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Can inflammatory plasma proteins predict Long COVID or Fatigue severity after SARS-CoV-2 infection?

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ABSTRACT

Objective: To investigate whether specific immune response plasma proteins can predict an elevated risk of developing Long COVID symptoms or fatigue severity after SARS-CoV-2 infection.
Methods: This study was based on 257 outpatients with test-confirmed SARS-CoV-2 infection between February 2020 and January 2021. At least 12 weeks after the acute infection, 92 plasma proteins were measured using the Olink Target 96 immune response panel (median time between acute infection and venous blood sampling was 38.8 [IQR: 24.0–48.0] weeks). The presence of Long COVID symptoms and fatigue severity was assessed 115.8 [92.5–118.6] weeks after the acute infection by a follow-up postal survey. Long COVID (yes/no) was defined as having one or more of the following symptoms: fatigue, shortness of breath, concentration or memory problems. The severity of fatigue was assessed using the Fatigue Assessment Scale (FAS). In multivariable-adjusted logistic and linear regression models the associations between each plasma protein (exposure) and Long COVID (yes/no) or severity of fatigue were investigated.
Results: Nine plasma proteins were significantly associated with Long COVID before, but not after adjusting for multiple testing (FDR-adjustment): DFFA, TRIM5, TRIM21, HEXIM1, SRPK2, PRDX5, PIK3AP1, IFNLR1 and HCLS1. Moreover, a total of 10 proteins were significantly associated with severity of fatigue before FDR-adjustment: SRPK2, ITGA6, CLEC4G, HEXIM1, PPP1R9B, PLXNA4, PRDX5, DAPP1, STC1 and HCLS1. Only SRPK2 and ITGA6 remained significantly associated after FDR-adjustment.
Conclusions: This study demonstrates that certain immune response plasma proteins might play an important role in the pathophysiology of Long COVID and severity of fatigue after SARS-CoV-2 infection.

1. Introduction

The acute phase of the worldwide COVID-19 pandemic is over, but the SARS-CoV-2 virus still infects thousands of people every day (WHO Coronavirus (COVID-19) Dashboard, 2024). Due to natural infection and vaccination the vast majority of the population worldwide is already immunized and less susceptible to severe disease or hospitalization (Markov et al., 2023). Nevertheless, in some individuals with SARS-CoV-2 infection, symptoms such as fatigue, shortness of breath, memory difficulties or concentration problems persist for many weeks or even months and years (Davis et al., 2023). This phenomenon is referred to as 'Long COVID' and probably will continue to be an issue in the future. Generally, the complex of potential symptoms is very heterogeneous and a specific characterization or definition is difficult (Michelen et al., 2021). Surprisingly, the severity of the symptoms at the acute event is likely not a good predictor for the development of Long COVID (Castanares-Zapatero et al., 2022). On the other hand, in patients with Post COVID fatigue (PCF), the number of different symptoms that occur during the acute infection is positively associated with the severity of PCF (Schmidbauer et al., 2023). A lot of research has been conducted to understand the underlying pathophysiological mechanisms and to identify risk factors, yet the phenomenon of Long COVID and persisting symptoms such as fatigue is far from being understood completely. Many studies indicated or proposed potential associations with inflammatory processes or autoimmunity caused by the virus (Astin et al., 2023; Castanares-Zapatero et al., 2022; Yong, 2021). Thus, the aim of the present study was to identify immune response plasma proteins that are prospectively associated with an increased risk of Long COVID or

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severity of fatigue.

2. Methods

2.1. Study population and data collection

The present analysis was based on patients from a prospective single-center study in the region of Augsburg, Germany. In this study, individuals with test confirmed SARS-CoV-2 infection between February 2020 and January 2021 were recruited in cooperation with the local health offices (potential study participants were asked to participate regardless of their Long COVID risk). The participants were examined in a baseline visit between November 2020 and May 2021 which was scheduled weeks or months after the acute infection (counting from the day of the first positive test). For the present analysis, participants were only included on the following additional conditions: no hospital treatment of the acute infection and a time gap of at least 12 weeks between acute infection and baseline examination visit. The baseline visit included a self-reporting questionnaire administered on a tablet personal computer including questions about the acute infection (duration, severity etc.), specific symptoms, fatigue and other topics. Additionally, venous blood samples were taken (mostly from elbow veins). The plasma samples were promptly processed (centrifugation, aliquoting and freezing at −80 °C). Approximately two years after the baseline visit, a postal follow-up questionnaire was sent to all participants in order to evaluate persisting symptoms, (chronic) fatigue, quality of life and other topics. Altogether, 361participants took part in the follow-up survey. For the final analyses, a total of 257 participants were included, thereof 257 with information on severity of fatigue and 254 with information on Long COVID. Fig. 1 displays the inclusion/exclusion process and the number of participants at each stage. Median time between acute infection and baseline examination was 38.8 weeks (IQR: 24.0–48.0 weeks); median time between acute infection and postal follow-up was 115.8 weeks (IQR: 92.5–118.6 weeks).

The data collection and study protocol has been approved by the ethics committee of the Ludwig Maximilians Universität, Munich (no. 20–735) and the study has also been registered at Clinical Trials (no. NCT04615026). The study was performed in accordance with the Declaration of Helsinki and all study participants have given written informed consent.

2.2. Clinical chemistry measurement

The measurements of the 92 protein markers were performed by Olink Proteomics (Uppsala, Sweden) using the Olink Target 96 immune response panel. The procedure of measurements is based on the Proximity Extension Assay (PEA). Detailed information on this process can be found at the website of Olink Proteomics (Olink Proteomics, 2016) or in a prior publication (Ponce-de-Leon et al., 2022). In the main manuscript we use short names or abbreviations to indicate the immune markers. Full names can be taken from table S1 of the supplementary material.

2.3. Outcome

The endpoint considered in this analysis was the self-reported presence of Long COVID and severity of fatigue approximately 2 years after acute SARS-CoV-2 infection. In a postal follow-up survey patients were asked whether they had the following symptoms/conditions during the past two weeks: fatigue, shortness of breath, concentration problems or memory problems. In supplemental table S4 we provide the actual questions (translated) that were asked in the follow-up survey. Long COVID was defined as the presence of one or more of these symptoms. Fatigue severity was assessed using the established and validated Fatigue Assessment Scale (FAS) (Michielsen et al., 2003). It includes 10 items (5 regarding physical fatigue and 5 regarding mental fatigue), each with possible response options from “never” (1 point) to “always” (5 points) resulting in a maximum total score of 50 (Michielsen et al., 2003).

2.4. Statistical analysis

For the comparison of categorical variables, Chi-square tests were performed and the results were presented as absolute frequencies with percentages. For normally-distributed continuous variables, Student’s *t*-tests were used. For continuous variables that were not normally-distributed we used nonparametric tests. The results are presented as mean and standard deviation (SD) or median and inter-quartile range (IQR).

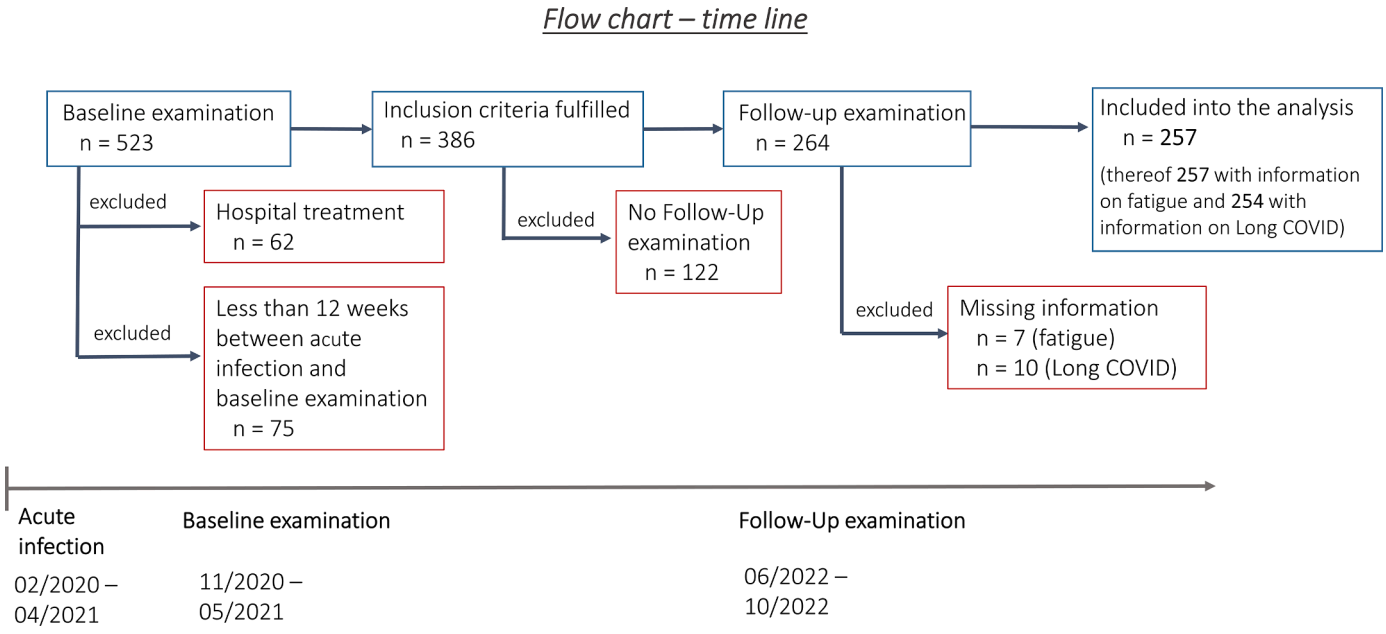

Fig. 1. flowchart and time line of the included participants and baseline examination as well as postal follow-up survey.

2.5. Logistic and linear regression models

We calculated logistic regression models in order to examine the association between each plasma protein (exposure) and the presence of Long COVID. Accordingly, we calculated linear regression models to analyze the associations between plasma proteins and severity of fatigue. Since for 26 plasma proteins there were more than 25 % of the determined values below the limit of detection, we did not calculate regression models for these proteins as the validity of these results would be questionable. For plasma proteins with less than 25 % values below limit of detection, we included all cases into the regression models and in case of values below limit of detection, we used the extrapolated values provided by Olink. According to literature review, all logistic and linear regression models were adjusted for sex, age, smoking status at follow-up survey (current smoker, ex-smoker, never smoker), depression at follow-up (yes/no), body mass index (BMI, kg/m^2) at follow-up and time lag between baseline examination and follow-up survey. For the linear regression model, normality of the residuals was checked graphically (histogram and Q-Q-Plot) and potential outliers were identified by calculating Cook's distance. In addition, we calculated robust regression models and median regression models as a sensitivity analysis. The results of those models confirmed the results of the linear models as the obtained results were very similar (results not shown).

Another sensitivity analysis was conducted with a different definition of Long COVID. Firstly, logistic regression models were calculated as described above, but an individual was assigned to the Long COVID group only if he/she had two or more of the four symptoms at follow-up (see figure S1, supplementary material). Secondly, ordinal regression models were calculated using three outcome categories: no Long COVID symptoms, one Long COVID symptom and two or more Long COVID symptoms (see figure S2, supplementary material).

Since this study is characterized mainly by an exploratory approach, we decided to provide the results without adjusting p-values and 95 % CIs for multiple testing. We additionally calculated FDR (false discovery rate)-adjusted p-values and indicated the corresponding protein markers with significant p-values after FDR-adjustment in the corresponding result figures (orange color). The alpha level of significance was set at 0.05.

All statistical analyses were performed using R statistic software version 4.2.1.

3. Results

Table 1 displays the baseline characteristics for the total sample and stratified for Long COVID at follow-up (approximately 2 years after infection). Mean age was 49.6 (SD: 14.8) years with no significant difference between the Long COVID and the No Long COVID group. A majority of 58 % were female, with a non-significantly higher percentage in the Long COVID group. Participants of the Long COVID group were significantly more likely to have depression, at baseline as well as at the time of follow-up. Likewise, the Long COVID group had significantly higher FAS scores at both time points. There was no difference in body mass index (BMI), diabetes mellitus, hypertension, autoimmune disease, chronic bronchitis/COPD and smoking status between the two groups. In table S4, supplementary material, the baseline characteristics stratified for the number of Long COVID symptoms (0 to 4) are presented.

Fig. 2 displays the association between immune response markers at baseline and the presence of Long COVID at follow-up. Before FDR-adjustment, 9 markers were significantly associated in multivariable adjusted logistic regression models: DFFA, TRIM5, TRIM21, HEXIM1, SRPK2, PRDX5, PIK3AP1, IFNLR1 and HCLS1. None of these 9 markers remained being significantly associated with Long COVID after FDR-adjustment.

Another 10 immune response markers were significantly associated with severity of fatigue in multivariable adjusted linear regression models (see Fig. 3): SRPK2, ITGA6, CLEC4G, HEXIM1, PPP1R9B, PLXNA4, PRDX5, DAPP1, STC1 and HCLS1. Of those, SRPK2 and ITGA6 remained significantly associated also after FDR-adjustment.

Tables S2 and S3 of the supplementary material display all results of the regression models in tabular form. Supplemental figures S1 and S2 show the results of the alternative logistic regression models (Long COVID definition: two ore more symptoms at follow-up) and the ordinal regression models. The overall results are quite similar and apart from PIK3AP1 and HCLS1, all markers of the original logistic models were confirmed by at least one of the alternative models.

4. Discussion

In this study, we investigated the associations between 92 immune response markers and Long COVID as well as severity of fatigue after SARS-CoV-2 infection. Many different risk factors for the development of Long COVID have previously been identified such as female sex and older age (Conti et al., 2023; Thompson et al., 2022), ethnicity

Table 1
Baseline characteristics for the total sample and stratified for Long Covid symptoms at follow-up. Continuous variables are presented as mean (SD) or median (IQR), categorical variables are shown as total numbers (%).

	Total sample (n = 257)	No Long COVID* (n = 99)	Long COVID* (n = 155)	p-value	Missing values
Age (years)	49.6 (14.8)	50.8 (14.9)	48.9 (14.8)	0.3153	0
Sex (male)	108 (42.0)	50 (50.5)	58 (37.4)	0.0540	0
Family status - married	183 (71.5)	65 (65.7)	116 (75.3)	0.312	1
Baseline depression (yes/no)	24 (9.3)	5 (5.1)	19 (12.3)	0.0393	8
Follow-up depression (yes/no)	13 (5.2)	1 (1)	12 (8)	0.0042	13
Baseline FAS score	19 (16 - 24)	16 (13 - 19)	22 (18 - 28)	<0.001	0
Follow-up FAS score	20 (15 - 25)	15 (13 - 17.5)	23 (19 - 31)	<0.001	0
Comorbidities (at baseline)					
BMI (kg/m^2)	24.7 (22.2–27.8)	24.7 (22.7–27.5)	24.7 (22.1–28.4)	0.8186	0
Diabetes mellitus	3 (5.1)	3 (3.1)	10 (6.5)	0.363	2
Hypertension	55 (22.1)	21 (21.9)	33 (22.0)	1	8
Autoimmune disease	23 (9.3)	7 (7.2)	15 (10.1)	0.580	9
Chronic bronchitis/COPD	17 (6.9)	4 (4.1)	13 (8.8)	0.251	9
Baseline smoking status				0.4267	0
never smoker	139 (54.1)	57 (57.6)	80 (51.6)		
current smoker	103 (40.1)	35 (35.4)	67 (43.2)		
ex-smoker	15 (5.8)	7 (7.1)	8 (5.2)		

FAS = Fatigue Assessment Scale.

* for 3 patients sufficient information on Long-Covid at follow-up (yes/no) was missing, but not so information on FAS score at follow-up. These 3 patients are included in the total sample (left column), but not in the columns stratified for Long-Covid at follow-up.

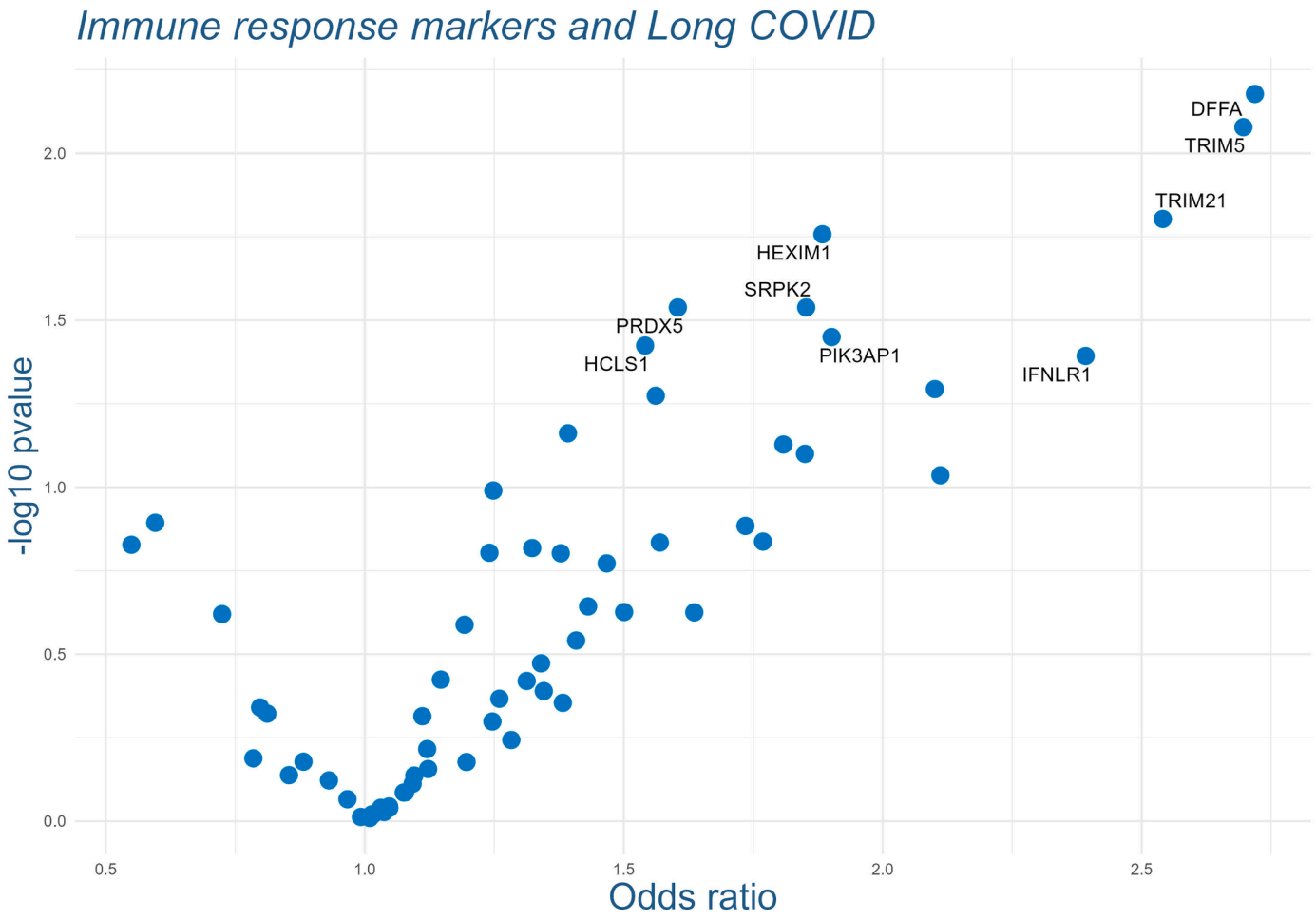

Fig. 2. Association between immune response markers and Long COVID symptoms (yes/no) at follow-up. The multivariable logistic regression models (n = 240) were adjusted for the following variables: sex, age, smoking status at follow-up visit, depression at follow-up, BMI at follow-up and time lag between baseline examination and follow-up survey. The values on the X-axis display the estimated odds ratios. The y-axis shows the corresponding, not FDR-adjusted p value for each marker. Only markers with less than 25 % values below limit of detection are displayed. Name-labeled markers represent significant association with Long COVID before FDR-adjustment. Markers displayed in orange had significant p-values after FDR adjustment (none in this figure).

(Thompson et al., 2022), lifestyle behaviors e.g. active smoking (Conti et al., 2023), overweight/obesity (Thompson et al., 2022) or biomarkers such as CRP or LDH (Conti et al., 2023). Also, alterations of the immune system have been reported to be associated with Long COVID including a variety of cytokines such as IL-6 and changes in immune cell counts or phenotypes (Conti et al., 2023). These results suggest, that immune system particularities and inflammation processes at the acute infection or shortly afterwards are connected to the subsequent persistence of some symptoms. The markers investigated in the present study are so called '*immune response markers*'. A total of 9 markers were associated with Long COVID, but only before adjusting for multiple testing. Some of these markers also belong to the 10 biomarkers that were associated with severity of fatigue. Only the proteins SRPK2 (SRSF protein kinase 2) and ITGA6 (Integrin Subunit Alpha 6) remained significantly associated with severity of fatigue after adjusting for multiple testing.

SRPKs are known to be involved into the regulation of several steps of mRNA splicing but also regulate various cellular activities by phosphorylation (Nikolakaki et al., 2022). SRPK2 in particular has been shown to promote either apoptosis or tumor growth, revealing diverging functions in different cell environments (Nikolakaki et al., 2022). The ITGA6 gene encodes the Integrin subunit alpha 6 (Human Protein Atlas, 2014). Integrins are transmembrane proteins that mediate interactions with other cells and the extracellular matrix (adhesion molecules), but they are also important in cell signal transduction (Takada et al., 2007). Prior studies reported its overexpression in different cancer types suggesting that ITGA6 promotes tumorigenesis and metastasis (Brooks et al., 2016; Jin et al., 2019; Stewart et al., 2016).

To date, many hypotheses have been suggested on the potential pathophysiological mechanism underlying the development of Long COVID. One of the current main hypotheses is a long-term tissue damage, especially in the lungs, that causes persisting symptoms (Castanares-Zapatero et al., 2022; Yong, 2021). In addition, vascular dysfunction, hyper-coagulation or mitochondrial dysfunction causing reduced tissue oxygen availability have been proposed as potential pathomechanisms (Astin et al., 2023). Another major theory claims an involvement of a pathological and prolonged inflammation (Astin et al., 2023; Castanares-Zapatero et al., 2022; Yong, 2021). One often suggested mechanism refers to the spike protein (Theoharides, 2022), which was suspected to cause perivascular inflammation in the brain (Theoharides, 2022). Other authors proposed autoimmunity triggered by the spike protein (Paladino et al., 2020), a hypothesis which is questioned by recent results (Scherlinger et al., 2023).

Overall, studies on the interconnection between immunological alterations and risk of Long COVID are very heterogeneous as different studies analyzed different aspects of the immune system and used different methodology (definition of Long COVID, inclusion criteria etc.). A weakness of many observational studies is a completely cross-sectional approach, not allowing to draw any conclusions about causality. The present study used a longitudinal design and the blood samples for the biomarker measurements were taken approximately two

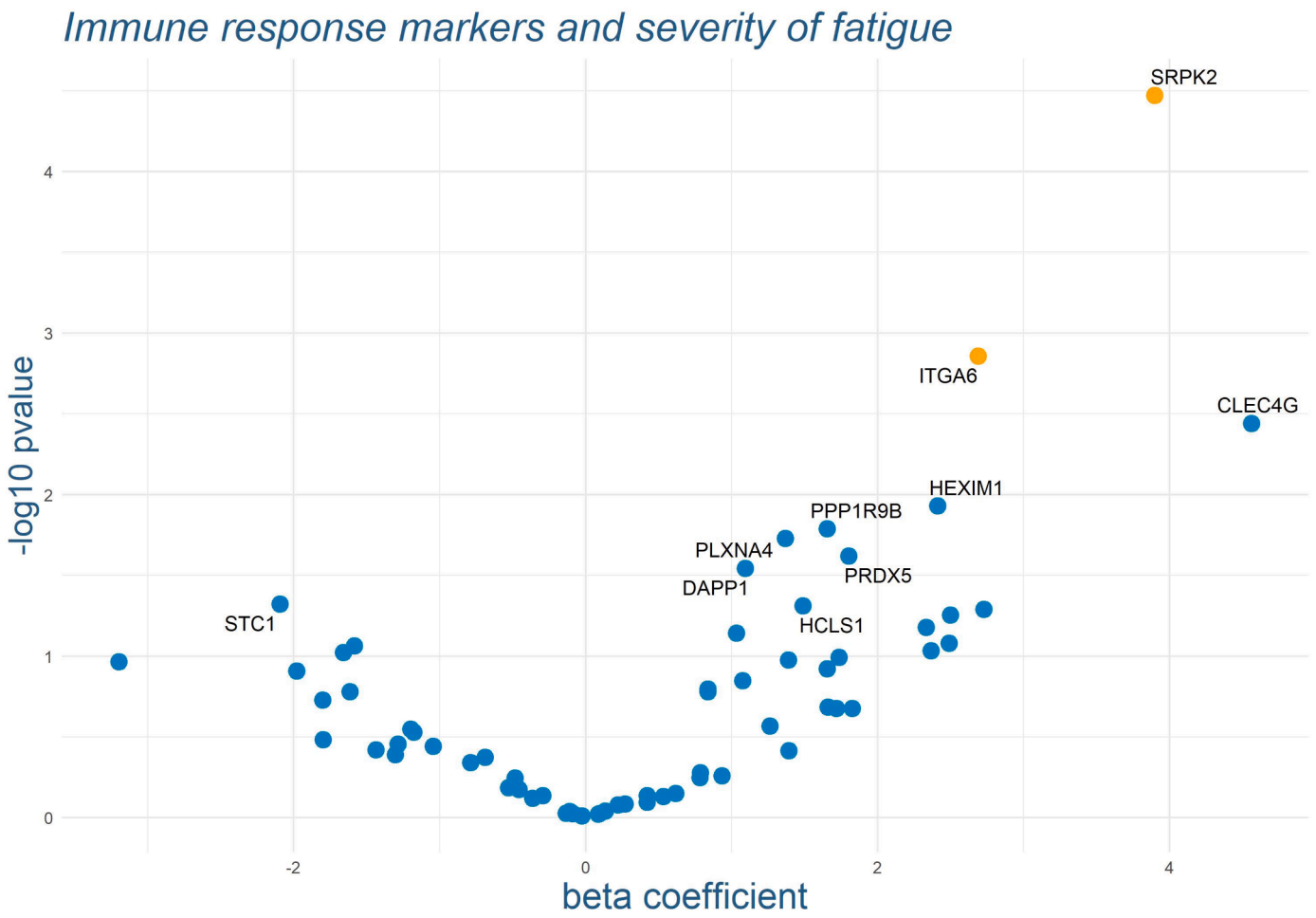

Fig. 3. Association between immune response markers and severity of fatigue at follow-up. The multivariable linear regression models ($n = 242$) were adjusted for the following variables: sex, age, smoking status at follow-up, depression at follow-up, BMI at follow-up and time lag between baseline and follow-up. The values on the X-axis display the estimated ß-coefficients. The y-axis shows the corresponding, not FDR-adjusted p value for each marker. Only markers with less than 25 % values below limit of detection are displayed. Name-labeled markers showed significant association with FAS score before FDR-adjustment. Markers displayed in orange had significant p-values after FDR adjustment.

years before the assessment of Long COVID or severity of fatigue. Some prior studies also examined the associations between plasma proteins and Long COVID. For example, Talla et al. measured, among other markers, the serum proteins of the Olink immune response panel (Talla et al., 2023). They used two-sided Wilcoxon tests to compared the values for a group of 55 COVID patients with persisting symptoms (≥60 days) and two control groups (COVID patients without persisting symptoms and uninfected individuals). They found several markers to be higher in the Long COVID group. Amongst these markers, there were six markers (DFFA, SRPK2, PIK3AP1, HCLS1, PRDX5, PPP1R95) that were also significantly associated with either Long COVID and/or severity of fatigue in the present study. Some other studies also analyzed the association between immune markers/plasma proteins (using other panels/methods than the OLINK immune response panel) and the persistence of symptoms after SARS-CoV-2 infection (Espín et al., 2023; Gu et al., 2023; Klein et al., 2023; Kovarik et al., 2023; Leung et al., 2024; Yong et al., 2023). Generally, the studies on this topic are difficult to compare as they examined different markers and used different methodology and definitions and so their overall results do not reveal a uniform picture.

The present study had an explorative approach, which is also the reason why we presented the results (p-values and 95 %CI) without adjusting for multiple testing. Anyhow, we consider these results to be well in line with the general hypothesis of a pathological inflammation underlying Long COVID. Except one, all markers showing noticeable associations were positively associated with Long COVID or fatigue severity indicating a general pro-inflammatory condition in the affected patients.

Remarkably, we found stronger associations between plasma proteins and severity of fatigue as for Long COVID. One reason might be the specific assessment of fatigue using the established FAS score. The presence of Long COVID was only determined by the presence of one or more of four self-reported symptoms, since so far no established and validated score is available that also assesses the extent of the Long COVID. Nevertheless, fatigue might indeed have stronger associations with certain plasma proteins than Long COVID in general. In a prior study we observed an association between SARS-CoV-2-specific T-cell responses and fatigue in women after mild acute COVID-19 disease (Meisinger et al., 2022), indicating an important interplay specifically between fatigue and immunological processes after SARS-CoV-2 infection. Even though many biomarkers have been investigated and discussed, a general association between (chronic) fatigue and specific circulating biomarkers in healthy individuals or patients with certain diseases remains rather unclear (Blundell et al., 2015; Klimas et al., 2012; Roerink et al., 2017).

4.1. *Strengths and limitation*

There are several strengths characterizing this study. First, all patients with non-hospitalized SARS-Cov-2 infection in the early phase of

the pandemics were uniformly recruited with the help of the local health offices. Another strength is the longitudinal approach with blood sampling after the acute infection (more than 3 months after the first positive test) and assessment of Long COVID and fatigue severity approximately 2 years later. The variety of information collected for each patient allowed to calculate well-adjusted regression models minimizing the effects caused by important confounders.

However, there are some limitations as well. First, there was no validation cohort from other studies to confirm the obtained results. Second, information on various symptoms were solely based on self-reported data and not on confirmed clinical diagnoses. We used a very broad definition of Long COVID (about 61 % of all individuals included were classified as having Long COVID at follow-up), which is higher than the frequencies generally reported. Nevertheless, the sensitivity analyses presented in the supplementary material suggest similar results for narrower definitions of Long COVID. As we only included persons treated on an outpatient basis, our results might not be applicable to patients with a severe course of the acute infection. No information on the plasma proteins at the time of the acute infection was available and there were also no repeated measurements of the respective markers. As this study is based on observational data, no conclusions about causality can be drawn (including the possibility of reverse causality). Finally, we might not have considered all potential confounders and results may not be generalized to all ethnicities.

5. Conclusion

Several immune response markers appear to be related to Long COVID and severity of fatigue. Especially SRPK2 and ITGA6 are strongly associated with severity of fatigue in patients with prior SARS-CoV-2 infection. These association might contribute to a better and more comprehensive understanding of the underlying pathophysiology of persistent symptoms like fatigue after SARS-CoV-2 infection.

Ethics approval and consent to participate

The data collection and study protocol has been approved by the ethics committee of the Ludwig Maximilian University of Munich (no. 20–735) and the study has also been registered with Clinical Trials (no. NCT04615026). The study was performed in accordance with the Declaration of Helsinki and all study participants have given written informed consent.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated during and/or analyzed in the current study are not publicly available due to data protection aspects but are available in an anonymized form from the corresponding author on reasonable request.

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CRediT authorship contribution statement

Timo Schmitz: Writing – original draft, Methodology, Formal analysis, Conceptualization. **Dennis Freuer:** Writing – review & editing, Methodology, Formal analysis. **Yvonne Goßlau:** Writing – review & editing, Resources, Methodology, Investigation, Conceptualization. **Tobias Dominik Warm:** Writing – review & editing, Resources, Methodology, Investigation. **Alexander Hyhlik-Dürr:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Conceptualization. **Jakob Linseisen:** Writing – review & editing, Resources, Project administration, Methodology, Conceptualization. **Christa Meisinger:** Writing – review & editing, Supervision, Project administration, Methodology, Formal analysis, Conceptualization. **Inge Kirchberger:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

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.

Data availability

The data that has been used is confidential.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2024.199363.

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