Inflammation Research (2023) 72:1933–1940
https://doi.org/10.1007/s00011-022-01670-3

Inflammation Research

BRIEF REPORT

A common variant close to the "tripwire" linker region of *NLRP1* contributes to severe COVID-19

Vinicius N. C. Leal[1] · Leandro M. Paulino[2] · Raylane A. G. Cambui[1] · Thiago G. Zupelli[1] · Suemy M. Yamada[1] · Leonardo A. T. Oliveira[1] · Valéria de F. Dutra[3] · Carolina B. Bub[3] · Araci M. Sakashita[3] · Ana Paula H. Yokoyama[3] · José M. Kutner[3] · Camila A. Vieira[2] · Wellyngton M. de S. Santiago[2] · Milena M. S. Andrade[4] · Franciane M. E. Teixeira[4] · Ricardo W. Alberca[4] · Sarah C. Gozzi-Silva[4] · Tatiana M. Yendo[5] · Lucas C. Netto[6] · Alberto J. S. Duarte[4,7] · Maria N. Sato[4] · James Venturini[2] · Alessandra Pontillo[1]

Received: 1 September 2022 / Revised: 26 October 2022 / Accepted: 9 November 2022 / Published online: 23 November 2022

Abstract

Objective and design The heterogeneity of response to SARS-CoV-2 infection is directly linked to the individual genetic background. Genetic variants of inflammasome-related genes have been pointed as risk factors for several inflammatory sterile and infectious disease. In the group of inflammasome receptors, NLRP1 stands out as a good novel candidate as severity factor for COVID-19 disease.

Methods To address this question, we performed an association study of *NLRP1*, *DPP9*, *CARD8*, *IL1B*, and *IL18* single nucleotide variants (SNVs) in a cohort of 945 COVID-19 patients.

Results The NLRP1 p.Leu155His in the linker region, target of viral protease, was significantly associated to COVID-19 severity, which could contribute to the excessive cytokine release reported in severe cases.

Conclusion Inflammasome genetic background contributes to individual response to SARS-CoV-2.

Keywords NLRP1 · COVID-19 · Inflammasome · SARS-CoV-2 · SNV

Responsible Editor: John Di Battista.

✉ Alessandra Pontillo
alepontillo@usp.br

1 Laboratório de Imunogenética, Departamento de Imunologia, Instituto de Ciências Biomédicas/ICB, Universidade de São Paulo/USP, São Paulo, SP, Brasil

2 Faculdade de Medicina, Universidade Federal de Mato Grosso do Sul, Campo grande, Mato Grosso do Sul, Brasil

3 Departamento de Hemoterapia, Hospital Israelita Albert Einstein, São Paulo, SP, Brasil

4 Laboratório de Investigação Médica em Dermatologia e Imunodeficiências (LIM-56, Departamento de Dermatologia, Hospital das Clínicas E Faculdade de Medicina/HCFMUSP, São Paulo, Brasil

5 Departamento de Dermatologia, Faculdade de Medicina, Instituto de Medicina Tropical, Universidade de São Paulo/FMUSP, São Paulo, Brasil

6 Unidade Terapia Intensiva, Hospital das Clínicas/FMUSP, São Paulo, Brasil

7 Departamento de Patologia, Faculdade de Medicina, Universidade de São Paulo/FMUSP, São Paulo, Brasil

Introduction

The individual response to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection is considerably variable in the general population, ranging from a flu-like disease to a severe acute respiratory syndrome. High mortality was associated with old age and male sex [1, 2], hypertension and diabetes [3].

SARS-CoV-2 infects airway and lung epithelial cells, and innate receptors may detect it and control its replication or inducing excessive inflammation and broad activation of neighboring cells, including leukocytes, platelets, and endothelium, therefore leading to endotheliitis and diffuse thromboembolism, complications observed in severe patients [3, 4].

Half a dozen genetic *loci* have been identified as possible risk factors for severe COVID-19 disease, including genes involved in virus-associated molecular patterns detection and in the type 1 interferon pathway (as recently reviewed in [5]).

The cytosolic complex inflammasome comprises an important response to viral infection, as it is responsible for the activation of caspase-1 and the subsequent cleavage and release of the two cytokines IL-1ß and IL-18, which are directly involved in antiviral innate response, and, indirectly, by inducing cell-mediated adaptive response [6]. Increased plasma level of IL-18, but not IL-1ß, was observed in all COVID-19 patients, with the highest titers in severe ones. On the other hand, IL-1ß is upregulated in bronchoalveolar lavage, and both cytokines are highly produced by lung macrophages, suggesting that inflammasome is greatly induced in lungs during SARS-CoV-2 infection [7].

Moreover, gasdermin-D (GSDMD), another substrate of caspase-1, and responsible for the inflammatory lithic cell death known as pyroptosis, has been shown to worsen the prognosis of COVID-19 [8].

How SARS-CoV-2 activates the inflammasome is not yet fully understood. Recent literature supports the hypothesis that the NACHT and LRRs containing receptor with a PYD domain (NLRP) 1 acts as a broad viral proteases' sensor and decoy factor [9, 10]. NLRP1 has been shown to be the target of some viral proteases, including the picornavirus and enterovirus 3C protease [9, 10], and also the SARS-CoV-2 3CL protease NSP5 [11]. Viral proteases cleave the amino-terminal portion of the NLRP1 within a "tripwire" region localized in the link sequence between the PYD and NACHT domains (Fig. 1). The cleavage leads to proteasomal digestion of the amino-terminal portion of NLRP1 and to the activation of the inflammasome [9, 10, 11]. In a previous activation model, NLRP1 was assumed to suffer self-cleavage in the FIIND domain (at Ser1312), resulting in the dissociation of the endogenous inhibitor dipeptidyl peptidase (DPP)-9 and proteasomal degradation of the amino-terminal portion of the receptor [12]. Whether this mechanism is involved also in the activation of NLRP1 by viral proteases has not been yet elucidated.

Of note, several gain-of-function missense variants and sites of positive selection are present in the NLRP1 linker region, likely related to past events of virus-mediated selective pressure [13].

All this considering, the NLRP1 inflammasome represents a good candidate as a severity factor in the COVID-19 disease. In an attempt to verify whether common variants in the *NLRP1* gene may affect host response to the SARS-CoV-2 virus, we performed an association study by analyzing the frequency distribution of eight well-known functional single nucleotide variants (SNVs) in a cohort of Brazilian COVID-19 patients. We have selected the two missense variants in NLRP1 p.Leu155His (rs12150220) and p.Met1184Val (rs11651270) which are known to increase IL-1ß processing in peripheral blood mononuclear cells [14], and their high frequency in the general population is hypothesized to be the consequence of a selective advantage against a viral pandemic [13]. Moreover, the nonsense variant p.Cys10Ter in CARD8 (rs2043211), another recently recognized viral proteases target [15], and whose activation is regulated by the interaction with DPP9 like NLRP1 [16], was also analyzed. Finally, taking in account that the inflammasome is a complex, and that SNV–SNV or gene–gene interaction are expected, we included in the study also the promoter enhancer variant rs16944 in *IL1B*, two eQTL SNVs in *IL18* (rs5744256 and rs1834481), known

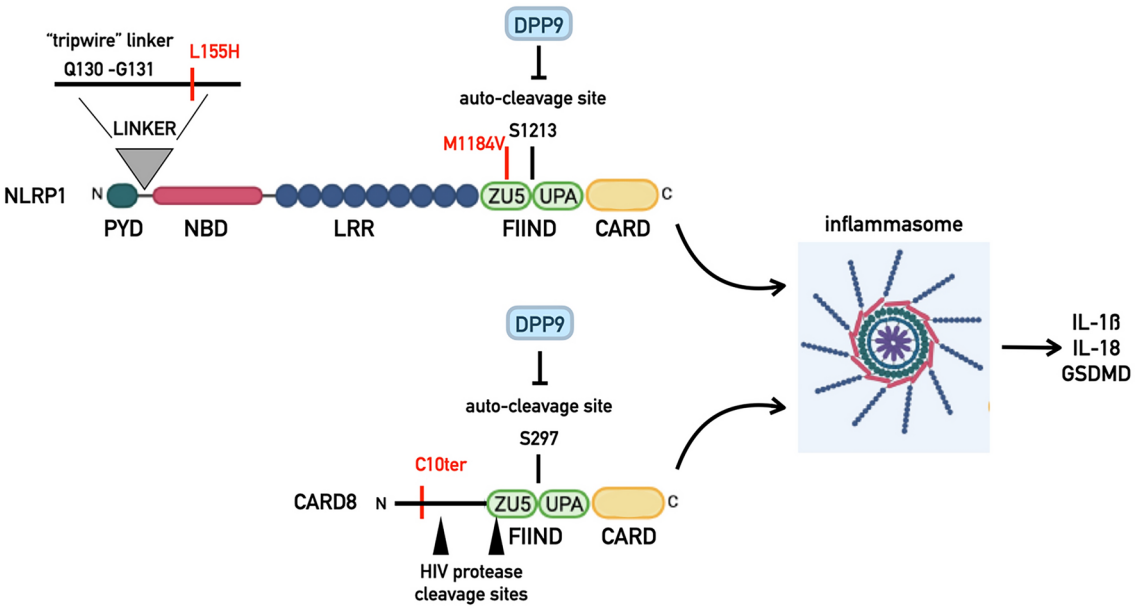

Fig. 1 Schematic representation of NLRP1 and CARD8 inflammasomes

to affect the cytokines' level [17, 18, 19], and two SNVs (rs12610495 and rs8101703) with opposite eQTL effect in the *DPP9* gene, as a possible upstream regulator of NLRP1 and CARD8 [16].

Domain architecture is specified for the NLRP1 and CARD8 proteins. The common mechanism of endogenous inhibition of the sensors is mediated by the interaction between dipeptidyl peptidase (DPP)-9 and the FIIND domain. DPP-9 hides the auto-cleavage site within the FIIND domain of NLRP1 and CARD8 (Ser1213 and Ser297, respectively). Viral proteases' digestion of the N-terminal regions of NLRP1 ("tripwire" linker sequence is evidenced) and CARD8 has been shown to be sufficient to induce the mounting and activation of the inflammasome and the consequent cleavage of its substrates pro-IL-1ß, pro-IL-18, and GSDMD, into their biologically active forms. Functional variants selected for this study are indicated in red: p.Leu155His (L155H; rs12150220), p.Met1184Val (M1184V; rs11651270), and p.Cys10ter (C10X; rs2043211).

Patients and methods

Study participants

Nine hundred and forty five patients ($n=945$; median age: 47; range: 19–95; males: 51%) positive for polymerase chain reaction (PCR) test for RNA of SARS-CoV-2 in nasopharyngeal swabs were recruited in three Brazilian hospitals: "Hospital das Clinicas/Faculdade de Medicina da Universidade de São Paulo" (HC/FMUSP) and "Hospital Albert Einstein" in São Paulo (state of São Paulo) ($n=249$ and 396, respectively), and "Hospital Universitario/Universidade Federal de Mato Grosso do Sul" (HU/UFMS) in Campo Grande (state of Mato Grosso do Sul) ($n=300$), between 2020 and 2021, before the availability of the vaccine. Two hundred and eighty subjects ($n=280$; 30%) are patients with severe COVID-19 (cases), which is defined as hospitalization with respiratory failure and in need of intensive care and mechanical ventilation. Six hundred and sixty-five participants ($n=665$; 70%) with a mild flu-like disease were classified as paucisymptomatic patients (controls). Hypertension and diabetes are present in 43 and 34% of the recruited subjects, respectively (Table 1). The study was approved by the Ethics Committee of each of the hospitals (protocols CAAE: 30,800,520.7.0000.0068 2020; 41,492,620.1.0000.0071; 32,466,020.8.0000.0021). Written informed consent was obtained, sometimes in a delayed fashion, from the patients at each center when possible.

Sample processing and genotyping

The extraction of DNA from peripheral blood was carried out using the "Salting Out" technique [20]. Eight functional SNVs were selected in the *NLRP1 (*rs12150220, rs11651270), *CARD8* (rs2043211), *IL1B (*rs16944), *IL18 (*rs1834481, rs5744256), and *DPP9* (rs12610495, rs8101703) genes. Their minor allele frequency (MAF) in the general population is greater than 10%. (Table 2) The genotyping of the selected SNVs was performed by the use of allele-specific Taqman® assays (*Applied Biosystems, Thermo Fisher Scientific*) and qPCR in a QuantStudio real-time PCR equipment (*Applied Biosystems, Thermo Fisher Scientific*).

Table 1 Overview of patients included in the study

	A $n=249$	B $n=396$	C $n=300$	All $n=945$
Median age (IQR)—yr	60 (23–82)	40 (19–95)*	46 (19–87)*#	47 (19–95)
Male sex—no. (%)	139 (56)	130 (33)*	127 (42)*#	482 (51)
Severe—no. (%)	74 (30)	56 (14)*	150 (50)*#	280 (30)
Paucisymptomatic—no. (%)	175 (70)	340 (86)*	150 (50)	665 (70)
Hypertension—no. (%)	237 (95)	32 (8)*	63 (21)*#	406 (43)
Diabetes—no. (%)	215 (86)	14 (4)*	37 (12)*#	321 (34)

IQR interquartile range. * A vs B, A vs C: $p<0.05$; # B vs C: $p<0.05$.

Demographic characteristics (age, sex) and clinical informations used for analysis were reported for all the subjects recruited for the study (all) and for patients according to the hospital of origin: A, B, or C

Hospital A is the "Hospital das Clinicas" FMUSP, hospital B is the "Hospital Israelita Albert Einstein", and hospital C is the "Hospital Universitario" UFMS

Severe category means patients who need respiratory support and intensive care unit internalization

Paucisymptomatic category denotes SARS-CoV-2 positive patients who were hospitalized with a mild clinical presentation but did not need respiratory support

Fisher test and one-way ANOVA test followed by a multi comparison post-test were used to compare the variables in the three groups A, B, and C

Table 2 Single nucleotide variants (SNVs) selected for the association study

SNP ID	Position	Alleles	Base pair change	Amino acid change	Functional effect
rs12150220	chr17:5,582,047	A > T	NC_000017.11: g.5582047A > T	NP_127497.1: p.Leu155His	Gain-of-funciton NLRP1
rs11651270	chr17:5,521,757	T > C	NC_000017.11: g.5521757 T > C	NP_127497.1: p.Met1184Val	Gain-of-funciton NLRP1
rs2043211	chr19:48,234,449	A > T	NC_000019.10: g.48234449A > T	NP_001338713.1: p.Cys10Ter	Loss-of-function CARD8
rs16944	chr2:112,837,290	A > G	NC_000002.12: g.112837290A > G	Upstream transcript variant	Increased expression *IL1B*
rs1834481	chr11:112,153,104	C > G	NC_000011.10: g.112153104C > G	Intron variant	Decrease plasma level IL-18
rs5744256	chr11:112,152,125	A > G	NC_000011.10: g.112152125A > G	Intron variant	Decrease plasma level IL-18
rs12610495	chr19:4,717,660	A > G	NC_000019.10: g.4717660A > G	Intron variant	eQTL: decreased expression *DPP9*
rs8101703	chr19:4,717,175	G > A	NC_000019.10: g.4717175G > A	Intron variant	eQTL: increased expression *DPP9*

eQTL expression quantitative trait locus, identification number (ID), chromosome position according to *Homo sapiens* (human) genome assembly GRCh38.p13 (hg38), alleles, base pair and amino acid change, and reported functional effect were resumed in the table

Statistical analysis

First, a raw analysis comparing the allelic distribution of the SNVs in the case/control groups was performed, and then a multivariate analysis for the genotypic distribution including variables previously described to affect the disease outcome [1, 2, 21]: age and sex (main analysis), hypertension and diabetes (corrected analysis) was performed. *SNPassoc,* an R package (http://www.r-project.org), was used for the analysis. *Haploview* software was used to derive the haplotypes. The commonly accepted threshold of 0.05/number of independent SNVs (Bonferroni correction for multiple comparisons) was implied to determine statistical significant differences ($p < 0.01$).

Results

The aim of this study was to evaluate whether common variants in NLRP1 inflammasome may contribute to COVID-19 severity. To this end, we recruited patients proceeding from one public and one private hospitals in São Paulo (capital, state of São Paulo), and one public hospital in Campo Grande (capital, state of Mato Grosso do Sul) between 2020 and 2021, before the availability of the vaccine. Demographic and main clinical data of recruited individuals are resumed in Table 1. The proportion of severe patients in each cohort is different and it does not represent the proportion of severe COVID-19 patients in the total number of SARS-CoV-2 infections. Our cohort is enriched of severe patients due to the statistical power calculation and to ensure enough strength of the case/control association analysis. We selected known functional SNVs in the *NLRP1* gene (rs12150220 and rs11651270), the main focus of this study, but also in the genes of the two cytokines, *IL1B* (rs16944) and *IL18* (rs5744256 and rs1834481), downstream effectors of the inflammasome activation, and in the *DPP9* gene (rs12610495 and rs8101703), as a possible upstream regulator of NLRP1. The nonsense SNV rs2043211 in the *CARD8* gene, was also included in the study due to the similarity of the proposed activation mechanism for CARD8 and NLRP1, including the viral protease cleavage and the interaction with DPP-9 [16].

In the raw analysis, we identified three SNVs significantly associated with COVID-19 severity. The minor alleles of *NLRP1* rs12150220 and *CARD8* rs2043211 variants were more frequent in severe than in paucisymptomatic patients (odds ratio: 1.36 for the minor allele; 95% confidence intervals: 1.12–1.65 and 1.11–1.69, respectively). Moreover, the *IL18* rs5744256 variant was significantly associated with the protection of SARS-CoV-2 infected individuals to develop severe disease (O.R.: 0.66 for the minor allele: 95% C.I.: 0.51–0.87) (Table 3).

The SNVs in the *IL18* and *DPP9* genes formed two haploblocks with a strong linkage disequilibrium (LD) (D' > 90); while SNVs in the *NLRP1* gene resulted in medium LD (D' = 65) in our cohort. (Supplementary File 1). Raw haplotypes' analysis revealed that *IL18* G-G haplotype was more frequent in paucisymptomatic than in severe COVID-19 patients ($p = 0.006$) (Supplementary File 2), corroborating the single allele association result.

Table 3 Raw association study results

SNV ID	MAF	Severe/mild	*p* value	Odds ratio (95% C.I.)
rs12150220	0.35	0.39/0.32	**0.0015**	1.36 (1.12–1.65)
rs11651270	0.43	0.45/0.41	0.0864	1.18 (0.98–1.42)
rs2043211	0.29	0.29/0.23	**0.0034**	1.36 (1.11–1.69)
rs16944	0.44	0.44/0.44	1	1 (0.83–1.20)
rs1834481	0.15	0.16/0.21	0.0134	0.71 (0.55–0.94)
rs5744256	0.16	0.12/0.17	**0.0022**	0.66 (0.51–0.87)
rs12610495	0.22	0.21/0.22	0.6552	0.95 (0.76- 1.18)
rs8101703	0.24	0.23/0.25	0.334	0.90 (0.72–1.11)

Raw analysis included 280 severe and 665 paucisymptomatic (mild) patients

Allele frequencies for the minor allele are shown for the whole cohort and for the severe and mild patients

Fisher test was applied to compare allele frequencies in the two groups

The *p* values and corresponding odds ratios and 95% confidence intervals (C.I.) are shown with respect to the minor allele

Differences with *p* value lower than 0.01 were considered statistically significant

Statistically significant *p* values are indicated in bold characters

Main analysis included origin, sex, and age as covariates and it was performed according to dominant and recessive models of inheritance for all the SNVs (Table 4). The *NLRP1* rs12150220 T/T genotype resulted more frequent in severe than in mild patients according to a recessive model of inheritance for the minor allele (O.R.: 2.42; 95% C.I.: 1.44–4.08).

When hypertension and diabetes were added as covariates in our analysis, the association of the *NLRP1* rs12150220 T/T genotype with COVID-19 severity was maintained (O.R.: 2.35; 95% C.I.: 1.39–3.99). Moreover, the *IL18* rs5744256 G-containing genotypes resulted significantly less frequently in severe than in mild patients according to a dominant model of inheritance for the minor allele (O.R.: 0.60; 95% C.I.: 0.41–0.89). (Table 4).

Given that the selected genes belong to the same biologic pathway, the NLRP1 inflammasome, we then realized an interaction analysis between SNVs localized in distinct genes (*NLRP1* and *IL1B,* or *IL18,* or *DPP9*). Possibly due to the relatively low frequency of rs5744256 variant (16%), the combination between rs12150220 and rs5744256 genotypes resulted in a not significant association after Bonferroni correction ($p = 0.040$).

Discussion

Using a candidate SNV approach, based on known common functional variants, and the cooperation of teams of three Brazilian hospitals, we performed an association study to evaluate the hypothesis that the newly identified cytosolic receptor of SARS-CoV-2, NLRP1 could represent a susceptibility gene for COVID-19 severity, in terms of respiratory failure and ICU internalization.

Table 4 Main association study results

	Main analysis		Adjusted for HAS and diabetes	
SNV ID	*p* value	Odds ratio (95% C.I.)	*p* value	Odds ratio (95% C.I.)
rs12150220	**9.5 exp-5**	**2.42 (1.44–4.08)**	**1.5 exp-4**	**2.35 (1.39–3.99)**
rs11651270	0.041	1.57 (1.02–2.41)	0.025	1.65 (1.07–2.57)
rs2043211	0.577	0.84 (0.45–1.56)	0.704	0.89 (0.47–1.6)
rs16944	0.769	0.93 (0.60–1.47)	0.721	0.92 (0.58–1.45)
rs1834481	0.068	0.62 (0.37–1.04) §	0.076	0.63 (0.37–1.06) §
rs5744256	0.015	0.62 (0.42–0.92) §	**0.010**	**0.60 (0.41–0.89) §**
rs12610495	0.377	0.69 (0.30–1.59)	0.475	0.74 (0.32–1.71)
rs8101703	0.929	0.96 (0.40–2.30)	0.844	0.92 (0.38–2.19)

Multivariate analysis included 280 severe (cases) and 665 paucisymptomatic/mild (controls) patients

Two case/control analyses were performed: a main analysis, which was corrected for origin, age, and sex, and a second analysis which was corrected for the presence of hypertension (HAS) and diabetes in addition to origin, age, and sex. The association results of the two analyses are shown according to the recessive model of inheritance, except for the *IL18* variants for which the results are reported according to the dominant model (§)

The *p* values and corresponding odds ratios and 95% confidence intervals (C.I.s) are shown with respect to the reference genotype, major allele homozygotes in the case of dominant model of inheritance (§), major allele homozygotes and heterozygotes in the case of recessive model of inheritance

Differences with *p* value lower than 0.01 were considered statistically significant

Statistically significant *p* values are indicated in bold characters

We detected a significant association of NLRP1 p.Leu155His missense variant (rs12150220) and severe COVID-19. This SNV is localized in the linker region between amino-terminal PYD and central NACHT domain, very close to the so-called "tripwire" region (a.a. 127–134), which is the target of viral proteases [10], including the Coronavirus 3CL protease NSP5 [11]. The known functional effect of p.Leu155His regards the increased activation of NLRP1 inflammasome and IL-1ß release in peripheral blood mononuclear cells [14]. Far from recent discoveries, Vasseur and colleagues have hypothesized that the high frequency of rs12150220 in the European population (about 50%) may suggest a rapid evolution in response to a viral epidemic [13], reinforcing the idea that this specific SNV could be related to the host antiviral response.

Intriguingly, the other missense variant in NLRP1, the p.Met1184Val substitution (rs11651270), which also had been identified in the study of Vasseur et al. [13] as target of a rapid positive selective pressure together with rs12150220, did not associate with the severity of COVID-19, at least in our cohort. It is interesting to emphasize, however, that p.Met1184Val is localized within the FIIND domain, not a direct target of SARS-CoV-2 protease, supporting a specific effect of rs12150220 in COVID-19.

We have included in the study also the nonsense variant *CARD8* rs2043211, due to the structural similarity of CARD8 and NLRP1, and the recent findings about the role of CARD8 as cytosolic target of HIV-1 protease [15]. However, this variant in the *CARD8* gene did not result associated to COVID-19 disease. Of note, the two variants in the *DPP9* gene did not associate with the disease, once more supporting the idea that, in this specific context of SARS-CoV-2 infection, the "tripwire" region is more determinant for NLRP1 activation than the DPP-9/FIIND mechanism.

As such, it is reasonable to conclude that NLRP1 p.Leu155His variant can be specifically involved in COVID-19 severity. We hypothesized that this polymorphism could affect the secondary structure of the "tripwire" sequence leading to increased accessibility to viral proteases and consequently to augment the activation of NLRP1 inflammasome. When we performed an "in silico" analysis using the "PredictProtein" online software (https://predictprotein.org/), we observed that the amino acid substitution Leu- > His modifies the predicted secondary structure of the N-terminal portion of NLRP1. A helix structure is lost in the "tripwire" region (a.a. 127–134) and the accessibility of the sequence (exposed versus buried residues) also is affected (Supplementary File 3), suggesting that the rs12150220 variant can result in a NLRP1 N-terminal region prone to proteases' digestion.

It is important to note that the association resulted significant in all the three steps of analysis, even when well-known risk variables, age, sex, hypertension, and diabetes, have been included in the multivariate analysis. As NLRP1 has been previously associated to Addison disease [22], and type-1diabetes [23, 24], we performed an association analysis using diabetes as main variable, instead of a covariate, however, at least in our cohort, none of the studied SNVs resulted associated to diabetes. The same result was obtained when hypertension was used as main variable, finally excluding a possible effect of rs12150220 on these two conditions in our cohort, and emphasizing the robustness of the main association analysis. The meaning of the association of NLRP1 p.Leu155His with COVID-19 severity may be related to excessive cytokines release, and literature have yet showed that both IL-1ß and IL-18 are dysregulated during SARS-CoV-2 response and in severe patients [8]. Although we expected to find a relationship between the SNVs in NLRP1and IL1B and/or IL18 genes, we did not observe any association between rs12150220 and the well-known variant -511 C>T (rs16944) in the promoter of the IL1B gene, which is related to the increase in expression and release of the cytokine [17]. On the other hand, the variants in the IL18 gene, and in particular, the rs5744256 SNV, were inversely associated with the severity of the COVID-19. This intronic variant has been described as an eQTL for reduced plasma IL-18 concentration [18], suggesting here that lower cytokine levels might be beneficial in severe disease. This result appears to be in contrast with the common view of IL-18 as an anti-viral factor [6] and with the elevated levels of IL-18 observed in SARS-CoV-2 infection [8]. However we can hypothesize that the role of the cytokine may or not be protective depending on the time of the disease. As genetic background has been pointed out as a risk factor for the development of severe COVID-19 disease [6], our findings added another piece of the complexpuzzle of the individual response to SARS-CoV-2, and at the same time confirm the key role of the NLRP1 sensor in antiviral response. Fundings: This study was funded by "Fundação de Apoio a Pesquisa do Estado de Sao Paulo" (FAPESP) process n.21/05420–4 (A.P.) and "Coordination for the Improvement of Higher Education Personnel" (CAPES) process number (n.) 88,887.503842/2020–00 (A.J.D.S.; M.N.S; A.P.), "Brazilian Ministry of Education, TED SESU/MEC nº 9233/2020" (J.V). Authors are supported by fellowship programs: Excellence in Research Fellowship from "National Council for Scientific and Technological Development" (CNPq) (A.P., M.N.S, A.J.S.D.); Postdoctoral Fellowship from São Paulo Research Foundation (FAPESP) n. 2021/13049–4 (V.N.C.L), PhD Fellowships n.2021/09161–3 (R.W.A), n. 2019/22448–0 (S.C.G.S), n. 2018/18230–6 (F.M.E.T); CAPES PhD Fellowships (n. 88,887.469122/2019–00; R.A.G.C); and Undergraduate Scholarship (SMY: PIBIC n.158842/2021–9).

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00011-022-01670-3.

Author contributions Conception and design: VNCL; AP, patients recruitment: LCN; JV; CBB; CAV; AMS; APHY; JMK; LMP; WMSS, acquisition of data: VNCL; MMSA; CAV; FMET; RAGC; SMY; RWA; SCGS; TMY; VFD; CBB; AMS; APHY; JMK; JV; LMP; WMSS. Analysis and interpretation of data: VNCL, AP. Writing of the manuscript: VNCL, AP. Critical reagents and manuscript editing: AP. Funding acquisition: AP, MNS, AJSD; JV.

Data availability All data needed to evaluate the conclusions in the paper are present in the paper or in the Supplementary Section.

Declarations

Conflict of interest The authors declare that they have no competing interests.

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