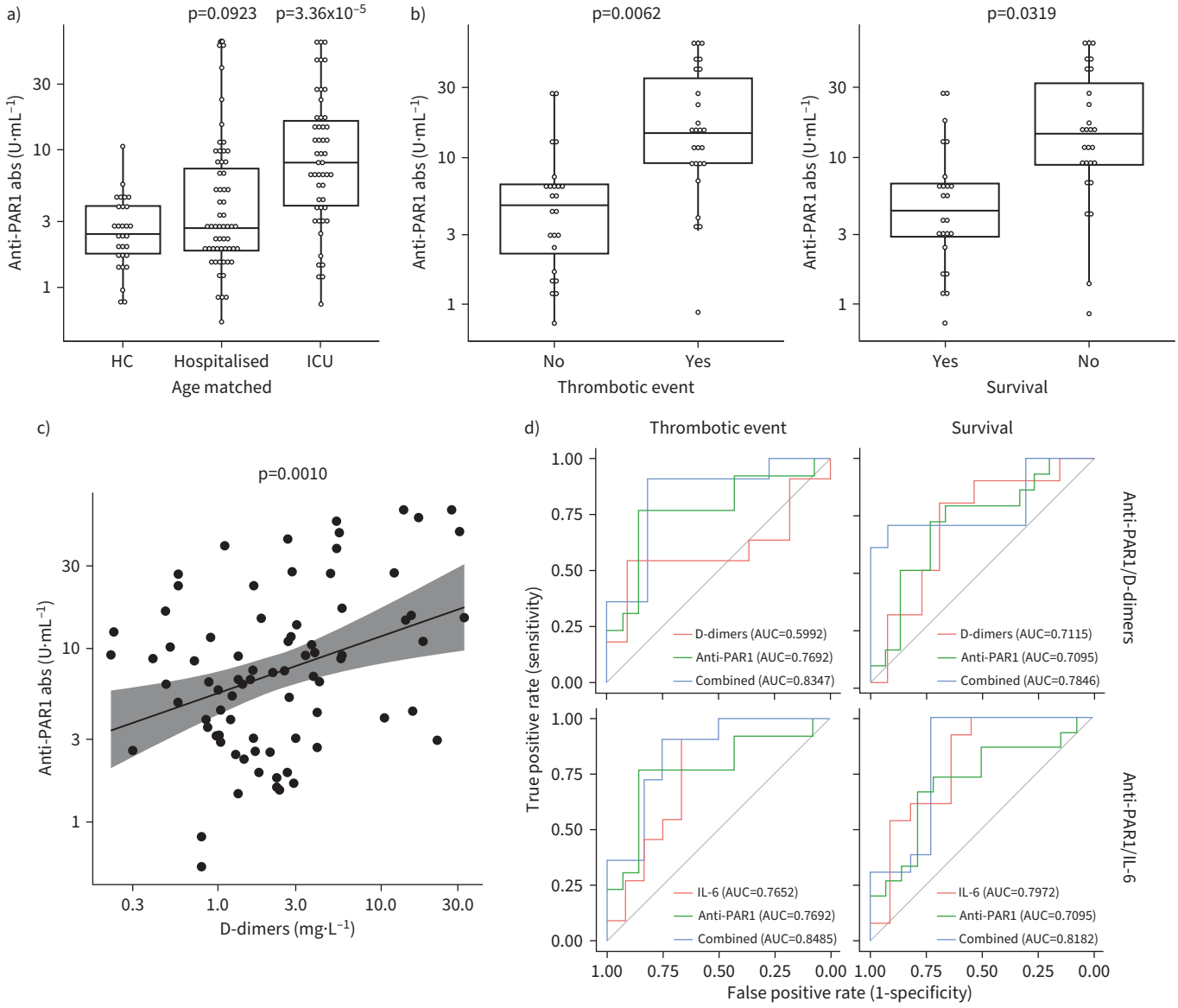

FIGURE 1 Anti-protease-activated receptor 1 (PAR1) antibodies (abs) correlated with disease severity and survival. a) anti-PAR1 ab levels in serum samples from healthy controls (HC) (29 samples, 29 individuals) and patients with coronavirus disease 2019 (COVID-19), either hospitalised/non-intensive care unit (ICU) (59 samples, 45 individuals) or ICU treatment (52 samples, 29 individuals); linear mixed model (LMM) p=0.092 and 3.36×10^{-5}, respectively; b) anti-PAR1 ab levels in serum samples from ICU-treated COVID-19 patients in the cohort (29 patients), stratified by the outcome thrombotic events and survival (LMM p=0.0062 and 0.0319, respectively); c) correlation analysis for anti-PAR1 abs in COVID-19 patients against D-dimers. The linear regression line with confidence interval is displayed; statistical analysis is based on LMM (p=0.0010); d) receiver operating characteristic (ROC) analysis of anti-PAR1 abs, D-dimers and combined (upper panels) as well as anti-PAR1 abs, interleukin (IL)-6 and combined (lower panels) for the clinical outcomes "thrombotic events" (left panels) and "survival" (right panels). Areas under the ROC curve (AUC) are indicated in the panels.

Core Team, Vienna, Austria) with the base package stats and packages LmerTest and pROC (versions 3.1-3 and 1.18.0, respectively).

Disease severity varied within the cohort from hospitalised moderate-to-severe COVID-19 following World Health Organization criteria. The median age (63 years) was identical in the COVID-19 cohort (range 20–93 years) and the controls (range 19–90 years). Circulating anti-PAR1 abs were markedly increased in COVID-19 patients who required ICU treatment (95% CI 0.60–1.59; $p=3.36\times10^{-5}$ in LMM, ICU escalation as fixed effect) in comparison to age-matched controls, but not in hospitalised patients without ICU treatment (95% CI −0.06–0.84; p=0.092 in LMM) (figure 1a). Importantly, increased levels of anti-PAR1 abs within the ICU-treated subcohort were associated with fatal outcome (95% CI 0.12–1.71; p=0.0319 in LMM, mortality as fixed effect (figure 1b) and occurrence of thromboembolic events (95% CI 0.42–2.00; p=0.0062 in LMM, thrombotic events as fixed effect). Circulating anti-PAR1 abs correlated with D-dimers (95% CI 0.32–1.14; p=0.0010 in LMM, D-dimers as fixed effect (figure 1c), further underscoring that anti-PAR1 abs are linked to coagulation processes in acute COVID-19, while significant correlation was neither found with platelet counts nor inflammatory markers like IL-6 (p=0.3467 in LMM, data not shown). Anti-PAR1 abs and D-dimers have similar area under ROC curves (AUROCs) for ICU patients for the end-point survival (AUROC 0.7095 *versus* 0.7115), while anti-PAR1 abs performed better in discrimination of thromboembolism (AUROC 0.7692 *versus* 0.5992) (figure 1d). Combination of both markers further increased the AUROC for both end-point survival (AUROC 0.7846) and thromboembolism (AUROC 0.8347). Anti-PAR1 abs do not have better predictive value compared to IL-6 (AUROC 0.7652 and 0.7972 for the end-points thrombotic events and survival, respectively) and CRP (AUROC 0.8132 and 0.6619 for thrombotic events and survival, respectively); however, anti-PAR1 does improve the predictive power of IL-6 when the analytes are combined in logistic regression (anti-PAR1 abs+IL-6 AUROC 0.8485 and 0.8182 for thrombotic events and survival, respectively, the highest AUROC of all combined analyses of two analytes).

Our data reveal an association between ICU treatment in severe COVID-19 and generation of anti-PAR1 abs, which are associated with poor outcome. Intriguingly, we found no association between anti-PAR1 abs and systemic IL-6, despite the suggested agonistic role of anti-PAR1 abs on PAR1/p70S6K/ERK-dependent IL-6 expression in endothelial cells [13]. This suggests that endothelium-derived IL-6 does not impact systemic IL-6 levels. Indeed, monocytes have been described as the main source of IL-6 in COVID-19 [14], which may explain why endothelial cell and serum IL-6 appear to be uncoupled. This would explain not only the lack of correlation between serum IL-6 and anti-PAR1 abs, but also the improved predictive power that results from the combination of the two analytes. Additionally, increased PAR1-dependent platelet activation in COVID-19 leads to elevated aggregation of circulating cells and collagen [15]. We hypothesise that anti-PAR1 abs in combination with dysregulated coagulation proteases like activated protein C or matrix metalloprotease 1 could activate PAR1-dependent signals in endothelial cells and platelets, thus contributing to immune-mediated microthrombosis as suggested by the correlation of anti-PAR1 abs with D-dimers. Stimulating platelets and endothelial cells with isolated IgG from COVID-19 patients with PAR1 inhibitors (*e.g.* vorapaxar) could provide evidence of anti-PAR1 ab involvement in COVID-19-related coagulopathy.

Our study has several limitations. For most ICU patients, first sampling was upon ICU admission, making prediction of ICU necessity impossible. Although we did not observe sex effects on anti-PAR1 abs in either COVID-19 (p=0.60 in LMM, sex as fixed effect) or control cohorts (p=0.90 in t-test), the sex ratios in the cohorts were skewed.

Our data suggest an association of anti-PAR1 abs with thrombotic complications and fatal outcome in COVID-19, warranting verification in larger cohorts. Functional assessment of anti-PAR1 abs is important to understand their molecular properties and pathophysiological role in thrombosis and endothelial dysfunction in COVID-19.

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