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IL-6 signalling biomarkers in hospitalised patients with moderate to severe SARS-CoV-2 infection in a single centre study in Sweden

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ABSTRACT

Background: COVID-19 disease severity and need for intensive care has been associated with profound immune disturbances in which interleukin 6 (IL-6) is central. IL-6 signals through two pathways: classical IL-6 signalling with C-reactive protein (CRP) as a product is pivotal in the acute immune response against pathogens while IL-6 *trans*-signalling is involved in prolonged inflammation. We measured biomarkers of the IL-6 classical and *trans*-signalling pathways in patients with moderate or severe COVID-19 in the first wave of the COVID-19 pandemic.
Method: In a longitudinal cohort study including patients admitted to Danderyd hospital, Stockholm, Sweden, with COVID-19 (n = 112), plasma IL-6 mirroring activity in both pathways, CRP as marker of classical signalling and the soluble IL-6 receptor (sIL-6R) and soluble glycoprotein 130 (sgp130) as markers of *trans*-signalling were analysed at baseline. Potential differences in biomarker levels between groups of moderate and severe COVID-19 defined by care level, level of respiratory support and one-month mortality was analysed, as was correlations between biomarkers. In addition, levels 4 months after hospital admission were compared to those at baseline.
Results: Levels of IL-6 and CRP were increased in severe COVID-19 whereas IL-6 *trans*-signalling markers (sIL-6R, sgp130) did not differ between the groups. CRP correlated positively with IL-6 in all patients while correlation with IL-6 could not be demonstrated for sIL-6R and sgp130 in either group. Levels of IL-6, CRP and sIL-6R were significantly decreased after 4 months whereas sgp130 levels increased.
Conclusion: Classical signalling is the dominating IL-6 pathway in moderate-severe COVID-19.

1. Introduction

The Corona Virus Disease 2019 (COVID-19) epidemic was declared a pandemic by the World Health Organisation on 11 March 2020 and has since challenged the world with great strain on health care systems and with many deaths during four large waves. Like with previous severe corona virus infections, Severe Acute Respiratory Syndrome (SARS) and the Middle East Respiratory Syndrome (MERS), serious cases of COVID-19 are characterised by acute respiratory distress syndrome (ARDS) and immune disturbances with high levels of circulating cytokines, above all interleukin 6 (IL-6) [1]. IL-6 is a pleiotropic cytokine with diverging properties depending on which of its two signalling pathways that is active. In classical IL-6 signalling, pivotal in the immune system, IL-6 signals through a membrane-bound receptor complex consisting of the ligand binding IL-6 receptor (IL-6R) and the signal transducing receptor glycoprotein 130 (gp130), see the **Graphical abstract** [2]. Hepatocytes and leukocytes express the IL-6R while most other somatic cells lack this expression and consequently are not responsive to IL-6 classical signalling [3]. C-reactive protein (CRP) is one of the end-products of IL-6 classical signalling. Pro-inflammatory IL-6 *trans*-signalling is on the other hand promoted by the soluble isoform of IL-6R (sIL-6R) creating a circulating IL-6:sIL-6R receptor complex able to bind gp130 and elicit the intracellular signal on most cells (**Graphical abstract**). The sIL-6R isoform is produced by proteolytic cleavage of IL-6R from the cell membrane of cells expressing the receptor [3]. IL-6 *trans*-signalling is associated with chronic inflammatory conditions [3]. In bacterial infections, it has been demonstrated that, depending on which of the two IL-6 pathways is dominating, inflammation will resolve or progress with sIL-6R driving the transition from a neutrophil dominated scenario to monocyte/macrophage domination and prolonged inflammation [4]. IL-6 *trans*-signalling is counteracted by a buffer system consisting of high affinity binding of the soluble gp130 isoform (sgp130) to the IL-6:sIL-6R

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complex creating the inactive IL-6:sIL-6R:sgp130 complex. Considering the biology of the SARS infections, IL-6 *trans*-signalling could potentially enhance and extend the systemic inflammatory reaction of severe COVID-19 and the associated immune disturbances.

IL-6 has, in experimental ARDS studies, been shown to contribute to alveolar inflammation and tissue damage, and IL-6 *trans*-signalling specifically increases vascular permeability [5]. At the same time, IL-6 is important in tissue regeneration after influenza-induced pulmonary damage/ARDS [6]. In COVID-19, high IL-6 levels correlate with a poor clinical outcome and increased mortality [1,7]. In two observational studies, IL-6 *trans*-signalling was upregulated in COVID-19 patients treated in intensive care unit (ICU) compared to healthy controls or patients treated in wards [8,9]. The importance of *trans*-signalling for disease severity or outcome was however not investigated.

1.1. Hypothesis and aim

We hypothesized that IL-6 classical signalling is active in the acute phase and milder stages of COVID-19 while IL-6 *trans*-signalling contributes to the hyperinflammatory state in severe cases of COVID-19 during the first wave of the pandemic.

The aim of the present study was therefore to measure plasma concentrations of IL-6 classical and *trans*-signalling components in COVID-19 patients with moderate or severe disease.

2. Materials and methods

2.1. Study population

As described previously, adult patients (>18 years of age) admitted to Danderyd Hospital in Stockholm, Sweden with COVID-19 were invited to participate in the COMMUNITY cohort study between 15 April and 8 June 2020 [10,11]. The included patients either had a reverse-transcriptase polymerase chain reaction (PCR) viral detection verified COVID-19 diagnosis upon inclusion or presented with typical clinical symptoms and radiological signs of COVID-19 on pulmonary computed tomography.

Information on demographics, medical history, current medication, clinical data such as respiratory support and routine laboratory tests were retrieved from the patients' hospital records.

In the present analysis, 112 of the originally 118 study participants were included. Reasons for exclusions were incorrect COVID-19 diagnosis (n = 1), already included in an interventional study (n = 3), lacking plasma sample (n = 1) and included after terminating ICU care (n = 1) as can be seen in the flow chart in Supplemental Fig. 1.

Patients who survived the hospital stay were invited to a follow-up visit four months after admission. Excluding two patients due to concurrent inclusion in an interventional study, plasma samples from 57 convalescent patients were analysed.

All study participants gave informed consent and in case of inability to do so, the consent was given by their next-of-kin. The study was approved by the Swedish Ethical Review Authority (reference number 2020–01653).

2.2. Blood sampling

Fasting blood samples were drawn in the morning within 14 days of admission to hospital (median 2 and interquartile range [IQR] 2–3 days). Within two hours from venepuncture, plasma in EDTA tubes was prepared by 20 min 2000 g centrifugation in room temperature and stored until analyses in −80°C. Samples from patients at the 4-month follow-up were prepared similarly.

2.3. Biochemical analyses

Plasma concentrations of IL-6, sIL-6R and sgp130 were analysed by enzyme-linked immunoassay (ELISA) using commercial kits from R&D Systems® (R&D Systems Minneapolis, MN, USA). Plasma samples were diluted 1:1 to measure IL-6 and 1:100 to measure sIL-6R and sgp130 levels. Concentrations were derived from a standard curve by interpolation and IL-6 was reported in picograms per millilitre (pg/mL) and sIL-6R and sgp130 in nanograms per millilitre (ng/mL), respectively. The lower limit of detection was 0.70 pg/mL for IL-6, 6.5 pg/mL for sIL-6R and 0.05 ng/mL for sgp130. For high concentrations, outside of the standard curve, samples were reanalysed with a higher dilution factor and/or extrapolated from the standard curve, when possible.

CRP was analysed as part of the routine laboratory tests.

2.4. Statistical analyses

Continuous variables are presented as median and IQR and binary variables as frequencies and proportions (%).

In cross sectional analyses, plasma concentrations of IL-6 classical signalling markers (IL-6, CRP) and IL-6 *trans*-signalling markers (IL-6, sIL-6R, sgp130) are presented in subgroups of disease severity and differences between groups are analysed using Kruskal Wallis test.

Three different indicators of disease severity were use: 1) care level at the time of blood sampling (ICU or intermediate care unit [IMCU] versus regular ward), 2) need for advanced respiratory support at the time of blood sampling (high flow nasal cannula [HFNC], non-invasive ventilation [NIV] or intubation versus none or only oxygen) and 3) in-hospital mortality.

Sensitivity analyses restricting patients with longer duration than 7 days from hospital admission to baseline were performed on all analyses without changing the results of the study (data not shown).

All analyses were performed using Stata Statistical Software: Release 14. College Station, TX: StataCorp LP.

3. Results

The clinical characteristics of the study population are presented in Table 1. The majority of study participants were male. Patients were overweight with a median BMI of 27.8, Diabetes mellitus was present in 24 % and 17 % had prevalent cardiovascular disease. A small proportion of the study participants were admitted to ICU or IMCU at baseline and an even smaller number of patients were in need of advanced respiratory support as seen in Table 1.

Table 1
Clinical characteristics of the study population & proportion severe COVID-19. Continuous variables are presented as median (interquartile range) and proportions as percentages. Advanced respiratory support included non-invasive or invasive mechanical ventilation or oxygen treatment with high flow nasal canula. Missing data on CRP (n = 4).

Clinical characteristics	
Age (years)	61 (50–69)
Male sex (n, %)	72 (64.3)
BMI (kg/m^2)	27.84 (24.6–31.56)
Smoking, active (n, %)	3 (2.7)
Diabetes mellitus (n, %)	27 (24.1)
Cardiovascular disease (n, %)	19 (17.0)
Proportion severe COVID-19 at baseline	
ICU/IMCU (n, %)	15 (13.4)
Advanced respiratory support (n, %)	7 (6.3)
30-day mortality (n, %)	13 (11.6)
IL-6 pathway markers at baseline	
IL-6 (pg/mL)	39.50 (18.88–101.05)
CRP (mg/L)	97.5 (62–169)
sIL-6R (ng/mL)	40.25 (30.92–51.24)
sgp130 (ng/mL)	241.31 (210.23–275.55)

3.1. IL-6 marker plasma levels in subgroups according to disease severity

Patients with severe COVID-19 according to ward level or 30-day mortality, had significantly higher IL-6 and CRP levels than patients with moderate disease (Figs. 1 and 2, panel A and B). In contrast, there were no significant differences in the IL-6 *trans*-signalling markers sIL-6R and sgp130 for these indicators of disease severity (Fig. 1 and Fig. 2, panel C and D). Complete data on plasma levels of the IL-6 signalling markers in different groups of COVID-19 severity are presented in the Supplemental Materials, **Supplemental** Table 1 and 2. The same pattern was seen in patients treated with advanced respiratory support compared to no or little support (**Supplemental** Table 3).

3.2. Correlation between IL-6 marker plasma levels in relation to disease severity

IL-6 was strongly correlated with CRP, but not with any of the IL-6 *trans*-signalling pathway markers as seen in Table 2. The correlation between IL-6 and CRP was slightly weaker in COVID-19 patients admitted to an ICU/IMCU as compared to in patients treated in wards. On the contrary, the negligible insignificant negative correlation between IL-6 and sIL-6R in patients on wards became stronger and the p-value lower, albeit still not significant, in patients with severe COVID-19 (Table 2).

3.3. IL-6 pathway markers after 4 months follow-up

Surviving study participants who accepted to participate were followed up after 4 months (n = 57). This subset was representative for the study population regarding the clinical characteristics and proportion receiving care in ICU/IMCU or advanced respiratory support as presented in **Supplemental Table 4**. After 4 months, IL-6 and sIL-6R levels were significantly decreased. Instead sgp130 had increased (Table 3).

3.4. Discussion

The present study from the first wave of the COVID-19 pandemic, suggests that classical signalling is the dominating IL-6 signalling pathway in hospitalised unvaccinated COVID-19 patients and even more so in severe compared to moderate disease. We observed higher IL-6 and CRP plasma concentrations in severely ill COVID-19 patients, but no differences in the circulating levels of the IL-6 *trans*-signalling markers. Moreover, CRP levels correlated with IL-6 while the IL-6 *trans*-signalling receptor levels did not.

In murine models of influenza and respiratory syncytial virus, IL-6 depletion is associated with impaired inflammatory resolution and subsequent deterioration and tissue damage [12,13]. In SARS-CoV-1 and SARS-CoV-2 instead, high IL-6 levels are associated with a more severe inflammatory reaction [1,14,15]. Thus, there seems to be a differential role for IL-6 in these infections possibly due to shifts in the balance between the two IL-6 signalling pathways.

Targeting both IL-6 signalling pathways with monoclonal IL-6R

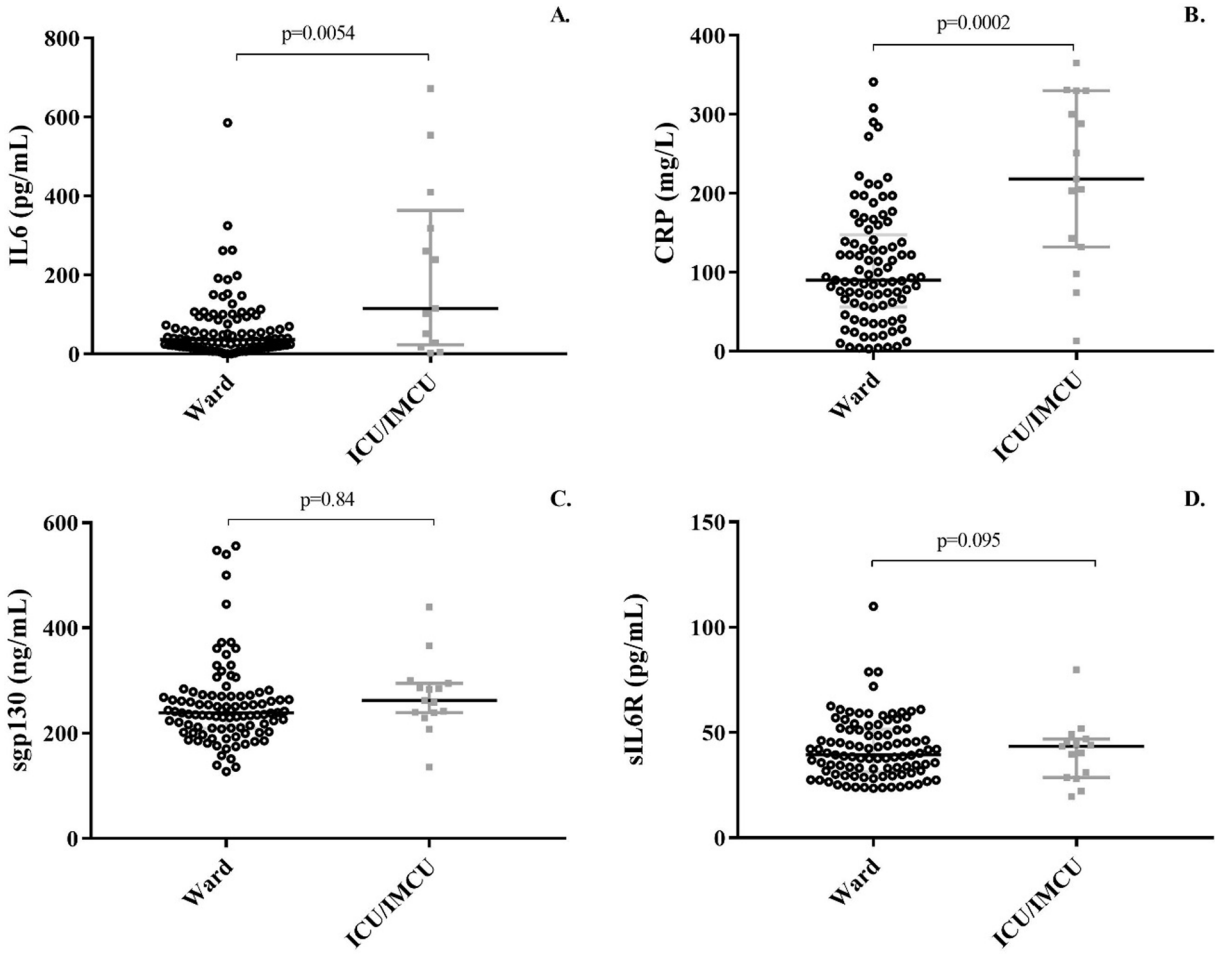

Fig. 1. Plasma levels of IL-6 signalling markers by care level. IL-6 signalling marker plasma concentrations are presented as medians. Differences between groups defined by care level were analysed using Kruskal Wallis test and expressed in p-values. For IL-6, two outliers with values > 1000 pg/mL were restricted from the graph.

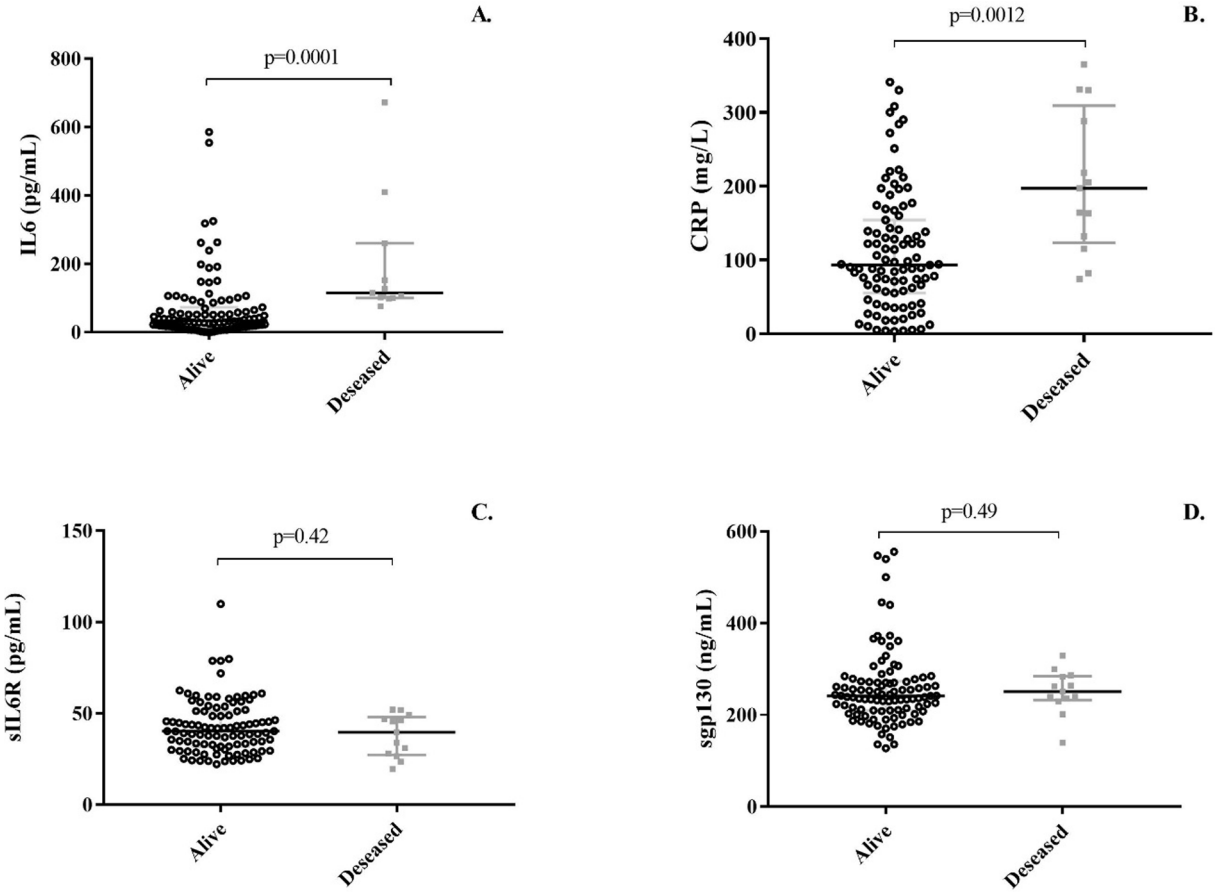

Fig. 2. Plasma levels of IL-6 signalling markers by 30-day mortality. IL-6 signalling marker plasma concentrations are presented as medians. Differences between groups defined by care level or deceased/alive at one month were analysed using Kruskal Wallis test and expressed in p-values. For IL-6, two outliers with values > 1000 pg/mL were restricted from the graph.

Table 2
Correlation between IL-6 and the IL-6 signalling pathway markers by care level. Correlation tested by Spearman correlation and presented as rho and p-value in the full cohort and stratified by care level at baseline. Missing data on CRP (n = 4).

All (n = 112)	**IL-6**	**Ward (n = 97)**	**IL-6**	**ICU/IMCU (n = 15)**	**IL-6**
CRP	0.67 p < 0.0001	**CRP**	0.61 p < 0.0001	**CRP**	0.49 p = 0.065
sIL-6R	−0.09 p = 0.32	**sIL-6R**	− 0.04 p = 0.71	**sIL-6R**	− 0.38 p = 0.16
sgp130	0.05 p = 0.62	**sgp130**	0.008 p = 0.94	**sgp130**	− 0.046 p = 0.87

Table 3
IL-6 trans-signalling pathway markers at baseline and after 4 months. Data are presented as median (IQR).

	Baseline (n = 112)	**4 months (n = 57)**	**P**
IL-6 (pg/mL)	39.50 (18.88–101.05)	1.53 (0.59–3.34)	0.0001
sIL-6R (ng/mL)	40.25 (30.92–51.24)	35.62 (28.11–41.36)	0.006
sgp130 (ng/mL)	241.31 (210.23–275.55)	271.7 (218.72–326.53)	0.027

antibodies, in particular tocilizumab, on top of standard care has been investigated in randomised controlled trials of hospitalised COVID-19 patients [16–20]. Of note is that standard care in most studies include the use of low-dose systemic glucocorticoids. Results have overall been inconclusive albeit with a trend towards protective effects as was also concluded in a Cochrane review [21]. The two largest of these randomised controlled trials did however both show improved clinical recovery and survival [17,19]. These studies indicate causality between COVID-19 clinical outcome and the IL-1-IL-6-CRP pathway but do not establish which of the IL-6 pathways is central. Targeting IL-6 *trans*-signalling specifically could be attractive due to the increased incidence of bacterial infections associated with inhibition of classical IL-6 signalling [22] our results do however not support this.

In line with previous studies, we saw significantly higher plasma levels of IL-6 and CRP in patients with severe compared to moderate COVID-19 [1,9,15]. The IL-6 *trans*-signalling pathway markers, sIL-6R and sgp130, on the other hand, did not differ between these two groups of patients. This finding is conflicting with the results in a small Italian case-control study with ICU-treated COVID-19 patients (n = 23) and healthy controls where cases had significantly higher sIL-6R and lower sgp130 circulating levels [8]. Considering that the control group was free of infection/inflammation, the differences in inflammation marker levels is not surprising. Of note, nearly half of the cases had sIL-6R levels comparable to those in the control group [8]. In addition, in an Australian study of COVID-19 patients (n = 85) with diverse severity and at different stages of the disease, sIL-6R along with IL-6 was higher in patients in the ICU but in line with the results from the present study IL-6 and sIL-6R levels did not correlate [9]. Moreover, sIL-6R was predictive of needing ICU care and more so than IL-6. The absolute sIL-6R plasma levels during active infection were however remarkably lower in our ICU/IMCU subgroup than the serum levels in the Australian study and in the group of patients with high sIL-6R levels presented in the Italian study [8,9]. Possible explanations for differences in absolute

levels between our study participants and in the above-mentioned studies could be that concentrations differ in serum and plasma, diverse pre-analytical conditions, and the different stages of the disease. We could however not find any difference in IL-6 *trans*-signalling marker levels with duration of the disease when looking at levels in relation to time passed from symptom onset to baseline sampling (data not shown). In addition, when scrutinising the few ICU/IMCU treated patients and especially those with extreme IL-6 levels (>500 pg/mL, n = 4), as a sign of very severe disease, we did not see a common pattern of higher sIL-6R concentrations (data not shown). Concentrations of sgp130 were lower in COVID-19 patients compared to healthy controls in the Di Spigna study [8] while we saw a trend towards higher levels in patients with severe disease albeit not statistically significant. The mechanisms that regulate sgp130 production are largely unknown and sgp130 circulating levels are increased in some and decreased in other inflammatory conditions compared to healthy individuals [23].

As is seen in other studies of inflammatory markers, levels of IL-6 and CRP varied within groups of disease severity most certainly due to the manyfold stimulators of inflammation in acute severe infectious disease. For this reason, these biomarkers should always be used together with clinical parameters to assess disease severity.

In the four-month follow-up, levels of sIL-6R were significantly lower and sgp130 levels significantly higher compared to in the acute phase of COVID-19. Hence, IL-6 *trans*-signalling markers were marginally affected by the infection but less than has been shown in infections in general and in COVID-19 specifically [3,8].

The finding that only CRP correlated with IL-6 regardless of care level again suggests that classical IL-6 signalling was the more dominant IL-6 pathway in both moderately and severe COVID-19 in our cohort. In line with the Australian cohort, a correlation between IL-6 and sIL-6R could not be demonstrated [9]. When stratifying for care level however, we found that the negative correlation coefficient was increased in patients on ICU/IMCU compared to in patients on wards albeit still not statistically significant. The finding of a potentially negative correlation between IL-6 and sIL-6R is surprising considering the previously described positive feedback mechanism with CRP stimulating shedding of IL-6R from the cell membrane [24]. On the other hand, the membrane-bound or soluble IL-6 receptor is rapidly internalised after binding the co-receptor gp130 and the IL-6:(s)IL-6R complex is then degraded in the cytosol while the cell is desensitised to IL-6 signalling by down-regulated IL-6R expression [25,26].

The present results confirm the central role of IL-6 in COVID-19 but reveals the complexity of the balance between IL6 classical signalling and *trans*-signalling. Regardless of the somewhat diverging results on IL-6 *trans*-signalling in the COVID-19 literature, IL-6 signalling is a suitable target with CRP and IL-6 as prognostic biomarkers while the role for IL-6 *trans*-signalling as a target and sIL-6R as a marker is questionable.

4. Strengths and limitations

This study is to date the largest cohort assessing the balance between the two IL-6 signalling pathways in consecutively included COVID-19 patients admitted to in-hospital care.

The study is limited by the unequal sample sizes in the different subgroups. With a small group treated in the ICU/IMCU and a possible lack of power there is a risk that we were not able to demonstrate potential differences between groups. On the other hand, there was enough power to show significant differences between moderate and severe COVID-19 for the classical IL-6 signalling markers indicating that lack of power did not explain the absence of significant differences between the groups.

Defining severe COVID-19 by care level or treatment with advanced respiratory support could be affected by the access to such level of care during a pandemic. To overcome this potential selection bias, we also included the criteria of 30-day mortality.

The analyses performed were not subject to multivariable analyses i.e., this study cannot demonstrate independent associations between exposure and outcome.

Plasma levels of the IL-6 signalling markers could differ from other studies due to differences in pre-analytical conditions. This would however only affect absolute differences, not affecting relative differences between subgroups of COVID-19 severity.

Finally, with the arrival of medical treatment, virus mutations, increasing proportions of immunised individuals either by vaccination or prior infection, a complex network of factors influences the outcome in COVID-19 patients. In the light of this, we cannot with certainty apply our results on the disease of today. We do however believe that the results merit attention owing to the central position of IL-6 in COVID-19 and the fact that the role of its two pathways has not been fully elucidated. Moreover, despite widely available and effective vaccines in the developed countries, large parts of these populations remain unvaccinated as do large parts of the developing countries. In addition, considering the similarities between the SARS, MERS and COVID-19 and the tendency of this type of viruses to return we need to stay ahead.

5. Conclusion

In this hospital-based cohort of patients with COVID-19 in the first wave of the pandemic, classical signalling was the dominating IL-6 signalling pathway in severe compared to moderate disease. Considering the diverging results of this study compared to the other existing clinical study on both IL-6 signalling pathways in COVID-19, more research into the mechanisms for IL-6 signalling in this setting is warranted.

CRediT authorship contribution statement

Louise Ziegler: Conceptualization, Formal analysis, Methodology, Visualization. **Annika Lundström:** Conceptualization, Formal analysis, Methodology, Visualization. **Sebastian Havervall:** Conceptualization, Data curation, Project administration, Resources. **Charlotte Thålin:** Conceptualization, Data curation, Funding acquisition, Project administration, Resources. **Bruna Gigante:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Visualization.

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

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