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Soluble TNF receptors predict acute kidney injury and mortality in critically ill COVID-19 patients: A prospective observational study

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ABSTRACT

Background: Although pneumonia is the hallmark of coronavirus disease 2019 (COVID-19), multiple organ failure may develop in severe disease. TNFα receptors in their soluble form (sTNFR) are involved in the immune cascade in other systemic inflammatory processes such as septic shock, and could mediate the inflammatory activation of distant organs. The aim of this study is to analyse plasma levels of sTNFR 1 and 2 in association with organ failure and outcome in critically ill patients with COVID-19.

Methods: After informed consent, we included 122 adult patients with PCR-confirmed COVID-19 at ICU admission. Demographic data, illness severity scores, organ failure and survival at 30 days were collected. Plasma sTNFR 1 and 2 levels were quantified during the first days after ICU admission. Twenty-five healthy blood donors were used as control group.

Results: Levels of sTNFR were higher in severe COVID-19 patients compared to controls ($p < 0.001$). Plasma levels of sTNFR were associated to illness severity scores (SAPS 3 and SOFA), inflammation biomarkers such as IL-6, ferritin and PCT as well as development of AKI during ICU stay. sTNFR 1 higher than 2.29 ng/mL and? sTNFR 2 higher than 11.7 ng/mL were identified as optimal cut-offs to discriminate survivors and non-survivors 30 days after ICU admission and had an area under the curve in receiver operating characteristic curve of 0.75 and 0.67 respectively.

Conclusion: Plasma levels of sTNFR 1 and 2 were higher in COVID-19 patients compared to controls and were strongly associated with other inflammatory biomarkers, severity of illness and acute kidney injury development during ICU stay. In addition, sTNFR 1 was an independent predictor of 30-day mortality after adjustment for age and respiratory failure.

1. Introduction

The coronavirus disease 2019 (COVID-19) pandemic, caused by severe acute respiratory syndrome coronavirus (SARS-CoV-2) [1], has resulted in high rates of hospitalization and admission to intensive care units (ICU). Moreover, the mortality rates among patients admitted to

Abbreviations: AKI, acute kidney injury; AUC, Area under the curve; 95%CI, 95% confidence interval; COVID-19, Coronavirus disease 2019; CRP, C-reactive protein; ELISA, Enzyme-Linked ImmunoSorbent Assay; HR, hazard ratio; ICU, intensive care unit; IL-6, Interleukin-6; IQR, Interquartile range; KDIGO, Kidney Disease Improving Global Outcomes; MAP, Median arterial blood pressure; PCR, polymerase chain reaction; PCT, Procalcitonin; PFR, PaO_2/FiO_2 ratio; ROC, receiver operating characteristic; RRT, renal replacement therapy; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2; SOFA, Sequential organ failure assessment; TNF, Tumor necrosis factor; TNFR, Tumor necrosis factor receptors; sTNFR, Soluble tumor necrosis factor receptors; sTNFR 1, Soluble tumor necrosis factor receptor 1; sTNFR 2, Soluble tumor necrosis factor receptor 2; SAPS 3, Simplified acute physiology score 3.

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the ICU are high [2,3]. Organ dysfunction in critically ill patients is common [4] and up to 90% of the patients requiring mechanical ventilation develop acute kidney injury (AKI) [5].

There is evidence suggesting that the pro-inflammatory response seen in severe COVID-19 is driven by activation of monocyte-derived macrophages [6]. Systemic levels of macrophage-related cytokines, such as tumor necrosis factor alpha (TNFα) and interleukin-10 (IL-10), are increased in severe cases compared to non-severe cases and have been shown to be associated with increased disease severity, organ failure and death [7,8].

TNFα is a pro-inflammatory cytokine essential for the host defense against infections and for regulating cell survival [9]. TNFα initiates cellular inflammatory responses primarily by binding to two cell-surface tumor necrosis factor receptors (TNFR): TNFR 1 and TNFR 2. TNFR 1 is the key mediator of TNF signaling and is expressed in most tissues, mediating apoptosis [10,11] whereas TNFR 2 is mainly expressed on immune cells and is associated with survival signaling, cellular activation and differentiation [11]. Proteolytic shedding of the membrane-bound TNFR 1 and TNFR 2 to soluble TNFR (sTNFR) has been observed in experimental sepsis [12] and inflammation [13]. sTNFR limits the effects of TNFα by neutralizing circulating TNFα through binding and thus dampening the inflammatory response [12,14], and by decreasing the number of available TNFR on cell surfaces. Although sTNFR may act as carriers of TNFα and even augment its effects by prolonging its function[15], increased levels of sTNFR have been observed in patients with sepsis and correlate with mortality and with development of AKI in patients with septic shock [16,17]. The levels of sTNFR have been shown to be elevated in patients with COVID-19 with the levels being higher in patients with severe disease [18,19].

We hypothesized that plasma levels of sTNFR 1 and sTNFR 2 increase as part of the inflammatory activation in COVID-19 and are markers of organ failure and death. Our primary endpoint was to investigate if sTNFR 1 and sTNFR 2 levels are elevated in critically ill COVID-19 patients. Our secondary endpoint was to study if the levels of sTNFR 1 and sTNFR 2 predict development of AKI and mortality.

2. Materials and methods

2.1. Setting

This prospective observational study was conducted at the ICU caring for COVID-19 patients at Uppsala University Hospital, Sweden.

2.2. Ethical approval

The study was conducted in accordance with the Declaration of Helsinki and its subsequent revisions and was approved by the Regional Ethical Review Board (EPN) in Uppsala (No. 2017-043 and amendment 2020-01623). We obtained informed consent from the patients, or from next of kin if the patient was too unwell or otherwise unable to give informed consent at the time of inclusion. The protocol of the study was registered a priori to initiation (Clinical Trials ID: NCT04316884). The study was performed according to the relevant guidelines and regulations and STROBE guidelines were followed in reporting the study results [20].

2.3. Data collection

Patients with SARS-CoV-2 infection admitted to the ICU in Uppsala University Hospital between 23th March and 28th September 2020 due to Covid-19 were screened for eligibility and included in the study. SARS-CoV-2 infection was confirmed with a positive polymerase chain reaction (PCR) testing of a nasopharyngeal sample. The patients in the study are a part of the Uppsala PRONMED-study cohort. Patients that were younger than 18 years old or pregnant were excluded. Number of days from onset of symptoms to admission to the ICU was recorded. Simplified acute physiology score 3 (SAPS 3) [21] were recorded on arrival, and clinical data and Sequential Organ Failure Assessment (SOFA) [22] score was recorded daily. Blood samples for the analysis of sTNFR 1 and sTNFR 2 were collected within 72 h from admission to the ICU, except for 7 patients where the blood samples were collected 4–6 days after admission. In some patients, multiple blood samples were collected over the first days on ICU, and in these cases the highest sTNFR 1 and sTNFR 2 values were used in the analyses. Blood samples for the analysis of C-reactive protein (CRP), ferritin, IL-6, D-dimer and procalcitonin (PCT) were collected regularly during the ICU stay. We recorded ICU day 1 creatinine values, maximum creatinine value and we identified patients who developed AKI during the ICU stay, defined according to the Kidney Disease Improving Global Outcomes (KDIGO) criteria [23]. Patients were followed for up to 30 days from ICU admission. Healthy blood donors were used as controls to compare sTNFR 1 and TNFR 2 levels.

2.4. Analysis of sTNFR 1 and sTNFR 2

Plasma levels of endogenous sTNFR 1 and sTNFR 2 were analyzed using commercial sandwich Enzyme-Linked ImmunoSorbent Assay (ELISA, DY225 and DY726, R&D Systems, Minneapolis, MN). The assays had a total coefficient of variation of 6%. Lower quantification limits for sTNFR 1 and sTNFR 2 were 12.5 pg/mL and 7.8 pg/mL. The laboratory tests were performed blinded without knowledge of clinical data at the time of the measurements.

2.5. Statistical analysis

Given the observational design and the exploratory nature of the study, no sample size calculation was performed. The proportion of missing data (Table S1) for key variables was less than 10%, no imputations were made. No case was lost to follow up. Data was tested for normal distribution. Data with log-normal distribution was log-transformed. Data is presented as mean ± SD or median (IQR) if not stated otherwise. Frequencies are presented as absolute numbers of total participants (% of study population). We applied T-test or Mann-Whitney as required for comparisons between quantitative continuous variables. One Way ANOVA or Kruskal-Wallis was used as appropriate for comparisons between several groups. Correlations were assessed with Pearson's correlation. Receiver operator characteristics (ROC) curves were plotted to assess sTNFR 1 and 2 as mortality predictors by their area under the curve (AUC) and defining optimal cut-off for these biomarkers using the maximum sensitivity and specificity threshold. We added a Kaplan-Meier plot and performed Logrank test for 30 days after ICU admission survival analysis. Univariable and multivariable hazard ratio were used to identify increases in mortality risk. We used age as a surrogate to adjust for comorbidities in the multivariable model. Rstudio software (Version 1.4.1103, A Language and Environment for Statistical Computing, R Core Team, R Foundation for Statistical Computing, Vienna, Austria, 2021) and Statistica (Version 13.5, Tibco Software Inc., Palo Alto, USA) used for the calculations. $p < 0.05$ was considered significant.

3. Results

3.1. Patient characteristics

The flowchart of patient selection is presented in Figure S1. We included 125 patients admitted to ICU due to clinical diagnosis of severe COVID-19 infection. Three patients were excluded from the analysis as the PCR test for SARS-COV-2 was negative.

The demographics and comorbidities of the cohort are presented in Table 1. Blood samples from healthy blood donors were used as controls to compare sTNFR 1 and sTNFR 2 levels with those in COVID-19 patients. We report only age and sex for blood donors according to the

Table 1
Patient demographics and clinical characteristics patients with COVID-19 and healthy controls. Mean ± SD.

	COVID-19 n = 122	Controls n = 25
Age (y)	60 ± 14	57 ± 5
Sex (n, % female)	28 (22)	6 (24)
Weight (kg)	88 ± 21	
Pulmonary disease, n (%)	33 (27)	
Arterial Hypertension, n (%)	65 (53)	
Diabetes, n (%)	33 (27)	
Heart failure, n (%)	5 (4)	
Malignant disease, n (%)	10 (8)	
Previous Steroid Treatment, n (%)	12 (10)	
During ICU stay		
SAPS 3	53 ± 10	
Invasive ventilation, n (%)	71 (58)	
AKI development, n (%)	69 (56)	
RRT, n (%)	17 (13)	
30 day mortality, n (%)	26 (22)	

Abbreviations: ICU, intensive care unit; AKI, acute kidney injury; RRT, renal replacement therapy.

ethical approval and they were similar to the main cohort. At ICU admission, patients had 11 ± 4 days of COVID symptoms. A majority of the patients were previously diagnosed with arterial hypertension.

SAPS 3 was used to assess illness severity, mean score was 53 ± 10 (Table 1). Median PaO_2/FiO_2 ratio (PFR) at admission was 17.9 (15.5 – 24.4) kPa, and lowest PFR during ICU stay was 10.6 (9.25 – 12.95) kPa. Seventy-one patients received invasive mechanical ventilation, with treatment length of 8 (3–15) days. Hemodynamic instability at ICU admission was unusual, mean arterial pressure (MAP) was 90 ± 16 mmHg at admission. However, 69 patients (56%) were treated with vasopressor drugs at some point during their ICU stay, with a mean length of treatment of 6 ± 4 days. ICU length of stay was on average 8 (4 – 16) days with 22% mortality 30 days after ICU admission.

3.2. sTNFR

Both sTNFR 1 and 2 levels were higher in COVID-19 patients compared to healthy blood donors ($p < 0.001$ for both; Fig. 1). sTNFR 1 values were also higher in those patients with history of previous malignant disease ($p = 0.038$) while no differences were found in sTNFR 2 values ($p = 0.10$). No other differences were found between sTNFR 1 and 2 values and comorbidities. There was a positive correlation between both sTNFR and age of the patient and SAPS 3 at admission (Table S2).

3.3. Biomarkers of inflammation

sTNFR values correlated both to IL-6 and ferritin levels during first ICU days, as well with their maximum values during ICU stay (Table S2). We also found an association between PCT on admission and both sTNFR 1 ($r = 0.34$) and sTNFR 2 values ($r = 0.46$). In comparison, no association was found between sTNFR 1 and 2 and other inflammatory biomarkers as CRP or D-dimer at admission. Maximum CRP values during ICU stay were only correlated with sTNFR 1 ($r = 0.3$), while PCT peak was well correlated with both sTNFR 1 ($r = 0.43$) and sTNFR 2 ($r = 0.49$).

3.4. Severity of illness and organ failure

Maximum SOFA score during ICU stay was correlated with both sTNFR 1 and 2 ($r = 0.45$ and $r = 0.41$ and $p < 0.001$, respectively). sTNFR levels were also associated with SAPS 3 ($r = 0.45$ and $r = 0.32$ respectively; Table S2).

Patients who received vasopressor drugs during ICU stay ($n = 69$) had higher sTNFR 1 than those who did not ($p = 0.009$), however no difference was found in sTNFR 2 values ($p = 0.14$).

PFR at admission was not correlated with higher levels of sTNFR 1 or 2. We did not find significant differences in sTNFR values between those patients who were treated with invasive mechanical ventilation ($n = 71$) and those who were not (sTNFR 1; $p = 0.17$; sTNFR 2; $p = 0.6$) or in patients who developed critical illness weakness (sTNFR 1; $p = 0.44$; sTNFR 2; $p = 0.43$). No differences in sTNFR 1 ($p = 0.29$) and 2 ($p = 0.31$) were found between the patients who were diagnosed with thromboembolic event during ICU stay ($n = 14$, 11%) and those who were not.

3.5. Acute kidney injury

Baseline creatinine was 71 (58–86) μmol/L. Twelve patients (10%) had increased baseline creatinine prior to ICU admission 123 (111–151) μmol/L, according to the hospitals reference interval (women < 90 μmol/L, men < 105 μmol/L), (Table S3). A majority ($n = 69$, 56%) of the patients developed or increased their previous AKI stage according to maximum creatinine reached, 97 (78–151) μmol/L, and/or oliguria and 17 patients (13%) were treated with renal replacement therapy (RRT).

Patients who developed AKI had higher sTNFR 1 and 2 levels compared with those without AKI. sTNFR 1 and 2 levels increased with increasing maximum AKI stage during the ICU stay (sTNFR 1; $p < 0.001$; sTNFR 2; $p = 0.02$; Fig. 2).

Plasma creatinine values on admission were associated with sTNFR 1 and 2 levels ($r = 0.55$ and $r = 0.43$, respectively). Correlation was also found between sTNFR 1 and 2 and the maximum creatinine level

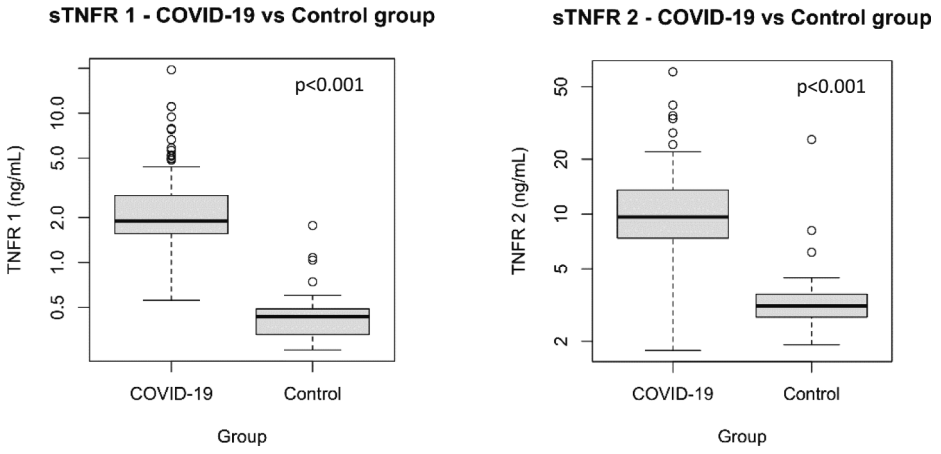

Fig. 1. sTNFR 1 and 2 levels in patients with COVID-19 and healthy controls. The p-values denotes the group differences.

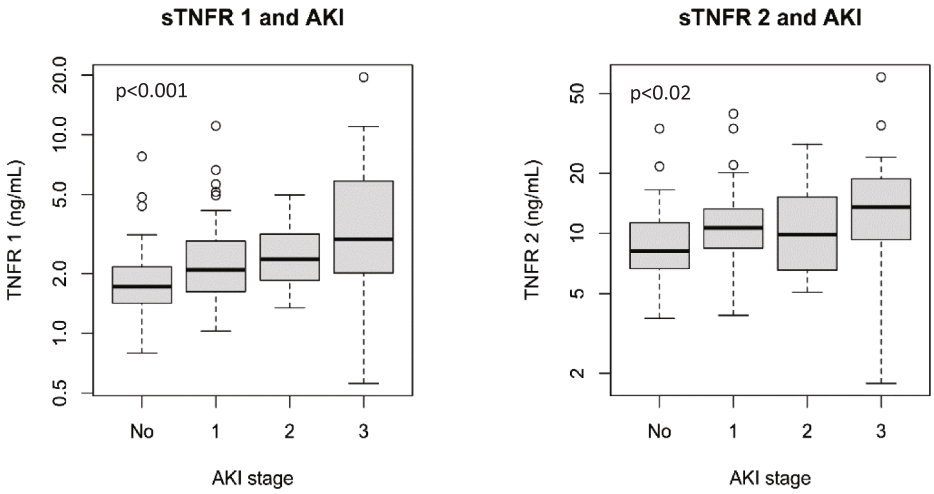

Fig. 2. sTNFR 1 and 2 levels in groups according to the maximum AKI stage of the patients during ICU stay. The p-value denotes increase in sTNFR 1 and 2 values over AKI stages.

reached during ICU (r = 0.46 and r = 0.32, respectively) (Table S2).

Patients treated with renal replacement therapy had higher sTNFR 1 (P = 0.001) and sTNFR 2 levels (P = 0.002) compared to patients not treated with RRT. Higher levels of both TNFR were also associated with less RRT free days (sTNFR 1: R = −0.33, P < 0.001; sTNFR 2: R = −0.27, P = 0.002).

3.6. Mortality

Both sTNFR 1 and 2 levels were higher in patients who died (p = 0.016 and p = 0.023 respectively; Fig. 3) within 30 days,

ROC plot for sTNFR 1 and 30 day mortality, had AUC 0.73 (95% confidence interval (95 %CI) = 0.62–0.84). We identified 2.29 ng/mL as cut-off for sTNFR 1 in plasma with the highest sensitivity (71%) and specificity (71%) to predict 30 day mortality. For a specificity of 90% the sTNFR 1 threshold was 2 ng/mL, with a consequent reduction of the sensitivity to 29%. (Fig. 4).

We also investigated sTNFR 2 as a predictor for 30 day mortality. The ROC plot had AUC 0.66 (95 %CI = 0.53–0.79), and the cut-off for plasma TNF 2 level with maximum sensitivity (62%) and specificity (73%) for sTNFR 2 was 11.7 ng/mL. When grouping patients according to sTNFR 1 cut-off of 2.29 ng/mL into two goups, patients with sTNFR 1 over 2.29 ng/mL were more likely to die during the 30 days follow-up, compared to those with sTNFR 1 lower than 2.29 ng/mL (Log-rank test p < 0.001; Fig. 5).

Patients who had sTNFR 1 over 2.29 ng/mL had a hazard ratio (HR) of 5.3 (95 %CI 2.2–13) and sTNFR 2 over 11.7 ng/mL had a HR 3.8 (95 %CI 1.6 – 8.6) for death at day 30 after ICU admission (p < 0.001). Age was also a risk factor for death at 30 days (p < 0.001) but not maximum plasma creatinine values or mechanical ventilation during ICU stay (p = 0.46, Table S4).

In the multivariable analysis, HR was 3.1 when adjusting sTNFR 1 cut-off for age (95 %CI 1.3 – 7.5, p = 0.013, Fig. 6). Adjusting for maximum creatinine HR was 5.5 (95 %CI 2.09–14.2, p < 0.001), and adjusting for mechanical ventilation HR was 5.1 (95 %CI 2.1 – 13, p=<0.001). Similarly, adjusting sTNFR 2 cut-off for maximum creatinine HR was 3.5 (95 %CI 1.4 – 8.4, p = 0.005), adjusting for age HR was 2.6 (95 %CI 1.1 – 6.1, p = 0.023), and HR was 3.6 (95 %CI 1.6 – 8.4, p = 0.002) when adjusting for mechanical ventiltion.

4. Discussion

4.1. Key findings

In this prospective observational study, sTNFR levels in plasma were higher in ICU patients with severe COVID-19 than in the control group and were associated with disease severity, acute kidney injury as well as inflammation biomarkers such as IL-6, ferritin and PCT with. sTNFR 1 higher than 2.29 ng/mL or sTNFR 2 higher than 11.7 ng/mL were identified as optimal cut-offs to discriminate patients alive and dead at 30 days after ICU admission. sTNFR 1 and sTNR2 were also independent predictors of death when adjusting for age and respiratory failure.

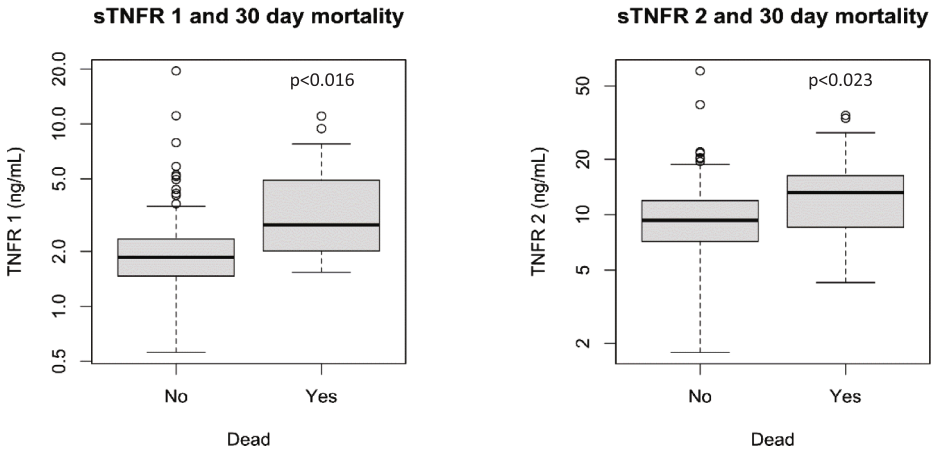

Fig. 3. sTNFR 1 and 2 levels comparing patients who died vs. those who survived at 30 days after ICU admission. The p-values denotes the group differences.

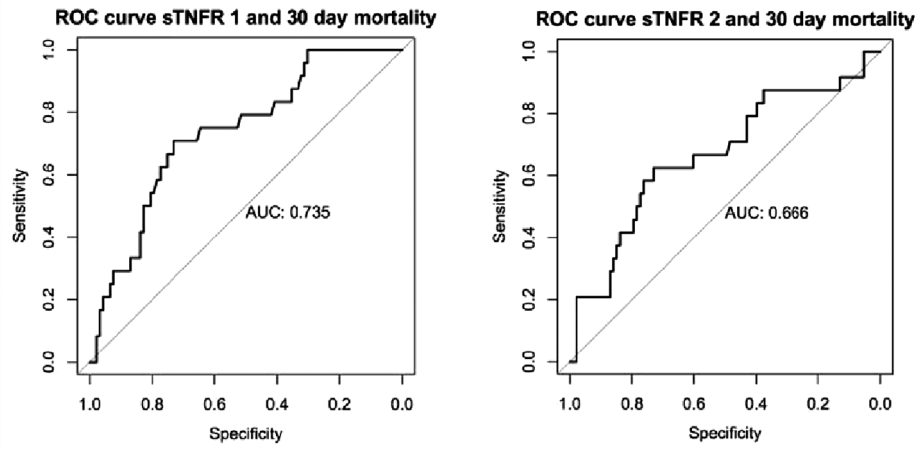

Fig. 4. ROC curves for sTNFR 1 and sTNFR 2 and 30 day mortality. Area under the curve (AUC) is shown in the graph.

4.2. Previous studies

TNFα is a key pro-inflammatory mediator that also regulates cell survival [24,25] by binding to TNFR expressed by a variety of cells. However, in some inflammatory processes TNFR are also released in their soluble form [16,26].

We report increased levels of sTNFR 1 and 2 in ICU patients with COVID-19 compared to matched controls, which is in line with a recent study in hospitalized patients [27]. In our cohort, sTNFR were associated with several inflammatory biomarkers. The correlation between sTNFR levels was strongest with IL-6, a proximal inflammatory mediator, possibly through sTNFR 1 triggering IL-6 synthesis [25] as a mechanism. We also found a weak association between sTNFR and early CRP, D-dimer and ferritin levels, but a strong correlation between the sTNFR and the maximum levels of these biomarkers. One possible explanation could be that CRP, D-dimer and ferritin are more distal mediators of the inflammatory cascade and thus take some time to increase. Alternatively, these mediators are released through pathways not directly linked to TNFR synthesis, as is the case of ferritin [28].

In addition to the association with the inflammatory response, both sTNFR were also associated with illness severity measured by SAPS 3 and the extent of organ failure seen in this ICU cohort. We also found an association between sTNFR and age. Since both acute illness severity and age are major contributors of the total SAPS 3, we also assessed the correlation between sTNFR and the age independent SOFA score, finding that sTNFR were related with organ failure per se.

Interestingly, despite the lungs being the focus of inflammation in COVID-19, plasma sTNFR levels were not linked to respiratory failure in terms of PFR or if the patients were treated with mechanical ventilation. These findings suggest that sTNFR pathways can be involved mainly in systemic inflammation and mediating distant organ failure instead of the primary site of inflammation.

Apart from the acute respiratory failure, COVID-19 patients frequently develop acute kidney injury as we have previously reported [5]. We found that sTNFR 1 and 2 were strongly correlated with creatinine values at ICU admission, the maximum creatinine level reached during ICU stay, and accordingly sTNFR were higher in patients who developed AKI compared to those who did not. sTNFR levels also increased with AKI stage during the ICU stay, based on both creatinine and urine output. Finally, sTNFR levels were higher in patients treated with RRT and higher sTNFR levels were correlated with longer RRT duration. Even though the current study presents the first data on the connection of plasma sTNFR and AKI development in COVID-19, sTNFRs have been linked to AKI previously in other systemic inflammation conditions such as septic shock [17,29] or cardiac surgery [30], as well as chronic kidney injury due to type 1 diabetes [31]. The mechanisms of plasma sTNFR involvement in AKI as a consequence of COVID-19 have not been previously explored. However, TNFα levels are associated with AKI in COVID-19 [8], and may exert its effects on membrane bound sTNFR 1 and 2 in the kidney as suggested by experimental data [16].

Other conditions associated to COVID-19 such as circulatory failure, thromboembolic events and critically illness weakness were not associated with higher sTNFR values. Circulatory failure as administration of vasopressors was related to sedation rather than shock in this cohort, and based on these data we cannot exclude an effect of sTNFR and thromboembolic events and critically illness weakness due to the limited number of observed events.

The acute dysfunction in vital organs contribute to mortality in COVID-19 [32]. We found higher plasma sTNFR levels in patients who did not survive to 30 days compared to those who did. Similar differences on survivors vs. non-survivors were reported recently in 175 hospitalized patients with COVID-19, of whom 70 were admitted to the ICU [27]. We identified cut-offs for both sTNFR 1 and 2 to identify patients at risk of death. Although both sensitivity and specificity were not higher than 71%, most patients who died were identified in the survival analysis by these biomarkers, and both sTNFRs were predictors of death also after adjustment for age used as a surrogate for inherent risk of death. These findings suggest that although plasma sTNFR do not accurately predict all patients at risk of death, sTNFR contributes to finding vulnerable patients early after admission by identifying risk of death that is not related to age or severe respiratory failure.

5. Strengths and limitations

As far as we know, we are the first to report, sTNFR 1 and 2 levels in a large ICU cohort of patients with COVID-19. An asset of the study is that samples were collected prospectively in consecutive patients. The large size of the cohort and the high detail of collected clinical data allowed us to assess the relation of sTNFR 1 and 2 with demography, illness severity, organ failure and death.

The study has some limitations. We did not measure the evolution of sTNFR 1 and 2 during ICU stay. The time between the sample and some of the outcomes described was not homogeneous, e.g. AKI occurred at different times in different patients after the sTNFR measurement. We compared our results in severe COVID-19 patients with 25 healthy controls. A larger study with more controls could have higher power, nevertheless, given the separation between controls and COVID-19 patients, the risk of type II error is low. The study does not include with mild or asymptomatic COVID-19 cases limiting the conclusions to ICU patients. Finally, due to low incidence of some characteristics and outcomes such as previous malignant disease and thromboembolism, we

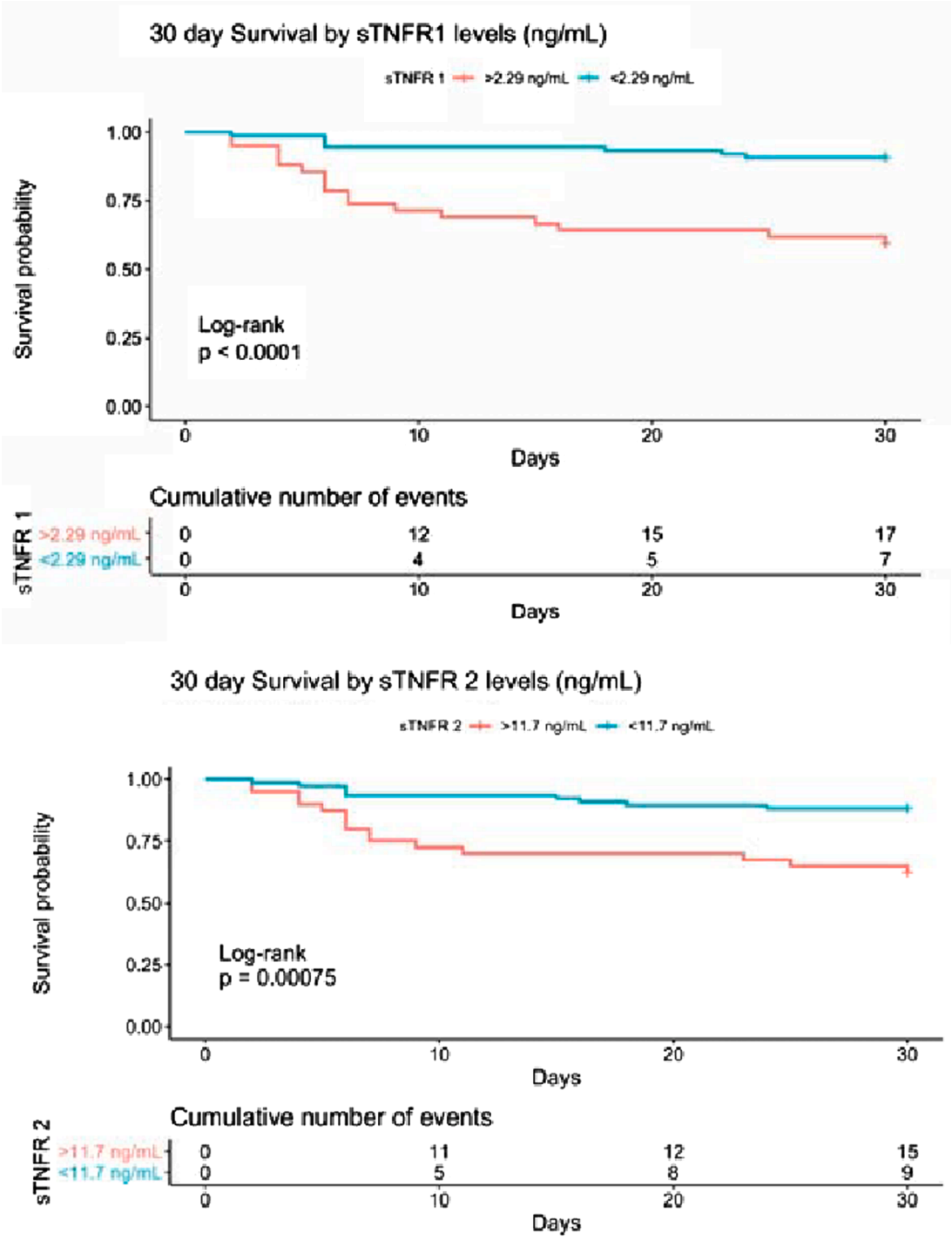

Fig. 5. Kaplan Meier plot depicting cumulative mortality incidence for sTNFR 1 and 2 values for cut-offs of 2.29 ng/mL and 11.7 ng/mL, respectively, during the 30 day's follow up.

are not able to make any conclusions on the association of these characteristics and outcomes and sTNFR.

5.1. Future studies

The association between sTNFR and AKI warrants further studies investigating whether sTNFR are mediators of AKI and thereby potential treatment targets or represent an epiphenomenon. Moreover, unlike short lived biomarkers such as TNFα, sTNFR have stabile elevated levels in hospitalized patients (9). Thus, investigating if sTNFR at hospital admission can predict which patients will be admitted to the ICU later on would be of interest.

6. Conclusions

In a cohort of critically ill patient COVID-19 patients, sTNFR 1 and 2 were strongly associated with inflammation, severity of illness. as well as development and the extent of AKI. Moreover, sTNFR 1 and 2 were predictors of death also after adjustment for age.

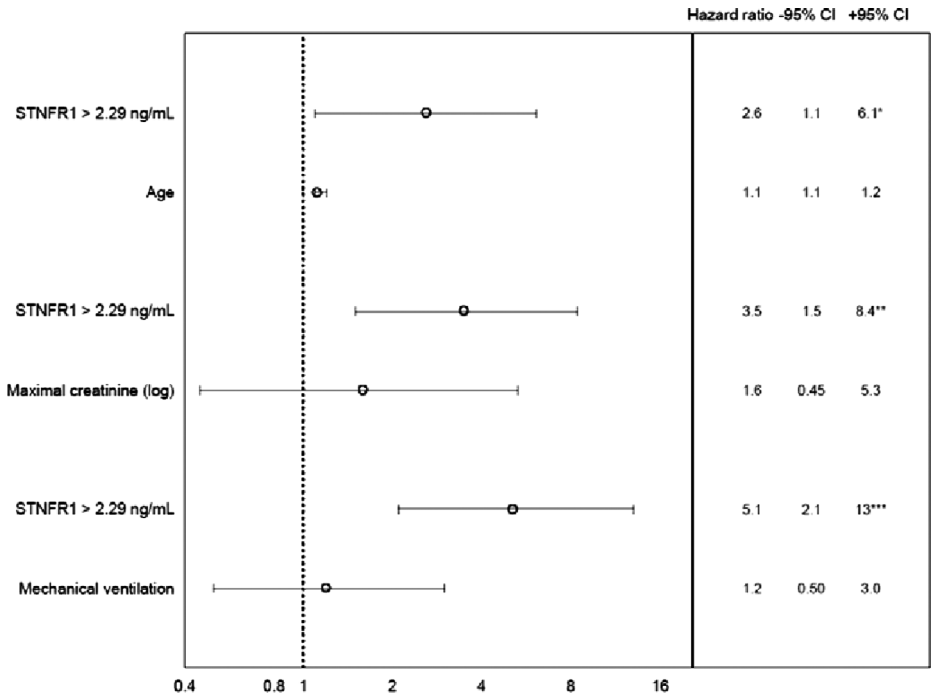

Fig. 6. Multivariable analysis showing sTNFR 1 optimal cut-off as independent predictor of mortality even when adjusted by age, maximum creatinine and mechanical ventilation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2021.155727.

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