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## Endothelial cell biomarkers in critically ill COVID-19-patients with encephalitis

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## Abstract

COVID-19 is associated with encephalitis in critically ill patients and endothelial dysfunction seems to contribute to this life-threatening complication. Our objective was to determine the hallmark of endothelial activation in COVID-19 related encephalitis.

In an observational study in intensive care unit (ICU), we compared vascular biomarkers of critically ill COVID-19 patients with or without encephalitis. To be classified in the Encephalitis group, patients had to have new onset of central neurologic symptom, and pathological findings on either brain magnetic resonance imaging (MRI) and/or electroencephalogram (EEG).

Among the 32 critically ill COVID-19 consecutive patients, 21 were categorized in the Control group and 11 in the Encephalitis group. Encephalitis patients had a longer ICU-stay than Control patients (median length [25<sup>th</sup>-75<sup>th</sup> percentile] of 52[16-79] versus 20.5[11-44] days respectively,  $p=0.04$ ). Nine-months overall follow-up mortality reached 21% (7/32 patients), with mortality rates in the Encephalitis-group and the Control group of 27% and 19% respectively. Encephalitis was associated with significant higher release of soluble endothelial activation markers (sE-selectin, Tumor-Necrosis-Factor- $\alpha$  (TNF- $\alpha$ ), Interleukin-6, Placental Growth Factor and Thrombomodulin), but these increases were correlated with TNF- $\alpha$  plasmatic levels. The hypoxia-inducible protein Angiopoietin-like-4 (ANGPTL4) was at significantly higher levels in Encephalitis patients compared to Control patients ( $p=0.0099$ ), and in contrary to the other increased factors, was not correlated with TNF- $\alpha$  levels ( $r=0.2832$ ,  $p=0.1163$ ).

Our findings suggest that COVID-19 related encephalitis is a cytokine-associated acute brain dysfunction. ANGPTL4 was the only elevated marker found in Encephalitis patients, which was not correlated with systemic inflammation, suggesting that ANGPTL4 might be a relevant factor to predict encephalitis in critically ill COVID-19 patients.

**Keywords:** COVID-19; acute respiratory distress syndrome; Encephalitis; Endothelial cell biomarker, Angiopoietin-like-4.

## Abbreviations:

A disintegrin and metalloprotease with thrombospondin type I repeats-13: ADAMTS13

Acute respiratory distress syndrome: ARDS

Acute disseminated encephalomyelitis: ADEM

Angiopoietin-1: ANGPT1

Angiopoietin-2: ANGPT2

Angiopoietin-like-4: ANGPTL4

CC chemokine ligand 20: CCL20

Chemokine Ligand-2: CXCL2

Coronavirus disease 2019: COVID-19

Electroencephalogram: EEG

Fluorescence Intensity: FI

Healthy donors: HD

Intensive care unit: ICU

Interleukin-6: IL-6

Interleukin-8: IL-8

Magnetic resonance imaging: MRI

Modified Rankin Score: mRS

Placental growth factor: PlGF

Severe acute respiratory syndrome coronavirus 2: SARS-CoV-2

Soluble E-selectin: sE-selectin

Soluble Fractalkine: CX3CL1

Soluble InterCellular Adhesion Molecule-1: sICAM-1

Soluble Vascular Cell Adhesion Molecule-1: sVCAM-1

Soluble Vascular endothelial growth factor receptor 2: sVEGFR2

Tumor Necrosis Factor- $\alpha$ : TNF- $\alpha$

Vascular endothelial growth factor: VEGF

Von Willebrand Factor-A2 domain: vWF-A2

## Introduction

Since December 2019, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is responsible for a worldwide outbreak of coronavirus disease 2019 (COVID-19). The most severe patients develop a systemic inflammatory phase characterised by respiratory failure and multiorgan dysfunction, with significant morbidity and mortality (Mehta *et al.* 2020). Among these complications, various central nervous system manifestations have been reported (Mao *et al.* 2020; Helms *et al.* 2020a), ranging from isolated anosmia, to COVID-19-related encephalitis (Paterson *et al.* 2020; Le Guennec *et al.* 2020; Cao *et al.* 2020). Meta-analysis have reported that the incidence of encephalitis in patients with COVID-19 was approximately around 0.6 and 2% since the beginning of the pandemic (D *et al.* 2021; He *et al.* 2021).

In patients with acute respiratory distress syndrome (ARDS) related to COVID-19, delirium and/or neurological symptoms have been reported in up to 84% of patients (Helms *et al.* 2020b). Moreover, among critically ill COVID-19 patients who had a magnetic resonance imaging (MRI) of the brain performed, 30% had a leukoencephalopathy and/or cerebral microbleeds, associated with a critical illness, worse functional outcome and higher mortality (Agarwal *et al.* 2020).

Brain biopsies and necropsies of COVID-19 related ARDS reported heterogenous findings, with brain lesions related to oxygen deprivation and severe systemic inflammation due to ARDS, such as hypoxic ischemic injury, neuronal loss and inflammatory changes (Matschke *et al.* 2020). In patients with COVID-19 related encephalitis, neuropathological examinations revealed several types of pathological lesions, including focal or diffuse perivascular parenchymal infiltrates of T-cell and macrophages, activated microglia and hypertrophic astrocytes associated with a marked axonal injury, and/or hemorrhagic white matter lesions with perivascular acute disseminated encephalomyelitis (ADEM)-like appearance (Younger 2020; Reichard *et al.* 2020; Lee *et al.* 2020). These lesions

most likely result from cerebrovascular insults, indicating that, together with the important systemic inflammation, dysfunction of the brain endothelium might play a central role in the neuropathological lesions.

Brain microvessels display specific structural and functional features, and form the blood brain barrier, which is essential to maintain the proper microenvironment for brain function and contribute to protect the brain from spontaneous bleeding and from invading pathogens (Abbott *et al.* 2010; Fisher 2013; Le Guennec *et al.* 2020). This barrier is formed by continuous non-fenestrated specialized endothelial cells expressing intercellular tight junction proteins that restrict paracellular diffusion, and specific transporters regulating the selective transport and metabolism of substances between the blood and the brain (Wolburg & Lippoldt 2002). Moreover, a thick basement membrane, the high coverage of pericytes and the astrocytic end feet processes constitute additional barriers sealing the vessels (Abbott *et al.* 2010).

To explore signs of endothelial dysfunction in COVID-19 patients, an analysis of soluble endothelial activation and angiogenic markers in plasma from COVID-19 patients has been previously performed (Smadja *et al.* 2020). High levels of the angiotensin-converting enzyme 2 (ACE2) and of soluble E-selectin (sE-selectin) were predictive of respiratory worsening and admission to intensive care unit (ICU) (Smadja *et al.* 2020). So far, no study has analysed these biomarkers in COVID-19 ARDS patients with neurological disorders, to determine whether soluble circulating endothelial and/or angiogenic markers associated with this neurological complication could predict a worse outcome.

In this study, we took advantage of our ICU specialized in neurocritically ill patients (repurposed as a COVID-ICU), which received COVID-19 patients with related encephalitis diagnosed during COVID-19 ARDS, to assess markers of endothelial cell activation in critically ill patients with or without COVID-19-related encephalitis.

## Methods

We performed a cross-sectional observational monocentric study of patients hospitalized between April 3<sup>rd</sup> and May 15<sup>th</sup>, 2020 in our ICU in the Hôpital Universitaire de la Pitié-Salpêtrière in Paris, France. All patients referred for COVID-19 related critical illness during this time frame, either for respiratory illness or to explore COVID-19-related encephalitis after COVID-19 ARDS, or both, were consecutively included. The study was exploratory, and no randomization methods were applied in the study due to its observational nature. No sample calculation was performed, and sample size was determined by the duration of the repurposing of our Neuro-ICU into a COVID-ICU (from April 3<sup>rd</sup> to May 15<sup>th</sup>, 2020). Exclusion criteria were: patients younger than 18 years old; a moribund state with mortality expected within the first 24h; critical illness related to other diagnosis than COVID-19; study refusal; absence of neurological examination until death due to neuromuscular blockers. Patients were managed in accordance with practices recommended at the time of the study during the first wave of the pandemic in France (*i.e.* without dexamethasone or tocilizumab early therapies).

### Ethics approval and consent to participate

The study was pre-registered on ClinicalTrials.gov (JOCOVID study Identifier: NCT04320017) and approved by the local ethics committee of the Sorbonne Université in Paris (reference#CER: 2020-14). To be included, the patient or their relatives must agree to participate study. The study was conducted in accordance with good clinical practices and was compliant with the European General Data Protection Regulation (GDPR) and the French Commission Nationale

de l'Informatique et des Libertés (CNIL) rules. All patients received written information about the research and consented to the use of their data. If oral consent for the use of clinic-biological parameters could not be obtained from a patient due to critical illness or in case of death, it was systematically confirmed by a relative, and in case of survival, after the critical stage by the patient itself.

### **Clinical and paraclinical definition of COVID-19 related encephalitis of critically ill patients**

COVID-19 diagnosis was based on a confirmed diagnosis of COVID-19 using real-time reverse transcription PCR SARS-CoV-2 test on nasopharyngeal swab samples or endotracheal aspirate. COVID-19 related critical illness was defined as any organ failure directly linked to COVID-19 and requiring ICU admission. Critically ill COVID-19 patients were retrospectively categorized in two groups according to whether or not they presented COVID-19 related encephalitis (Control group and Encephalitis group). Electroencephalogram (EEG) and brain MRI were motivated by changes in daily routine neurological examinations, performed by neuro-intensivists, and any unexpected event (focal neurological deficits, delirium, confusion, seizure, delayed awakening after sedation withdrawal...). Delirium was not considered as a COVID-19 related encephalitis if alone. Diagnosis of COVID-19 related encephalitis was performed by a multidisciplinary team composed of neurologists, intensivists, electrophysiologists and neuroradiologists. To be classified as COVID-19 related encephalitis (*i.e* in the Encephalitis group), patients had to have new onset of central neurologic clinical findings on patient examination not attributable to another cause, and pathological findings on either brain MRI and/or EEG. Data were collected prospectively by investigators in accordance with JOCOVID study (see above section). Follow-up was performed for 9 months. Items collected included demographic data, medical and treatment history, comorbidities, symptoms, date of COVID-19 onset and ICU admission, ICU organ support, neurological symptoms, neurological examination, date of ICU and hospital discharge, MRI and EEG. Modified Rankin Score (mRS), which measures degree of disability/dependence after acute brain injury, was performed at 9 month-follow up and was collected during physician visit and/or by telephone interview.

### **Sample preparation**

For plasma obtention process, patient samples were collected on sodium citrate tubes (Greiner Bio-One International – Vacuette Tube 3.5 ml - 9NC Coagulation Sodium Citrate 3.2%- 13x75 – ref #474327), and centrifuged twice at 2500 g for 10 min, to separate plasma supernatant, which was transferred to a plastic transport tube and kept frozen at -80 °C.

### **Blinding of samples**

Blood samples were collected and stored at patient admission in our Neuro-ICU from remaining plasma after coagulation testing, and plasmatic soluble biomarkers of endothelial cell activation were measured after patient categorization in either Control or Neuro-ICU COVID group. The experimenter, who tested the blood samples, was unaware of study groups during experimentation and during data analysis.

### **Blood sampling and data analysis**

We measured (i) plasma markers of vascular inflammation including the release of soluble endothelial cell adhesion molecules: sE-selectin, soluble InterCellular Adhesion Molecule-1 (sICAM-1), soluble Vascular Cell Adhesion Molecule-1 (sVCAM-1) and of inflammatory cytokines: Interleukin-8 (IL-8), Interleukin-6 (IL-6), soluble Fractalkine (CX3CL1), CC chemokine ligand 20 (CCL20), Chemokine Ligand-2 (CXCL2), Tumor Necrosis Factor- $\alpha$  (TNF- $\alpha$ ); (ii) angiogenic factors: Vascular endothelial growth factor (VEGF), soluble Vascular endothelial growth factor receptor 2 (sVEGFR2), Angiopoietin-1 (ANGPT1), Angiopoietin-2 (ANGPT2), soluble Endoglin, Placental growth factor (PlGF); (iii) hemostasis factors: soluble Thrombomodulin, von Willebrand Factor-A2 domain (vWF-A2); and finally (iv) a marker of cellular response to hypoxia: Angiopoietin-like-4 (ANGPTL4). These markers were measured with a Human Magnetic Luminex Assay from BioTechne (R&D system) (Catalogue number #LXSAHM), and data were analyzed within the CYBIO platform at the Cochin Institute (Paris, France) with the Bioplex 200 system (Luminex) using Bioplex Manager 6.2 software (Bio-Rad) (RRID:SCR\_014330).

Human Magnetic Luminex Assay was done on September 15<sup>th</sup>, 2020, after patient categorization in either Control or Encephalitis group. Plasma samples were measured from three healthy community donors (median age 30 years ([Full range] 25-37)) negative for SARS-CoV-2 to serve as an internal control. Results were expressed in Fluorescence Intensity (FI) and were normalized to the FI of healthy donors and given as percentage of the healthy donor (HD) group. No test for outliers was conducted and no data point was excluded.

ADAMTS13 activity (a disintegrin and metalloprotease with thrombospondin type I repeats-13), and vWF (von Willebrand Factor) activity and antigen were measured in our clinical laboratory, in parallel with other routine laboratory examinations, as previously described (Bianchi *et al.* 2002; Furlan *et al.* 1998) and reported as percentages of healthy donors activity.

### Statistical analysis

Both groups were compared for their characteristics, at their ICU admission, and at the time of the cross-sectional study (*i.e* the day of the biomarker of endothelial cell activation assay). Data are expressed as medians [25<sup>th</sup>-75<sup>th</sup> percentile]. In this descriptive study that sought to measure endothelial cell biomarkers in critically ill COVID-19 patients, we examined the differences between endothelial cell markers in patients with or without COVID-19-related encephalitis using the unpaired two-tailed Mann-Whitney U test, as samples did not satisfy the normal distribution. In the need for non-parametric analysis, data were normalized from healthy donors (HD) blood samples, which served as an internal control. Between-group comparisons were analyzed using the two-tailed Mann-Whitney U-test for continuous variables and two-sided Fischer's exact test for categorical variables. Each correlation was assessed using the Pearson's "r" coefficient correlation test. For the correlation matrix, we calculated the Pearson correlation for inter-relationships between biomarkers of endothelial cell activation for all patients, patients without encephalitis, and Encephalitis patients. The variables were clustered by minimizing the single linkage distance. A *p*-value below 0.05 was chosen for statistical significance. All statistical analyses were done using GraphPad Prism (version 8.4.2; GraphPad Software, San Diego, CA, USA). The correlation matrix was performed with Statgraphics (version 19.01.003; Statgraphics Technologies, Inc Software, The Plains, VA, USA).

## Results

### **COVID-19-related encephalitis was associated with a prolonged stay in Intensive Care Unit with no increased in mortality**

During the study period (April 3<sup>rd</sup> to May 15<sup>th</sup>, 2020), 36 patients were admitted in our ICU with a suspected diagnosis of COVID-19 (Figure 1). Among those, four were excluded; three had no neurological examination until death due to neuromuscular blocking agents use and one had a critical illness related to other diagnosis than COVID-19 with a negative SARS-CoV2 result. Of the remaining 32 patients retained for the analysis, 21 were classified in the Control group (critically ill COVID-19 patients without encephalitis), and 11 in the Encephalitis group. Of the 11 Encephalitis patients, 6 were addressed from another ICU to explore neurological symptoms and/or persistent coma after sedation withdrawal. Clinical neurological symptoms leading to COVID-19 related encephalitis diagnosis are shown in supplementary Table S1. Briefly, brainstem impairment (i.e pathological findings during cranial nerve clinical examination), movement disorder and coma after sedation withdrawal were the most frequent symptoms observed. For Encephalitis patients, detailed clinical presentations, complementary exploration findings and outcomes are available for each patient in supplementary Table S1. All Encephalitis patients were explored with EEG and Brain MRI, and 10 patients (90%) had a lumbar puncture. Brain MRI results are shown in supplementary Figure S1.

Baseline patient characteristics according to the presence or the absence of COVID-19 related encephalitis are reported in Table 1. There were no significant differences between groups for age, sex, past medical history, therapy prior to ICU admission, patient characteristics and severity scores at ICU admission.

Overall mortality during ICU stay reached 15.6% of the patients (5/32 patients), with mortality rates of 19% and 9% in the Control group and the Encephalitis group, respectively, with no statistically significant difference between groups. Increased in the length of stay in ICU was observed in Encephalitis patients compared to patients with no encephalitis, with medians length of stay of 52 [16-79] days and 20.5 [11-44] respectively. Nine-months overall follow-up mortality reached 21% (7/32 patients), with mortality rates of 19% and 27% in the Control group and the Encephalitis group, respectively, with no statistically significant difference between groups. Modified Rankin Score (mRS), which measures degree of disability/dependence after acute brain injury, measured at 9 month-follow up was at 1 [1-3] in the Control -Group and at 3 [1-6] in the Encephalitis group, with no statistically significant difference between groups.

These results suggest that COVID-19-related encephalitis is not associated with a worse functional outcome or a higher mortality, even if it is associated with a prolonged length of stay in ICU.

### **COVID-19-related encephalitis was associated with higher release of endothelial dysfunction biomarkers**

We then analyzed plasma levels of biomarkers of endothelial activation or damage/dysfunction (Table 2). A correlation matrix between endothelial cell biomarkers in the overall population was performed to assess if regulation processes usually expected in coagulopathy and inflammatory conditions were observed in this population (Supplementary Figure S2A). Among expected correlations, we found a significant negative correlation between levels of ANGPT1 and ANGPT2 ( $r=-0.39$ ,  $p<0.05$ ), two proteins with antagonistic actions on vascular permeability (Maisonpierre *et al.* 1997; Karamysheva 2008) and a significant positive correlation between levels of IL-8 and

ANGPT2 ( $r=0.46$ ,  $p<0.05$ ), two proteins released from Weibel Palade Bodies (Lowenstein *et al.* 2005), providing signs of endothelial activation and dysfunction.

Between group comparison showed statistically significant differences between Control and Encephalitis patients, with higher median plasma levels in the Encephalitis group of sE-selectin (212 [152-395] *versus* 142 [120-217] % of HD); TNF- $\alpha$  (379 [298-389] *versus* 235 [182-277] % of HD); IL-6 (1566 [798-5488] *versus* 588 [359-2289] % of HD); PIGF (155 [131-214] *versus* 131 [90-158] % of HD) and Thrombomodulin (216 [150-376] *versus* 129 [113-208] % of HD) (Figure 2). In the overall population, concentrations of sE-selectin, PIGF and Thrombomodulin were significantly correlated with TNF- $\alpha$  levels, with a Pearson correlation at respectively at  $r=0.5106$  ( $p=0.0028$ ),  $r=0.4102$  ( $p=0.0197$ ) and  $r=0.5089$  ( $p=0.0029$ ) (Figure 3), but not with IL-6 levels, with a Pearson correlation at respectively at  $r=-0.06760$  ( $p=0.7132$ ),  $r=0.03149$  ( $p=0.8642$ ) and  $r=0.07671$  ( $p=0.6765$ ) (Supplementary Figure S3), strongly suggesting of a widespread cerebral endothelial activation in response to circulating inflammatory cytokines.

In addition, Encephalitis patients had a significantly lower activity of the ADAMTS13, compared to Control patients, with a median activity respectively at 47 [24-42] *versus* 82 [72-97] per cent. This factor (also known as vWF-cleaving protease), together with vWF, plays a key role in microvascular thrombosis in COVID-19 patients (Philippe *et al.* 2021). In agreement, the median ADAMTS13 activity to vWF-A2 ratio was significantly lower in Encephalitis patients compared to Control patients, respectively at 8 [6-10] *versus* 14 [12-19], suggesting a higher risk of microvascular thrombosis in Encephalitis patients.

#### **Angiopoietin-like 4 level is a discriminant biomarker of COVID-19-related encephalitis**

Among the tested markers, we found that ANGPTL4 was increased in Encephalitis patients compared to Control patients (452 [380-614] *versus* 298 [162-352] % of HD ( $p=0.0099$ )). Interestingly, on the contrary of the other increased factors, ANGPTL4 concentrations in the overall population was not correlated with TNF- $\alpha$  levels ( $r=0.2832$ ,  $p=0.1163$ ), nor with IL-6 levels ( $r=-0.06475$ ,  $p=0.7248$ ) (Figure S3). In addition, ANGPTL4 and IL-8 levels were correlated in the Control group ( $r=0.63$ ,  $p<0.05$ , Supplementary Figure S2B), but not in the Encephalitis group (" $r$ " is not significantly different from zero,  $p>0.05$ , Supplementary Figure S2C).

As ANGPTL4 is described to be increased during hypoxia, we have investigated if ANGPTL-4 was correlated to the acute respiratory distress syndrome (*i.e.* PaO<sub>2</sub>/FiO<sub>2</sub> ratio), or with the procoagulant state of the patient (Fibrinogen level, and d-dimers level) (Supplementary Figure S2 A, B and C). None of these factors were correlated with ANGPTL-4 in the Control group or in the Encephalitis group. These results suggest that ANGPTL4 was the most relevant factor to predict encephalitis.

## **Discussion**

The pathophysiology of COVID-19 related encephalitis is still unclear and debated, as multifactorial mechanisms might account for it. Endothelial cell dysfunction and impaired microcirculatory function contribute markedly to life-threatening complications of COVID-19, and neuropathological examination of critically ill COVID-19 patients have

frequently found perivascular findings with leukoencephalopathy and/or cerebral microbleeds (Agarwal *et al.* 2020). In this study, we found that COVID-19 related encephalitis in patients with brain MRI findings and/or neurological impairment is associated with increased levels of soluble endothelial markers of activation/dysfunction, which correlated with levels of inflammatory cytokines, while soluble ANGPTL4 was associated with COVID-19-related encephalitis.

It is important to point out that this study has been performed during the first wave of the pandemic and, as a matter of fact, prior to new therapeutic recommendations, which appeared during the second wave enjoining to treat COVID-19 at the early stage of respiratory onset with dexamethasone in patients requiring oxygen above 3 liters per minutes (Horby *et al.* 2021), and with tocilizumab in patients without clinical improvement 48h after dexamethasone and/or requiring ICU (RECOVERY 2021). However, a recent meta-analysis has shown that, despite these novel recommendations, the incidence of encephalitis in COVID-19 patients remains around 2% (He *et al.* 2021), hence highlighting that this neurological complication remains a matter of grave concern at the time of this writing.

In agreement with a previous published study, we observed that COVID-19-related encephalitis was associated with a longer ICU-stay with no increase in mortality (Helms *et al.* 2020b). This longer length of stay in patients with encephalitis might be the consequence of a longer phase of awakening after sedation withdrawal and the neurological disability associated with a more difficult mechanical ventilation weaning phase.

As the definition of COVID-19 related encephalitis in critically ill patients is not clear in literature (Helms *et al.* 2020b; Ellul *et al.* 2020), in this study we did not consider as Encephalitis patients those presenting with isolated ICU-related delirium without other focal neurological signs, in order to avoid a overestimation of neurological symptoms in the overall population. Even with this tight definition of COVID-19 related encephalitis, our results were not associated with increased in ICU mortality nor with long-term functional outcome alteration. These findings therefore suggest that the occurrence of COVID-19-related encephalitis after COVID-19 ARDS should not motivate any limitation in active patient care. Although Encephalitis patients had a worse mRS median score at 9 month-follow-up compared to Control patients, we did not find any statistically significant differences in between group comparison for this outcome. However, we had only assessed the 9 months outcome of our patients by mRS scoring, and an extensive long-term analysis with cognitive assessment is mandatory to specifically assess long-term functional outcome of this population.

Regarding patient blood examinations, we found that prominent biomarkers of endothelial activation or dysfunction/damage were significantly elevated in the plasma of Encephalitis group compared to Control group. This observation is consistent with the higher prevalence of cerebrovascular lesions and vascular neuropathological findings observed in severe COVID patients (Matschke *et al.* 2020; Agarwal *et al.* 2020; Younger 2020; Reichard *et al.* 2020; Lee *et al.* 2020). The high correlation between endothelial markers and TNF- $\alpha$  levels strongly suggests that encephalitis during or after COVID-ARDS is a cytokine-associated acute brain dysfunction related to sepsis. This hypothesis is reinforced by recent observations of high levels of proinflammatory cytokines (TNF- $\alpha$ , IL-8 and IL-6) in the cerebrospinal fluid of Neuro-COVID patients suggesting a cytokine-release syndrome as the main inflammatory mechanism of SARS-CoV-2 related encephalitis (Pilotto *et al.* 2021; Bernard-Valnet *et al.* 2021).

To date, autopsy series have mostly reported a systemic endothelitis, with capillary damages particularly within the pulmonary and the renal vasculature (Carsana *et al.* 2020; Ackermann *et al.* 2020; Varga *et al.* 2020; Mauri

*et al.* 2020). SARS-CoV-2 invades host cells through the angiotensin-converting enzyme 2 receptor, Neuropilin-1 and CD147/Basigin, which are largely expressed in endothelial cells (Daly *et al.* 2020; Ferrario *et al.* 2005; Monteil *et al.* 2020a; Qanadli *et al.* 2020; Wang *et al.* 2020). Hence, there is direct evidence that SARS-CoV-2 can infect endothelial cells (Varga *et al.* 2020; Trevisan *et al.* 2021; Monteil *et al.* 2020b). SARS-CoV-2 elements were detected in endothelial cells of COVID-19 patients, together with an accumulation of inflammatory cells and evidence of endothelial and inflammatory cell death (Varga *et al.* 2020). As the endothelium is a major actor of cytokine synthesis and release (Ranta *et al.* 1999; de Vries *et al.* 1996), its infection and/or activation might take part in the process leading to the systemic inflammatory phase and to the capillary leak syndrome observed in ARDS (Maniatis *et al.* 2008). Brain lesions might occur secondary and seem to be related to oxygen deprivation and to the severe systemic inflammation due to ARDS, with hypoxic-ischemic injury, neuronal loss and inflammatory changes (Matschke *et al.* 2020; Singh *et al.* 2021; Thepmankorn *et al.* 2021).

Of most interest, we found that ANGPTL4 was at significantly higher levels in Encephalitis patients compared to Control patients, and, in contrary to the other increased factors, ANGPTL4 levels were not correlated with TNF- $\alpha$ , IL-6 nor IL-8 levels, suggesting that ANGPTL4 production was associated with cerebral lesions. Previous studies have reported that ANGPTL4 was increased during ARDS (Hu *et al.* 2021; Li *et al.* 2015). Similarly to what was observed in these studies, we also found here that ANGPTL-4 was markedly increased in the ARDS group without encephalitis (Figure 2). This secreted glycoprotein of the angiopoietin-like family is known to be upregulated when cells are exposed to severe hypoxia (Galaup *et al.* 2012; Huang *et al.* 2011b; Galaup *et al.* 2006), and it is produced by vascular cells in ischemic pathologies (Galaup *et al.* 2012; Huang *et al.* 2011b; Galaup *et al.* 2006). Recent studies indicate that ANGPTL4 exhibits multifunctional properties; it regulates tumorigenesis, angiogenesis, vascular permeability, glucose homeostasis, lipid metabolism, cell differentiation, energy homeostasis and inflammation (Yang *et al.* 2018). To date, its role in vascular integrity remains unclear. Several studies have shown that ANGPTL4 promotes destabilization of the endothelial cell junctions. This effect was attributed to its C-terminal fibrinogen-like domain, which can interact with many receptors, including integrins or junctional proteins, thereby promoting integrin signalling and disruption of intercellular VE-cadherin and claudin-5 clusters (Padua *et al.* 2008; Huang *et al.* 2011a; Tan *et al.* 2012; Yang *et al.* 2018). On the other hand, ANGPTL4 was shown to inhibit acute VEGF- or histamine-induced vascular hyperpermeability (Zhang *et al.* 2017; Galaup *et al.* 2006; Bouleti *et al.* 2013) and to stabilize cell junctions by modulating VEGF/VEGFR2 signalling and providing maintenance of the VEGFR2/VE-cadherin complex at adherent junctions (Bouleti *et al.* 2013 ; Gomez Perdiguero *et al.* 2016), suggesting it might be a key modulator of vascular function. Interestingly, ANGPTL4 was shown to reduce cerebral leakage following a brain ischemic stroke in a mice model, indicating that it could preserve cerebral vascular integrity (Zhang *et al.* 2017; Bouleti *et al.* 2013). It is then tempting to speculate that the higher levels of ANGPTL4 measured in Encephalitis patients could be linked to a physiological biological repair process contributing to stabilize endothelial intercellular junctions during brain injury due to COVID-19 ARDS.

Altogether, this study suggest that ANGPTL-4 dosage associated with clinics, brain imaging, and EEG could participate in the diagnosis of physicians and their decision to start therapy such as plasma exchange, corticosteroids or intravenous immunoglobulins in these patients, as neurological responses with good outcomes have been reported

with these treatments in several case series (Cao *et al.* 2020; Manganotti *et al.* 2021; Freire-Álvarez *et al.* 2020; Dogan *et al.* 2020).

Our study has several limitations including its small sample size, its monocentric nature, and the fact that our measurements were performed once during the patients' ICU stay. Moreover, 3 patients in the Control group had higher value of ANGPTL4 (Figure 2), including 2 outliers patients with values above all Encephalitis patients. As a point of interest, these 2 patients, free from any other neurological complication, later died in ICU.

Further studies with serial sampling over time until ICU discharge or death are mandatory to confirm our findings and to assess if elevated ANGPTL4 levels in COVID-19 related encephalitis is a cause or a consequence of brain injuries/neurological dysfunctions observed in these patients.

## Conclusion

Blood biomarkers of endothelial cell activation are more elevated in critically ill COVID-19 patients with encephalitis and are correlated with pro-inflammatory cytokine levels. Therefore, vascular alterations and associated lesions observed in COVID-19 related encephalitis in patients with brain MRI findings and/or neurological impairment following a COVID-19 ARDS might be a consequence of the cytokine storm, putting into question the specificity of COVID-related neurological impairment after ARDS.

ANGPTL4 was the only elevated marker found in critically ill COVID-19 patients with encephalitis, which was not correlated to systemic inflammation, suggesting an association with cerebrovascular lesions. However, our study design did not allow us to answer whether ANGPTL4 is a cause, or a consequence of brain injuries observed in these patients. In the hypothesis that ANGPTL4 is involved in the repair process, it may represent an interesting molecule to prevent or treat vascular damages or to restore vessel integrity after COVID-19-related brain injury. Further investigations in a larger cohort are warranted to assess if this factor could be a surrogate marker of encephalitis in critically ill COVID-19 patients.

## Declarations:

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**Competing interests:** Authors have no conflicts of interest to declare in relationship with this manuscript.

**Data availability:** The author has full access to all the data; and have the right to publish all data separate and apart from any sponsor.

**Authors' contributions:** V.A, S.B. and L.L.G. were responsible for the study conception and design, performed the data analysis, and were responsible for drafting of the manuscript; J.Z measured and analysed plasma markers; C.F., J.E.S., N.W., A.C., C.M., B.R., and S.D. made critical revisions to the manuscript for important intellectual content. C.F. organised the sample collection.

## Figure legends

**Table 1: Admission characteristics in the first ICU and outcomes of COVID-19 critically ill patient according to neurological complication status**

**Table 2: Biological parameters of Covid-19 patients according to neurological complication status**

**Figure 1: Flowchart of the study.**

**Figure 2: Comparisons of biomarkers of endothelial cell activation and of inflammation in Critically ill COVID-19 patient with or without neurological impairment.** Datapoints indicate individual measurements, whereas horizontal bars show median (IQR) for sE-selectin, PIGF, TNF-  $\alpha$ , sThrombomodulin, Interleukin-6 and

Angiopoietin-like-4. Results were expressed in Fluorescence Intensity (FI) and were normalized to the FI of healthy donors and given as percentage of the healthy donor (HD) group.

Abbreviations: n, number of patients; sE-selectin, soluble Endothelial-selectin; PlGF, Placental growth factor; TNF  $\alpha$ , Tumor Necrosis Factor- $\alpha$ ; sThrombomodulin, soluble Thrombomodulin; IL-6, Interleukin-6; ANGPTL4, Angiopoietin-like-4

**Figure 3: Correlations between plasma TNF- $\alpha$  and (A) sE-selectin levels; (B) PlGF levels (C) Angiopoietin-like-4 levels and (D) thrombomodulin levels in all critically ill COVID-19 patients.** r for Pearson correlation coefficient.

Abbreviations: n, number of patients; Tumor Necrosis Factor- $\alpha$  ; sE-selectin, soluble Endothelial-selectin; PlGF, Placental growth factor ; ANGPTL4, Angiopoietin-like-4; sThrombomodulin, soluble Thrombomodulin; Results were expressed in Fluorescence Intensity (FI) and were normalized to the FI of healthy donors and given as percentage of the healthy donor (HD) group.

## Supplementary Material

**Supplementary Table 1: Clinical and complementary explorations findings in the Encephalitis group**

**Supplementary Figure 1: Encephalitis patients with Brain MRI findings**

Patient #1: (A) Deep hemispherical white matter lesions on T2/FLAIR with (B) gadolinium enhancement on T1. (C) Left thalamic microhemorrhage on SWAN.

Patient #2: (A) Several right hemispherical deep and supratentorial white matter lesions on T2/FLAIR with (B) gadolinium enhancement on T1.

Patient #3: (A) Pontine lesions on T2/FLAIR with (B) multiple pontine microhemorrhages on SWAN. Bilateral insular lesion on T2/FLAIR with (D) gadolinium enhancement on T1.

Patient #5: (A) Several confluent periventricular and deep supratentorial white matter lesions on T2/FLAIR with (B, C) gadolinium enhancement on T1

Patient #6: (A) Bilateral diffuse lesions of the deep subcortical white matter on T2/FLAIR without gadolinium enhancement on T1 (not shown). (B) Multiple microhemorrhages of the corpus callosum on SWAN,

Patient #7: (A) Right hemispherical supratentorial white matter lesions on T2/FLAIR and (B) DWI with low ADC, without gadolinium enhancement on T1 (not shown).

Patient #10: (A) Several confluent periventricular and deep supratentorial white matter lesions on T2/FLAIR without gadolinium enhancement on T1 (not shown).

**Supplementary Figure 2:** (A) Correlation matrix between biomarkers of endothelial cell activation of all patients. Shades of red indicates a positive correlation. The variables were clustered by minimizing the single linkage distance; (B) Correlation matrix between biomarkers of endothelial cell activation of Control patients. Shades of red indicates a positive correlation. The variables were clustered by minimizing the single linkage distance; (C) Correlation matrix between biomarkers of endothelial cell activation of Encephalitis patients. Shades of red indicates a positive correlation. The variables were clustered by minimizing the single linkage distance.

**Supplementary Figure 3:** Correlations between plasma Interleukin-6 and (A) sE-selectin levels; PIGF levels (C) Angiopoietin-like-4 levels and (D) thrombomodulin levels in all critically ill COVID-19 patients.  $r$  for Pearson correlation coefficient.

Abbreviations: n, number of patients; Interleukin-6, IL-6 ; sE-selectin, soluble Endothelial-selectin; PIGF, Placental growth factor ; ANGPTL4, Angiopoietin-like-4; sThrombomodulin, soluble Thrombomodulin; Results were expressed in Fluorescence Intensity (FI) and were normalized to the FI of healthy donors and given as percentage of the healthy donor (HD) group.

**Table 1. Admission characteristics in the first ICU and outcomes of COVID-19 critically ill patient according to neurological complication status**

Characteristics	Control group (n =21)	Encephalitis group (n =11)	p value
Age, years	58 [51-68]	60 [51-67]	0.7355
Females (%)	4 (19)	2 (18)	0.9525
Body mass index, kg/m <sup>2</sup>	25.8 [23-31]	28.7 [26-29]	0.1527
Medical history (%)			
Hypertension	8 (38)	5 (45)	0.6872
Diabetes	2 (9.5)	4 (36)	0.0647
Previous history of stroke	1 (4.7)	1 (9)	0.6309
Ischemic cardiomyopathy	3 (14)	2 (18)	0.7374
Atrial fibrillation	1 (4.7)	0	0.4621
Chronic kidney disease	3 (14)	3 (27)	0.1891
Kidney transplant	1 (4.7)	1 (9)	0.6309
Heart transplant	1 (4.7)	0	0.4621
Cancer	1 (4.7)	0	0.4621
Treatments (%)			
Antiplatelet drug	6 (28)	2 (18)	0.5191
Anticoagulant drug	1	0	0.4621
β-blocker	7 (14)	3 (27)	0.7254
Angiotensin conversion enzyme inhibitor	1 (4.7)	3 (27)	0.0674
Antagonist of angiotensin 2 receptor	5 (23)	1 (9)	0.3110
Calcium channel blockers	4 (19)	2 (18)	0.9525
Oral antidiabetic agents	2 (9.5)	4 (36)	0.0647
Therapy prior ICU admission (%)			
Hydroxychloroquine	4 (19)	2 (18)	0.9525
Lopinavir–ritonavir	2 (9.5)	0	0.2905
Tocilizumab	1 (4.7)	1 (9)	0.6309
Reason for ICU (%)			
Acute respiratory failure	18 (100)	10 (90)	0.9347
Status epilepticus	0	1 (9)	0.1604
At ICU admission			
SAPS II score	52 [49-58]	58 [51-68]	0.1251
SOFA score	5 [4-7]	7 [5-9]	0.1112
Organ failure (%)			
Respiratory	18 (86)	10 (91)	0.9347

Circulation	7 (33)	4 (36)	0.8639
Hepatic	0	0	
Renal	7 (33)	4 (36)	0.8639
Haematological	0	1 (9)	0.1604
Neurological	0	1 (9)	0.1604
Left ventricular ejection fraction <sup>b</sup>	60 [50-60]	45 [40-54]	0.1697
Velocity time integral <sup>b</sup>	19 [15-24]	19 [15-25]	0.8969
<b>During ICU stay</b>			
Duration before neurological event, days	–	15 [8-25]	
Invasive mechanical ventilation (%)	18 (86)	11 (100)	0.2782
Acute respiratory distress syndrome <sup>a</sup> (%)	15 (71)	9 (81)	0.5191
Prone positioning (%)	13 (61)	6 (54)	0.6872
Renal replacement therapy (%)	5 (23)	5 (45)	0.2096
Vasopressor use (%)	18 (86)	10 (90)	0.9347
Thrombotic event in ICU (%)	6 (28)	3 (27)	0.9381
ICU length of stay, days	20.5 [11-44]	52 [16-79]	0.0442
ICU mortality	4 (19)	1 (9)	0.4613
<b>Follow-up</b>			
9 months follow-up mortality	4 (19)	3 (27)	0.5929
9 months mRS score	1 [1-3]	3 [1-6]	0.1772

Results are expressed as number (%) or median [27<sup>th</sup>;75th percentile]. Abbreviations: n, number of patients; ICU, Intensive Care Unit; SAPS, Simplified Acute Physiology Score; SOFA, sequential organ failure assessment; mRS, Modified Rankin Scale.

<sup>a</sup>According to Berlin criteria [ADT Force *et al*, JAMA, 2012] <sup>b</sup>Values are missing for 16 patients: 9 with no encephalitis, 7 with encephalitis.

**Table 2. Biological parameters of Covid-19 patients according to neurological complication status**

Biological parameters	Control group (n =21)	Encephalitis group (n =11)	p value
<b>At ICU admission</b>			
Fibrinogen (g/l)	6.9 [5.7-8.3]	6.2 [5.2-7.7]	0.3566
D-dimers (ng/ml)	2690 [1275-4675]	2320 [1430-6710]	0.7598
<b>The day of endothelial cell biomarker measurement</b>			
Delay between first ICU admission and measurement, day	12 [3-25]	16 [5-33]	0.3500
PaO <sub>2</sub> /FiO <sub>2</sub> Ratio	228 [164-301]	222 [125-315]	0.8768
<b>Adhesion molecules (% of Healthy donors):</b>			
sE-selectin	142 [120-217]	212 [152-395]	0.0355
sICAM-1	190 [123-256]	209 [152-251]	0.6060

<b>sVCAM-1</b>	335 [186-806]	349 [295-930]	0.5256
<b>Cytokines (% of Healthy donors):</b>			
<b>TNF-<math>\alpha</math></b>	235 [182-277]	379 [298-389]	0.0002
<b>sFractalkin</b>	135 [80-186]	194 [99-233]	0.2579
<b>Interleukin-8</b>	278 [173-455]	347 [260-772]	0.2113
<b>Interleukin-6</b>	588 [359-2289]	1566 [798-5488]	0.0430
<b>CCL20</b>	538 [243-980]	749 [413-2916]	0.0809
<b>CXCL2</b>	184 [94-256]	165 [146-195]	0.8117
<b>Vascular permeability (% of Healthy donors):</b>			
<b>VEGF</b>	921 [400-2300]	2062 [640-3240]	0.2244
<b>sVEGFR2</b>	83 [58-105]	79 [63-138]	0.7812
<b>PIGF</b>	131 [90-158]	155 [131-214]	0.0306
<b>Angiopoietin-1</b>	245 [134-525]	303 [128-474]	0.9525
<b>Angiopoietin-2</b>	173 [141-354]	264 [152-713]	0.3410
<b>Angiopoietin-like-4</b>	298 [162-352]	452 [380-614]	0.0099
<b>sEndoglin</b>	83 [71-105]	95 [86-117]	0.1212
<b>Hemostasis regulation:</b>			
<b>sThrombomodulin (% of Healthy donors)</b>	129 [113-208]	216 [150-376]	0.0473
<b>vWF-A2 (% of control)</b>	603 [479-659]	544 [419-659]	0.2840
<b>ADAMTS13 activity (%)<sup>a</sup></b>	82 [72-97]	47 [24-42]	0.0185
<b>Platelets count (/mm<sup>3</sup>)</b>	270 [218-425]	293 [156-375]	0.7107
<b>Lactate dehydrogenase (UI/l) <sup>b</sup></b>	342.5 [281-528]	358 [266-620]	0.2411
<b>Haptoglobin (g/l)</b>	2.57 [1.76-4.47]	3 [2.5-4.6]	0.9138
<b>Fibrinogen (g/l)</b>	6.6 [5-8]	6.7 [5-7]	0.9052
<b>D-dimers (ng/ml)</b>	1150 [1295-3865]	2285 [1700-3740]	0.4382
<b>vWF activity (%)</b>	334 [261-390]	276 [183-493]	0.8571
<b>vWFAntigen (UI/ml)</b>	395 [294-487]	477 [344-615]	0.2910
<b>Ratios of:</b>			
<b>Angiopoietin-1: Angiopoietin-2</b>	1.3 [0.5-3.2]	2.2 [0.6-8.3]	0.5786
<b>VEGF: VEGFR2</b>	936 [487-2897]	2330 [404-4809]	0.3023
<b>ADAMTS13: vWF-A2</b>	14 [12-19]	8 [6-10]	0.0044

Results are expressed as median [27<sup>th</sup>;75<sup>th</sup> percentile]. Abbreviations: n, number of patients; ICU, Intensive Care Unit; FI, Fluorescence Intensity; sE-selectin, soluble Endothelial-selectin; VEGF, Vascular endothelial growth factor; sVEGFR2, soluble Vascular endothelial growth factor receptor 2; sEndoglin, soluble Endoglin; PIGF, Placental growth factor; sICAM-1, soluble InterCellular Adhesion Molecule-1; sVCAM-1, soluble Vascular Cell Adhesion Molecule-1; vWF-A2, von Willebrand Factor-A2 domain; sThrombomodulin, soluble Thrombomodulin; sFractalkin, soluble Fractalkin; CCL20, CC chemokine ligand 20; CXCL2,

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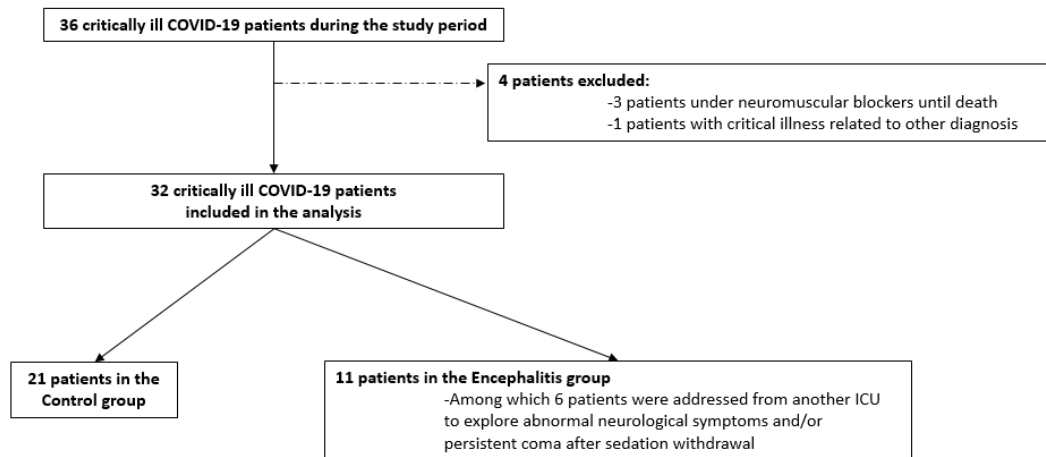
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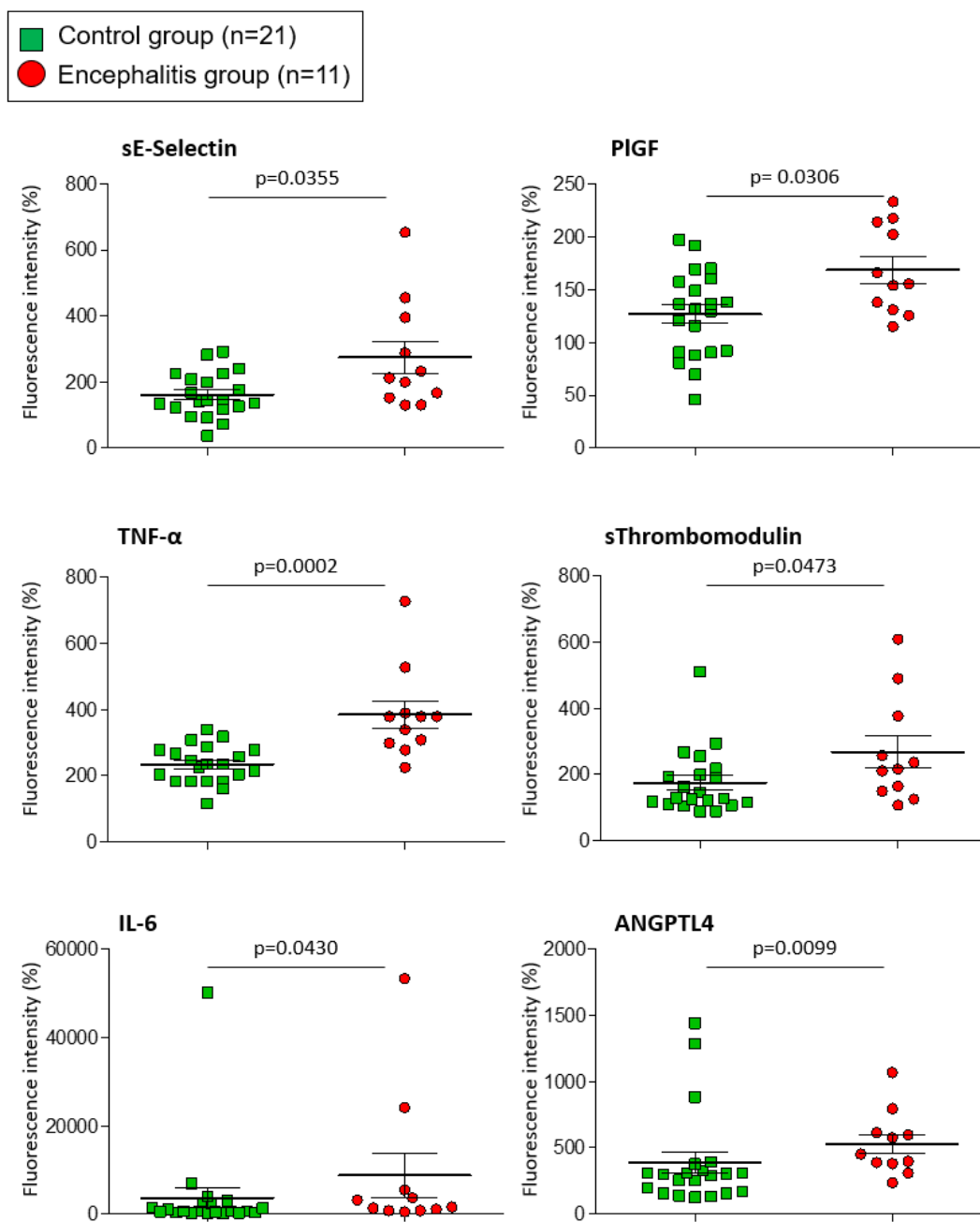
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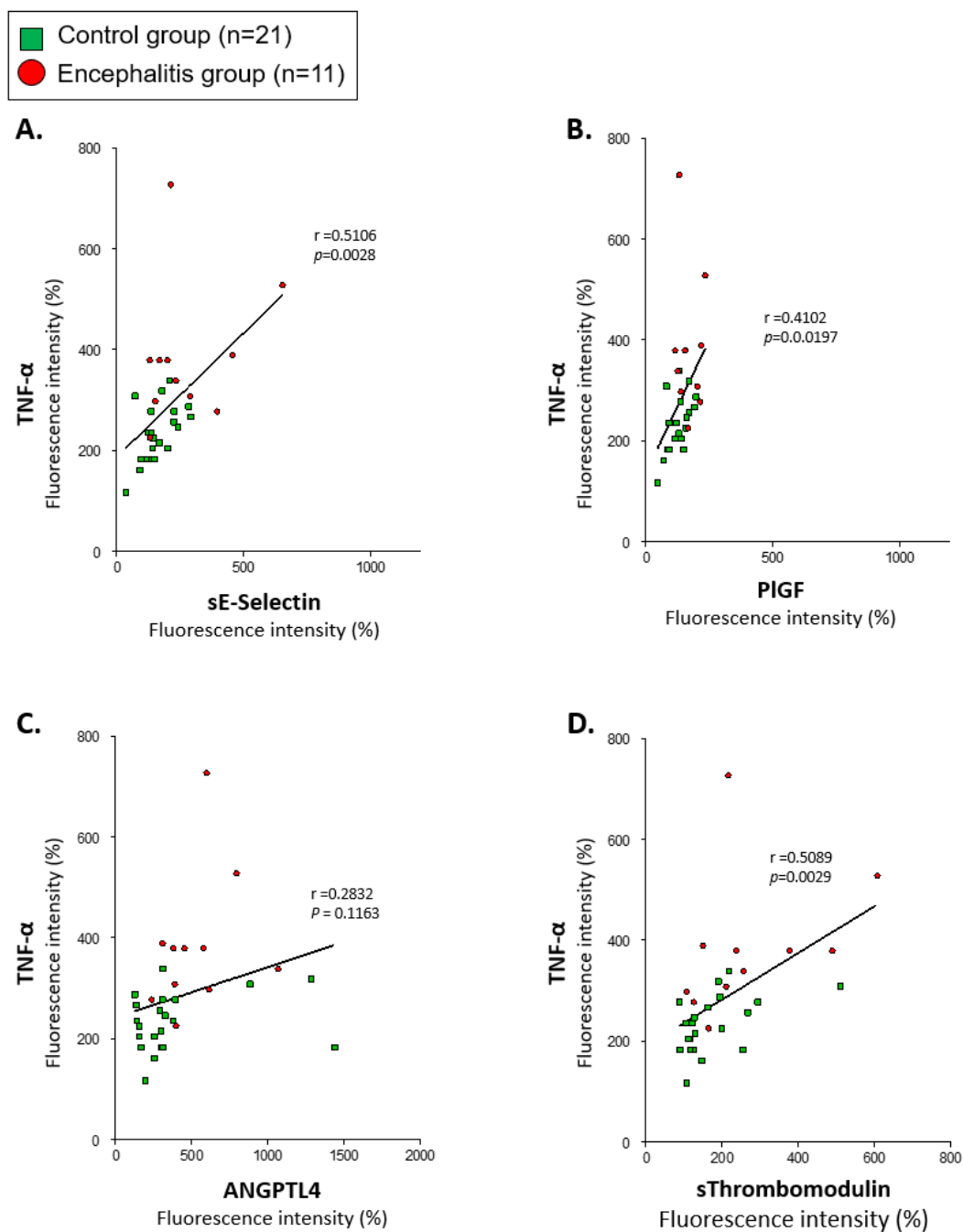
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