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Imbalance between alpha-1-antitrypsin and interleukin 6 is associated with in-hospital mortality and thrombosis during COVID-19

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ABSTRACT

Thrombosis is a hallmark of severe COVID-19. Alpha-1-antitrypsin (AAT), an inflammation-inducible serpin with anti-inflammatory, tissue protective and anticoagulant properties may be involved in severe COVID-19 pathophysiology including thrombosis onset. In this study, we examined AAT ability to predict occurrence of thrombosis and in-hospital mortality during COVID-19. To do so, we performed a monocentric cross-sectional study of 137 hospitalized patients with COVID-19 of whom 56 (41%) were critically ill and 33 (22.4%) suffered from thrombosis during hospitalization. We measured AAT and IL-6 plasma levels in all patients and phenotyped AAT in a subset of patients with or without thrombosis paired for age, sex and COVID-19 severity. We observed that AAT levels at admission were higher in both non-survivors and thrombosis patients than in survivors and non-thrombosis patients. AAT: IL-6 ratio was lower in non-survivors and thrombosis patients. In a logistic regression multivariable analysis model adjusted on age, BMI and D-dimer levels, a higher AAT: IL-6 was a protective factor of both in-hospital mortality (Odds ratio, OR: 0.07 95%CI [0.02−0.25], $p < 0.001$) and thrombosis (OR 0.36 95%CI [0.14 −0.82], $p = 0.02$). AAT phenotyping did not show a higher proportion of AAT abnormal variants in thrombosis patients.Our findings suggest an insufficient production of AAT regarding inflammation intensity during severe COVID-19. AAT appeared as a powerful predictive marker of severity, mortality and thrombosis mirroring the imbalance between harmful inflammation and protective counter-balancing

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mechanism in COVID-19. Restoring the balance between AAT and inflammation could offer therapeutic opportunities in severe COVID-19.

1. Introduction

The coronavirus disease 2019 (COVID-19) pandemic is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). In most patients, the clinical course of COVID-19 is limited to mild to moderate symptoms. However, some patients develop a progressive respiratory failure that requires cardio-respiratory support and potentially leads to death [1]. Severe COVID-19 is associated with an overwhelming inflammatory response causing widespread alveolar and endothelial lesions that trigger unregulated coagulation activation and subsequent *in situ* micro-and macro-thrombosis, which likely contribute to the hypoxemia and transition to respiratory failure [2,3]. Consistently, increased levels of circulating biomarkers reflecting systemic inflammation and coagulation activation (e.g., interleukin 6 [IL-6] and D-dimer) are independently associated with poor outcomes and a greater risk of respiratory failure, thrombosis and death [4,5].

Alpha-1-antitrypsin (AAT) is the most abundant serine protease inhibitor in human plasma that plays an important immunomodulatory and tissue-protective role. This multifunctional protein can protect against inflammation and has also anticoagulant effects. As a result, AAT inborn deficiency is associated with increased risk of chronic obstructive pulmonary disease, liver disease and venous thrombosis [6]. During COVID-19, an inappropriate AAT acute phase response to inflammation (resulting in a decreased AAT:IL-6 ratio) has been recently highlighted in a limited cohort of COVID-19 patients [7]. The objective of the present study is to assess to which extent the balance between AAT and IL-6 levels at admission is predictive of in-hospital mortality and thrombosis in a cohort of 137 hospitalized patients with COVID-19.

2. Materials and methods

2.1. Study design and participants

We performed a monocentric cross-sectional study of adult (≥18 years old) COVID-19 hospitalized patients in the French European Georges Pompidou hospital between March 13 and April 10, 2020. The study was performed in accordance with the Declaration of Helsinki. All patients included, or their trusted relatives signed a written consent form at the time of enrollment (NCT04624997). All included patients had a diagnosis of SARS-CoV-2 infection confirmed by a RT-PCR test on nasopharyngeal swab samples (Allplex™ 2019-nCoV Assay, Seegene, SK). Patients were classified according to World Health Organization (WHO) guidance as non-critical (i.e., requiring oxygen supplementation WHO score range 5–6) or critical (i.e., requiring mechanical ventilation; WHO score range 7–9) based on their clinical condition on the day of their hospitalization [8]. All non-critical patients were initially admitted in medical wards and all critical patients were immediately treated in intensive care unit following their admission. Patient characteristics, including age, sex, comorbidities and treatment at admission, were recorded in the REDCAP software (Vanderbilt University, USA). The primary outcome was COVID-19 in-hospital mortality, and the secondary outcome was the occurrence of symptomatic venous thrombosis, defined as either pulmonary embolism, deep vein thrombosis, or jugular thrombosis throughout hospital stay.

2.2. Procedures

Blood sampling using 129 M trisodium citrate tubes was performed at hospital admission (within 48h following hospitalization). AAT circulating plasma concentration was measured using an immunonephelometric assay on IMMAGE 800 analyzer (Beckman-Coulter, USA). AAT genotype was performed using Taqman allelic discrimination fluorescent probes (Thermofisher, France) and AAT phenotype analysis was performed by isoelectrofocusing agarose gels with specific immunological detection using the Hydragel 18 AAT Isofocusing kit with the semiautomatic Hydrasys System (Sebia, France), as described previously [9]. Phenotypes of serum A1AT samples were determined by comparing their migration patterns with control samples allowing the identification of the most frequent AAT alleles: the common M alleles and the two variant S and Z alleles associated with severe AAT deficiency. Elastase inhibitory capacity (EIC) was determined by a kinetic spectrophotometric method, as described previously [10]. IL-6 plasma levels were measured using a magnetic beads assay analyzed on a BioPle×2200 analyzer (Bio-Rad, USA).

2.3. Statistical analyses

Continuous data were expressed as median (interquartile range, IQR) and categorical data as proportions. Continuous and categorical variables were compared using the Mann-Whitney test and the Fisher exact test, respectively. Spearman rank coefficient correlation was used to determine the correlation between AAT levels and inflammatory biomarkers IL-6 and CRP. In the multivariate analysis, we assessed the association between admission AAT:IL-6 ratio and clinical endpoints (in-hospital mortality and thrombosis) using the logistic regression model adjusted for age, male gender, body mass index (BMI) and D-dimer level. All analyses were two-sided, and a p-value<0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism version 9.0.0 for Windows (GraphPad Software, USA).

3. Results

3.1. Study population

The cohort consisted of 137 hospitalized COVID-19 patients (93 males and 44 females) with a median age of 63 years (range: 23 to 99). 56 (41%) patients were classified as critical and 81 (59%) as non-critical based on their admission clinical severity. COVID-19 patients had higher BMI (median 28.5 kg/m^2, IQR 26.0–33.6) than non-critical patients (25.8 kg/m^2, 23.6–28.8, $p < 0.001$), while age and sex ratios were not significantly different between the two groups. As expected, most patients had underlying co-morbidities of which the most frequent were hypertension (54.0%), dyslipidemia (27.0%), diabetes (26.3%), and chronic kidney failure (16.1%). Patients' clinical characteristics, comorbidities, biological characteristics and outcomes are summarized in Table 1. Detailed characteristics about patients hospitalization (duration of hospitalization, delay between hospitalization and death and proportion of patients admitted to ICU even later during hospitalization) are also summarized in Table 1.

Table 1
Patients clinical characteristics, comorbidities, clinical outcomes and biological parameter levels according to COVID-19 severity. BMI: Body mass index; VTE: Venous thromboembolism; AAT: Alpha-1 antitrypsin; IL-6: Interleukin 6; CRP: C-reactive protein.

	Non-critical (n = 81)	Critical (n = 56)	p-value
Male sex – n (%)	53 (65.4)	40 (71.4)	0.58
Age – years	65.0 [53.0–78.0]	62.0 [53.0–71.0]	0.14
Median – [IQR]			
BMI – kg/m^2	25.8 [23.6–28.8]	28.5 [26.0–33.6]	**<0.001**
Median – [IQR]			
Delay between first symptoms and hospitalization—days	5.0 [3.0–10.0]	7.0 [6.0–10.0]	0.03
Median – [IQR]			
Comorbidities			
Obesity—n (%)	27 (33.3)	24 (42.9)	0.28
Hypertension—n (%)	44 (54.3)	30 (53.6)	0.98
Dyslipidemia—n (%)	22 (27.2)	17 (30.4)	0.70
Diabetes—n (%)	19 (23.5)	16 (28.6)	0.55
Chronic kidney disease —n (%)	17 (21.0)	10 (17.9)	0.83
Active malignancy—n (%)	10 (12.3)	3 (5.36)	0.24
History of VTE—n (%)	5 (6.17)	3 (5.36)	1
Biological parameters			
AAT – g/L	1.57 [1.37–1.80]	2.30 [1.90–3.21]	**<0.001**
Median - IQR			
IL-6 – pg/mL	13.5 [5.5–22.9]	51.0 [26.4–190.2]	**<0.001**
Median - IQR			
CRP – pg/mL	94.1 [49.3–123.4]	207.6 [123.1–270.0]	**<0.001**
Median - IQR			
D-Dimer – ng/mL	999 [730–1748]	1542 [1100–2241]	**<0.001**
Median - IQR			
Clinical outcomes			
Length of hospitalization—days median [IQR]	**11.0 [4.5–16.0]**	**28.0 [15.3–42.3]**	**<0.001**
ICU admission—n (%)	**9 (11.1)**	**56 (100)**	**<0.001**
Mechanical ventilation —n (%)	9 (11.1)	56 (100)	**<0.001**
Delay between hospitalization and death—days	**13.5 [9.0–16.0]**	**14.5 [8.0–22.0]**	**0.81**
Median – [IQR]			
In-hospital mortality—n (%)	6 (7.41)	24 (42.9)	**<0.001**
Thrombosis —n (%)	8 (9.88)	25 (44.6)	**<0.001**

3.2. Critical COVID-19 patients display an inappropriate AAT response to inflammation

At admission, critical patients displayed significantly higher AAT plasma levels (median 2.30 g/L, IQR 1.90–3.21) than non-critical patients (1.57 g/L, 1.37–1.80, $p < 0.001$, Fig. 1-A). Critical patients displayed significantly higher CRP (median 208 mg/L, IQR 123–270) and IL-6 (51.0 pg/mL, 26.4–190.2) levels in comparison with non-critical patients (94 mg/L, 49–123, $p < 0.001$; 13.5 pg/mL, 5.5–22.9, $p < 0.001$ for CRP and IL-6 respectively). AAT levels were strongly correlated with both IL-6 levels and CRP ($r = 0.49$ and $r = 0.45$, respectively, $p < 0.001$ for both), consistent with the onset of an acute AAT response to inflammation.

However, AAT: IL-6 ratio at admission was significantly lower in critical patients (median 0.04, IQR 0.01–0.09) than non-critical patients (0.13, 0.07–0.30, $p < 0.001$), in favor of a blunted AAT response to inflammation in critical patients (Fig. 1-B). Thus, at the same IL-6 level, there was approximately three times more circulating AAT in non-critical patients than in critical patients.

3.3. AAT: IL-6 ratio is highly predictive of in-hospital mortality

We assessed the predictive value of admission AAT level to discriminate between the 107 COVID-19 survivors (78%) and 30 non-survivors (22%) in our cohort. Median AAT admission levels were significantly higher in non-survivors (2.21 g/L, IQR 1.73–3.07) compared to survivors (1.73 g/L, 1.47–2.14, $p = 0.002$**).** Median admission IL-6 levels were significantly higher in non-survivors (59.78 pg/mL, IQR 29.14–200.6) compared to survivors (17.03 pg/mL, 6.24–32.6, $p < 0.001$). Median AAT: IL-6 ratio values were significantly lower in non-survivors (0.03, IQR 0.01–0.06) than in survivors (0.11, 0.07–0.24, $p < 0.001$), in favor of an association between insufficient AAT response to inflammation and COVID-19-related-death (Fig. 1-C). In a logistic regression model adjusted for age, BMI, and D-dimer, a ratio AAT: IL-6 >0.08 (whole cohort median), was a protective factor against mortality (**odds ratio [OR] 0.09 95% confidence interval [CI] 0.02–0.35, p<0.001** Fig. 1-D), meaning that at given IL-6 level, the highest AAT is the lower the mortality risk.

3.4. AAT: IL-6 ratio is highly predictive of in-hospital thrombosis

In our cohort, 33 (17.6%) patients displayed symptomatic thrombotic events, including 19 (57.6%) pulmonary embolism and 14 (42.4%) jugular thrombosis. AAT admission levels were higher in patients who suffered from thrombosis during their hospitalization (median 2.24 g/L, IQR 1.76–2.96) than those who did not (1.70 g/L, 1.50–2.10, $p = 0.001$). Median IL-6 levels were significantly higher in thrombosis (42.39 pg/mL, 17.54–139.2) compared to non-thrombosis patients (17.71 pg/mL 6.16–36.22). AAT: IL-6 ratio was found significantly lower in patients who suffered from venous thromboembolism (VTE) (median 0.05, IQR 0.02–0.11) than those who did not (0.09, IQR 0.05–0.26, $p < 0.001$, Fig. 1-E), suggesting that an insufficient AAT production was associated with thrombosis occurrence. A logistic regression analysis model adjusted for age, BMI, and D-dimer showed that an AAT: IL-6 ratio > **0.08** (whole cohort median) was associated with a significantly lower risk of in-hospital thrombosis (**OR 0.37 95%CI 0.16–0.83, $p = 0.02$,** Fig. 1-F), in favor of an association between thrombosis onset and inadequate AAT levels adjustment to inflammation.

To fully explore the association between AAT and thrombosis during COVID-19, we assessed the prevalence of S and Z mutant alleles, associated with congenital AAT deficiency via genotyping of AAT in all patients. Ten patients (7.9%) were identified as

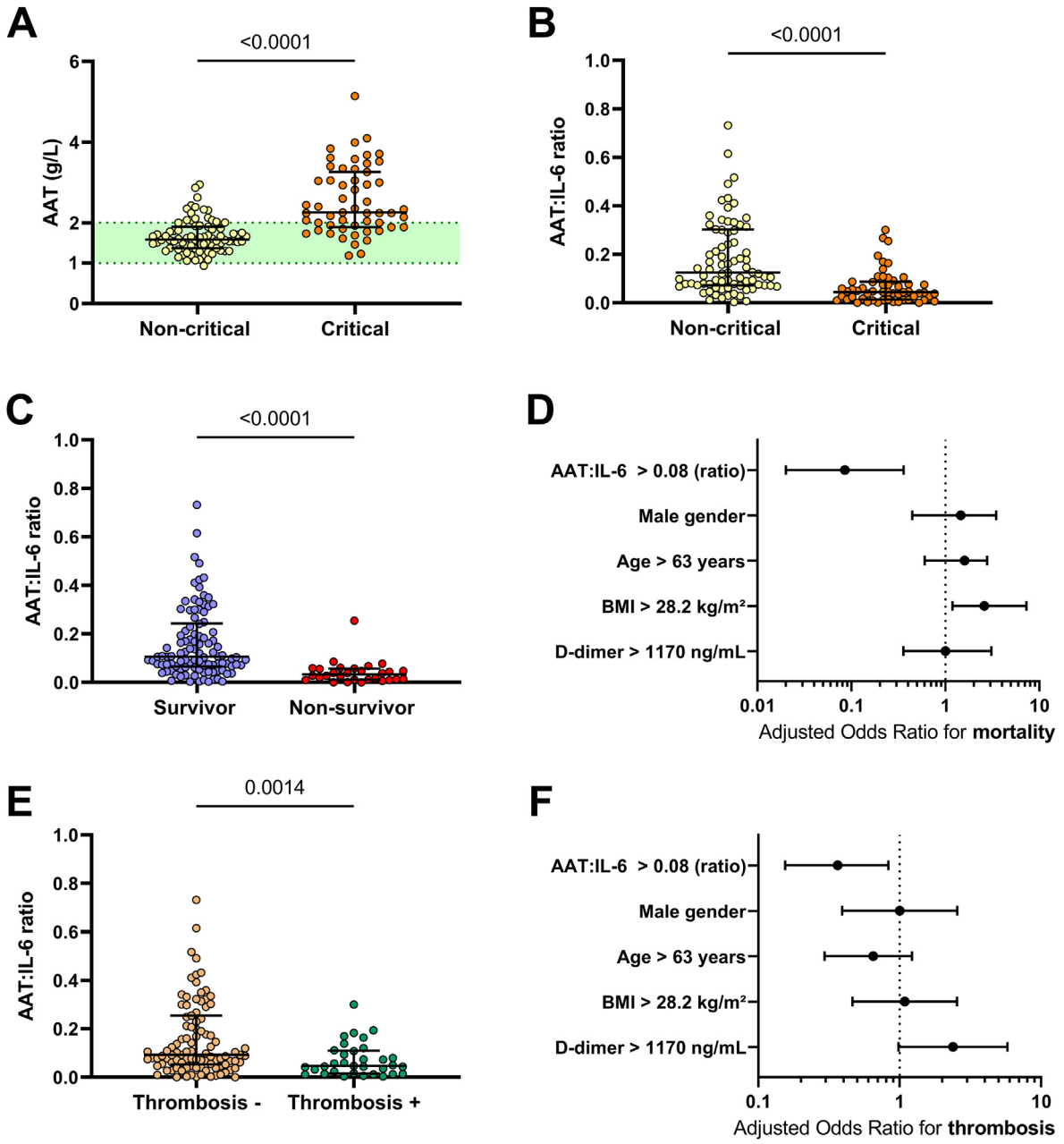

Fig. 1. Alpha-1 antitrypsin and alpha-1 antitrypsin: Interleukin 6 ratio at admission in 137 hospitalized COVID-19 patients: association with clinical severity, in-hospital mortality and thrombosis.

In dot plots, data points indicate individual measurements, whereas horizontal bars represent the medians with interquartile ranges. Green shaded areas indicate the laboratory normal range of alpha-1 antitrypsin (AAT) (1.0–2.0 g/L). In forest plots, cut-offs of variables were set as the median value of the whole cohort. Measurement of D-dimer was performed using the Vidas d-dimers® assay (Biomérieux, Marcy-Etoile, France) according to the manufacturer's instruction.

A: Levels of AAT at admission in critical and non-critical COVID-19 patients. **B**: Alpha-1 antitrypsin: interleukin 6 (AAT: IL-6) ratio at admission in critical and non-critical COVID-19 patients; **C**: AAT: IL-6 ratio at admission in survivors and non-survivors COVID-19 patients. **D**: Multivariable logistic regression model examining admission AAT: IL-6 ratio association with in-hospital mortality with adjustment on age, male gender, body mass index (BMI) and D-dimer levels. **E**: AAT: IL-6 ratio at admission in COVID-19 patients with and without thrombosis during hospitalization. **F**: Multivariable logistic regression model examining admission AAT: IL-6 ratio association with thrombosis hospitalized COVID-19 patients with adjustment on age, male gender, BMI and D-dimer levels.

heterozygous carriers of AAT variants alleles (9MS and 1MZ). Of these, 9 (6.6%; 8MS and 1MZ) were in the group without thrombosis and 1 (3.0%; MS) experienced thrombosis, no patient homozygous carrier for S or Z deficient allele was identified in our cohort. Moreover, phenotyping of AAT and EIC was performed for the 33 patients with thrombosis and a control group of 33 patients without thrombosis matched for age, sex, and clinical severity. No patient with rare variant or low anti-elastase activity was identified in this subgroup.

4. Discussion

In this study, our findings argue in favor of an insufficient production or increased consumption of AAT in response to COVID-19 hyper-inflammatory state, assessed in our cohort using the AAT: IL-6 ratio. Indeed, a decreased AAT: IL-6 ratio, which may reflect an insufficient AAT response with respect to IL-6 production, is associated with poor survival in COVID-19 inpatients. In physiological

conditions, the primary role of AAT is to mitigate the collateral self-tissue damage induced by the inflammatory response, mainly via its anti-neutrophil elastase activity [6]. Indeed, the lack of regulation of the proteolytic activity of neutrophil elastase leads to a range of detrimental effects, including increased cleavage of extracellular matrix or connective tissue molecules, enhanced rate of neutrophil reactive oxygen species production and decreased efficiency of neutrophils phagocytosis, which all converge toward tissue chronic inflammation and lesion [7]. Beyond its anti-elastase activity, AAT also display direct anti-inflammatory and immunomodulatory properties, notably by reducing the release of TNF-α and IL-1β from monocytes trough NF-κB activation inhibition [11]. In COVID-19, the observed inability of AAT response to counter the massive inflammatory syndrome could trigger and/or amplify widespread tissue damage, in particular diffuse alveolar damage in lungs, a hallmark of severe COVID-19 and an important event in the transition to critical illness and death [12]. A cohort of 40 COVID-19 hospitalized patients, investigated by McElvaney OJ et al. also reported that a decreased AAT:IL-6 ratio was associated with increased in-hospital mortality [13]. Here, we confirmed this association between inadequate response to AAT and in-hospital mortality in patients with COVID-19 in a larger cohort, allowing us to perform a robust survival analysis adjusted on other known factors associated with COVID-19-associated-mortality, such as D-dimer level and BMI.

In addition, AAT was suggested to protect from SARS-CoV-2 infection by inhibiting TMPRSS2 and TMPRSS4, two proteases facilitating the entry of SARS-CoV-2 into the host cells [14]. SARS-CoV-2 viral load, whether in plasma or nasopharyngeal swabs, has been positively associated with increased disease severity and mortality [15]. Thus, an AAT inadequate response to hyper-inflammatory stress may additionally promote COVID-19 patients worsening via the loss of efficiency of this anti-viral mechanism.

Furthermore, we describe here for the first time that a decreased AAT: IL-6 ratio was associated with a higher rate of COVID-19-associated-thrombosis, even after adjustment for D-dimer level. AAT is able to inhibit numerous serine protease of the blood coagulation, notably the serine protease thrombin, the end-product of the coagulation cascade [16]. In line with this, Gupta et al. described the first case of a congenital AAT severe deficiency associated with recurrent pulmonary thrombosis [17]. Recent studies showed that subjects with AAT deficiency, particularly in cases of severe deficiency, have increased risk of developing venous thrombosis compared with control subjects [18,19]. COVID-19 critically ill patients are at high risk for thrombosis despite receiving standard-dose pharmacologic thromboprophylaxis [2]. Thrombosis onset in COVID-19 is commonly explained by the COVID-19-associated lesions of the physiologically anticoagulant endothelium, whether from harmful inflammatory cytokine or direct viral invasion, thus resulting in a procoagulant state [3]. However, based on our results, we hypothesise that there may be inappropriate production of AAT, not only in response to endothelial hyperinflammation, but also in the context of a COVID-19-associated procoagulant state which may also contribute to the increased thrombotic risk observed. In the absence of excess of inborn AAT deficiency among the patients with thrombosis, our results rather suggest an inadequate AAT response to SARS-CoV-2 infection than a constitutive low AAT production.

Our findings highlight that an imbalance between inflammation and circulating AAT in COVID-19, evaluated through the AAT: IL-6 ratio, may allow an early identification of patients with higher risk of in-hospital mortality and thrombosis. Further studies are required to evaluate the AAT: IL6 ratio as biological parameter to select the most vulnerable patients with severe COVID-19 for new therapeutic strategies aiming at restoring the balance between unregulated inflammation and AAT levels through AAT injection.

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Authors contributions

AP, MP, CN, DMS and MAL interpreted data, conceived and supervised the study and wrote the manuscript. MB carried out the assays. RC analyzed the data and supervised statistical analysis and supervised the study. All other authors' included patients, reviewed all patients' characteristics.

Author agreement statement

We the undersigned declare that this manuscript is original, has not been published before and isnot currently being considered for publication elsewhere.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed.

We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We understand that the Corresponding Author is the sole contact for the Editorial process.

He/she is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs.

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