MINISTÉRIO DA SAÚDE
FUNDAÇÃO OSWALDO CRUZ
INSTITUTO OSWALDO CRUZ

Mestrado em Programa de Pós-Graduação em Biologia Parasitária

PAPEL DOS INFLAMASSOMAS NA INFECÇÃO POR SARS-COV-2: POLIMORFISMOS DE BASE ÚNICA (SNP) E NÍVEIS DE EXPRESSÃO GÊNICA COMO POTENCIAIS BIOMARCADORES PARA OS DESFECHOS CLÍNICOS DA COVID-19.

MILENA NEIRA GUIMARÃES GOULART

Rio de Janeiro
Dezembro de 2022

Ministério da Saúde

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Papel dos inflamassomas na infecção por SARS-CoV-2: polimorfismos de base única (SNP) e níveis de expressão gênica como potenciais biomarcadores para os desfechos clínicos da covid-19.

Dissertação apresentada ao Instituto Oswaldo Cruz como parte dos requisitos para obtenção do título de Mestre em Ciências

Orientador (es): Prof. Dra. Mariza Gonçalves Morgado

RIO DE JANEIRO
Dezembro de 2022

Neira, Milena.

PAPEL DOS INFLAMASSOMAS NA INFECÇÃO POR SARS-COV-2: POLIMORFISMOS DE BASE ÚNICA (SNP) E NÍVEIS DE EXPRESSÃO GÊNICA COMO POTENCIAIS BIOMARCADORES PARA OS DESFECHOS CLÍNICOS DA COVID-19 / Milena Neira. - Rio de Janeiro, 2022.
120 f.

Dissertação (Mestrado) – Instituto Oswaldo Cruz, Pós-Graduação em Biologia Parasitária, 2022.

Orientador: Mariza Morgado.

Bibliografia: f. 96-106

1. Polimorfismos de nucleotídeo único (SNPs). 2. Inflamassoma. 3. COVID-19. I. Título.

Elaborado pelo Sistema de Geração Automática de Ficha Catalográfica da Biblioteca de Manguinhos/Icict/Fiocruz com os dados fornecidos pelo(a) autor(a), sob a responsabilidade de Igor Falce Dias de Lima - CRB-7/6930.

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Aprovada em: _13 /_ 12 _/ 2022

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Rio de Janeiro,13 de Dezembro de 2022

Ata da defesa de dissertação de mestrado acadêmico em Biologia Parasitária de **Milena Neira Guimarães Goulart**, sob orientação da Drª. Mariza Gonçalves Morgado . Ao décimo terceiro dia do mês de dezembro de dois mil e vinte e dois, realizou-se às nove horas, de forma síncrona remota, o exame da dissertação de mestrado acadêmico intitulada: "**Papel dos inflamassomas na infecção por SARS-COV-2:polimorfismos de base única (SNP) e níveis de expressão gênica como potenciais biomarcadores para os desfechos clínicos da COVID-19"**, no programa de Pós-graduação em Biologia Parasitária do Instituto Oswaldo Cruz, como parte dos requisitos para obtenção do título de Mestre em Ciências - área de concentração: Imunologia e Patogenia, na linha de pesquisa: Imunologia e Patogênese de Doenças Infecciosas e Parasitárias. A banca examinadora foi constituída pelos Professores: Drª Roberta Olmo Pinheiro – IOC/FIOCRUZ (Presidente), Dr. Mário Campos Júnior – IOC/FIOCRUZ, Dr. Átila Duque Rossi – UFRJ/RJ, e como suplentes: Dr. Josué da Costa Lima Júnior - IOC/FIOCRUZ e Drª. Cleonice Alves de Mello Bento - UNIRIO/RJ. Após arguir a candidata e considerando que a mesma demonstrou capacidade no trato do tema escolhido e sistematização da apresentação dos dados, a banca examinadora pronunciou-se pela Aprovação da defesa da dissertação de mestrado acadêmico. De acordo com o regulamento do Curso de Pós-Graduação em Biologia Parasitária do Instituto Oswaldo Cruz, a outorga do título de Mestre em Ciências está condicionada à emissão de documento comprobatório de conclusão do curso. Uma vez encerrado o exame, o Presidente da Banca atesta a decisão e a participação da aluna e de todos o membros da banca de forma síncrona remota. O Coordenador do Programa, Dr. André Luiz Rodrigues Roque, assinou a presente ata tomando ciência da decisão dos membros da banca examinadora. Rio de Janeiro, 13 de dezembro de 2022.

Drª Roberta Olmo Pinheiro (Presidente da Banca)

Dr. André Luiz Rodrigues Roque (Coordenador do Programa):

Dedico esta dissertação à minha família que me incentivou e foi essencial em todas as etapas até aqui.

AGRADECIMENTOS

Agradeço a Deus por ter me permitido chegar até aqui.

Aos meus pais e meu irmão, por serem os meus maiores incentivadores e minha fortaleza. Nada disso seria possível sem todo o suporte de vocês. Tudo o que fiz e continuo fazendo é por vocês. Muito obrigada! Amo muito vocês.

À minha avó por ser minha maior fã e me apoiar em todos os momentos. Já falei que te amo hoje?

Ao meu namorado, Vinicius, que foi essencial em todo o percurso. Obrigada por toda paciência, dedicação e por ser o meu menino do TI. Você acompanhou cada passo dessa conquista e foi fundamental em cada etapa. Te amo.

À minha orientadora Dra. Mariza Morgado pela orientação exemplar. Obrigada pela confiança e por todos os ensinamentos ao longo desse percurso, eles foram essenciais para o meu crescimento profissional. Foi uma honra poder ser orientada por você.

À Dra. Nathalia de Sá, que apesar de oficialmente não ser minha coorientadora, cumpriu esse papel com maestria e de forma integral. Palavras não são suficientes para demonstrar minha gratidão pela sua generosidade em me ensinar em cada etapa. Admiro profundamente a profissional que você é, e foi um privilégio ter você diariamente me acompanhando e orientando. Muito obrigada por tanto!

Ao Dr. Marcelo Ribeiro-Alves por todo o auxílio nas análises estatísticas e esclarecimentos prestados.

Gostaria de agradecer imensamente a Karine Venegas, por toda a parceria nesse último ano me auxiliando em todo o processo para obtenção dos resultados de expressão gênica. Sua amizade foi uma grata surpresa durante essa caminhada e tornou ela muito mais leve e divertida.

À Plataforma de PCR em Tempo Real e a Angélica, pela disponibilidade e utilização do equipamento.

À Dra Cristiana Couto Garcia, Dra Ohanna Cavalcanti de Lima Bezerra, Dra. Marilda Mendonça Siqueira e Dra. Larissa Rodrigues pela colaboração no trabalho referente ao Artigo 1.

Ao Dr. Hugo Perazzo, Dra. Kim Mattos Geraldo, Dra. Maria Pia Diniz Ribeiro, Dra. Sandra Wagner Cardoso, Dra Beatriz Grinsztejn, Dra Valdiléa G. Veloso e todo o time de profissionais envolvidos no estudo RECOVER desenvolvido no Instituto Nacional de Infectologia (INI/FIOCRUZ) por toda a colaboração.

Gostaria de agradecer imensamente ao grupo COVID-19, Dra. Fernanda Heloise Côrtes, Dra Carmem Beatriz Wagner Giacoia-Gripp, Dra. Dalziza Victalina de Almeida

e Andressa da Silva Cazote por todo auxílio no projeto, participação na montagem do banco de amostras biológicas e suporte prestado. Vocês foram fundamentais.

À equipe do Laboratório de AIDS e Imunologia Molecular, por todos os ensinamentos e trocas, aprendi muito com cada um de vocês.

Agradeço aos voluntários que aceitaram participar deste trabalho, sem os mesmos essa dissertação não seria possível.

Ao Programa de Pós-graduação em Biologia Parasitaria, pela oportunidade de realizar o mestrado.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES pela bolsa concedida ao longo destes 2 anos e meio.

Aos órgãos de fomento pelo apoio financeiro.

A todos que estiveram presentes em minha vida colaborando para o desenvolvimento deste trabalho, muito obrigada!

Há duas formas para viver a sua vida. Uma é acreditar que não existe milagre. A outra é acreditar que todas as coisas são um milagre. *Albert Einstein*

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RESUMO

DISSERTAÇÃO DE MESTRADO EM BIOLOGIA PARASITÁRIA

Milena Neira Guimarães Goulart

A COVID-19 tem um amplo espectro de manifestações clínicas e os mecanismos subjacentes à sua evolução clínica ainda não são claros. Nesse estudo, nós investigamos o impacto de polimorfismos em genes que codificam proteínas do complexo multiproteico inflamassoma e correlacionamos com a expressão em indivíduos com diferentes perfis clínicos de severidade da COVID-19. Na primeira abordagem (Artigo 1), os pacientes foram divididos em 2 grupos de acordo com a severidade da doença baseado na Escala de Progressão Clínica da Organização Mundial de Saúde (OMS). O grupo 1 incluiu pacientes hospitalizados ou não, com doença leve/moderada (OMS<6; n=76) e o grupo 2 incluiu pacientes hospitalizados com quadros severos/críticos da COVID-19 (OMS≥6; n=357). Os pacientes hospitalizados foram recrutados no Centro Hospitalar para a Pandemia de COVID-19 – Instituto Nacional de Infectologia (INI/FIOCRUZ) de junho de 2020 a março de 2021. Os pacientes com doença leve foram recrutados no Instituto Oswaldo Cruz (IOC/FIOCRUZ) em agosto de 2020. A estimativa de risco e proteção foi realizada utilizando o modelo de regressão logística incondicional. Na segunda abordagem (Artigo 2), foram incluídos 451 indivíduos hospitalizados, a partir dos quais analisamos fatores de risco de progressão para ventilação mecânica (n=174 [38.6%]) ou óbito (n=175 [38.8%]). A genotipagem de 11 SNPs de inflamassoma foi feita por PCR em tempo real. Para a terceira abordagem verificamos a expressão de *NLRP3* e *CASP1* em 77 indivíduos, sendo 17 classificados como moderados (OMS < 6) e 60 como graves/críticos (OMS ≥ 6). Esses indivíduos representam um recorte dos pacientes hospitalizados, recrutados para as análises anteriores. Na primeira abordagem, a proteção contra a severidade da doença foi associada ao genótipo A/A (OR_{adj}=0.36;*P*=0.032), alelo A (OR_{adj}=0.93;*P*=0.010), ou carrear-A (OR_{adj}=0.45;*P*=0.027) no polimorfismo rs1539019 do gene *NLRP3*; e o genótipo A/T (OR_{adj}=0.5;*P*=0.045), alelo T (OR_{adj}=0.93;*P*=0.018), ou carrear-T (OR_{adj}=0.48;*P*=0.029) no polimorfismo rs2043211 do gene *CARD8*. Os haplótipos das variantes do gene *NLRP3* incluídas A-C-G-C-C (OR_{adj}=0.11;*P*=0.018), A-C-G-C-G (OR_{adj}=0.23;*P*=0.003), C-C-G-C-C (OR_{adj}=0.37;*P*=0.021) e C-T-G-A-C (OR_{adj}=0,04;*P*=0.0473) também foram associados à proteção. Na segunda abordagem a progressão lenta para suporte ventilatório mecânico (SVM) foi associada ao alelo G (aHR=0.66;P=0.005) ou o genótipo G/G (aHR=0.391;P=0.006) no polimorfismo rs10754558 do gene *NLRP3*, ou o alelo G (aHR=0.309;P=0.004) no polimorfismo rs1143634 no gene *IL1β*. O alelo C do polimorfismo rs4612666 do gene *NLRP3* (aHR=2.342;P=0.006) e o de rs10754558 (aHR=2.957;P=0.005) foram associados a uma progressão mais rápida ao óbito. Progressão mais lenta para o óbito foram associadas às presenças do alelo G (aHR=0.563;P=0.006) ou o genótipo A/G (aHR=0.537;P=0.005) rs6509365 do gene *CARD8*; ao genótipo A/C do rs1101996 do gene *IFI16* (aHR=0.569;P=0.011); ao genótipo T/T (aHR=0.394;P=0.004) ou alelo T (aHR=0.68;P=0.006) do rs4612666 do gene *NLRP3*, e ao genótipo G/G (aHR=0.326;P=0.005) ou alelo G (aHR=0,68;P=0.014) rs10754558 do gene *NLRP3*. A expressão de *CASP1* e *NLRP3* não apresentou nenhuma associação significativa com os desfechos clínicos estudados. No entanto, foi observada uma correlação positiva, embora fraca, da expressão desses genes (rho= 0,249; P=0,0298), em pacientes com COVID-19. Nosso trabalho ressalta a importância da análise de variações genéticas nos genes do inflamassoma como fatores de risco na evolução clínica da COVID-19.

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ROLE OF INFLAMASOMES IN SARS-COV-2 INFECTION: SINGLE NUCLEOTIDE POLYMORPHISM (SNP) AND GENE EXPRESSION LEVELS AS POTENTIAL BIOMARKERS FOR CLINICAL OUTCOMES OF COVID-19.

ABSTRACT

MASTER DISSERTATION IN PARASITE BIOLOGY

Milena Neira Guimarães Goulart

COVID-19 has a broad spectrum of clinical manifestations and the mechanisms underlying its clinical course are still unclear. In this study, we investigated the impact of polymorphisms in genes encoding inflammasome multiprotein complex proteins and correlated them with expression in individuals with different clinical profiles of COVID-19 severity. In the first approach (Article 1), patients were divided into 2 groups according to disease severity based on the World Health Organization (WHO) Clinical Progression Scale. Group 1 included hospitalized and non-hospitalized patients with mild/moderate disease (WHO<6; n=76) and group 2 included hospitalized patients with severe/critical cases of COVID-19 (WHO≥6; n=357). Hospitalized patients were recruited at the Hospital Center for the COVID-19 Pandemic – National Institute of Infectious Diseases (INI/FIOCRUZ) from June 2020 to March 2021. Patients with mild illness were recruited at Instituto Oswaldo Cruz (IOC/FIOCRUZ) in August 2020. The risk and protection estimate were performed using the unconditional logistic regression model. In the second approach (Article 2), 451 hospitalized individuals were included, from which we analyzed risk factors for progression to mechanical ventilation (n=174 [38.6%]) or death (n=175 [38.8%]). Genotyping of 11 inflammasome SNPs was performed by real-time PCR. For the third approach, we verified the expression of NLRP3 and CASP1 in 77 individuals, 17 of which were classified as moderate (WHO < 6) and 60 as severe/critical (WHO ≥ 6). These individuals represent a selection of hospitalized patients recruited for the previous analyses..In the first approach, protection against disease severity was associated with genotype A/A (ORadj=0.36; P=0.032), allele A (ORadj=0.93; P=0.010), or carry-A (ORadj=0.45; P =0.027) in the rs1539019 polymorphism of the NLRP3 gene; and the A/T genotype (ORadj=0.5; P=0.045), T allele (ORadj=0.93; P=0.018), or carrier-T (ORadj=0.48; P=0.029) in the rs2043211 polymorphism of the CARD8 gene. The haplotypes of the NLRP3 gene variants included A-C-G-C-C (ORadj=0.11; P=0.018), A-C-G-C-G (ORadj=0.23; P=0.003), C-C-G-C-C (ORadj=0.37; P=0.021) and C-T-G-A-C (ORadj=0.04; P =0.0473) were also associated with protection. In the second approach, slow progression to mechanical ventilation support (MVS) was associated with the G allele (aHR=0.66;P=0.005) or the G/G genotype (aHR=0.391;P=0.006) in the NLRP3 rs10754558 polymorphism, or the G allele (aHR=0.309;P=0.004) in the rs1143634 polymorphism in the IL1β gene. The C allele in the NLRP3 rs4612666 polymorphism (aHR=2342;P=0.006) and that of rs10754558 (aHR=2957;P=0.005) were associated with a faster progression to death. Slower progression to death was associated with the presence of the allele G (aHR=0.563;P=0.006) or the A/G genotype (aHR=0.537;P=0.005) in the CARD8 rs6509365 polymorphism; to the A/C genotype of the rs1101996 of the IFI16 gene (aHR=0.569;P=0.011); to the T/T genotype (aHR=0.394;P=0.004) or T allele (aHR=0.68;P=0.006) of the NLRP3 rs4612666 polymorphism, and to the G/G genotype (aHR=0.326;P=0.005) or G allele (aHR=0.68;P=0.014) of NLRP3 rs10754558 polymorphism. The gene expression of *CASP-1* and *NLRP3* did not show any significant association with the clinical outcomes studied. However, a positive, albeit weak, correlation was observed in the expression of these genes (rho=0.249; P=0.0298) in patients with COVID-19. Our work highlights the importance of analyzing genetic variations in inflammasome genes as risk factors in the clinical course of COVID-19.

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LISTA DE SIGLAS E ABREVIATURAS

ACE2	Enzima Conversora de Angiotensina 2
ACTB	*β*-actina
AIM2	Ausente no melanoma 2
ASC	Molécula adaptadora contendo um domínio CARD
AT2	Angiotensina-2
ATP	Adenosina trifosfato
CARD8	A proteína 8 contendo domínio de recrutamento de caspase
CASP-1	Caspase-1
CASP-11	Caspase-11
cDNA	DNA complementar
COVID-19	Doença do Coronavírus 2019
DEPC	Dicarbonato de dietila
DNA	Ácido Desoxirribonucleico
GAPDH	Gliceraldeído-3-fosfato desidrogenase
gDNA	DNA genômico
HCoVs	Coronavírus humanos
HLA	Antígeno leucocitário humano
IDH	Índice de Desenvolvimento Humano
IFI16	Ativador transcricional de diferenciação mielóide induzida por interferon
IL-17	Interleucina-17
IL-18	Interleucina-18
IL-1β	Interleucina-1β
LogF	Log na base 2 de Fold-change
LPS	Lipopolissacarideos
MAF	Alelo de menor frequência
MERS	Síndrome Respiratória do Oriente Médio
MIQE	Informações mínimas para publicação de experimentos quantitativos de PCR em tempo real
NF-κB	Fator nuclear-κB
NLRP1	Família NLR contendo domínio de pirina da proteína 1
NLRP2	Família NLR contendo domínio de pirina da proteína 1
NLRP3	Família NLR contendo domínio de pirina da proteína 3
No-RT	Sem a reacao em cadeia da polimerase em tempo real
OMS	Organização Mundial da Saúde
PAMPs	Padrões moleculares associados a patógenos
PBMCs	Células mononucleares de sangue periférico
Pró-IL-18	Pró-interleucina-18
Pró-IL-1β	Pró-interleucina-1β
PRR	Receptor de reconhecimento de padrão
PYD	Domínio pirina
RIN	Número de integridade do RNA
RNA	Acido ribonucleico
RPMI	Do inglês - *Roswell Park Memorial Institute medium*
RT-PCR	Reacao em cadeia da polimerase em tempo real
SARS	Síndrome Respiratória Aguda Grave,
SARS-CoV-1	Coronavírus da Síndrome Respiratória Aguda Grave 1
SARS-CoV-2	Coronavírus da Síndrome Respiratória Aguda Grave 2

SFB	Soro fetal bovino
SNP	Polimorfismos de base única
SVM	Suporte ventilatório mecânico
Th1	T helper 1
Th17	T helper 17
Th2	T helper 2
TLR	Receptor Toll-like
TLR7	Receptor Toll-like 7
TMPRSS2	Serina Protease 2 Transmembrana
VNI	Ventilação não invasiva
VOCs	Variantes de preocupação

1. INTRODUÇÃO

No final de dezembro de 2019 surgiu uma nova doença, descrita inicialmente como um surto de casos de pneumonia viral, em indivíduos que viviam na região de Wuhan, na China (LIU; KUO; SHIH, 2020). A recém-surgida doença, Doença do Coronavírus 2019 (COVID-19), é uma infecção viral altamente transmissível, causada por um novo coronavírus zoonótico chamado SARS-CoV-2, que ocasionou uma pandemia desafiadora e ameaçadora, ocasionando um enorme impacto na vida das pessoas, especialmente nas populações mais vulneráveis (MEO et al., 2021). Atualmente, apesar da implementação das vacinas ter sido um fator essencial para a desaceleração da pandemia, a COVID-19 continua sendo fonte de preocupação e, até outubro de 2022, mais de 12 bilhões de doses foram administradas em todo o mundo (OMS, 2022).

Recentemente, variantes de preocupação (VOCs) surgiram e elas têm sido associadas ao aumento na transmissão, severidade ou mortalidade na COVID-19 e podem escapar da imunidade quando comparadas à cepa original (CHALLEN et al., 2021; EDARA et al., 2021). Além das novas variantes, fatores de risco como idade, sexo, hipertensão, obesidade e diabetes já foram identificados por sua associação com formas mais severas da COVID-19, porém eles sozinhos não explicam totalmente as complicações causadas pela doença (GAO et al., 2021a) .

Já foi demonstrado que o vírus causa em alguns indivíduos uma resposta hiperinflamatória, conhecida como tempestade de citocinas, com elevada produção de citocinas pró-inflamatórias, sendo um processo em que o desencadeamento de uma resposta imune descontrolada é responsável por complicações graves observadas durante o curso da infecção (VAN EIJK et al., 2021). Na infecção por SARS-CoV-2 se os efeitos da replicação viral e do dano tecidual se somarem aos efeitos pró-inflamatórios, pode haver uma liberação generalizada de citocinas e quimiocinas que podem iniciar esse quadro de hiperinflamação e explicar a extensa lesão local e sistêmica (FREEMAN; SWARTZ, 2020).

Os inflamassomas fazem parte dessa cascata de inflamação que é ocasionada após o reconhecimento de fragmentos do vírus, conhecidos como padrões moleculares associados a patógenos (PAMPs), por receptores de reconhecimento de padrão (PRR) presentes na célula hospedeira (THEOBALD et al., 2020). Os inflamassomas são complexos multiproteicos citosólicos, que agem em

resposta aos PAMPs gerados durante a infecção. Eles interagem com uma proteína adaptadora denominada ASC (molécula adaptadora contendo um domínio CARD), levando à ativação da caspase-1, que por sua vez irá clivar citocinas pró-inflamatórias como a IL -1β e IL-18 (BIASIZZO; KOPITAR-JERALA, 2020; KETELUT-CARNEIRO; FITZGERALD, 2020). Muitos estudos já demonstraram que mutações nos genes do complexo inflamassoma estão relacionadas com distúrbios inflamatórios graves (FENINI et al., 2020; MALIK; KANNEGANTI, 2017).

Apesar de alguns estudos já terem relacionado a ativação do inflamassoma com a COVID-19 (LÓPEZ-REYES et al., 2020; RODRIGUES et al., 2020; YAP; MORIYAMA; IWASAKI, 2020) e, mais recentemente, ter sido demonstrada a associação dos polimorfismos rs10157379 e rs10754558 do gene *NLRP3* com a gravidade da doença (MAES et al., 2022), a análise do papel de polimorfismos nos genes do complexo inflamassoma e sua associação com os diferentes tipos de evolução clínica da doença continua ainda pouco estudado. Portanto, no presente estudo, investigamos o papel de 11 polimorfismos de base única (SNP) dos genes *NLRP3*, *CARD8*, *AIM2*, *CASP-1*, *IFI16* e *IL1β* do inflamassoma em indivíduos infectados por SARS-CoV-2, bem como a expressão dos genes *NLRP3* e *CASP1* em pacientes com diferentes formas clínicas da COVID-19.

1.1 Aspectos epidemiológicos da COVID-19

Após o surto de SARS e MERS, a COVID-19 é o terceiro grande surto de coronavírus, que provou ser o mais mortal entre todos os surtos anteriores, devida a sua transmissão rápida e ampla, resultando em uma crise global (FANI; TEIMOORI; GHAFARI, 2020).

O patógeno causador do surto de COVID-19 em 2020 foi identificado como um novo coronavírus, do gênero *Betacoronavírus*, tendo sido denominado Coronavírus da Síndrome Respiratória Aguda Grave 2 (SARS-CoV-2) pelo Comitê Internacional de Taxonomia de Vírus (SARIOL; PERLMAN, 2020). A doença associada foi denominada COVID-19 pela Organização Mundial da Saúde (OMS) em 11 de fevereiro de 2020 (OMS, 2022; SEYED HOSSEINI et al., 2020). Devido a sua rápida dispersão pelo mundo, a COVID-19 foi elevada à categoria de pandemia pela OMS em março de 2020 e, até o de outubro de 2022, já foram relatados mais de 620 milhões de casos

da doença e cerca de 6,5 milhões de óbitos ao redor do mundo, como demonstrado na Figura 1 (OMS, 2022).

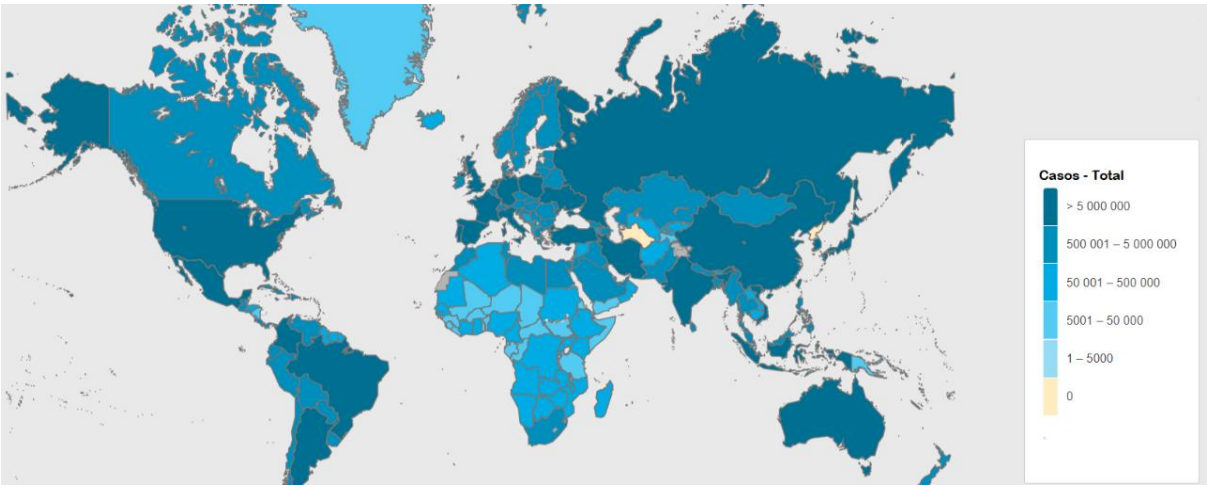

Figura 1: Total de casos de COVID-19 no mundo até 14 de outubro de 2022. A figura descreve o total de casos de COVID-19 confirmados no mundo até 14 de outubro de 2022. Fonte: OMS; 2022.

Atualmente os Estados Unidos da América (EUA), Índia e Brasil são os líderes em número de casos cumulativos no mundo. No Brasil, até início de outubro de 2022, mais de 34 milhões de casos de COVID-19 foram confirmados e mais de 686 mil pessoas foram a óbito pela doença (OMS, 2022).

As 5 principais variantes de preocupação do vírus que circularam pelo Brasil foram: Alpha, Beta, Gama, Delta e Ômicron, além de suas diversas subvariantes descendentes (GARCIA-BELTRAN et al., 2021). No Brasil, foi quando a variante Gama estava em circulação que se obteve um número recorde de óbitos, em abril de 2021, com mais de 4 mil óbitos em 24h pela COVID-19 (GIOVANETTI et al., 2022). Vale ressaltar que no Brasil a vacinação começou em janeiro de 2021, portanto em abril deste mesmo ano, o esquema vacinal estava longe de estar completo, com apenas aproximadamente 903 mil doses aplicadas (MATHIEU et al., 2020). Em janeiro de 2022, devido a emergência da variante Ômicron tivemos mais de 4 milhões de casos confirmados em 24h, um número recorde no país. Essa variante se destacou por ter um número maior de mutações concentradas na proteína S e essas mutações podem levar ao escape da imunidade induzida por infecção natural ou vacinação anterior, podendo causar um grande número de infecções ou reinfecções (WANG et al., 2022). No entanto, o número de óbitos continua diminuindo, muito provavelmente

em consequência das mais de 455 milhões de doses administradas de vacinas em todo território brasileiro (OMS, 2022).

No estado do Rio de Janeiro, em outubro de 2022, a letalidade da COVID-19 era de 3,0% acumulando mais de 75 mil óbitos e 2,5 milhões de casos confirmados da doença, como mostra a Figura 2 (“Painel de monitoramento Covid-19 - Governo do Estado do Rio de Janeiro”).

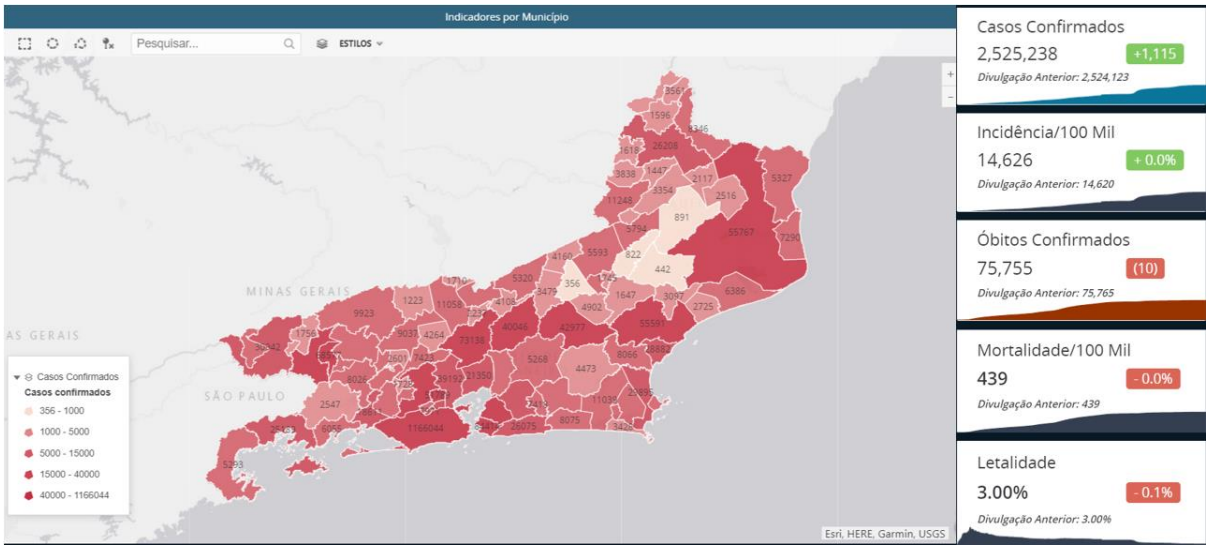

Figura 2: Painel COVID-19 no Rio de Janeiro. A figura descreve o número de casos confirmados, incidência, óbitos confirmados, mortalidade e letalidade da COVID-19 no Estado do Rio de Janeiro em outubro de 2022. Fonte: Governo do Estado do Rio de Janeiro; 2022 (https://painel.saude.rj.gov.br/monitoramento/covid19.html; acesso 14 de outubro de 2022).

Dados do boletim epidemiológico do estado do Rio de Janeiro confirmam que pessoas com baixa escolaridade estão inseridas no grupo de maior mortalidade e menor número de recuperações. Os dados também mostram que existe um maior número de vítimas fatais dentro da classe de etnia preta/parda que residem em municípios do Rio de Janeiro com baixos níveis de Índice de Desenvolvimento Humano (IDH) (“CTC PUC-Rio - Centro Técnico Científico”).

1.2 Características gerais de SARS-CoV-2

Os coronavírus pertencem à família Coronaviridae e causam infecção respiratória em mamíferos e em algumas espécies de aves (GONG; BAO, 2018). O primeiro coronavírus foi isolado em 1965, quando se verificou experimentalmente ser o causador de resfriados em humanos (MAHASE, 2020). Até o momento sete coronavírus humanos (HCoVs) são conhecidos, dentre os mais conhecidos e comumente identificados estão os *Alpha Coronavírus* 229E e NL63 e os

Betacoronavírus OC43, HKU1, além do SARS-CoV-1, causador da Síndrome Respiratória Aguda Grave (SARS), MERS-CoV, causador da Síndrome Respiratória do Oriente Médio (MERS) e SARS-CoV-2, causador da COVID-19 (MALIK, 2020).

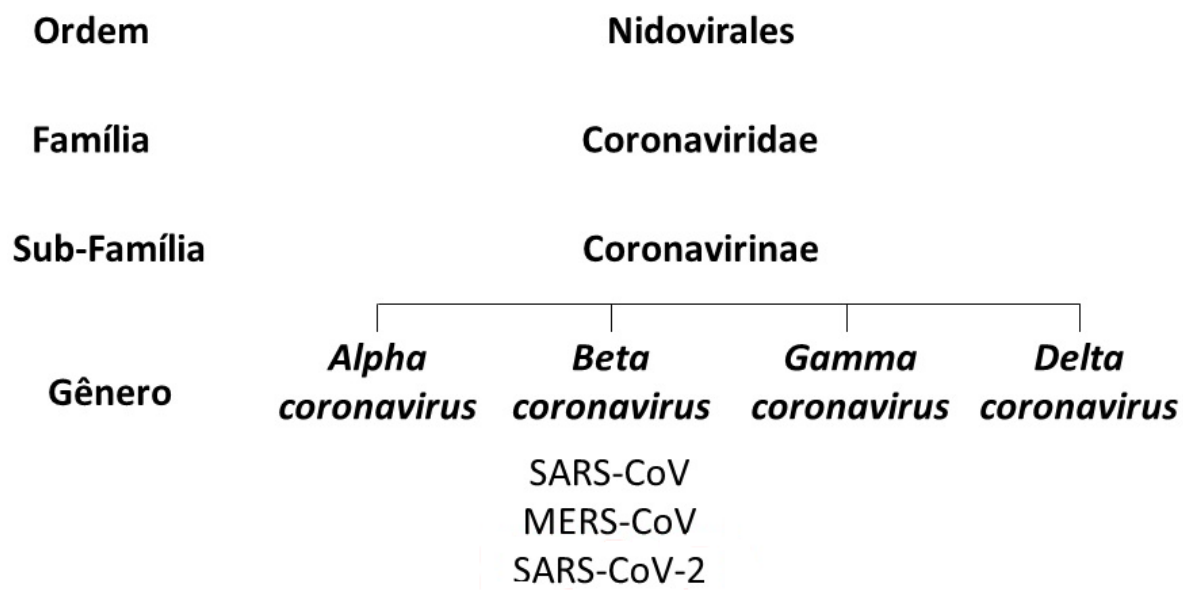

Figura 3: Taxonomia dos coronavírus. Fonte: Monteil *et al;* 2020.

Como mostrado na Figura 4, SARS-CoV-2 é um vírus envelopado com espículas na superfície, chamadas de *Spikes*. Perto da extremidade 3' do genoma, os genes S, E, M e N codificam as principais proteínas estruturais encontradas no vírion maduro (WU et al., 2020b). A espícula (S) forma trímeros na superfície do vírion e se liga ao receptor humano da Enzima Conversora de Angiotensina 2 (ACE2) para entrada na célula do hospedeiro (YAN et al., 2020). Ela contém duas subunidades, S1 e S2, que permite a clivagem efetiva pela furina e outras proteases (ANDERSEN et al., 2020). O sítio de clivagem S2 é clivado pela Serina Protease 2 Transmembrana (TMPRSS2) e a proteína S também contém os principais epítopos imunogênicos (BESTLE et al., 2020). A proteína do envelope (E) forma uma viroporina, que é importante para a montagem e liberação do vírus, e é um suposto determinante de virulência. A proteína de membrana (M) é uma proteína estrutural abundantemente expressa dentro do envelope lipídico, sendo também importante para a morfogênese viral e supressão do Interferon, um importante mecanismo antiviral (ZHENG et al., 2020). Finalmente, a proteína do nucleocapsídeo (N) estabiliza o genoma de RNA em um complexo helicoidal e serve como alvo chave da imunidade adaptativa do hospedeiro (PENG et al., 2020).

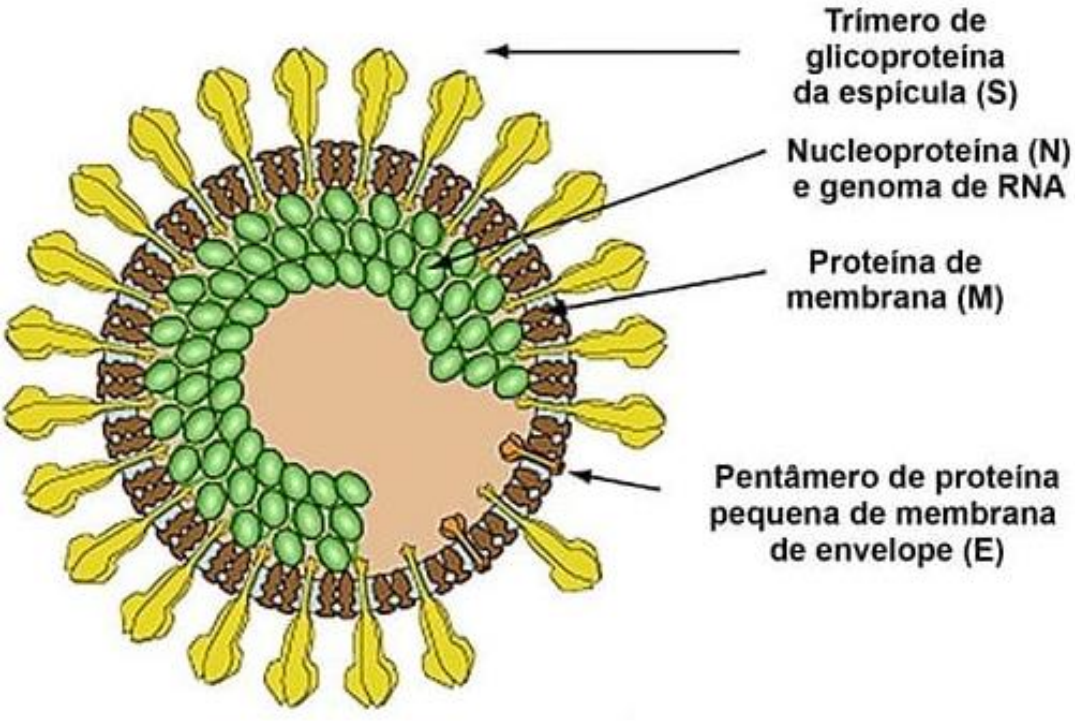

Figura 4: Estrutura da partícula viral de um coronavírus. Fonte: ViralZone; 2020.

1.3 Aspectos clínicos

A COVID-19 possui um amplo espectro de manifestações clínicas, que vão desde casos assintomáticos ou leves, apresentando tosse seca, dor de cabeça, perda do olfato e paladar, até casos mais graves, com o desenvolvimento de desconforto respiratório, síndrome respiratória aguda grave (SRAG), tromboembolismo, sepse, dentre outros (RITCHIE HANNAH, 2021; ROSSI et al., 2021). Segundo a OMS a gravidade da COVID-19 pode ser classificada de acordo com quadros clínicos pré-estabelecidos, como mostrado na figura 5.

Estado do paciente	Descrição	
Não infectado	Não infectado, RNA viral não detectado	0
Ambulatorial; doença leve	Assintomático; RNA viral detectado	1
	Sintomático; independente	2
	Sintomático; necessidade de assistência	3
Hospitalizado; doença moderada	Hospitalizado; sem oxigenoterapia	4
	Hospitalizado; oxigenação por máscara ou cateter nasal	5
Hospitalizado; doença grave	Hospitalizado; oxigenação por VNI ou alto fluxo	6
	Intubação e ventilação mecânica; $pO_2/FiO_2 \geq 150$ ou $SpO_2/FiO_2 \geq 200$	7
	Ventilação mecânica $pO_2/FiO_2 < 150$ ($SpO_2/FiO_2 < 200$) ou vasopressores	8
	Ventilação mecânica $pO_2/FiO_2 < 150$ e vasopressores, diálise ou ECMO	9
Crítico; Óbito	Óbito	10

Figura 5: Escala de progressão clínica da OMS. A figura descreve os 4 estados de gravidade de pacientes com COVID-19, sendo eles: assintomático ou doença leve, moderado, grave ou crítico, de acordo com parâmetros clínicos pré-estabelecidos. Fonte: Marshal et al., 2020

Pacientes com fatores de risco, como idade avançada, sexo masculino, obesidade, comorbidades adjacentes como câncer e internação em UTI, têm maiores riscos de desenvolverem formas graves da doença (GAO et al., 2021b).

Os vírus respiratórios têm como principais vias de transmissão as gotículas e aerossóis (TADA; NOHARA; KAWASHIRI, 2019). Em geral, as pessoas infectadas espalham partículas virais sempre que falam, respiram, tossem ou espirram. SARS-CoV-2 é transmitido primordialmente por gotículas e pode ser transmitido a uma pessoa saudável se ela tiver contato com a pessoa infectada ou qualquer um de seus pertences (SALIAN et al., 2021).

O período de incubação do vírus é em média de 5-7 dias, dependendo da variante do vírus (GUAN et al., 2020). A linfopenia é frequentemente identificada entre os pacientes com COVID-19 e níveis séricos de ferritina e proteína C reativa, bem como velocidade de hemossedimentação, podem estar elevados em associação com níveis alterados de citocinas pró-inflamatórias e quimiocinas circulantes.

Atualmente o diagnóstico clínico de infecção por SARS-CoV-2, na maioria dos casos, é realizado com espécimes de swab nasofaríngeo e/ou orofaríngeo em testes

rápidos de identificação de antígenos que estão sendo amplamente utilizados, pelo menor custo e maior rapidez no resultado, melhorando assim o manejo clínico (YAMAYOSHI et al., 2020). No entanto, o RT-PCR continua sendo o padrão-ouro para a detecção de SARS-CoV-2 devido à sua maior sensibilidade na detecção de partes genômicas virais, em vez dos biomarcadores secundários, como anticorpos (YÜCE; FILIZTEKIN; ÖZKAYA, 2021).

As fases clínicas da infecção por SARS-CoV-2 podem ser categorizadas em três fases: a “fase de infecção”, “fase pulmonar e “fase de hiperinflamação”, como mostrado na figura 6 (SIDDIQI; MEHRA, 2020). A fase de infecção para a maioria das pessoas está associada a sintomas leves e durante esse período SARS-CoV-2 se multiplica, focando principalmente no sistema respiratório, devido a abundância dos receptores pulmonares ACE2 e a afinidade do vírus pelo mesmo. Portanto, a infecção por SARS-CoV-2, de forma geral, se apresenta inicialmente com sintomas respiratórios (WAN et al., 2020). No segundo estágio, a doença pulmonar já está estabelecida e a multiplicação viral e a inflamação localizada no pulmão estão agravadas. É nesse momento que a maioria dos pacientes com COVID-19, que avançam para esse estágio, geralmente precisam ser hospitalizados para observação e gerenciamento de perto, onde são divididos em estágio 2A (sem hipóxia) e 2B (com hipóxia) (ELROBAA; NEW, 2021; SIDDIQI; MEHRA, 2020). Por fim, na terceira fase e mais grave estágio, onde poucos pacientes farão essa transição, a doença se manifesta como uma síndrome de hiperinflamação sistêmica extrapulmonar, por isso os marcadores de inflamação sistêmica nesse estágio se apresentam bastante elevados. No geral, o prognóstico e a recuperação desse estágio crítico da doença são ruins, e o rápido reconhecimento e implantação de terapias podem elevar as chances de recuperação (SIDDIQI; MEHRA, 2020; WU et al., 2020a).

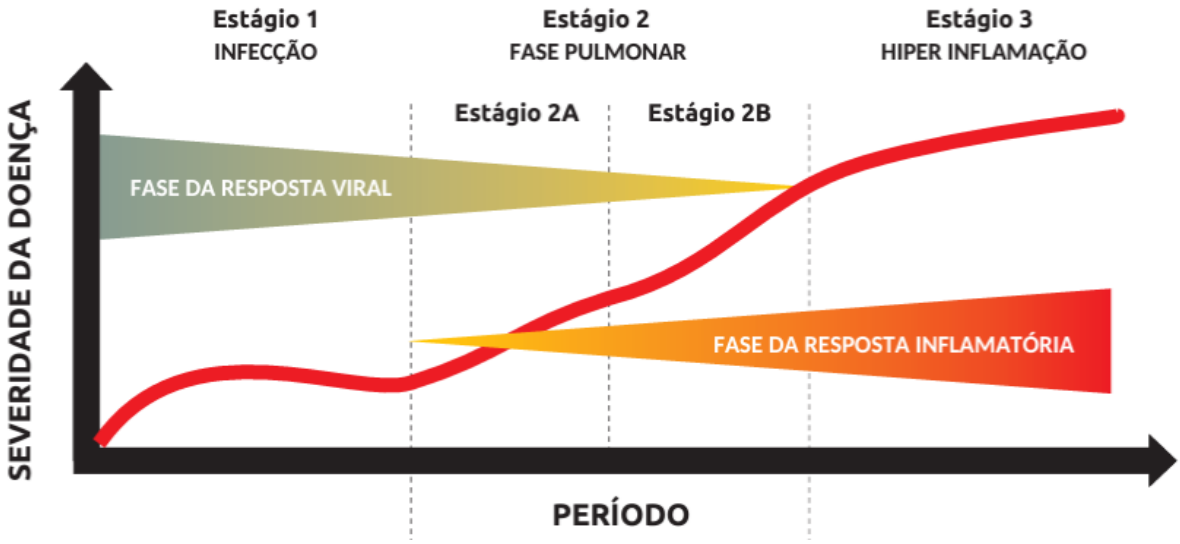

Figura 6: Fases clínicas da COVID-19. A figura descreve as 3 fases crescentes da progressão da doença COVID-19. O estágio 1 está associado a sintomas leves, focados principalmente no sistema respiratório. No estágio 2 a doença pulmonar já está estabelecida e é dividido em estágio 2A, onde o paciente não apresenta hipóxia e estágio 2B, onde a hipóxia está presente no quadro clínico. O estágio 3 é o mais grave, onde a doença se manifesta como uma síndrome de hiperinflamação sistêmica extrapulmonar. Fonte: Siddgi e Mehra; 2020.

As opções atuais de tratamento para COVID-19 são estratificadas em duas categorias, a dos antivirais e a dos imunomoduladores, não existindo ainda um medicamento específico para a doença, mas muitos estudos ainda estão em andamento (FERNANDES et al., 2022). Como os tratamentos farmacológicos para COVID-19 são direcionados para manejar ou evitar complicações graves da doença, a vacinação que se iniciou em 2020 no mundo se tornou essencial para controle da pandemia e diminuição dos casos graves (VITIELLO et al., 2021). Atualmente, temos quatro tipos diferentes de tecnologias de vacinas monovalentes disponíveis aprovadas contra SARS-CoV-2, a de tecnologia de vírus inativado, a de vetores virais não replicantes, a de RNA mensageiro (mRNA) e vacinas à base de subunidades de proteínas (FIOLET et al., 2022). Com mutações no genoma do SARS-CoV-2 e o surgimento de novas variantes de preocupação, houve uma redução da eficácia das vacinas disponíveis e mesmo com as doses de reforço a eficácia das vacinas contra a variante Ômicron especificamente ainda segue menor do que as demais. Por essa razão, vacinas bivalentes começaram a ser estudadas, demonstrando uma forte resposta imune e que podem ser podem ser uma nova ferramenta na resposta a variantes emergentes (CHALKIAS et al., 2022; TSENG et al., 2022). Em novembro de 2022 a Anvisa aprovou para uso emergencial a utilização de vacinas bivalentes na

população acima de 12 anos, mas a vacina monovalente original continua sendo importante instrumento no combate à Covid-19 (“Anvisa aprova vacinas bivalentes para dose de reforço contra Covid-19 — Português (Brasil)").

1.4 Imunopatogênese

A replicação do vírus começa quando a glicoproteína viral S se liga ao receptor ACE2 das células alvo e aproximadamente 83% desses receptores são expressos na superfície de células epiteliais alveolares tipo II, tornando-os reservatórios primários da invasão viral (SCIALO et al., 2020). Além disso, os receptores ACE2 também estão amplamente distribuídos no organismo, como no coração, rim, endotélio e intestino, o que explica a susceptibilidade desses órgãos a infecção (ZHAO et al., 2020). A ligação do vírus ao receptor ACE2 causa uma desregulação, promovendo assim um aumento da produção de angiotensina-2 (AT2). Esse aumento da produção da AT2 pode aumentar a permeabilidade vascular pulmonar, causando uma lesão pulmonar, o que pode ser uma das razões para este ser um dos órgãos mais afetados durante o curso da doença (MEDINA-ENRÍQUEZ et al., 2020)

Assim que o vírus entra na célula ele é reconhecido por sensores que recrutam proteínas adaptadoras capazes de induzir uma cascata de sinalização, levando à ativação de fatores de transcrição como o NF-κB e subsequente produção de interferons e citocinas pró-inflamatórias (CHATTERJEE et al., 2021). As citocinas liberadas pelas células infectadas vão modular a resposta imune, recrutando e ativando outras células, como macrófagos, células B e T para orquestrar a eliminação do vírus. No entanto, todas essas vias vão culminar em uma extensa resposta inflamatória e antiviral (SETTE; CROTTY, 2021). Essa superprodução de citocinas pró-inflamatórias vão resultar no que foi descrito como uma tempestade de citocinas, que seria uma reação hiperinflamatória, levando a um risco aumentado de hiperpermeabilidade vascular, falência de múltiplos órgãos e, eventualmente, morte (HU; HUANG; YIN, 2021).

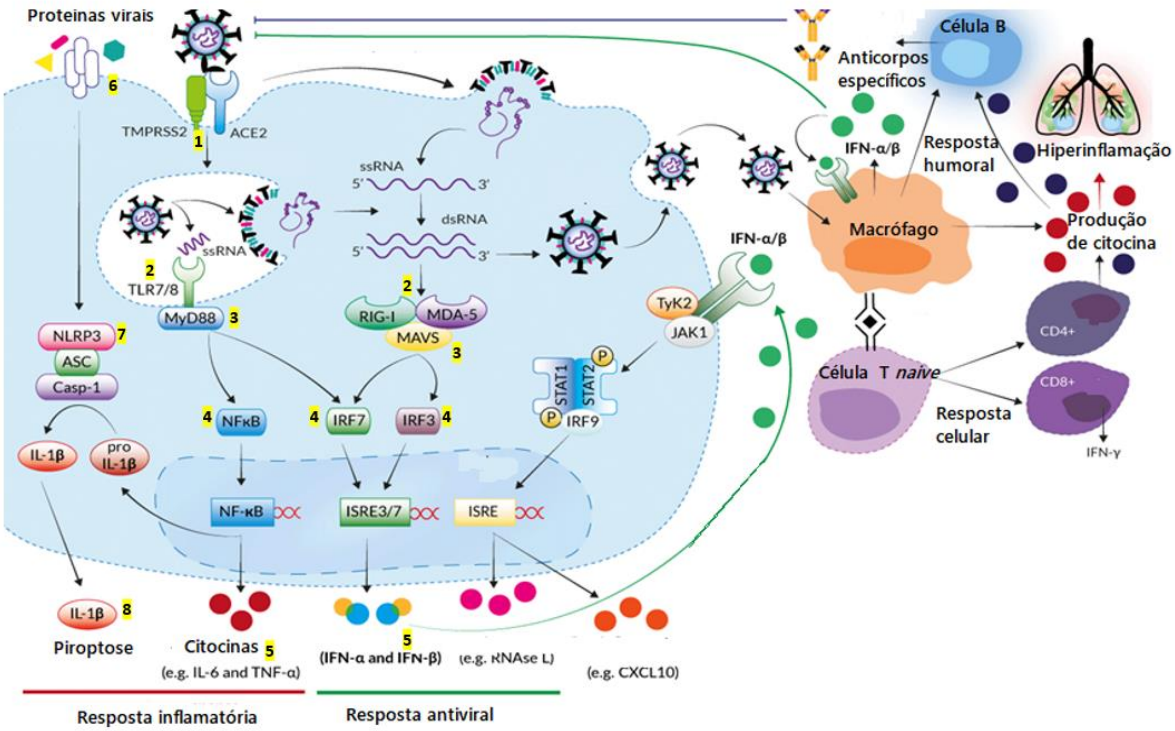

Figura 7: Resposta imune frente a COVID-19 e a tempestade de citocinas. A figura demonstra através de um esquema como a resposta imune inata se desenvolve desde o momento da entrada do vírus no epitélio alveolar, via receptores ACE-2 e TMPRSS2 (1), onde PRRs, incluindo TLR7/8 e RIG-I/MDA-5 vão reconhecer o genoma viral de RNA e seus intermediários de replicação do vírus (2). Após o reconhecimento, esses sensores recrutam as proteínas adaptadoras, MyD88 e MAVS, respectivamente, e induzem uma sinalização (3). Em última análise, isso leva à ativação dos fatores de transcrição, IRF3/7 e NF-κB (4) e à subsequente produção de IFN-α e IFN-β e citocinas pró-inflamatórias como IL-6 e TNF-α, respectivamente (4). Além disso, proteínas virais (6) ativam o sensor do inflamassoma NLRP3 (7), resultando na secreção da citocina altamente inflamatória IL-1β (6). Adaptado de InvivoGen, 2020.

Uma das vias que também será ativada é a dos inflamassomas, que são complexos multiproteicos formados após o reconhecimento de um conjunto diversificado de estímulos indutores de inflamação que incluem padrões moleculares associados ao patógeno (PAMPs) e padrões moleculares associados ao dano (DAMPs) (STROWIG et al., 2012). Muitos complexos de inflamassomas já foram identificados, incluindo o NLRP1, NLRP2, NLRP3, AIM2 e IFI16 (OZAKI; CAMPBELL; DOYLE, 2015). O melhor caracterizado é o inflamassoma NLRP3, também conhecido como “Família NLR contendo domínio de pirina da proteína 3”, que pertence a família de receptores do tipo NOD (NLRs) (EIGENBROD; DALPKE, 2015).

O complexo do inflamassoma NLRP3 é formado com a junção de um NLR, um adaptador ASC e uma caspase-1 ativa (MOOSSAVI et al., 2018). Para o funcionamento de toda essa estrutura são necessários dois sinais, o primeiro, se dá

por receptores de reconhecimento de padrões (PRRs), através do reconhecimento de PAMPs ou DAMPs, que incluem componentes como da parede celular microbiana, ácidos nucleicos, toxinas formadoras de poros e sinais de perigo endógenos como ATP e cristais de ácido úrico entre outras móleculas (MAN; KANNEGANTI, 2015). Na ativação canônica do inflamassoma NLRP3 há ativação de um ligante do receptor Toll-like (TLR), como o LPS, que vai resultar na ativação do fator nuclear-κB (NF-κB), estimulando a transcrição e a expressão de componentes do inflamassoma NLRP3, como a pró-interleucina-1β e pró interleucina-18, que irão permanecer inativas até o segundo sinal (BAUERNFEIND et al., 2009). No segundo sinal ocorre o acoplamento da proteína adaptadora ASC, onde essa molécula adaptadora liga sua porção de domínio de pirina (PYD) na porção PYD do NLRP3, deixando livre sua porção CARD, onde a pró-caspase-1 se liga, formando o inflamassoma. A pró-caspase 1 vai sofrer uma ativação autoproteolítica e a sua forma ativa vai atuar sobre a pró-IL-1β e a pró-IL-18, clivando-as em interleucina-1β (IL-1β) e (IL-18) ativas, sendo então liberadas para o meio extracelular.

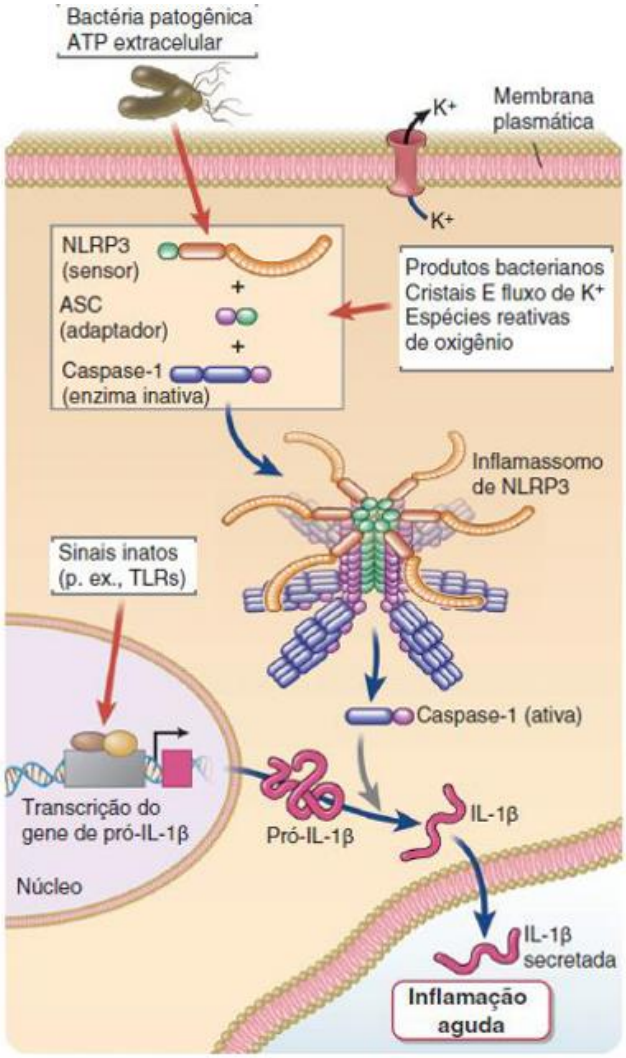

Figura 8: Mecanismo de ativação do inflamassoma NLRP3. Adaptado de Abbas, Lichtman, Pillai, 9a. Edição, 2019. A figura descreve o complexo do inflamassoma NLRP3, que é formado com a junção de um NLR, um adaptador ASC e uma caspase-1 e a sua ativação por dois sinais. O primeiro se dá por PRRs, através do reconhecimento de PAMPs ou DAMPs, onde posteriormente a pró-interleucina-1β e pró interleucina-18 serão expressas. No segundo sinal a proteína adaptadora ASC se acopla e recruta a pró-caspase 1, que irá sofrer uma ativação autoproteolítica e na sua forma ativa vai atuar sobre a pró-IL-1β e a pró-IL-18, clivando-as em IL-1β e IL-18 ativas e serão secretadas para o meio extracelular.

Além dos inflamassomas serem responsáveis pela produção de citocinas pró-inflamatórias importantes, verificou-se que regulam outros aspectos importantes da inflamação e reparação de tecidos, como a piroptose, uma forma de morte celular (BROZ; DIXIT, 2016). As células que morrem por piroptose exibem inchaço citoplasmático e ruptura da membrana plasmática, requerendo a atividade enzimática da CASP-1 ou CASP-11 (KAYAGAKI et al., 2011). A piroptose emergiu como uma defesa chave contra infecções microbianas. Acredita-se que este fenômeno

interrompa a replicação de patógenos intracelulares, eliminando células imunes infectadas, ao mesmo tempo em que promove a destruição de agentes microbianos sobreviventes. Além disso, a piroptose pode influenciar a imunidade adaptativa contra agentes infecciosos (AACHOUI et al., 2013; MIAO et al., 2010).

A CASP-1 induz piroptose mediada pela proteína da família gasdermina (GSDM) e processa as citocinas IL-1β e IL-18, onde a IL-1β é uma citocina mediadora da inflamação pulmonar, febre e fibrose, que induz uma cascata de citocinas, contribuindo para diferentes efeitos inflamatórios (ZHAO; DI; XU, 2021). Ela também promove imunidade adaptativa T helper 1 (Th1), Th17 e imunidade humoral. A IL-18 é importante para a expressão de IL-17 pelas células Th17 e pode polarizar as células T para perfis Th1 ou Th2 em combinação com outras citocinas (DINARELLO, 2009).

Os inflamassomas fazem parte dessa cascata de sinalização que leva à tempestade de citocinas na COVID-19 e, de fato, ele já teve sua ativação relacionada a casos moderados e graves da COVID-19, assim como citocinas derivadas deste inflamassoma também foram relacionadas a quadros clínicos graves (RODRIGUES et al., 2020). A ativação excessiva de citocinas pró-inflamatórias tanto pela imunidade inata, quanto adquirida, contribuem para uma piora no quadro clínico do paciente (WIERSINGA et al., 2020). Um inflamassoma que já teve seu envolvimento na infecção por SARS-CoV-2 estudado foi o NLRP1, onde em um estudo recente foi identificado como um sensor de infecção por SARS-CoV-2 no epitélio pulmonar e a sua ativação após a infecção em células epiteliais in vitro sugeriram que a inflamação causada por NLRP1 pode ser tanto benéfica, quanto prejudicial em pacientes com COVID-19 (PLANÈS et al., 2022). Um outro estudo também relacionou o aumento da ativação do inflamassoma NLRP3 microglial mediado por SARS-CoV-2 a diminuição da sobrevida em camundongos. Esses resultados apoiam um possível mecanismo de ativação imune inata microglial por SARS-CoV-2, o que poderia explicar a maior vulnerabilidade ao desenvolvimento de sintomas neurológicos em indivíduos com COVID-19 (ALBORNOZ et al., 2022).

1.5 Genética do hospedeiro na COVID-19

Já foi visto que a expressão de *NLRP3* e *CASP1* foram significativamente mais altas em células sanguíneas e derivadas de tecido pulmonar de pacientes com COVID-19 e a ativação dos inflamassomas é uma das principais hipóteses que

explicam a tempestade de citocinas que ocorre na COVID-19 (RODRIGUES et al., 2020; THEOBALD et al., 2021). No entanto, muitos fatores desempenham um papel na determinação da gravidade da doença e na taxa de infecção em uma população (CLEMENTI; GIANANTONIO, 2006).

Desde o sequenciamento do genoma humano em 2001 (LANDER et al., 2001), estudos genéticos em larga escala têm desempenhado um papel cada vez mais importante no delineamento da patogênese da doença humana (CHAPMAN; HILL, 2012). Compreender o curso da infecção de doenças através do estudo da genética do hospedeiro tem sido fundamental para entender a sua imunopatogênese. Além disso, complementa a compreensão in vitro da biologia da infecção, o que pode levar ao desenvolvimento de medicamentos (NARANBHAI; CARRINGTON, 2017). Muitos estudos já relacionaram polimorfismos em genes do hospedeiro relacionados a susceptibilidade e/ou gravidade de diversas doenças, como tuberculose (CAI et al., 2019; DE SÁ et al., 2022a), malária (KARIUKI; WILLIAMS, 2020), hepatite C (RAUCH et al., 2010), HIV (PEREYRA et al., 2010), dentre outras.

Na COVID-19 não seria diferente, por esse motivo alguns genes importantes envolvidos na patogênese da doença já foram estudados e associados a susceptibilidade e/ou severidade da doença. Polimorfismos nos genes *ACE2*, *TMPRRS2*, *TLR7*, locus *ABO*, *HLA* e outros já foram associados à proteção e/ou susceptibilidade em pacientes infectados (HOU et al., 2020; YILDIRIM et al., 2021). Esses achados indicam que *SNPs* de muitos outros genes envolvidos na resposta inflamatória também podem impactar na susceptibilidade e/ou proteção na infecção pelo vírus ou no curso da doença.

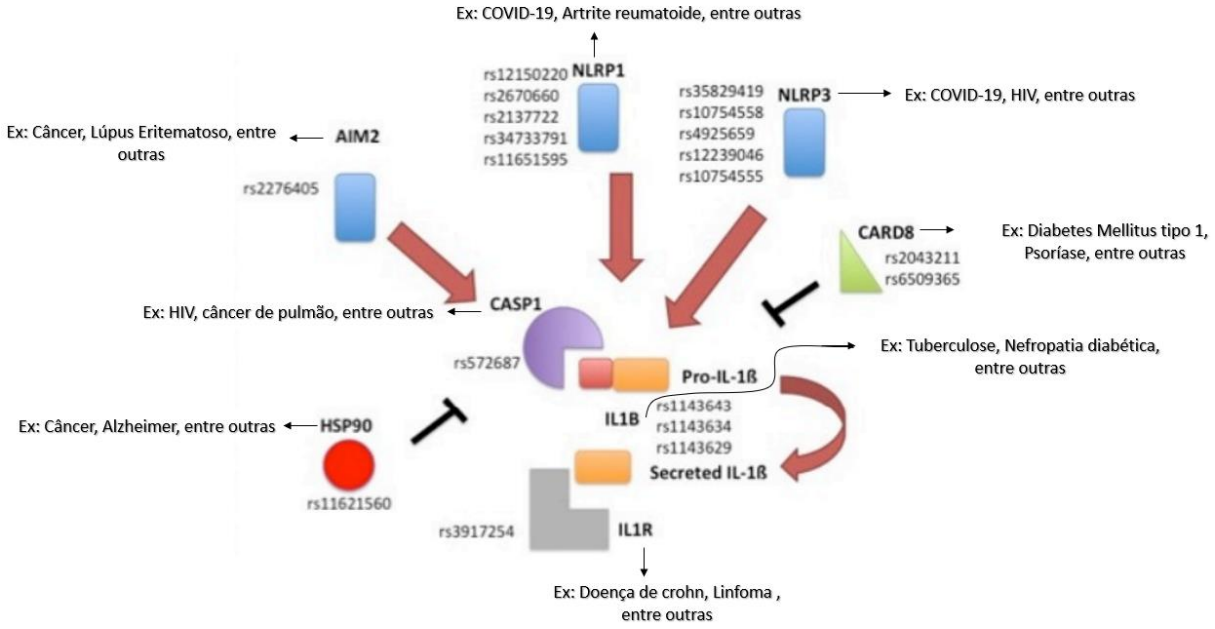

Figura 9: Polimorfismos em genes do inflamassoma que já foram associados à patogênese de diversas doenças. A figura descreve genes do inflamassoma e algum de seus polimorfismos que já foram associados a patogênese de doenças e seus respectivos exemplos, inclusive na COVID-19. Fonte: Adaptado Pontillo, et al., 2010.

Os polimorfismos em genes dos inflamassomas já foram relacionados a diversas doenças, como HIV (PONTILLO et al., 2012a), HPV (PONTILLO et al., 2016), hepatite C (TORO et al., 2021), doenças cardiovasculares (SCHUNK et al., 2021), diabetes mellitus (PANG et al., 2021), psoríase (JUNEBLAD et al., 2021) e meningite (GELDHOFF et al., 2013), dentre outras.

Um estudo recente mostrou que dois polimorfismos (rs10754558 e rs10157379) do gene *NLRP3* estão envolvidos com quadros graves da COVID-19 (MAES et al., 2022). A busca por fatores de risco e/ou de proteção na infecção pelo SARS-CoV-2, assim como para desfechos clínicos graves na COVID-19 é relevante para o manejo clínico e merece mais investigação.

1.6 Justificativa

A hiperinflamação ocasionada pela COVID-19 é um dos principais motivos apontados para a piora de quadros clínicos no curso da doença (LI et al., 2020). Os inflamassomas já foram apontados como um dos principais responsáveis pela tempestade de citocinas desencadeada pelo vírus e alguns artigos já observaram que a alteração no padrão de expressão do inflamassoma NLRP3 influencia diretamente

na gravidade da doença ao longo do curso da infecção (AMIN et al., 2022; BATIHA et al., 2022; SEFIK et al., 2022)

É bem consolidado que o *background* genético do hospedeiro pode contribuir significativamente para a suscetibilidade e/ou proteção às infeções e fatores de risco como algumas comorbidades, idade avançada e gênero não explicam completamente o curso de gravidade da doença (SHI et al., 2020).

Polimorfismos em genes dos inflamassomas já foram associados a inúmeras doenças, principalmente o inflamassoma NLRP3, que foi relacionado a muitos distúrbios e sintomas, incluindo doenças virais, neurológicas, inflamatórias, febre, mialgia, diabetes mellitus, *Alzheimer*, aterosclerose, hipertensão, doenças cardíacas, acidente vascular cerebral e obesidade ("DisGeNET - a database of gene-disease associations"). Portanto, se faz necessário a condução de mais estudos buscando entender o papel de polimorfismos do inflamassoma durante o curso da COVID-19, tanto para um melhor entendimento da ocorrência dos diferentes desfechos clínicos, como também para analisar como potencial alvo terapêutico, visando reduzir o processo inflamatório da COVID-19.

Diante do exposto, no presente estudo, investigamos o impacto de 11 SNPs em 6 genes do inflamassoma descritos na Tabela 1, em indivíduos infectados pelo SARS-CoV-2 com diferentes classificações clínicas e tempo de progressão para desfechos graves (uso de ventilação mecância e óbito), em um hospital público de referência para COVID-19 no Rio de Janeiro, Brasil. Além disso, analisamos a expressão de dois genes do inflamassoma, *NLRP3* e *CASP1*, em pacientes moderados e graves/críticos, visando a identificação de possíveis biomarcadores inflamatórios da COVID-19.

Tabela 1: Características dos SNPs incluídos no estudo:

Genes	SNP	Localização
CARD8	rs2043211	exônica
CARD8	rs6509365	intrônica
AIM2	rs2276405	intrônica
IFI16	rs1101996	intrônica
CASP1	rs572687	intrônica
IL-1β	rs1143634	exônica
NLRP3	rs3806268	exônica
NLRP3	rs35829419	exônica
NLRP3	rs4612666	intrônica
NLRP3	rs15390193	intrônica
NLRP3	rs10754558	3′UTR

2. OBJETIVOS

2.1 Objetivo Geral

Determinar a existência de potenciais biomarcadores inflamatórios associados com a gravidade de COVID-19, através da análise de expressão e polimorfismos de genes que codificam proteínas da via dos inflamassomas.

2.2 Objetivos Específicos

- Analisar os polimorfismos de base única (SNP) nos genes que codificam moléculas do inflamassoma (*NLRP3, CARD8, AIM2, IFI16, CASP-1 e IL-1β*) em todos os indivíduos incluídos no estudo, a fim de verificar a distribuição destes SNP nos grupos estudados (Artigo 1);
- Avaliar, a partir da distribuição dos SNP, as possíveis associações entre estes marcadores genéticos do hospedeiro com as formas graves/moderadas e leves da COVID-19 (Artigo 1);
- Avaliar, a partir da distribuição dos SNP, as possíveis associações entre estes marcadores genéticos do hospedeiro e a gravidade da COVID-19, medida através do tempo de evolução para o uso de ventilação mecânica ou para o óbito (Artigo 2);
- Determinar a ativação do inflamassoma *NLRP3* e *CASP-1*, através da quantificação do RNAm nos grupos de indivíduos com quadro moderado, grave/crítico de COVID-19 e sua associação com os polimorfismos estudados;
- Com base nos dados de ativação do inflamassoma *NLRP3* e *CASP-1*, discutir os seus papéis potenciais como alvos terapêuticos, visando reduzir o processo inflamatório da COVID-19;

3. RESULTADOS

Os dados obtidos foram organizados em três partes. A primeira foi inserida em um artigo, que para fins didáticos intitulamos “Artigo 1”, publicado na *BioMed Research International* em 6 de setembro de 2022. A segunda parte foi incluída em um outro artigo, que chamamos de “Artigo 2”, submetido na revista *Gene* em 17 de novembro de 2022. Na terceira parte se encontram os dados obtidos e ainda não publicados.

3.1. ARTIGO 1 – *“Inflammasomes genetic variants are associated with protection to clinical severity of COVID-19 among patients from Rio de Janeiro, Brazil.”*

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[a] Esses autores contribuíram igualmente e dividem a primeira autoria.

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Periódico: Trabalho aceito para publicação em 6 de setembro de 2022 na *BioMed Research International* | DOI: 10.1155/2022/9082455.

Resumo

A COVID-19 possui um grande número de manifestações clínicas, desde leves ou asssintomáticas a formas severas. Os mecanismos por trás desses perfis de evolução clínica ainda não foram completamente elucidados. Na infecção pelo SARS-CoV-2 o inflamassoma é ativado na presença do vírus dentro das células infectadas. Nesse estudo, nós investigamos o impacto de SNPs do inflamassoma em indivíduos infectados pelo SARS-CoV-2 com perfis clínicos de severidade distintos. Os pacientes foram divididos em 2 grupos de acordo com a severidade da doença baseado na Escala de Progressão Clínica da OMS. O grupo 1 incluiu pacientes com doença leve/moderada (OMS<6; n=76), e o grupo 2 incluiu pacientes com quadros

severos/críticos (OMS≥6; n=357). Pacientes internados com quadros moderados e severos/críticos foram recrutados e acompanhados no Centro Hospitalar para a pandemia de COVID-19 – Instituto Nacional de Infectologia (INI/FIOCRUZ) de junho de 2020 a março de 2021. Pacientes com doença leve foram recrutados no Instituto Oswaldo Cruz (IOC/FIOCRUZ) em agosto de 2020. A genotipagem de 11 SNPs do inflamassoma foi feita por PCR em tempo real. Estimativa de risco e proteção foi realizada utilizando o modelo de regressão logística incondicional. Diferenças significativas no polimorfismo rs1539019 do gene *NLRP3* e rs2043211 do gene *CARD8* foram observadas entre os 2 grupos. Proteção contra a severidade da doença foi associada ao genótipo A/A (OR_{adj}=0.36; *P*=0.032), alelo A (OR_{adj}=0.93; *P*=0.010), ou carrear-A (OR_{adj}=0.45; *P*=0.027) no polimorfismo rs1539019 do gene *NLRP3*; Genótipo A/T (OR_{adj}=0.5; *P*=0.045), alelo T (OR_{adj}=0.93; *P*=0.018), ou carrear-T (OR_{adj}=0.48; *P*=0.029) no polimorfismo rs2043211 do gene *CARD8*. Os haplótipos das variates do gene *NLRP3* incluídas A-C-G-C-C (OR_{adj}=0.11; *P*=0.018), A-C-G-C-G (OR_{adj}=0.23; *P*=0.003), C-C-G-C-C (OR_{adj}=0.37; *P*=0.021) e C-T-G-A-C (OR_{adj}=0,04; *P*=0.0473) também foram asssociados à proteção. Os demais polimorfismos não apresentaram nenhuma associação significativa. Nosso estudo demonstra associação entre variantes dos genes do inflamassoma *CARD8* e *NLRP3* com a proteção contra a severidade da COVID-19, contribuindo para a discussão do impacto dos inflamassomas nos diferentes desfechos da doença.

Hindawi
BioMed Research International
Volume 2022, Article ID 9082455, 15 pages
https://doi.org/10.1155/2022/9082455

Research Article

Inflammasome Genetic Variants Are Associated with Protection to Clinical Severity of COVID-19 among Patients from Rio de Janeiro, Brazil

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Received 4 May 2022; Revised 18 August 2022; Accepted 24 August 2022; Published 5 September 2022

Academic Editor: Horacio Bach

COVID-19 has a broad spectrum of clinical manifestations, from asymptomatic or mild/moderate symptoms to severe symptoms and death. The mechanisms underlying its clinical evolution are still unclear. Upon SARS-CoV-2 infection, host factors, such as the inflammasome system, are activated by the presence of the virus inside host cells. The search for COVID-19 risk factors is of relevance for clinical management. In this study, we investigated the impact of inflammasome single-nucleotide polymorphisms (SNPs) in SARS-CoV-2-infected individuals with distinct severity profiles at clinical presentation. Patients were divided into two groups according to disease severity at clinical presentation based on the WHO Clinical Progression Scale. Group 1 included patients with mild/moderate disease (WHO < 6; $n = 76$), and group 2 included patients with severe/critical COVID-19 (WHO ≥ 6; $n = 357$). Inpatients with moderate to severe/critical profiles were recruited and followed-up at Hospital Center for COVID-19 Pandemic – National Institute of Infectology (INI)/FIOCRUZ, RJ, Brazil, from June 2020 to March 2021. Patients with mild disease were recruited at Oswaldo Cruz Institute (IOC)/FIOCRUZ, RJ, Brazil, in August 2020. Genotyping of 11 inflammasome SNPs was determined by real-time PCR. Protection and risk estimation were performed using unconditional logistic regression models. Significant differences in NLRP3 rs1539019 and CARD8 rs2043211 were observed between the two groups. Protection against disease severity was associated with the A/A genotype ($OR_{adj} = 0.36$; $P = 0.032$), allele A ($OR_{adj} = 0.93$; $P = 0.010$), or carrier-A ($OR_{adj} = 0.45$; $P = 0.027$) in the NLRP3 rs1539019 polymorphism; A/T genotype ($OR_{adj} = 0.5$; $P = 0.045$), allele T ($OR_{adj} = 0.93$; $P = 0.018$), or carrier-T ($OR_{adj} = 0.48$; $P = 0.029$) in the CARD8 rs2043211 polymorphism; and the A-C-G-C-C ($OR_{adj} = 0.11$; $P = 0.018$), A-C-G-C-G ($OR_{adj} = 0.23$; $P = 0.003$), C-C-G-C-C ($OR_{adj} = 0.37$; $P = 0.021$), and C-T-G-A-C ($OR_{adj} = 0.04$; $P = 0.0473$) in NLRP3 genetic haplotype variants. No significant associations were observed for the other polymorphisms. To the best of our knowledge, this is the first study demonstrating an association between CARD8 and NLRP3 inflammasome genetic variants and protection against COVID-19 severity, contributing to the discussion of the impact of inflammasomes on COVID-19 outcomes.

1. Introduction

At the end of 2019, a new disease emerged, described initially as an outbreak of viral pneumonia in individuals living in Wuhan, China [1]. Researchers identified a new coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), as the pathogen causing the outbreak *[2]*. The World Health Organization (WHO) named the associated coronavirus disease COVID-19 [3], which was raised to the pandemic category in March 2020 due to its fast dispersion around the world [4]. Globally, the mortality and incidence of SARS-CoV-2 have increased rapidly. Currently, the Americas are the continents most affected by the COVID-19 pandemic, and the United States of America (USA) and Brazil are the leaders in the numbers of cases to date [5]. According to official data from the WHO, more than 478 million individuals are already infected by SARS-CoV-2, and more than 6 million subjects have died due to COVID-19 worldwide [5, 6]. In Brazil, the first case was reported on February 26, 2020, and the first community transmission was identified on March 13, 2020. The country has accumulated more than 29 million reported cases, with more than 658,000 deaths, as of March 2022 [6]. SARS-CoV-2 vaccination started in January 2021 but was initially restricted to health care workers and elderly people. By April 2022, a total of 163 million individuals had been fully vaccinated, and more than 80 million subjects had received a booster vaccine dose, leading to a decrease in the incidence of severe disease and mortality [5].

The clinical presentation of COVID-19 can range from asymptomatic or mild/moderate flu-like symptoms to critical symptoms, such as severe acute respiratory syndrome (SARS), thromboembolism, sepsis, multiple organ failure, and death [6]. Although COVID-19 mortality rates vary among countries, older age and the presence of comorbidities have been strongly associated with more severe disease and death. The relationship between host genetics and the mechanisms underlying SARS-CoV-2 infection with the worst clinical evolution remains unclear [7–10].

In fact, during SARS-CoV-2 infection, host factors are activated by the presence of the virus inside host cells. Pattern recognition receptors (PRRs) recognize conserved virus fragments, known as pathogen-associated molecular patterns (PAMPs), and trigger the activation of several cellular components [11, 12]. Among the large family of PRRs are NOD-like receptors (NLRs), retinoic acid-inducible gene-I-(RIG-I-) like receptors (RLRs), and Toll-like receptors (TLRs) [13, 14]. Some studies have already shown that the RLR family is an important PRR in the detection of coronaviruses [15, 16]. In addition, NLR receptors stand out due to their wide recognition of intrinsic or extrinsic stimuli, operating principally as cytoplasmic sensors [17]. These receivers lead to activation of the NF-κB signaling pathway, which culminates in the transcription of several molecules, such as gasdermin-D (GSDM-D), pro-IL-1β, and pro-IL-18, among others [18, 19]. These released molecules cause a wave of local inflammation involving increased secretion of proinflammatory cytokines and chemokines (e.g., IL-6, IFN-γ, CCL2, and CXCL10) [20, 21]. These and other cytokines have already been observed to be increased in SARS-CoV-2 infection, especially in more severe cases [20, 22].

The primary function of NLRs is to form a multiprotein complex known as the inflammasome. Inflammasomes are cytosolic multiprotein oligomers of the innate immune system that interact with several adapter proteins and are responsible for the activation of inflammatory responses, leading to activation of caspase-1 and inducing the release of the proinflammatory cytokines IL-1β and IL-18 [23]. Different PRRs (e.g., NLRP1 and NLRP3) can activate inflammasome assembly in response to specific stimuli, leading to inflammation and triggering the innate immune response. Inflammasome activation is strictly regulated by endogenous host proteins (e.g., CARD8 and HSP90) and by a variety of transcriptional and posttranscriptional mechanisms [24]. NLR inflammasomes comprise at least three components: a protein sensor (e.g., NLRP1 and NLRP3), an inflammatory caspase (e.g., caspase-1 and caspase-11), and an adapter molecule containing a CARD domain (e.g., ASC) [25]. In addition, twenty-two members of the NLR family have been described in humans and can be divided into four categories based on their functions: inflammasome formation, signal transduction, transcription activation, and autophagy [26]. The association between dysregulated inflammasome activity and the occurrence of certain human inflammatory diseases highlights the importance of this pathway in innate immune responses. As a regulatory mechanism, activation of inflammasome sensors (NLRP3 and AIM2) induces autophagy that subsequently impacts negatively the inflammasome function by inhibiting the formation and production of cytokines, such as IL1β, and degrading the inflammasome complex. Thus, autophagy accompanies inflammasome activation to limit inflammation by eliminating active inflammasomes [27]. As discussed in Sargazi et al., a potential correlation between SARS-CoV-2 and other coronavirus pathogens and autophagy has been suggested, indicating the relevance of targeting the autophagy pathway in the development of therapies for COVID-19 [28]. It has been shown that the SARS-CoV-2 proteins E, ORF3a, and ORF8b activate the NLRP3 inflammasome [17]. Mutations in inflammasome genes may lead to inflammatory disorders, such as chronic inflammation, autoimmunity, and viral infections [29–31]. For example, SNPs in the NLRP3 gene were found to be associated with a group of inflammatory disorders of genetic origin with exaggerated secretion of IL-1β [32].

Studies have already noted a relationship between inflammasome activation and COVID-19 *[13, 33–36]*. Inflammasome activation is one of the main theories to explain the cytokine storm that can occur during COVID-19, causing severe disease [13, 36]. NLRP3 activation in COVID-19 has already been described in tissues of COVID-19 patients. Additionally, higher levels of the inflammasome products IL-18 and Casp1p20 in COVID-19 patients were associated with severe disease [36]. Recently, it has been demonstrated that lung-resident macrophages infected with SARS-CoV-2 activate inflammasomes and release IL-1 and IL-18, leading to pyroptosis, which might contribute to lung inflammation [37].

However, data exploring the role of inflammasomes in SARS-CoV-2 infection remain scarce. Genetic factors contributing to the outcome of SARS-CoV-2 infection remain unclear; however, variants in specific sites of the ACE2 and TMPRSS2 genes, as well as the ABO locus, have already been considered genetic risk factors for COVID-19 outcomes [38–42]. Currently, new candidate genes have been described in the literature as influencing susceptibility to COVID-19. In this respect, Nia et al. showed that TNFα/TNFβ polymorphisms might substantially affect COVID-19 susceptibility [43]. In a case-control study, Rokni et al. reported that carrying the A allele in TNFA-rs361525, the C allele in IL1RN-rs419598, and the A allele in IL6R-rs2228145 was related to susceptibility to developing COVID-19 [44]. These findings indicate that SNPs in several other candidate genes involved in the inflammatory response might also impact susceptibility to COVID-19. A recent study showed that two NLRP3 variants play an important role in severe and critical COVID-19 [45]. The search for risk and/or protection factors in severe COVID-19 is relevant for clinical management and deserves more investigation. Thus, in the present study, we investigated the impact of 11 single-nucleotide polymorphisms (SNPs) in NLRP3 rs10754558 (3′UTR), rs4612666 (intronic region), rs1539019 (intronic region), rs3806268 (exonic region), and rs35829419 (exonic region); CARD8 rs2043211 (exonic region) and rs6509365 (intronic region); AIM2 rs2276405 (intronic region); CASP-1 rs572687 (intronic region); IFI16 rs1101996 (intronic region); and IL-1β rs1143634 (exonic region) inflammasome genes in SARS-CoV-2-infected individuals at distinct severity stages at clinical presentation in a public reference center for COVID-19 in Rio de Janeiro, Brazil.

2. Materials and Methods

2.1. Study Design and Population. This is a case-control genetic study nested in the RECOVER-SUS study (NCT04807699), which is a prospective multicenter study that includes participants with SARS-CoV-2 infection who were hospitalized due to COVID-19 at "Instituto Nacional de Infectologia Evandro Chagas" of the "Fundação Oswaldo Cruz" (INI/FIOCRUZ). Patients 18 years or older with confirmed SARS-CoV-2 infection presenting moderate, severe, or critical COVID-19 profiles based on the WHO severity classification at clinical presentation were enrolled in the RECOVER-SUS cohort from June 2020 to March 2021. Details regarding patient eligibility, enrollment, inclusion/exclusion criteria, and the study design of the RECOVER-SUS clinical cohort study have been previously described [46]. For the present study, we analyzed a subset of the RECOVER-SUS cohort, including 451 patients who agreed to participate in the substudy, gave biological samples for the genetic analyses, and were recruited from June to October 2020 and from February to March 2021.

Additionally, a group with mild COVID-19 composed of 43 individuals 18 years or older with SARS-CoV-2 infection confirmed by RT-PCR with asymptomatic or mild disease severity (outpatients with COVID-19) were recruited in August 2020 by the Laboratory of Respiratory Virus and Measles – IOC/FIOCRUZ, Rio de Janeiro, Brazil, after disease resolution for blood collection.

For this study, both outpatients and hospitalized individuals without suspected, probable, or RT-PCR-confirmed SARS-CoV-2 infection, according to the WHO COVID-19 case definition, or those who did not sign the consent form were excluded.

This study was approved by the Ethics Committee of National Institute of Infectology Evandro Chagas (INI)/Oswaldo Cruz Foundation (FIOCRUZ), Rio de Janeiro, Brazil, under the approval number CAAE 32449420.4.1001.5262 and the Oswaldo Cruz Institute (IOC)/Oswaldo Cruz Foundation (FIOCRUZ), Rio de Janeiro, Brazil, under the approval number CAAE 68118417.6.0000.5248. All participants or their legal representatives signed an informed consent form prior to enrollment in the study. All methods were performed in accordance with the relevant guidelines and regulations.

Demographic and clinical data and blood samples were collected at the study entry visit (baseline). Skin color was self-declared following the classification system employed by the Brazilian Institute of Geography and Statistics (IBGE) [47]. IBGE is an entity linked to the Brazilian Federal Government and is responsible for collecting Brazilian statistical, geographic, cartographic, geodetic, and environmental information.

2.2. Clinical Profiles at Presentation. Clinical presentation was defined as mild, moderate, severe, or critical COVID-19 according to the WHO severity classification [48]. The mild group (WHO < 4) included asymptomatic outpatients or those with mild symptoms, such as cough, chest pain, coryza, dyspnea, odynophagia, anosmia, ageusia, digestive symptoms, headache, and/or myalgia. The moderate group (WHO 4-5 classification) included hospitalized symptomatic patients with no need for oxygen therapy or oxygen by mask or nasal prong. The severe group (WHO 6-8 classification) included hospitalized patients requiring oxygen via NIV (noninvasive ventilation) or high flow, intubation, and mechanical ventilation $pO_2/FiO_2 \geq 150$ or $SpO_2/FiO_2 \geq 200$, mechanical ventilation $pO_2/FiO_2 < 150$ ($SpO_2/FiO_2 < 200$) or vasopressors. The critical group (WHO 9-10 classification) included patients requiring mechanical ventilation $pO_2/FiO_2 < 150$ and vasopressors, dialysis, or extracorporeal membrane oxygenation (ECMO) and patients who died.

2.3. Genomic DNA Extraction. DNA was extracted from whole blood using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Nordrhein-Westfalen, Germany) following the manufacturer's instructions. The DNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The filtrates containing the isolated DNA were stored at -20°C until the genomics analyses.

2.4. Single-Nucleotide Polymorphism Selection and Genotyping. We selected 11 SNPs in six inflammasome genes [49–51], considering the relevance of each gene in

the inflammasome pathway: CARD8 (rs2043211 and rs6509365), AIM2 (rs2276405), IFI16 (rs1101996), CASP-1 (rs572687), IL-1β (rs1143634), and NLRP3 (rs10754558, rs1539019, rs4612666, rs3806268, and rs35829419). SNP genotyping was performed using commercially available TaqMan assays (Applied Biosystems/AB and Life Technologies) according to the manufacturer's instructions (Applied Biosystems/AB and Life Technologies). Briefly, for qPCR, a final volume of 10 μL was used, and 4.50 μL of the genomic DNA, which was adjusted to 0.2 ng/μL, was placed in each well of a 96-well fast plate with 5 μL of the reaction buffer 2X TaqMan Master Mix and 0.5 μL of the 20X Assay Working Stock of each gene target. Genotyping was conducted with a reaction of one cycle of 95°C for 10 min for polymerase activation, one cycle of 95°C for 15 seconds for denaturation, and 60°C for 1 min for annealing/extension in an ABI7500 Real-Time PCR platform. Allelic discrimination was carried out using Thermo Fisher Connect Software. The SNP characteristics are listed in Supplementary Table 1.

2.5. Statistical Analyses. For statistical analyses, we divided the COVID-19 patients into two groups according to WHO scores < 6 or ≥6. Group 1 included patients with mild and moderate COVID-19 ($n = 76$; WHO < 6), and group 2 included patients with severe and critical COVID-19 ($n = 357$; WHO ≥ 6).

The Mann–Whitney *U* tests were used to compare baseline demographic and clinical, continuous numerical variables, and Fisher's exact tests were used for categorical variables. In the SNP analyses, the frequencies of genotypes were determined by direct count, and χ^2 tests assessed deviations from HWE. Pairwise LD patterns were determined for each gene using r^2 statistics (cutoff of $r^2 \geq 0.8$). The homozygous genotypes of the allele with the major frequency in our sample were compared with the other genotypes including the minor allele frequency allele (carriers) to better observe the differences caused by the variation. The protection/risk estimate is presented as adjusted odds ratios (aORs) with a 95% CI for each SNP and estimated through unconditional logistic regression models. We included any clinical phenotypic marker associated with COVID-19 as a confounder in modeling all other genotypic analyses to eliminate any possible bias. Haplotype frequencies were estimated by maximum likelihood, and phase uncertainty was included in statistical models applied for association analyses. The most frequent haplotypes of the NLRP3 and CARD8 genes were considered references for the haplotype analyses. Multiple comparisons were corrected by estimations of false-discovery rates (FDRs). All statistical analyses were performed using R version 4.1.1 (R Core Team, 2021).

We post hoc estimated the statistical power of the study considering (a) the observed minor allele frequencies (MAFs) observed in our sample's controls (i.e., those with WHO < 6) for the studied gene variants (SNPs); (b) the prevalence of severe COVID-19 prevaccination cases of 36.17% previously reported for the Brazilian population in the same period [52]; and (c) the ratio between controls and cases of 1 : 4.7 (or 76/357). We conducted simulations assuming that gene variants were under a genetic additive model with a 95% association with the disease genotype, given by the D' linkage-disequilibrium measure. Simulations were conducted with R and packages "genetics" and "GeneticsDesign."

3. Results

3.1. Sociodemographic and Clinical Characteristics. A total of 494 individuals with COVID-19 were included in this study. Of those, 61 patients with missing data needed for classification according to the WHO severity classification were excluded from the analysis. Thus, 433 individuals were included in this study and divided into two major groups according to the WHO severity classification, and their sociodemographic and clinical characteristics are depicted in Table 1. The mean age was 58 years (IQR = 21.79), with a mean of 50 years (IQR = 24.86) among the mild and moderate patients (WHO < 6 group) and 58 years (IQR = 22.64) among the severe and critical patients (WHO ≥ 6 group). Overall, 256 individuals (51.8%) were male, with 33 (43.4%) in the mild and moderate group and 189 (52.9%) in the severe and critical group. Regarding schooling, 145 (29.4%) of the individuals had a high school degree, with 15 (19.7%) in the mild and moderate group and 109 (30.5%) in the severe and critical group. The most common symptoms (>80%) in both groups were chest pain, diarrhea, abdominal pain, and nausea. In addition, the severe and critical groups presented a high frequency of (>80%) coryza, odynophagia, anosmia, loss of taste, headache, and myalgia as the most frequent symptoms. Most individuals included in this study ($n = 323$; 74.6%) were classified as having WHO severity scores between 6 and 8. The schooling, skin color, the comorbidity coronary artery disease, and some symptoms (fever, odynophagia, anosmia, loss of taste, and diarrhea) differed significantly between the groups. After correction by age, gender, skin color, schooling, diabetes mellitus, coronary artery disease, and obesity or previous bariatric disease comorbidities, wherever applicable, only oxygen supplementation or use of ventilatory support and a saturation level below 95% were significantly different between groups (Table 1).

3.2. Alleles, Genotypes, and Haplotype of Inflammasome Genes

3.2.1. Association of Alleles and Genotypes between the Groups. The genotypes, alleles, and carrier frequencies of all the studied SNPs associated with disease severity protection or risk are shown in Table 2. Genotype frequencies associated with the 11 SNPs analyzed were in the Hardy-Weinberg equilibrium in both groups (Supplementary Table 1).

In this study, both NLRP3 rs1539019 and CARD8 rs2043211 polymorphisms were associated with protection against disease severity in SARS-CoV-2-infected individuals (Table 2). For NLRP3 rs1539019, the association was with carrying the A/A genotype ($OR_{adj} = 0.36$ [95% CI, 0.14-0.92], $P = 0.033$), allele A ($OR_{adj} = 0.93$ [95% CI, 0.88-0.98], $P =$

Table 1: Sociodemographic and clinical features associated with presentation of either mild and moderate COVID-19 symptoms or severe and critical COVID-19 symptoms ($n = 433$).

Features		WHO scale		aOR[a] (95% CI)	P value[b]	Adjusted P value[b]
		Mild and moderate group $N = 76$	Severe and critical group $N = 357$			
Gender; n (%)	Female	43 (56.58%)	168 (47.06%)		Reference	
	Male	33 (43.42%)	189 (52.94%)	1.58 (0.82-3.02)	0.169	1
Skin color; n (%)	White	36 (47.37%)	64 (17.93%)		Reference	
	Brown	32 (42.11%)	229 (64.15%)	2.76 (1.32-5.77)	0.014	0.562
	Other	8 (10.53%)	64 (17.93%)	2.78 (0.95-8.16)	0.063	1
Age; n (%)	40-60	25 (36.76%)	135 (39.59%)		Reference	
	18-40	22 (32.35%)	39 (11.44%)	0.49 (0.13-1.91)	0.914	1
	60-80	18 (26.47%)	139 (40.76%)	1 (0.25-4.06)	1	1
	80-90.6	3 (4.41%)	28 (8.21%)	2.22 (0.11-43.98)	1	1
Schooling; n (%)	University education	38 (54.29%)	45 (15.62%)		Reference	
	High school	15 (21.43%)	109 (37.85%)	2.77 (1.19-6.46)	0.036	1
	Low education	17 (24.29%)	134 (46.53%)	2.14 (0.92-4.99)	0.078	1
Diabetes mellitus; n (%)	No	66 (86.84%)	235 (65.83%)		Reference	
	Yes	10 (13.16%)	122 (34.17%)	2.01 (0.8-5.05)	0.135	1
Systemic arterial hypertension; n (%)	No	59 (77.63%)	177 (49.58%)		Reference	
	Yes	17 (22.37%)	180 (50.42%)	1.57 (0.7-3.51)	0.270	1
Coronary artery disease; n (%)	No	67 (88.16%)	347 (97.2%)		Reference	
	Yes	9 (11.84%)	10 (2.8%)	0.21 (0.05-0.89)	0.033	1
Obesity or previous bariatric surgery; n (%)	No	69 (90.79%)	291 (81.51%)		Reference	
	Yes	7 (9.21%)	66 (18.49%)	1.14 (0.45-2.87)	0.786	1
O_2 supplementation or ventilatory support; n (%)	No	45 (59.21%)	47 (13.17%)		Reference	
	Yes	31 (40.79%)	310 (86.83%)	5.24 (2.34-11.76)	<0.001	0.002
Saturation below 95%; n (%)	No	67 (88.16%)	166 (46.5%)		Reference	
	Yes	9 (11.84%)	191 (53.5%)	8.06 (3.12-20.82)	<0.001	0.001
Fever; n (%)	No	42 (55.26%)	149 (41.74%)		Reference	
	Yes	34 (44.74%)	208 (58.26%)	2.05 (1.05-4.01)	0.035	1
Cough; n (%)	Yes	45 (59.21%)	225 (63.03%)		Reference	
	No	31 (40.79%)	132 (36.97%)	0.97 (0.49-1.9)	0.925	1
Chest pain; n (%)	No	64 (84.21%)	325 (91.04%)		Reference	
	Yes	12 (15.79%)	32 (8.96%)	0.55 (0.22-1.36)	0.195	1
Coryza; n (%)	No	59 (77.63%)	327 (91.6%)		Reference	

Table 1: Continued.

Features		WHO scale Mild and moderate group $N = 76$	Severe and critical group $N = 357$	aOR[a] (95% CI)	P value[b]	Adjusted P value[b]
Dyspnea; n (%)	Yes	17 (22.37%)	30 (8.4%)	0.8 (0.31-2.03)	0.635	1
	No	40 (52.63%)	101 (28.29%)		Reference	
	Yes	36 (47.37%)	256 (71.71%)	1.67 (0.84-3.34)	0.143	1
Odynophagia; n (%)	No	59 (77.63%)	343 (96.08%)		Reference	
	Yes	17 (22.37%)	14 (3.92%)	0.35 (0.13-0.98)	0.046	1
Anosmia; n (%)	No	46 (60.53%)	314 (87.96%)		Reference	
	Yes	30 (39.47%)	43 (12.04%)	0.34 (0.16-0.74)	0.006	0.266
Loss of taste; n (%)	No	50 (65.79%)	320 (89.64%)		Reference	
	Yes	26 (34.21%)	37 (10.36%)	0.42 (0.19-0.94)	0.034	1
Diarrhea; n (%)	No	61 (80.26%)	326 (91.32%)		Reference	
	Yes	15 (19.74%)	31 (8.68%)	0.3 (0.12-0.73)	0.008	0.343
Abdominal pain; n (%)	No	68 (89.47%)	346 (96.92%)		Reference	
	Yes	8 (10.53%)	11 (3.08%)	0.39 (0.11-1.32)	0.129	1
Nausea; n (%)	No	65 (85.53%)	338 (94.68%)		Reference	
	Yes	11 (14.47%)	19 (5.32%)	0.47 (0.15-1.46)	0.193	1
Headache; n (%)	No	48 (63.16%)	298 (83.47%)		Reference	
	Yes	28 (36.84%)	59 (16.53%)	0.5 (0.24-1.04)	0.063	1
Myalgia; n (%)	No	44 (57.89%)	272 (76.19%)		Reference	
	Yes	32 (42.11%)	85 (23.81%)	0.69 (0.34-1.37)	0.288	1
Outcomes; n (%)	No hospt	43 (56.58%)	0 (0%)		Reference	
	Hospt	33 (43.42%)	357 (100%)		NC	

[a]Odds ratios were adjusted by skin color, schooling, gender, age, and associated comorbidities, such as diabetes mellitus, coronary artery disease, and obesity or previous bariatric disease. [b]P values were calculated using the unconditional logistic regression model. Associations were considered significant at $^*P < 0.05$. n: number of individuals in each group; aOR: adjusted odds ratio; 95% CI: 95% confidence interval; NC: not calculated; Hospt: hospitalized; No hospt: not hospitalized.

0.010), or carrier-A ($OR_{adj} = 0.45$ [95% CI, 0.22-0.91], $P = 0.027$). For CARD8 rs2043211, the association was with carrying the A/T genotype ($OR_{adj} = 0.5$ [95% CI, 0.25-0.99], $P = 0.046$), allele T ($OR_{adj} = 0.93$ [95% CI, 0.88-0.99], $P = 0.018$), or carrier-T ($OR_{adj} = 0.48$ [95% CI, 0.25-0.93], $P = 0.029$).

The frequency of the T allele in the NLRP3 rs4612666 polymorphism was slightly different between the group of patients with mild and moderate disease (29.61%) and those with severe to critical disease (38.52%) ($OR_{adj} = 1.05$ [95% CI, 1-1.11], $P = 0.062$). Additionally, the T/T genotype showed a frequency of 5.26% in the mild and moderate group and 16.81% in the severe and critical group, constituting a genetic marker with a trend for the risk of disease severity in SARS-CoV-2-infected individuals ($OR_{adj} = 3.41$ [95% CI, 0.93-12.59], $P = 0.065$).

The SNPs in CARD8 (rs6509365), IFI16 (rs1101996), CASP-1 (rs572687), IL-1β (rs1143634), AIM2 (rs2276405), and NLRP3 (rs3806268, rs35829419, and rs10754558) did not reveal any significant associations with disease severity risk and/or protection in SARS-CoV-2-infected individuals.

3.2.2. Association of Haplotypes between the Groups. With respect to the NLRP3 genetic haplotype variants (rs1539019 - rs4612666 - rs3806268 - rs35829419 - rs10754558), carrying the A-C-G-C-G ($OR_{adj} = 0.11$ [95% CI, 0.02-0.69], $P = 0.018$), A-C-G-C-G ($OR_{adj} = 0.23$ [95% CI, 0.09-0.62], $P = 0.004$), C-C-G-C-C ($OR_{adj} = 0.37$ [95% CI, 0.16-0.86], $P = 0.022$), and/or C-T-G-A-C ($OR_{adj} = 0.04$ [95% CI, 0-0.96], $P = 0.047$) haplotypes were associated with protection against disease severity in SARS-CoV-2-infected

Table 2: Unconditional logistic multiple regression model of risk and protective genetic factors for disease severity in SARS-CoV-2-infected individuals ($n = 433$).

Genes SNP (rs)	Alleles and genotypes	Mild and moderate group $N = 76$	Severe and critical group $N = 357$	aOR[a] (95% CI)	P value[b]
CARD8 rs2043211	A/A	35 (46.05)	202 (56.58)	Reference	
	A/T	32 (42.11)	130 (36.41)	0.5 (0.25-0.99)	0.046
	T/T	9 (11.84)	25 (7)	0.42 (0.13-1.4)	0.157
	A	102 (67.11)	534 (74.79)	Reference	
	T	50 (32.89)	180 (25.21)	0.93 (0.88-0.99)	0.018
	Noncarrier-A	9 (11.84)	25 (7)	Reference	
	Carrier-A	67 (88.16)	332 (93)	1.76 (0.56-5.54)	0.337
	Noncarrier-T	35 (46.05)	202 (56.58)	Reference	
	Carrier-T	41 (53.95)	155 (43.42)	0.48 (0.25-0.93)	0.029
CARD8 rs6509365	A/A	35 (46.05)	180 (50.42)	Reference	
	A/G	31 (40.79)	142 (39.78)	0.7 (0.35-1.4)	0.314
	G/G	10 (13.16)	35 (9.8)	0.53 (0.18-1.57)	0.253
	A	101 (66.45)	502 (70.31)	Reference	
	G	51 (33.55)	212 (29.69)	0.96 (0.9-1.01)	0.135
	Noncarrier-A	10 (13.16)	35 (9.8)	Reference	
	Carrier-A	66 (86.84)	322 (90.2)	1.6 (0.57-4.47)	0.372
	Noncarrier-G	35 (46.05)	180 (50.42)	Reference	
	Carrier-G	41 (53.95)	177 (49.58)	0.66 (0.35-1.27)	0.217
AIM2 rs2276405	C/C	73 (96.05)	345 (96.64)	Reference	
	C/T	3 (3.95)	12 (3.36)	0.95 (0.19-4.74)	0.951
	C	149 (98.03)	702 (98.32)	Reference	
	T	3 (1.97)	12 (1.68)	1.02 (0.83-1.24)	0.878
	Noncarrier-C	76 (100)	76 (100)	Reference	
	Carrier-C	357 (100)	357 (100)	0.3055	0.306
	Noncarrier-T	73 (96.05)	345 (96.64)	Reference	
	Carrier-T	3 (3.95)	12 (3.36)	0.95 (0.19-4.74)	0.951
IFI16 rs1101996	C/C	38 (50)	166 (46.5)	Reference	
	A/A	9 (11.84)	42 (11.76)	0.62 (0.22-1.78)	0.376
	C/A	29 (38.16)	149 (41.74)	0.72 (0.35-1.46)	0.360
	C	105 (69.08)	481 (67.37)	Reference	
	A	47 (30.92)	233 (32.63)	0.98 (0.92-1.04)	0.445
	Noncarrier-C	9 (11.84)	42 (11.76)	Reference	
	Carrier-C	67 (88.16)	315 (88.24)	1.37 (0.51-3.68)	0.533
	Noncarrier-A	38 (50)	166 (46.5)	Reference	
	Carrier-A	38 (50)	191 (53.5)	0.7 (0.36-1.36)	0.287
CASP1 rs572687	G/G	52 (68.42)	243 (68.07)	Reference	
	A/A	5 (6.58)	14 (3.92)	1.44 (0.26-7.86)	0.675
	G/A	19 (25)	100 (28.01)	1.57 (0.75-3.28)	0.228
	G	123 (80.92)	586 (82.07)	Reference	
	A	29 (19.08)	128 (17.93)	1.03 (0.97-1.11)	0.347
	Noncarrier-G	5 (6.58)	14 (3.92)	Reference	
	Carrier-G	71 (93.42)	343 (96.08)	0.8 (0.15-4.28)	0.796
IL-1β rs1143634[c]	Noncarrier-A	52 (68.42)	243 (68.07)	Reference	
	Carrier-A	24 (31.58)	114 (31.93)	1.56 (0.77-3.15)	0.219
	G/G	47 (61.84)	228 (63.87)	Reference	
	A/A	2 (2.63)	9 (2.52)	0.53 (0.08-3.39)	0.503
	G/A	27 (35.53)	119 (33.33)	1.17 (0.58-2.39)	0.660

Table 2: Continued.

Genes SNP (rs)	Alleles and genotypes	Mild and moderate group $N = 76$	Severe and critical group $N = 357$	aOR[a] (95% CI)	P value[b]
	G	121 (79.61)	575 (80.53)	Reference	
	A	31 (20.39)	137 (19.19)	1 (0.93-1.07)	
	0.925				
	Noncarrier-G	2 (2.63)	10 (2.8)	Reference	
	Carrier-G	74 (97.37)	347 (97.2)	1.98 (0.31-12.49)	0.467
	Noncarrier-A	47 (61.84)	229 (64.15)	Reference	
Carrier-A	29 (38.16)	128 (35.85)	1.1 (0.55-2.18)	0.79	
	C/C	24 (31.58)	146 (40.9)	Reference	
	A/A	17 (22.37)	48 (13.45)	0.36 (0.14-0.92)	0.033
	C/A	35 (46.05)	163 (45.66)	0.48 (0.23-1.03)	0.061
	C	83 (54.61)	455 (63.73)	Reference	
NLRP3 rs1539019	A	69 (45.39)	259 (36.27)	0.93 (0.88-0.98)	0.010
	Noncarrier-C	17 (22.37)	48 (13.45)	Reference	
	Carrier-C	59 (77.63)	309 (86.55)	1.79 (0.81-3.97)	0.152
	Noncarrier-A	24 (31.58)	146 (40.9)	Reference	
	Carrier-A	52 (68.42)	211 (59.1)	0.45 (0.22-0.91)	0.027
	C/C	35 (46.05)	142 (39.78)	Reference	
	C/T	37 (48.68)	155 (43.42)	1.42 (0.72-2.82)	0.316
	T/T	4 (5.26)	60 (16.81)	3.41 (0.93-12.59)	0.065
	C	107 (70.39)	439 (61.48)	Reference	
NLRP3 rs4612666	T	45 (29.61)	275 (38.52)	1.05 (1-1.11)	0.062
	Noncarrier-C	4 (5.26)	60 (16.81)	Reference	
	Carrier-C	72 (94.74)	297 (83.19)	0.35 (0.1-1.24)	0.103
	Noncarrier-T	35 (46.05)	142 (39.78)	Reference	
	Carrier-T	41 (53.95)	215 (60.22)	1.66 (0.86-3.21)	0.131
	G/G	31 (40.79)	135 (37.82)	Reference	
	A/A	10 (13.16)	50 (14.01)	1.34 (0.46-3.94)	0.593
	G/A	35 (46.05)	172 (48.18)	1.4 (0.7-2.79)	0.342
	G	97 (63.82)	442 (61.9)	Reference	
NLRP3 rs3806268	A	55 (36.18)	272 (38.1)	1.02 (0.97-1.08)	0.476
	Noncarrier-G	10 (13.16)	50 (14.01)	Reference	
	Carrier-G	66 (86.84)	307 (85.99)	0.9 (0.33-2.46)	0.835
	Noncarrier-A	31 (40.79)	135 (37.82)	Reference	
	Carrier-A	45 (59.21)	222 (62.18)	1.39 (0.72-2.68)	0.332
	C/C	73 (96.05)	340 (95.24)	Reference	
	A/A	0 (0)	1 (0.28)	NC	
	C/A	3 (3.95)	16 (4.48)	0.73 (0.18-3.02)	0.665
	C	149 (98.03)	696 (97.48)	Reference	
NLRP3 rs35829419	A	3 (1.97)	18 (2.52)	0.98 (0.84-1.15)	0.827
	Noncarrier-C	0 (0)	1 (0.28)	Reference	
	Carrier-C	76 (100)	356 (99.72)	NC	
	Noncarrier-A	73 (96.05)	340 (95.24)	Reference	
	Carrier-A	3 (3.95)	17 (4.76)	0.75 (0.18-3.08)	0.689
	C/C	26 (34.21)	149 (41.74)	Reference	
NLRP3 rs10754558	C/G	43 (56.58)	164 (45.94)	0.7 (0.35-1.4)	0.313
	G/G	7 (9.21)	44 (12.32)	1.14 (0.38-3.4)	0.819
	C	95 (62.5)	462 (64.71)	Reference	

Table 2: Continued.

Genes SNP (rs)	Alleles and genotypes	Mild and moderate group $N = 76$	Severe and critical group $N = 357$	aOR[a] (95% CI)	P value[b]
	G	57 (37.5)	252 (35.29)	0.99 (0.94-1.04)	0.711
	Noncarrier-C	7 (9.21)	44 (12.32)	Reference	
	Carrier-C	69 (90.79)	313 (87.68)	0.71 (0.26-1.96)	0.510
	Noncarrier-G	26 (34.21)	149 (41.74)	Reference	
	Carrier-G	50 (65.79)	208 (58.26)	0.77 (0.39-1.5)	0.435

[a]Odds ratios were adjusted by skin color, schooling, gender, age, and associated comorbidities, such as diabetes mellitus, coronary artery disease, and obesity or previous bariatric disease. [b]P values were calculated using the unconditional logistic regression model. Associations were considered significant at a value of $^*P < 0.05$. [c]The rs1143634 polymorphism in the IL-1β gene determination was not possible for one individual in the hospitalized group. n: number of individuals in each group; aOR: adjusted odds ratio; 95% CI: 95% confidence interval; NC: not calculated; A, T, G, and C: each allele count, irrespective of the genotype; Carrier-A: total of genotypes with the A allele; Carrier-T: total of genotypes with the T allele; Carrier-C: total of genotypes with the C allele; Carrier-G: total of genotypes with the G allele; Noncarrier-A: total of genotypes without the A allele; Noncarrier-T: total of genotypes without the T allele; Noncarrier-C: total of genotypes without the C allele; Noncarrier-G: total of genotypes without the G allele.

individuals (Table 3). No haplotype of the CARD8 genetic variants was associated with risk and/or protection against disease severity in COVID-19 (Table 3). These analyses were performed considering the most frequent haplotype of the NLRP3 (C-T-G-C-C haplotype) and CARD8 (AA) genes as references.

Considering the observed minor allele frequencies (MAFs) of 0.02 (rs2276405/T and rs35829419/A), 0.19 (rs572687/A), 0.20 (rs1143634/A), 0.30 (rs4612666/T), 0.31 (rs1101996/C), 0.33 (rs2043211/T), 0.34 (rs6509365/G), 0.36 (rs3806268/A), 0.38 (rs10754558/G), and 0.45 (rs1539019/A) observed in our sample's controls (i.e., those with WHO < 6) for the studied gene variants (SNPs) and the prevalence of severe COVID-19 prevaccination cases of 36.17% previously reported for the Brazilian population in the same period [52], we estimated statistical powers for the study under a genetic additive model framework of 80% for MAFs between 0.19 and 0.34 to accept aORs ≥ 2.6 (or ≤0.38). For MAFs, between 0.36 and 0.45 was sufficient to accept aORs greater or equal to 2.8 (or ≤0.36) with equal estimated power. Only for the variant with an extremely low MAF, of 0.02, the sample size was insufficient. Indeed, we estimated 80% statistical power for this MAF only for aORs > 4 (<0.25).

3.3. Inflammasome Gene Polymorphisms and COVID-19-Associated Comorbidities. As the individuals included in the study had several comorbidities (Table 1), e.g., diabetes mellitus (DM), systemic arterial hypertension (SAH), coronary artery disease (CAD), and obesity or previous bariatric surgery (Ob), they were taken into consideration in all analyses between the groups (mild and moderate group vs. severe and critical group), as shown in the footnotes of all tables. Furthermore, to make sure that the results found were not influenced by the associated comorbidities, we performed an analysis with the general population of our study and the comorbidities present in the cohort.

The association of the major comorbidities identified in the COVID-19 individuals included in the present study with the inflammasome SNPs analyzed here is presented in Supplementary Tables 2S-4S. Briefly, carrier-A in the CARD8 rs2043211 polymorphisms ($OR_{adj} = 0.17$ [95% CI, 0.04-0.85], $P = 0.031$) was associated with protection against CAD (Table 2S). Similarly, protection against DM was associated with carrier-C ($OR_{adj} = 0.47$ [95% CI, 0.24-0.91], $P = 0.024$) in the NLRP3 rs1539019 polymorphism (Table 3S). Protection against obesity was associated with carrying the G/A genotype ($OR_{adj} = 0.42$ [95% CI, 0.23-0.78], $P = 0.006$) or carrier-A ($OR_{adj} = 0.48$ [95% CI, 0.27-0.85], $P = 0.012$) in the NLRP3 rs3806268 polymorphisms, whereas a slightly increased risk for obesity was observed for those carrying the A allele in the NLRP3 rs35829419 polymorphism ($OR_{adj} = 1.21$ [95% CI, 1.02-1.44], $P = 0.029$) (Table 4S). Concerning the analysis of the inflammasome haplotypes (Table 5S), carriers of the NLRP3 C-T-G-C-G haplotype had an increased risk for CAD ($OR_{adj} = 11.82$ [95% CI, 2.43-57.59], $P = 0.002$). No other association between comorbidities and inflammasome haplotypes was observed (data not shown). No significant associations between hypertension and the SNPs included in this study were observed (data not shown).

It is important to point out that none of the inflammasome polymorphisms found to be associated with CAD, DM, or obesity comorbidities showed any significant association when comparing the COVID-19 mild/moderate group with the severe/critical group (Tables 2 and 3).

4. Discussion

Innate immune receptors are essential in the sensing of infectious organisms, continuously monitoring the extracellular milieu as well as intracellular compartments. The inflammatory process in cells is often mediated by inflammasomes, which are cytosolic multiprotein oligomers of the innate immune system [53]. Inflammasomes tend to aggregate in response to various endogenous or exogenous stimuli and orchestrate the development of local and/or systemic inflammation [53, 54]. The mechanism of inflammasome activation in COVID-19 is still poorly explored. However, Rodrigues et al. showed that the NLRP3 inflammasome is activated in hospitalized patients infected with SARS-CoV-2 [36]. This suggests a role of the NLRP3 inflammasome in the pathophysiology of the disease, as a marker

Table 3: Association analyses among NLRP3 and CARD8 inflammasome haplotype frequencies and risk/protection factors for disease severity in SARS-CoV-2-infected individuals.

Genes SNP (rs)	Haplotypes	Mild and moderate group $N = 76$	Severe and critical group $N = 357$	Adjusted model aOR[a] (95% CI)	P value[b]
NLRP3 rs1539019 rs4612666 rs3806268 rs35829419 rs10754558	CTGCC	26 (17.11)	181 (25.71)	Reference	
	ACACC	10 (6.58)	54 (7.67)	0.78 (0.24-2.56)	0.677
	ACACG	21 (13.82)	112 (15.91)	0.58 (0.25-1.38)	0.220
	ACGCC	4 (2.63)	6 (0.85)	0.11 (0.02-0.69)	0.018
	ACGCG	20 (13.16)	37 (5.26)	0.23 (0.09-0.62)	0.004
	ATGAG	2 (1.32)	2 (0.28)	0.1 (0.01-1.62)	0.104
	ATGCC	12 (7.89)	41 (5.82)	0.35 (0.11-1.12)	0.077
	ATGCG	0 (0)	2 (0.28)	NC	
	CCACC	17 (11.18)	77 (10.94)	0.77 (0.28-2.1)	0.605
	CCACG	5 (3.29)	21 (2.98)	0.34 (0.07-1.69)	0.186
	CCGAC	0 (0)	1 (0.14)	NC	
	CCGCC	24 (15.79)	92 (13.07)	0.37 (0.16-0.86)	0.022
	CCGCG	6 (3.95)	35 (4.97)	1.87 (0.32-11.02)	0.487
	CTACC	1 (0.66)	4 (0.57)	0.67 (0.01-76.67)	0.87
	CTACG	1 (0.66)	1 (0.14)	1.28 (0.01-164.14)	0.921
	CTGAC	1 (0.66)	1 (0.14)	0.04 (0-0.96)	0.047
	CTGAG	0 (0)	10 (1.42)	NC	
	CTGCG	2 (1.32)	27 (3.84)	1.96 (0.3-12.75)	0.480
CARD8 rs2043211 rs6509365	AA	99 (65.13)	501 (70.17)	Reference	
	AG	3 (1.97)	33 (4.62)	1.7 (0.43-6.75)	0.449
	TA	2 (1.32)	1 (0.14)	NC	
	TG	48 (31.58)	179 (25.07)	0.61 (0.37-1.02)	0.062

[a]Odds ratios were adjusted by skin color, schooling, gender, age, and associated comorbidities, such as diabetes mellitus, coronary artery disease, and obesity or previous bariatric disease. [b]P values were calculated using the unconditional logistic regression model. Associations were considered significant at a value of $^*P < 0.05$. aOR: adjusted odds ratio; 95% CI: 95% confidence interval; NC: not calculated; n: number of individuals in each group.

of disease severity and a potential therapeutic target for COVID-19. Toldo et al. identified the presence of inflammasomes in the lungs of patients with fatal COVID-19 [55]. On the other hand, several studies of the genes involved in assembling inflammasome complexes have attempted to explain their role in the heterogeneity of disease. For the same infection, some individuals are more susceptible to developing the disease, while others remain asymptomatic [25]. The present study demonstrated that genetically specific profiles (alleles, genotypes, and haplotypes) of NLRP3 rs1539019 and CARD8 rs2043211 polymorphisms were associated with protection against disease severity in SARS-CoV-2-infected individuals. Our data suggest that these SNPs might modulate inflammasome activation, contributing to protection against disease severity. Indeed, in a recent study [45], two other NLRP3 SNPs, NLRP3 rs10157379 and rs10754558 polymorphisms, were associated with an important role in severe acute respiratory syndrome (SARS) and severe and critical COVID-19 [45]. There was no significant association between NLRP3 rs10754558 and decreased

COVID-19 severity risk or protection in our cohort. Although both studies included Brazilian individuals, our study was focused on people from the Southeast and North regions, whereas the study of Maes et al. included COVID-19 patients from one city in South Brazil, with a predominance of Caucasian ethnicity in their study group, while self-declared brown individuals predominated in our cohort. We do not know if this difference in ethnicity predominance between the two studies contributed to the differences in our results.

The NLRP3 rs1539019 polymorphism is an intronic variation whose function is still not entirely understood. However, several studies have reported that intronic polymorphisms may be associated with susceptibility/resistance to several diseases, such as rheumatoid arthritis [56], type II diabetes [57], and coronary artery disease [58]. One explanation for this is that many transcription factors bind to intronic sites that may play a role in regulating gene expression. In a study by Chung et al., the C allele of the NLRP3 rs1539019 polymorphism was found to be associated with the risk of renal cell carcinoma [59]. Additionally, Estfanous et al. reported that rs1539019 is associated with susceptibility to hepatitis C and a lower response to IFN treatment, depending on the allele and/or genotype. Moreover, Dehghan et al. reported a statistically significant association between the NLRP3 rs1539019 polymorphism and the risk of cardiovascular disease [60, 61]. To the best of our knowledge, our study is the first to demonstrate an association of the genotype A/A, allele A, or carrier-A in the NLRP3 rs1539019 variant with protection against disease severity in SARS-CoV-2-infected individuals. What is still unclear is the exact molecular mechanisms by which the NLRP3 rs1539019 polymorphism plays a protective effect in the outcome of COVID-19. It is possible that many transcription factors bind to intronic sites that may play a role in regulating gene expression [62]; therefore, we suggest that this polymorphism may involve an area containing a positive regulatory sequence. However, this hypothesis needs to be confirmed in further functional investigations.

The NLRP3 inflammasome, also known as NALP3 and cryopyrin, is currently the most studied inflammasome and is considered the main study model of these cytoplasmic complexes. The NLRP3 gene is located on the long arm of chromosome 1q44 and reacts to a diverse set of endogenous or exogenous stimuli [63]. NLRP3 has been linked to the pathogenesis of several diseases, including [1] metabolic disorders, such as type 2 diabetes [64], obesity [65], and autoimmune and inflammatory diseases [66–68]; neurological diseases [69]; and [2] diseases caused by viral pathogens, such as HIV [50], influenza A [70], and SARS-CoV [71]. SNPs in the *NLRP3* gene have already been associated with a group of inflammatory disorders of genetic origin [32, 72]. Other inflammasomes and molecules related to the activation cascade (e.g., CARD8, AIM2, IFI16, CASP-1, and IL-1β) have also been found to be associated with a variety of infections and metabolic diseases. They may affect the function of the NLRP3 inflammasome [73, 74]. From a functional perspective, the results of another study showed that SARS-CoV-2 upregulates the expression of genes involved in inflammatory processes, such as NLRP3, while downregulating the genes in the autophagic pathway [28].

Caspase recruitment domain–containing protein (CARD) 8 mediates inflammasome activation in response to various pathogen-associated signals [24]. CARD8 plays an important role in apoptosis regulation, inhibition of the activation of NF-κB and caspase-1, and cytokine regulation [75]. CARD8 polymorphisms have been associated with several diseases, such as HIV-1 [76], ischemic stroke [77], and type 2 diabetes mellitus [78].

The CARD8 rs2043211 polymorphism has already been associated with risk and/or protection against several diseases, such as cardiovascular disease [79], atherosclerotic coronary artery disease [80], and inflammatory diseases, such as inflammatory bowel disease [81]. The rs2043211 variant of CARD8 is an A > T transversion on the template strand that introduces a premature stop codon, which results in the expression of a severely truncated CARD8 protein; therefore, this variant is unable to suppress NF-KB activity, which leads to high constitutive levels of pro-IL-1β [82]. In a recent study by our group, we verified an association between the CARD8 variant rs2043211 and protection against immune reconstitution inflammatory syndrome (IRIS) associated with HIV-TB coinfection (de Sá et al., 2022 unpublished data). Although COVID-19 has an important inflammatory profile [83] and constitutive increases in pro-IL-1β contribute to the cytokine storm that worsens the clinical status of patients [84], in our study, we found that the allele T, carrier-T, or genotype A/T in CARD8 rs2043211 polymorphisms is associated with protection against disease severity in individuals infected with SARS-CoV-2. One explanation for this is the interaction between CARD8 and NLRP3 [85]. Roberts et al. reported that a combination of CARD8 rs2043211 and NLRP3 rs35829419 has a protective effect against Crohn's disease, which is an inflammatory disease, by preventing the NLRP3 inflammasome from excessively producing interleukin-1β [85]. In our study, both CARD8 rs2043211 polymorphisms and NLRP3 rs1539019 polymorphisms had a protective effect against disease severity; thus, we hypothesized that this protective effect could be explained by their interaction, although the mechanism underlying this positive association with protection against disease severity in SARS-CoV-2 is not fully elucidated.

To the best of our knowledge, this is one of the first studies demonstrating an association between CARD8 genetic variants and protection against disease severity in SARS-CoV-2-infected individuals. Studies linking CARD8, NLRP3, and SARS-CoV-2 infection are still scarce due to the recent emergence of this pathogen. One recent study showed that the inflammasome is robustly activated in SARS-CoV-2-infected hospitalized individuals [36]. In addition, several studies have indicated that the inflammasome may be involved in the pathogenesis of the disease [13, 33–36].

In the present study, we classified patients at presentation according to the WHO severity classification and identified CARD8 and NLRP3 polymorphisms associated with protection against COVID-19 severity. No polymorphisms

associated with a higher risk of disease severity were observed in our analyses.

Although selected inflammasome polymorphisms were associated with protection/susceptibility to some comorbidities observed in our study group (coronary artery disease, diabetes mellitus, and obesity), none of them showed a significant association when comparing the mild/moderate COVID-19 group with the severe/critical COVID-19 group. In our study, we found that the CARD8 rs2043211 polymorphism was associated with protection against coronary artery disease. Several studies have tried to demonstrate the role of this SNP in CAD, but no consistent association has been described thus far [80, 86, 87]. We also found that carrier-C in the NLRP3 rs1539019 polymorphism and the G/A genotype of the NLRP3 rs3806268 polymorphism were associated with protection against diabetes mellitus and obesity, respectively; on the other hand, carrying the A allele in the NLRP3 rs35829419 polymorphism was associated with a risk of obesity. To the best of our knowledge, this is the first time that these polymorphisms have been associated with these comorbidities.

Some limitations of the current study should be noted, mainly concerning the limited sample size of the mild/moderate group. Moreover, although the Brazilian population has an extensive mixture of ethnic/racial origins, the frequency of these and other SNPs is not consistent throughout the different populations in the world, justifying large further international studies or meta-analyses using already published data from different countries to assess the associations of genetic background with COVID-19 clinical profiles and outcomes. Future studies combining inflammasome genetic polymorphisms and functional analysis will be of foremost relevance to better understand the role of this cytoplasmic protein complex and its downstream effector inflammatory factors in the outcomes of SARS-CoV-2 infection.

Concerning the impact of the COVID-19 vaccination in the individuals analyzed in the present group, it is important to note that the participants included in 2020 were not vaccinated. For those recruited in 2021 (until March), we have no information on vaccination status [46]; however, the inpatients recruited in this period had WHO scores of 8-9, which eliminated any bias potentially caused by vaccination in the association between SNPs and disease protection observed in our study. Moreover, in Brazil, administration of the COVID-19 vaccine began in 2021 (end of January) exclusively for elderly people (>80 years) and health care workers, and only 2.0% of the Brazilian population was fully vaccinated on the date of censure for this analysis (March 31, 2021) [88].

5. Conclusion

The present study is the first to report an association between the NLRP3 rs1539019 polymorphism and CARD8 rs2043211 polymorphisms and protection against disease severity in SARS-CoV-2-infected individuals. We conclude that inflammasome genetic variants influence the COVID-19 clinical outcomes among the patients included in our study. Our work highlights the importance of genetic variations in inflammasome genes in the clinical evolution of COVID-19.

Data Availability

The databases used and/or analyzed during the current study would be available from the corresponding author on reasonable request after anonymization.

Disclosure

The funding agencies played no role in the design of the study, data collection, analysis, or interpretation, nor in writing the manuscript.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

NBRDS, MNG, ASC, OCLB, CCG, ASC, and LRG organized the samples and databank and performed the experiments. NBRDS, MNG, MRA, and MGM analyzed and interpreted data. MRA performed the statistical analyses. NBRDS, MNG, MRA, and MGM wrote the manuscript. HP, KMG, MPDR, SWC, CCG, BG, and VGV contributed to the acquisition of data for the patients. HP, KMG, MPDR, SWC, BG, VGV, AC, MMS, OCLB, CCG, LRG, ASC, DVA, CBGG, and FHC revised the manuscript. NBRDS and MGM designed the experiments. MGM and FHC conceived, supervised, and provided infrastructure for the entire study. All authors read and agreed with the contents and submission of this manuscript. Nathalia Beatriz Ramos de Sá and Milena Neira-Goulart contributed equally to this work.

Acknowledgments

The authors are thankful to all patients who agreed to participate in this study and their families, the frontline health care workers at INI/FIOCRUZ Hospital, and the RECOVER study team in Rio de Janeiro. We are also in debt to Sylvia Lopes Maia Teixeira for helpful discussion. The study was supported by the Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) (Grant numbers SEI-260003/002689/2020 and SEI-260003/013002/2021) and INOVA FIOCRUZ/Fundação Oswaldo Cruz (Grant numbers 48401996705881 and 48402179262880). NBRS and CCG are recipients of INOVA FIOCRUZ/Fundação Oswaldo Cruz, HP is recipient of FAPERJ (E-26/201.351/2021), MGM is recipient of CNPQ (314064/2018-4) and FAPERJ (E-26/201.177/2021), and BG is recipient of CNPQ (305789/2019-8) and FAPERJ (E-26/202.915/2018).

Supplementary Materials

Table S1: characteristics of inflammasome SNPs included in the study. Table S2: unconditional logistic multiple

regression model of risk and protection genetic factors for coronary artery disease in SARS-CoV-2-infected individuals in our cohort ($n = 433$). Table S3: unconditional logistic multiple regression model of risk and protection genetic factors for diabetes mellitus in SARS-CoV-2-infected individuals in our cohort ($n = 433$). Table S4: unconditional logistic multiple regression model of risk and protection genetic factors for obesity or previous bariatric disease in SARS-CoV-2-infected individuals in our cohort ($n = 433$). Table S5: association analyses among NLRP3 and CARD8 inflammasome haplotype frequencies and risk/protection factors for coronary artery disease in SARS-CoV-2-infected individuals. *(Supplementary Materials)*

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Supplementary material

Table S1: Characteristics of inflammasome SNPs included in the study.

Genes	SNP ID	Chromosomes	Positions	Localizations	Ancestral allele	Variation allele	*P*-value HWE[a]
CARD8	rs2043211	19	48234449	exonic	A	T	1
CARD8	rs6509365	19	48240212	intron	A	G	0.814
AIM2	rs2276405	1	159073406	intron	C	T	1
IFI16	rs1101996	1	159028236	intron	A	C	0.814
CASP1	rs572687	11	105032992	intron	A	G	0.442
IL-1β	rs1143634	2	112832813	exonic	G	A	0.780
NLRP3	rs3806268	1	247424175	exonic	A	G	1
NLRP3	rs35829419	1	247425556	exonic	A	C	0.442
NLRP3	rs4612666	1	247435768	intron	C	T	1
NLRP3	rs15390193	1	247436999	intron	A	C	1
NLRP3	rs10754558	1	247448734	3′UTR	C	G	0.442

SNP: single nucleotide polymorphism; CARD8: Caspase Recruitment Domain-Containing Protein 8; AIM2: Absent In Melanoma 2; IFI16: Interferon Gamma Inducible Protein 16; CASP1: Caspase 1; IL-1β: Interleukin 1 Beta; NLRP3: NLR family Pyrin Domain Containing 3. [b]P-value of the Hardy- Weinberg equilibrium.

Table S2: Unconditional logistic multiple regression model of risk and protection genetic factors for coronary artery disease in SARS-CoV-2 infected individuals in our cohort (n=433).

Genes SNP (rs)	Alleles and Genotypes	With Coronary Artery Disease	Without Coronary Artery Disease	aOR[a] (CI 95%)	*P*-value[b]
CARD8 rs2043211	A/A	261 (54.95)	9 (47.37)	Reference	
	A/T	179 (37.68)	6 (31.58)	0.73 (0.2-2.72)	0.641
	T/T	35 (7.37)	4 (21.05)	5.01 (0.94-26.57)	0.058
	A	701 (73.79)	24 (63.16)	Reference	
	T	249 (26.21)	14 (36.84)	1.01 (0.98-1.05)	0.426
	NonCarrier-A	35 (7.37)	4 (21.05)	Reference	
	Carrier-A	440 (92.63)	15 (78.95)	0.17 (0.04-0.85)	**0.031**
	NonCarrier-T	261 (54.95)	9 (47.37)	Reference	
	Carrier-T	214 (45.05)	10 (52.63)	1.14 (0.37-3.53)	0.816
CARD8 rs6509365	A/A	236 (49.68)	8 (42.11)	Reference	
	A/G	194 (40.84)	6 (31.58)	0.83 (0.23-2.98)	0.777
	G/G	45 (9.47)	5 (26.32)	3.16 (0.65-15.31)	0.152
	A	666 (70.11)	22 (57.89)	Reference	
	G	284 (29.89)	16 (42.11)	1.01 (0.98-1.04)	0.466
	NonCarrier-A	45 (9.47)	5 (26.32)	Reference	
	Carrier-A	430 (90.53)	14 (73.68)	0.29 (0.07-1.31)	0.108
	NonCarrier-G	236 (49.68)	8 (42.11)	Reference	
	Carrier-G	239 (50.32)	11 (57.89)	1.2 (0.39-3.69)	0.757
AIM2 rs2276405	C/C	457 (96.21)	18 (94.74)	Reference	
	C/T	18 (3.79)	1 (5.26)	1.05 (0.1-11.16)	0.968
	C	932 (98.11)	37 (97.37)	Reference	
	T	18 (1.89)	1 (2.63)	1.02 (0.93-1.13)	0.653
	NonCarrier-C	475 (100)	475 (100)	Reference	
	Carrier-C	19 (100)	19 (100)	0	0
	NonCarrier-T	457 (96.21)	18 (94.74)	Reference	
	Carrier-T	18 (3.79)	1 (5.26)	1.05 (0.1-11.16)	0.968
IFI16 rs1101996	C/C	222 (46.74)	8 (42.11)	Reference	
	A/A	58 (12.21)	3 (15.79)	1.05 (0.19-5.84)	0.956
	C/A	195 (41.05)	8 (42.11)	1.23 (0.37-4.09)	0.734
	C	639 (67.26)	24 (63.16)	Reference	
	A	311 (32.74)	14 (36.84)	1.01 (0.98-1.04)	0.681
	NonCarrier-C	58 (12.21)	3 (15.79)	Reference	
	Carrier-C	417 (87.79)	16 (84.21)	1.04 (0.21-5.3)	0.958
	NonCarrier-A	222 (46.74)	8 (42.11)	Reference	
	Carrier-A	253 (53.26)	11 (57.89)	1.18 (0.39-3.6)	0.771
CASP1 rs572687	G/G	324 (68.21)	12 (63.16)	Reference	
	A/A	20 (4.21)	1 (5.26)	3.66 (0.35-38.44)	0.279

	G/A	131 (27.58)	6 (31.58)	1.27 (0.38-4.25)	0.698
	G	779 (82)	30 (78.95)	Reference	
	A	171 (18)	8 (21.05)	1.01 (0.97-1.05)	0.608
	NonCarrier-G	20 (4.21)	1 (5.26)	Reference	
	Carrier-G	455 (95.79)	18 (94.74)	0.3 (0.03-2.98)	0.303
	NonCarrier-A	324 (68.21)	12 (63.16)	Reference	
	Carrier-A	151 (31.79)	7 (36.84)	1.44 (0.46-4.51)	0.536
IL-1β rs1143634[c]	G/G	302 (63.71)	12 (63.16)	Reference	
	A/A	13 (2.74)	0 (0)	0 (0-Inf)	0.996
	G/A	159 (33.54)	7 (36.84)	0.93 (0.3-2.95)	0.908
	G	763 (80.49)	31 (81.58)	Reference	
	A	185 (19.51)	7 (18.42)	0.99 (0.96-1.03)	0.718
	NonCarrier-G	13 (2.74)	0 (0)	Reference	
	Carrier-G	461 (97.26)	19 (100)	8952448.82 (0-Inf)	0.996
	NonCarrier-A	302 (63.71)	12 (63.16)	Reference	
	Carrier-A	172 (36.29)	7 (36.84)	0.88 (0.28-2.78)	0.829
NLRP3 rs1539019	C/C	195 (41.05)	9 (47.37)	Reference	
	A/A	70 (14.74)	2 (10.53)	0.28 (0.03-2.48)	0.251
	C/A	210 (44.21)	8 (42.11)	0.83 (0.26-2.66)	0.750
	C	600 (63.16)	26 (68.42)	Reference	
	A	350 (36.84)	12 (31.58)	0.98 (0.95-1.01)	0.224
	NonCarrier-C	70 (14.74)	2 (10.53)	Reference	
	Carrier-C	405 (85.26)	17 (89.47)	3.26 (0.4-26.71)	0.272
	NonCarrier-A	195 (41.05)	9 (47.37)	Reference	
	Carrier-A	280 (58.95)	10 (52.63)	0.66 (0.21-2.02)	0.467
NLRP3 rs4612666	C/C	200 (42.11)	4 (21.05)	Reference	
	C/T	203 (42.74)	11 (57.89)	3.74 (0.93-14.99)	0.063
	T/T	72 (15.16)	4 (21.05)	2.53 (0.35-18.36)	0.359
	C	603 (63.47)	19 (50)	Reference	
	T	347 (36.53)	19 (50)	1.02 (0.99-1.05)	0.150
	NonCarrier-C	72 (15.16)	4 (21.05)	Reference	
	Carrier-C	403 (84.84)	15 (78.95)	0.92 (0.17-4.96)	0.919
	NonCarrier-T	200 (42.11)	4 (21.05)	Reference	
	Carrier-T	275 (57.89)	15 (78.95)	3.48 (0.89-13.56)	0.072
NLRP3 rs3806268	G/G	185 (38.95)	8 (42.11)	Reference	
	A/A	69 (14.53)	2 (10.53)	0.94 (0.16-5.53)	0.950
	G/A	221 (46.53)	9 (47.37)	0.73 (0.22-2.42)	0.612
	G	591 (62.21)	25 (65.79)	Reference	
	A	359 (37.79)	13 (34.21)	1 (0.97-1.02)	0.770
	NonCarrier-G	69 (14.53)	2 (10.53)	Reference	
	Carrier-G	406 (85.47)	17 (89.47)	0.88 (0.17-4.5)	0.880
	NonCarrier-A	185 (38.95)	8 (42.11)	Reference	
	Carrier-A	290 (61.05)	11 (57.89)	0.77 (0.25-2.41)	0.655

NLRP3 rs35829419	C/C	457 (96.21)	17 (89.47)	Reference	
	A/A	1 (0.21)	0 (0)	0.67 (0-Inf)	1
	C/A	17 (3.58)	2 (10.53)	3.32 (0.31-35.17)	0.319
	C	931 (98)	36 (94.74)	Reference	
	A	19 (2)	2 (5.26)	1.02 (0.93-1.12)	0.649
	NonCarrier-C	1 (0.21)	0 (0)	Reference	
	Carrier-C	474 (99.79)	19 (100)	2.16 (0-Inf)	1
	NonCarrier-A	457 (96.21)	17 (89.47)	Reference	
	Carrier-A	18 (3.79)	2 (10.53)	3.32 (0.31-35.17)	0.319
NLRP3 rs10754558	C/C	202 (42.53)	5 (26.32)	Reference	
	C/G	218 (45.89)	10 (52.63)	1.98 (0.48-8.13)	0.345
	G/G	55 (11.58)	4 (21.05)	4.29 (0.83-22.15)	0.082
	C	95 (62.5)	462 (64.71)	Reference	
	G	57 (37.5)	252 (35.29)	1.02 (1-1.05)	0.097
	NonCarrier-C	7 (9.21)	44 (12.32)	Reference	
	Carrier-C	69 (90.79)	313 (87.68)	0.37 (0.1-1.32)	0.125
	NonCarrier-G	26 (34.21)	149 (41.74)	Reference	
	Carrier-G	50 (65.79)	208 (58.26)	2.41 (0.62-9.29)	0.202

[a]Odds ratios were adjusted by skin color, schooling, gender, age, and associated comorbidities such as diabetes mellitus, and obesity or previous bariatric disease. [b]*P*-values were calculated using the unconditional logistic regression model. Associations were considered significant with a value of * $P < 0.05$. [c]The rs1143634 polymorphism in the IL-1β gene determination was not possible for one individual from the hospitalized group. N: number of individuals in each group; aOR: adjusted odds ratio; 95% CI: 95% confidence interval; A, T, G, and C = each allele count, irrespective of the genotype. Carrier-A = total of genotypes with the A allele; Carrier-T = total of genotypes with T allele; Carrier-C = total of genotypes with the C allele; Carrier-G = total of genotypes with the G allele; Non-Carrier-A = total of genotypes without the A allele; Non-Carrier-T = total of genotypes without the T allele; Non-Carrier-C = total of genotypes without the C allele; Non-Carrier-G = total of genotypes without the G allele.

Table S3: Unconditional logistic multiple regression model of risk and protection genetic factors for diabetes mellitus in SARS-CoV-2 infected individuals in our cohort (n=433).

Genes SNP (rs)	Alleles and Genotypes	With Diabetes Mellitus	Without Diabetes Mellitus	aOR[a] (CI 95%)	*P*-value[b]
CARD8 rs2043211	A/A	197 (55.65)	73 (52.14)	Reference	
	A/T	132 (37.29)	53 (37.86)	1.27 (0.75-2.16)	0.368
	T/T	25 (7.06)	14 (10)	1.43 (0.54-3.75)	0.468
	A	526 (74.29)	199 (71.07)	Reference	
	T	182 (25.71)	81 (28.93)	1.04 (0.97-1.11)	0.330
	NonCarrier-A	25 (7.06)	14 (10)	Reference	
	Carrier-A	329 (92.94)	126 (90)	0.78 (0.3-1.98)	0.595
	NonCarrier-T	197 (55.65)	73 (52.14)	Reference	
	Carrier-T	157 (44.35)	67 (47.86)	1.3 (0.79-2.14)	0.309
CARD8 rs6509365	A/A	178 (50.28)	66 (47.14)	Reference	
	A/G	141 (39.83)	59 (42.14)	1.39 (0.82-2.36)	0.225
	G/G	35 (9.89)	15 (10.71)	0.93 (0.38-2.24)	0.866
	A	497 (70.2)	191 (68.21)	Reference	
	G	211 (29.8)	89 (31.79)	1.01 (0.95-1.08)	0.679
	NonCarrier-A	35 (9.89)	15 (10.71)	Reference	
	Carrier-A	319 (90.11)	125 (89.29)	1.26 (0.54-2.93)	0.591
	NonCarrier-G	178 (50.28)	66 (47.14)	Reference	
	Carrier-G	176 (49.72)	74 (52.86)	1.28 (0.78-2.12)	0.331
AIM2 rs2276405	C/C	338 (95.48)	137 (97.86)	Reference	
	C/T	16 (4.52)	3 (2.14)	0.43 (0.09-2.16)	0.306
	C	692 (97.74)	277 (98.93)	Reference	
	T	16 (2.26)	3 (1.07)	0.9 (0.73-1.12)	0.354
	NonCarrier-C	354 (100)	354 (100)	Reference	
	Carrier-C	140 (100)	140 (100)	0	0
	NonCarrier-T	338 (95.48)	137 (97.86)	Reference	
	Carrier-T	16 (4.52)	3 (2.14)	0.43 (0.09-2.16)	0.306
IFI16 rs1101996	C/C	177 (50)	53 (37.86)	Reference	
	A/A	41 (11.58)	20 (14.29)	1.46 (0.63-3.37)	0.373
	C/A	136 (38.42)	67 (47.86)	1.29 (0.76-2.19)	0.353
	C	490 (69.21)	173 (61.79)	Reference	
	A	218 (30.79)	107 (38.21)	1.04 (0.97-1.11)	0.271
	NonCarrier-C	41 (11.58)	20 (14.29)	Reference	
	Carrier-C	313 (88.42)	120 (85.71)	0.78 (0.36-1.72)	0.540
	NonCarrier-A	177 (50)	53 (37.86)	Reference	
	Carrier-A	177 (50)	87 (62.14)	1.32 (0.79-2.19)	0.286
CASP1 rs572687	G/G	246 (69.49)	90 (64.29)	Reference	
	A/A	16 (4.52)	5 (3.57)	1.47 (0.35-6.18)	0.599
	G/A	92 (25.99)	45 (32.14)	1.53 (0.89-2.61)	0.122

	G	584 (82.49)	225 (80.36)	Reference	
	A	124 (17.51)	55 (19.64)	1.06 (0.98-1.15)	0.155
	NonCarrier-G	16 (4.52)	5 (3.57)	Reference	
	Carrier-G	338 (95.48)	135 (96.43)	0.78 (0.19-3.22)	0.727
	NonCarrier-A	246 (69.49)	90 (64.29)	Reference	
	Carrier-A	108 (30.51)	50 (35.71)	1.52 (0.9-2.56)	0.114
IL-1β rs1143634[c]	G/G	225 (63.74)	89 (63.57)	Reference	
	A/A	10 (2.83)	3 (2.14)	0.65 (0.15-2.8)	0.567
	G/A	118 (33.43)	48 (34.29)	1.08 (0.64-1.84)	0.766
	G	568 (80.45)	226 (80.71)	Reference	
	A	138 (19.55)	54 (19.29)	1 (0.92-1.08)	0.953
	NonCarrier-G	10 (2.83)	3 (2.14)	Reference	
	Carrier-G	343 (97.17)	137 (97.86)	1.57 (0.37-6.62)	0.542
	NonCarrier-A	225 (63.74)	89 (63.57)	Reference	
	Carrier-A	128 (36.26)	51 (36.43)	1.03 (0.62-1.72)	0.898
NLRP3 rs1539019	C/C	146 (41.24)	58 (41.43)	Reference	
	A/A	48 (13.56)	24 (17.14)	1.99 (0.98-4.06)	0.057
	C/A	160 (45.2)	58 (41.43)	0.88 (0.51-1.52)	0.645
	C	452 (63.84)	174 (62.14)	Reference	
	A	256 (36.16)	106 (37.86)	1.05 (0.98-1.12)	0.138
	NonCarrier-C	48 (13.56)	24 (17.14)	Reference	
	Carrier-C	306 (86.44)	116 (82.86)	0.47 (0.24-0.91)	**0.024**
	NonCarrier-A	146 (41.24)	58 (41.43)	Reference	
	Carrier-A	208 (58.76)	82 (58.57)	1.11 (0.67-1.83)	0.692
NLRP3 rs4612666	C/C	137 (38.7)	67 (47.86)	Reference	
	C/T	166 (46.89)	48 (34.29)	0.64 (0.37-1.11)	0.110
	T/T	51 (14.41)	25 (17.86)	0.72 (0.35-1.5)	0.387
	C	440 (62.15)	182 (65)	Reference	
	T	268 (37.85)	98 (35)	0.96 (0.9-1.02)	0.173
	NonCarrier-C	51 (14.41)	25 (17.86)	Reference	
	Carrier-C	303 (85.59)	115 (82.14)	1.12 (0.56-2.23)	0.741
	NonCarrier-T	137 (38.7)	67 (47.86)	Reference	
	Carrier-T	217 (61.3)	73 (52.14)	0.66 (0.4-1.1)	0.109
NLRP3 rs3806268	G/G	140 (39.55)	53 (37.86)	Reference	
	A/A	47 (13.28)	24 (17.14)	1.76 (0.81-3.84)	0.153
	G/A	167 (47.18)	63 (45)	1.19 (0.69-2.07)	0.528
	G	447 (63.14)	169 (60.36)	Reference	
	A	261 (36.86)	111 (39.64)	1.04 (0.98-1.11)	0.190
	NonCarrier-G	47 (13.28)	24 (17.14)	Reference	
	Carrier-G	307 (86.72)	116 (82.86)	0.63 (0.31-1.28)	0.198
	NonCarrier-A	140 (39.55)	53 (37.86)	Reference	
	Carrier-A	214 (60.45)	87 (62.14)	1.3 (0.77-2.2)	0.324
NLRP3	C/C	344 (97.18)	130 (92.86)	Reference	

rs35829419	A/A	0 (0)	1 (0.71)	1840875.47 (0-Inf)	0.987
	C/A	10 (2.82)	9 (6.43)	2.17 (0.74-6.34)	0.158
	C	698 (98.59)	269 (96.07)	Reference	
	A	10 (1.41)	11 (3.93)	1.19 (0.98-1.44)	0.078
	NonCarrier-C	0 (0)	1 (0.71)	Reference	
	Carrier-C	354 (100)	139 (99.29)	0 (0-Inf)	0.987
	NonCarrier-A	344 (97.18)	130 (92.86)	Reference	
	Carrier-A	10 (2.82)	10 (7.14)	2.33 (0.82-6.63)	0.111
NLRP3 rs10754558	C/C	151 (42.66)	56 (40)	Reference	
	C/G	161 (45.48)	67 (47.86)	1.29 (0.75-2.21)	0.360
	G/G	42 (11.86)	17 (12.14)	1.45 (0.65-3.25)	0.362
	C	463 (65.4)	179 (63.93)	Reference	
	G	245 (34.6)	101 (36.07)	1.04 (0.97-1.1)	0.282
	NonCarrier-C	42 (11.86)	17 (12.14)	Reference	
	Carrier-C	312 (88.14)	123 (87.86)	0.8 (0.38-1.66)	0.544
	NonCarrier-G	151 (42.66)	56 (40)	Reference	
	Carrier-G	203 (57.34)	84 (60)	1.32 (0.79-2.21)	0.294

[a]Odds ratios were adjusted by skin color, schooling, gender, age, and associated comorbidities such as coronary artery disease and obesity or previous bariatric disease. [b]*P*-values were calculated using the unconditional logistic regression model. Associations were considered significant with a value of * $P < 0.05$. [c]The rs1143634 polymorphism in the IL-1β gene determination was not possible for one individual from the hospitalized group. N: number of individuals in each group; aOR: adjusted odds ratio; 95% CI: 95% confidence interval; A, T, G, and C = each allele count, irrespective of the genotype. Carrier-A = total of genotypes with the A allele; Carrier-T = total of genotypes with T allele; Carrier-C = total of genotypes with the C allele; Carrier-G = total of genotypes with the G allele; Non-Carrier-A = total of genotypes without the A allele; Non-Carrier-T = total of genotypes without the T allele; Non-Carrier-C = total of genotypes without the C allele; Non-Carrier-G = total of genotypes without the G allele.

Table S4: Unconditional logistic multiple regression model of risk and protection genetic factors for obesity or previous bariatric disease in SARS-CoV-2 infected individuals in our cohort (n=433).

Genes SNP (rs)	Alleles and Genotypes	With obesity or previous bariatric disease	Without obesity or previous bariatric disease	aOR[a] (CI 95%)	*P*-value[b]
CARD8 rs2043211	A/A	224 (54.37)	46 (56.1)	Reference	
	A/T	155 (37.62)	30 (36.59)	0.88 (0.48-1.58)	0.659
	T/T	33 (8.01)	6 (7.32)	0.74 (0.23-2.4)	0.618
	A	526 (74.29)	199 (71.07)	Reference	
	T	603 (73.18)	122 (74.39)	0.98 (0.92-1.04)	0.496
	NonCarrier-A	221 (26.82)	42 (25.61)	Reference	
	Carrier-A	33 (8.01)	6 (7.32)	1.28 (0.4-4.04)	0.677
	NonCarrier-T	379 (91.99)	76 (92.68)	Reference	
	Carrier-T	224 (54.37)	46 (56.1)	0.85 (0.48-1.5)	0.583
CARD8 rs6509365	A/A	202 (49.03)	42 (51.22)	Reference	
	A/G	170 (41.26)	30 (36.59)	0.75 (0.41-1.38)	0.362
	G/G	40 (9.71)	10 (12.2)	1.12 (0.45-2.8)	0.801
	A	574 (69.66)	114 (69.51)	Reference	
	G	250 (30.34)	50 (30.49)	0.99 (0.93-1.05)	0.747
	NonCarrier-A	40 (9.71)	10 (12.2)	Reference	
	Carrier-A	372 (90.29)	72 (87.8)	0.78 (0.33-1.88)	0.587
	NonCarrier-G	202 (49.03)	42 (51.22)	Reference	
	Carrier-G	210 (50.97)	40 (48.78)	0.82 (0.47-1.44)	0.498
AIM2 rs2276405	C/C	396 (96.12)	79 (96.34)	Reference	
	C/T	16 (3.88)	3 (3.66)	1.23 (0.31-4.87)	0.767
	C	808 (98.06)	161 (98.17)	Reference	
	T	16 (1.94)	3 (1.83)	1.03 (0.85-1.25)	0.740
	NonCarrier-C	412 (100)	412 (100)	Reference	
	Carrier-C	82 (100)	82 (100)	0.1513	0.151
	NonCarrier-T	396 (96.12)	79 (96.34)	Reference	
	Carrier-T	16 (3.88)	3 (3.66)	1.23 (0.31-4.87)	0.767
IFI16 rs1101996	C/C	191 (46.36)	39 (47.56)	Reference	
	A/A	50 (12.14)	11 (13.41)	0.88 (0.33-2.37)	0.799
	C/A	171 (41.5)	32 (39.02)	0.92 (0.51-1.67)	0.794
	C	553 (67.11)	110 (67.07)	Reference	
	A	271 (32.89)	54 (32.93)	0.99 (0.93-1.05)	0.732
	NonCarrier-C	50 (12.14)	11 (13.41)	Reference	
	Carrier-C	362 (87.86)	71 (86.59)	1.09 (0.43-2.79)	0.857
	NonCarrier-A	191 (46.36)	39 (47.56)	Reference	
	Carrier-A	221 (53.64)	43 (52.44)	0.92 (0.52-1.62)	0.763
CASP1	G/G	275 (66.75)	61 (74.39)	Reference	

rs572687	A/A	18 (4.37)	3 (3.66)	1.02 (0.2-5.17)	0.984
	G/A	119 (28.88)	18 (21.95)	0.77 (0.4-1.46)	0.416
	G	669 (81.19)	140 (85.37)	Reference	
	A	155 (18.81)	24 (14.63)	0.98 (0.91-1.05)	0.554
	NonCarrier-G	18 (4.37)	3 (3.66)	Reference	
	Carrier-G	394 (95.63)	79 (96.34)	0.92 (0.18-4.64)	0.918
	NonCarrier-A	275 (66.75)	61 (74.39)	Reference	
	Carrier-A	137 (33.25)	21 (25.61)	0.79 (0.43-1.46)	0.449
IL-1β rs1143634[c]	G/G	258 (62.77)	56 (68.29)	Reference	
	A/A	9 (2.19)	4 (4.88)	1.14 (0.27-4.88)	0.857
	G/A	144 (35.04)	22 (26.83)	0.78 (0.42-1.44)	0.427
	G	660 (80.29)	134 (81.71)	Reference	
	A	162 (19.71)	30 (18.29)	0.98 (0.92-1.05)	0.589
	NonCarrier-G	9 (2.19)	4 (4.88)	Reference	
	Carrier-G	402 (97.81)	78 (95.12)	0.81 (0.19-3.45)	0.780
	NonCarrier-A	258 (62.77)	56 (68.29)	Reference	
	Carrier-A	153 (37.23)	26 (31.71)	0.81 (0.45-1.47)	0.492
NLRP3 rs1539019	C/C	164 (39.81)	40 (48.78)	Reference	
	A/A	63 (15.29)	9 (10.98)	0.54 (0.22-1.31)	0.174
	C/A	185 (44.9)	33 (40.24)	0.71 (0.39-1.28)	0.256
	C	513 (62.26)	113 (68.9)	Reference	
	A	311 (37.74)	51 (31.1)	0.95 (0.9-1.01)	0.102
	NonCarrier-C	63 (15.29)	9 (10.98)	Reference	
	Carrier-C	349 (84.71)	73 (89.02)	1.56 (0.68-3.63)	0.297
	NonCarrier-A	164 (39.81)	40 (48.78)	Reference	
	Carrier-A	248 (60.19)	42 (51.22)	0.66 (0.38-1.15)	0.146
NLRP3 rs4612666	C/C	163 (39.56)	41 (50)	Reference	
	C/T	187 (45.39)	27 (32.93)	0.93 (0.5-1.74)	0.829
	T/T	62 (15.05)	14 (17.07)	1.37 (0.63-2.98)	0.431
	C	513 (62.26)	109 (66.46)	Reference	
	T	311 (37.74)	55 (33.54)	1.02 (0.96-1.08)	0.570
	NonCarrier-C	62 (15.05)	14 (17.07)	Reference	
	Carrier-C	350 (84.95)	68 (82.93)	0.71 (0.34-1.46)	0.350
	NonCarrier-T	163 (39.56)	41 (50)	Reference	
	Carrier-T	249 (60.44)	41 (50)	1.05 (0.6-1.85)	0.866
NLRP3 rs3806268	G/G	156 (37.86)	37 (45.12)	Reference	
	A/A	57 (13.83)	14 (17.07)	0.73 (0.31-1.72)	0.474
	G/A	199 (48.3)	31 (37.8)	0.42 (0.23-0.78)	**0.006**
	G	511 (62.01)	105 (64.02)	Reference	
	A	313 (37.99)	59 (35.98)	0.96 (0.91-1.01)	0.130
	NonCarrier-G	57 (13.83)	14 (17.07)	Reference	
	Carrier-G	355 (86.17)	68 (82.93)	0.86 (0.39-1.91)	0.716
	NonCarrier-A	156 (37.86)	37 (45.12)	Reference	

	Carrier-A	256 (62.14)	45 (54.88)	0.48 (0.27-0.85)	**0.012**
NLRP3 rs35829419	C/C	398 (96.6)	76 (92.68)	Reference	
	A/A	0 (0)	1 (1.22)	136985966.82 (0-Inf)	0.996
	C/A	14 (3.4)	5 (6.1)	2.3 (0.73-7.27)	0.156
	C	810 (98.3)	157 (95.73)	Reference	
	A	14 (1.7)	7 (4.27)	1.21 (1.02-1.44)	**0.029**
	NonCarrier-C	0 (0)	1 (1.22)	Reference	
	Carrier-C	412 (100)	81 (98.78)	0 (0-Inf)	0.996
	NonCarrier-A	398 (96.6)	76 (92.68)	Reference	
	Carrier-A	14 (3.4)	6 (7.32)	2.72 (0.91-8.11)	0.073
NLRP3 rs10754558	C/C	168 (40.78)	39 (47.56)	Reference	
	C/G	192 (46.6)	36 (43.9)	0.76 (0.43-1.37)	0.369
	G/G	52 (12.62)	7 (8.54)	0.54 (0.2-1.44)	0.216
	C	528 (64.08)	114 (69.51)	Reference	
	G	296 (35.92)	50 (30.49)	0.96 (0.91-1.02)	0.158
	NonCarrier-C	52 (12.62)	7 (8.54)	Reference	
	Carrier-C	360 (87.38)	75 (91.46)	1.62 (0.63-4.15)	0.314
	NonCarrier-G	168 (40.78)	39 (47.56)	Reference	
	Carrier-G	244 (59.22)	43 (52.44)	0.71 (0.41-1.25)	0.237

[a]Odds ratios were adjusted by skin color, schooling, gender, age, and associated comorbidities such as coronary artery disease and diabetes mellitus. [b]*P*-values were calculated using the unconditional logistic regression model. Associations were considered significant with a value of * $P < 0.05$. [c]The rs1143634 polymorphism in the IL-1β gene determination was not possible for one individual from the hospitalized group. N: number of individuals in each group; aOR: adjusted odds ratio; 95% CI: 95% confidence interval; A, T, G, and C = each allele count, irrespective of the genotype. Carrier-A = total of genotypes with the A allele; Carrier-T = total of genotypes with T allele; Carrier-C = total of genotypes with the C allele; Carrier-G = total of genotypes with the G allele; Non-Carrier-A = total of genotypes without the A allele; Non-Carrier-T = total of genotypes without the T allele; Non-Carrier-C = total of genotypes without the C allele; Non-Carrier-G = total of genotypes without the G allele.

Table S5: Association analyses among NLRP3 and CARD8 inflammasome haplotypes frequencies and risk/protection factors for coronary artery disease in SARS-CoV-2 infected individuals.

Genes SNP (rs)	Haplotypes	With coronary artery disease	Without coronary artery disease	Adjusted model	
				aOR[a] (CI95%)	*P*-value[b]
NLRP3 rs1539019 rs4612666 rs3806268 rs35829419 rs10754558	CTGCC	227 (24.1)	8 (21.05)	Reference	
	ACACC	53 (5.63)	2 (5.26)	0.51 (0.03-8.97)	0.646
	ACACG	155 (16.45)	5 (13.16)	1.01 (0.24-4.16)	0.989
	ACGCC	23 (2.44)	0 (0)	0 (0-Inf)	0.997
	ACGCG	46 (4.88)	3 (7.89)	1.5 (0.29-7.63)	0.627
	ATGAG	4 (0.42)	0 (0)	0 (0-Inf)	0.999
	ATGCC	62 (6.58)	2 (5.26)	0 (0-Inf)	0.995
	ATGCG	3 (0.32)	0 (0)	0 (0-Inf)	0.999
	CCACC	110 (11.68)	4 (10.53)	1.11 (0.21-5.78)	0.903
	CCACG	31 (3.29)	0 (0)	0 (0-Inf)	0.996
	CCGAC	1 (0.11)	0 (0)	0.43 (0-Inf)	1
	CCGCC	135 (14.33)	3 (7.89)	0.66 (0.12-3.48)	0.623
	CCGCG	45 (4.78)	2 (5.26)	1.79 (0.18-18.18)	0.622
	CTACC	5 (0.53)	1 (2.63)	9.99 (0.71-140.45)	0.088
	CTACG	3 (0.32)	1 (2.63)	2.37 (0.08-72.29)	0.620
	CTGAC	2 (0.21)	0 (0)	0 (0-Inf)	0.999
	CTGAG	8 (0.85)	2 (5.26)	12.25 (0.69-217.99)	0.088
	CTGCG	29 (3.08)	5 (13.16)	11.82 (2.43-57.59)	**0.002**
CARD8 rs2043211 rs6509365	AA	664 (69.89)	21 (55.26)	Reference	
	AG	37 (3.89)	3 (7.89)	1.93 (0.35-10.61)	0.449
	TA	2 (0.21)	1 (2.63)	32777812.85 (0-Inf)	0.989
	TG	247 (26)	13 (34.21)	1.51 (0.63-3.64)	0.354

[a]Odds ratios were adjusted by skin color, schooling, gender, age, and associated comorbidities such as diabetes mellitus and obesity or previous bariatric disease; [b]*P*-values were calculated using the unconditional logistic regression model. Associations were considered significant with a value of * $P < 0.05$. aOR: adjusted odds ratio; 95% CI: 95% confidence interval; NC: not calculated; N: number of individuals in each group.

3.2. ARTIGO 2 – *"Inflammasome genes polymorphisms are associated with progression to mechanical ventilation and death in a cohort of hospitalized COVID-19 patients in a reference hospital in Rio de Janeiro, Brazil."*

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Periódico: Trabalho aceito para publicação em 28 de fevereiro de 2023 na *Gene* | DOI 10.1016/j.gene.2023.147325

Resumo

COVID-19 tem um amplo espectro de manifestações clínicas. Neste trabalho avaliamos o impacto de SNPs de genes do inflamassoma como fatores de risco para progressão de desfechos clínicos graves na COVID-19, como a utilização de ventilação mecânica ou óbito. O estudo incluiu 451 indivíduos hospitalizados recrutados pelo Instituto Nacional de Infectologia (INI/FIOCRUZ), Rio de Janeiro, Brasil de junho de 2020 a março de 2021. A genotipagem dos SNPs foi realizada por PCR em tempo real. Fatores de risco para progressão para ventilação mecânica (n=174[38.6%]) ou óbito (n=175[38.8%]) foram analisados em pacientes internados. As análises foram feitas pelo modelo de risco proporcional de Cox. A progressão lenta para ventilação mecânica foi associado ao alelo G (aHR=0.66;P=0.005) ou o genótipo G/G (aHR=0.391;P=0.006) no polimorfismo rs10754558 do gene *NLRP3* ou alelo G (aHR=0.309;P=0.004) no polimorfismo rs1143634 no gene *IL1β*. O alelo C do polimorfismo rs4612666 do gene *NLRP3* (aHR=2.342;P=0.006) quanto do rs10754558 (aHR=2.957;P=0.005) foram associados a uma progressão mais rápida ao óbito. Progressão mais lenta para o óbito foi associada ao alelo G (aHR=0.563;P=0.006) ou o genótipo A/G (aHR=0.537;P=0.005) rs6509365 do gene CARD8; o genótipo A/C do rs1101996 do gene *IFI16* (aHR=0.569;P=0.011); o genótipo T/T (aHR=0.394;P=0.004) ou alelo T (aHR=0.68;P=0.006) do rs4612666 in do gene *NLRP3*, e o genótipo G/G (aHR=0.326;P=0.005) ou alelo G (aHR=0,68;P=0.014) rs10754558 do gene *NLRP3*. Nossos resultados sugerem que

variantes genéticas dos inflamassomas podem influenciar um curso clínico crítico na COVID-19.

Gene 865 (2023) 147325

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

Inflammasome genes polymorphisms are associated with progression to mechanical ventilation and death in a cohort of hospitalized COVID-19 patients in a reference hospital in Rio de Janeiro, Brazil

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ARTICLE INFO

Edited by Negar Azarpira

Keywords:
COVID-19
Inflammasome single nucleotide polymorphisms (SNPs)
Risk factors

ABSTRACT

COVID-19 has a broad spectrum of clinical manifestations. We assessed the impact of single nucleotide polymorphisms (SNPs) of inflammasome genes as risk factors for progression to COVID-19 critical outcomes, such as mechanical ventilation support (MVS) or death. The study included 451 hospitalized individuals followed up at the INI/FIOCRUZ, Rio de Janeiro, Brazil, from 06/2020 to 03/2021. SNPs genotyping was determined by Real-Time PCR. We analyzed risk factors for progression to MVS (n = 174[38.6 %]) or death (n = 175[38.8 %]) as a result of COVID-19 by Cox proportional hazard models. Slower progression to MVS was associated with allele G (aHR = 0.66;P = 0.005) or the genotype G/G (aHR = 0.391;P = 0.006) in the NLRP3 rs10754558 or the allele G (aHR = 0.309;P = 0.004) in the IL1β rs1143634, while C allele in the NLRP3 rs4612666 (aHR = 2.342;P = 0.006) or in the rs10754558 (aHR = 2.957;P = 0.005) were associated with faster progression to death. Slower progression to death was associated to allele G (aHR = 0.563;P = 0.006) or the genotype A/G (aHR = 0.537;P = 0.005) in the CARD8 rs6509365; the genotype A/C in the IFI16 rs1101996 (aHR = 0.569;P = 0.011); the genotype T/T (aHR = 0.394;P = 0.004) or allele T (aHR = 0.68;P = 0.006) in the NLRP3 rs4612666, and the genotype G/G (aHR = 0.326;P = 0.005) or allele G (aHR = 0,68;P = 0.014) in the NLRP3 rs10754558. Our results suggest that inflammasome genetic variations might influence the critical clinical course of COVID-19.

Abbreviations: ACE2, Angiotensin-converting enzyme 2; AIM2, Absent in melanoma 2; ARDS, acute respiratory distress syndrome; CARD8, Caspase recruitment domain-containing protein 8; CASP-1, Interleukin-1 converting enzyme; CFTR, Cystic fibrosis transmembrane conductance regulator; COPD, Chronic obstructive pulmonary disease; COVID-19, coronavirus disease-2019; DNA, Deoxyribonucleic acid; GSDM-D, Gasdermin-D; HR, Hazard ratios; IFI16, Gamma-interferon-inducible protein 16; IFNγ, Interferon gamma; IL-18, Interleukin-18; IL-1α, Interleukin-1 alpha; IL-1β, Interleukin 1-beta; IL-23, Interleukin-23; IL-4, Interleukin-4; IL-6, Interleukin-6; IP-10, Interferon gamma-induced protein 10; MCP1, Monocyte chemoattractant protein 1; MVS, Mechanical ventilation support; NF-kB, Nuclear factor kappa B; NLRP3, Pyrin domain-containing 3; PRR, pattern recognition receptors; SAPS-III, Simplified Acute Physiology Score III; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2; SNPs, Single nucleotide polymorphisms; SOFA, Sequential Organ Failure Assessment; Th17, T helper 17; TLR3, Toll-like receptor 3; TLR7, Toll-like receptor 7; TLRs, Toll-like receptors; TMPRSS2, transmembrane serine protease 2; WHO, World Health Organization.

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https://doi.org/10.1016/j.gene.2023.147325
Received 17 November 2022; Received in revised form 23 January 2023; Accepted 27 February 2023
Available online 2 March 2023

1. Introduction

An outbreak of viral pneumonia caused by a new coronavirus, named SARS-CoV-2, was firstly described in Wuhan (China) in December 2019 (Zhu et al., 2020; Zhou et al., 2020). In March 2020, the World Health Organization (WHO) classified this outbreak as the coronavirus disease-2019 (COVID-19) pandemic. As June of 2022, more than 530 million individuals have been infected, and more than 6.3 million deaths occurred due to COVID-19 worldwide (WHO, 2022). Globally, mortality rates and incidence of SARS-CoV-2 have been increasing over time, especially in Europe and the region of the Americas. The United States of America (USA), India, and Brazil have been the top leaders in numbers of cumulative cases of COVID-19 (WHO, 2022). As a strategy of harm reduction of COVID-19, anti-SARS-CoV-2 vaccines became available by the end of 2020 in few countries. However, difficulty in implementing a policy for rapid population vaccination, especially in large low-to-middle income countries leads to the emergence of new virus variants (Karim and Karim, 2021; WHO, 2021).

The first SARS-CoV-2 infection case in Brazil was described on February 26, 2020, and the presence of community transmissions was reported as of March 13, 2020. Since the first case, Brazil has become an epicenter of COVID-19. Up to June 2022, Brazilian health authorities has reported more than 31 million reported cases, including more than 667,000 deaths (Ministério da Saúde, 2022; Delatorre et al., 2020). Mortality rates due to COVID-19 extremely varied among countries (Abou Ghayda, 2022). Older age and presence of comorbidities were the main factors associated with higher mortality in people with SARS-CoV-2 infection (Djaharuddin et al., 2021). In a prospective study conducted by our group, Perazzo *et al.* (2022) reported a high in-hospital high mortality rate (27 %) from June 2020 to March 2021, mainly driven by older age, need of significant ventilation support and high severity scores at hospital admission (Perazzo et al., 2022).

COVID-19 has a wide spectrum of clinical manifestations, ranging from asymptomatic or with mild symptoms to more severe forms that lead to death. Most of the patients with COVID-19 have mild or moderate diseases. However, 5–10 % might have severe and life-threatening disease courses (Gavriatopoulou et al., 2021). Severe disease can be characterized by dysregulated cytokine release (cytokine storm), pneumonia, and acute lung injury that rapidly progresses to acute respiratory distress syndrome (ARDS) and need of mechanical ventilation. Individuals with severe COVID-19 might also have thromboembolism, sepsis, multiple organ failure, and conditions that lead to death (NIH, 2022). Such a wide clinical spectrum suggests the influence of the host genetic background on disease susceptibility/resistance to SARS-CoV-2 infection and/or clinical evolution in distinct populations (Velavan et al., 2021; Ellinghaus et al., 2020; Fricke-Galindo and Falfán-Valencia, 2021). Previous analyses described that NLRP3, TLR7, CFTR, ACE2, TMPRSS2, and TLR3 polymorphisms might be associated with susceptibility to SARS-CoV-2 infection and/or COVID-19 disease severity (Devaux et al., 2020; Zguro et al., 2022).

The human ACE-2 (angiotensin-converting enzyme 2) molecule that is associated with TMPRSS2 (transmembrane serine protease 2) from the host cell interacts with the protein S (Spike) of the viral envelope, facilitating the entry of SARS-CoV-2 in target cells (Hoffmann et al., 2020). Host factors are activated by the presence of viruses in the cells. Among them cytosolic pattern recognition receptors (PRR) recognize virus fragments and trigger the activation of cellular components (Shi et al., 2015; Zhang et al., 2020), which may lead to activation of the NF-kB pathway, culminating in the transcription of several inflammasome molecules, such as the Pyrin domain-containing 3 (NLRP3), gasdermin-D (GSDM-D), pro-IL-1β, and pro-IL-18, among others (Tay et al., 2020; Tang et al., 2020). These released molecules cause a wave of local inflammation involving increased secretion of proinflammatory cytokines and chemokines (e.g., IL-6, IFNγ, MCP1, and IP-10) at inflammatory sites (Chen et al., 2020; Huang et al., 2020), culminating with the cytokine storm associated with the worsening with the disease and death (Hu et al., 2021). The activation of TLRs (Toll-like receptors) induces proinflammatory cytokines, such as IL-1α, IL-1β, IL-4, and IL-6, as well as interferons. The TLR-7 and TLR-3, endosomal pattern recognition receptors for viral RNA, have already been involved in the control of innate immunity during lung SARS-CoV-2 infection (Bortolotti et al., 2021).

The role of inflammasome activation was already demonstrated in the COVID-19 (Rodrigues, 2020). Therefore, inflammasome genetics can impact in outcomes of people with COVID-19. Mutations in the inflammasome genes may lead to inflammatory disorders, linked to constitutively high levels of IL-1β contributing to chronic inflammation (Keyel, 2014), including viral infections, such as HIV, tuberculosis, and hepatitis C (Pontillo et al., 2012; Toro et al., 2021; De Sá et al., 2022). Single nucleotide polymorphisms (SNPs) in the NLRP3 gene have already been associated with a group of inflammatory disorders of genetic origin with the exaggerated secretion of IL-1β and acting in synergy with IL-6 and IL-23, to induce the differentiation of Th17 cells (Lasigliè, 2011). Recent studies show that NLPR3 rs10157379, NLPR3 rs10754558, NLRP3 rs1539019, and CARD8 rs2043211 variants play an important role in severe and critical COVID-19 (Lasigliè, 2011; Maes et al., 2022). However, the role of SNPs in the progression to severe COVID-19 remains unclear. Thus, in the present study, we investigated the association of 11 SNPs of the NLRP3, Caspase recruitment domain-containing protein 8 (CARD8), Absent in melanoma 2 (AIM2), Interleukin-1 converting enzyme (CASP-1), Gamma-interferon-inducible protein 16 (IFI16), and Interleukin 1-beta (IL-1β) inflammasome genes with risk of progression to severe outcomes in subjects hospitalized with SARS-CoV-2 infection in a public reference hospital for COVID-19 in Rio de Janeiro, Brazil.

2. Materials and methods

This is a time-to-event study nested in the RECOVER-SUS study [NCT04807699], which is a prospective multicenter study coordinated by the Evandro Chagas National Institute of Infectious Diseases of the Oswaldo Cruz Foundation (INI/FIOCRUZ). The RECOVER-SUS study has been including individuals who were hospitalized with moderate, severe and critical COVID-19 in seven centers in Brazil. The RECOVER-SUS clinical cohort study and details of patient eligibility, enrollment, inclusion/exclusion criteria, and study design have been previously described (Perazzo et al., 2022). For the present study, we analyzed a subset of the RECOVER-SUS cohort of participants hospitalized at INI/FIOCRUZ from June 2020 to March 2021. Overall, data from 451 individuals hospitalized with COVID-19 at the INI-FIOCRUZ who agreed to provide biological samples for research analyses. Among them, 174 evolved to the use of mechanical ventilatory support, and 175 evolved to death. The individuals who were discharged without using MVS during hospital stay were analyzed as control groups in the descriptive analyses. However, for the time-to-event analyses all individuals were evaluated from symptoms onset to either any outcome (e.g., MVS, death) or hospital discharge.

The study protocol was approved by the Ethics Committee of the National Institute of Infectology Evandro Chagas (INI)/Oswaldo Cruz Foundation (FIOCRUZ), Rio de Janeiro, Brazil, under approval number CAAE: 32449420.4.1001.5262. All participants or their guardians signed an informed consent before enrolling in the study.

2.1. Clinical profiles at presentation

Clinical presentation was defined according to the WHO COVID-19 severity classification (Marshall et al., 2020), as moderate, severe, and critical COVID-19 within the first 24 hs of hospitalization. Moderate cases (WHO 4–5 classification) included in-hospital individuals with no oxygen therapy or oxygen by mask or nasal prong. Individuals who were hospitalized requiring oxygen support by NIV (noninvasive ventilation) or high flow, intubation, and mechanical ventilation, pO2/FiO2 $\geq$ 150

or SpO2/FiO2 ≥ 200, and mechanical ventilation pO2/FiO2 < 150 (SpO2/FiO2 < 200), or vasopressors were classified as severe (WHO 6–8 classification). Were classified as critical (WHO 9–10 classification) individuals requiring mechanical ventilation pO2/FiO2 < 150 and vasopressors, dialysis, and death, within the first 24 hs of hospitalization.

2.2. Genomic DNA extraction and quantification

The genomic DNA was extracted from the patient's whole blood cells, using QIAamp DNA Blood Mini Kit (Qiagen, Germany), according to the manufacturer's guidelines, and the degree of DNA purity and concentration in each sample was measured by spectrophotometry. Samples were stored at −20 °C.

2.3. Genotyping of SNPs in the inflammasome genes

We selected 11 SNPs in six inflammasome genes: CARD8 (rs2043211, rs6509365), AIM2 (rs2276405), IFI16 (rs1101996), CASP-1 (rs572687), IL-1β (rs1143634), and NLRP3 (rs10754558, rs1539019, rs4612666, rs3806268, and rs35829419). SNPs were genotyped through Taq Man assays using commercial kits (Applied Biosystems/AB and Vida Technologies, São Paulo, Brazil) at the ABI7500 Real-Time platform (Applied Biosystems/AB and Life Technologies). Allelic discrimination was carried out by employing the Thermo Fisher Connect Software.

2.4. Statistical analyses

For descriptive analyses, Mann-Whitney U tests were used to compare continuous numerical variables, and Fisher's exact tests were used for categorical demographic and baseline clinical variables.

Genotype and allele frequencies of each variant were determined by direct counting, and Hardy-Weinberg Estimation (HWE) deviations were evaluated by chi-square tests. Pairwise LD patterns were determined for each gene using r^2 statistics (cut from $r^2 \geq 0.8$). The haplotype frequencies, or even the allelic phase determination, were estimated by the expectation–maximization algorithm (EM algorithm). The estimation uncertainty was included in the statistical models applied for association analysis in the form of weights. Haplotype analyses similarly used the most common haplotype in our sample as a reference.

The incidence of mechanical ventilation use and death were analyzed based on the hospital medical record and on the "person-years" (pY) at risk based on time follow-up from the date of the first symptoms of COVID-19 to the date of the outcomes of death or use of mechanical ventilatory support. The incidences and their 95 % confidence intervals (95 % CI) were estimated according to asymptotic standard errors calculated from a Gamma distribution. The observation start date for time-to-event analyzes was defined as the onset of symptoms of COVID-19. Individuals who remained hospitalized without the need for mechanical ventilatory support or death were censored at the time of hospital discharge. The results of the time-to-event analyzes were presented in the form of hazard ratios (HR) with their 95 % CI, and the risks of progression for the events described above were estimated using Cox's proportional hazard models. In time-to-event analysis, the effects of the genetic traits of interest were corrected for phenotypic traits with at least one suggestion of association (p-value ≤ 0.1) with the outcome of interest, and marginal effects presented in the form of adjusted-HR (aHR). Multiple comparisons were corrected by the false discovery ratio (FDR) estimate. The risk proportionality assumption was tested using correlation analysis and chi-square tests based on the Schoenfeld scaled residuals and transformed survival times.

Statistical power for the Cox proportional hazard analyses was estimated for the different events (i.e., clinical outcomes) of interest in our sample assuming the minor allele frequencies among controls (individuals who had the best outcome) of the observed genetic variants (SNPs) and significance level of 0.05 (Chow et al., 2008).

Statistical analyzes were performed using the R software (version 4.1.1) and its "genetics" and "survival" packages.

3. Results

3.1. Sociodemographic and clinical characteristics

Clinical and sociodemographic characteristics of participants at hospital admission comparing discharge and death outcomes according to the use or not of MVS are described in Table 1.

Data from 451 participants were analyzed, where 277 (61.4 %) were discharged and 174 (38.6 %) died during the hospital stay. Overall, most of the participants were male (53.2 %). The overall median age was 60 years (IQR = 21.84), with a median of 55 years (IQR = 20.83) in the group of individuals who entered MVS and were discharged and 67 years (IQR = 17.05) in the group of individuals who entered MVS and died. The frequencies of the individuals according to gender, skin color, and schooling were not distinct among the groups. Among the comorbidities, the frequencies of chronic obstructive pulmonary disease (COPD), HIV infection, hepatic cirrhosis, and transplant were equally distributed between the discharge and death outcomes, according to the use or not of MVS. Overall, 374 individuals (82.9 %) required some use of oxygen support at clinical presentation, with significant differences among groups (p-value < 0.001). Several common symptoms at clinical presentation (greater than50 %) were observed for the COVID-19 individuals (Table 1), but only dyspnea (312 individuals; 69.2 %), fever (243 individuals; 53.9 %), and headache (70 individuals; 15.5 %) showed statistically significant differences among groups.

Accordingly, to the mortality predictor scores performed at clinical presentation, the group of individuals who entered MVS and died also had the highest number of patients with the highest SOFA and SAPS-III scores. In SAPS-III scores, this same group was the one with the highest number of individuals with scores greater than 46 with 106 patients (77.9 %), which predicts a higher mortality rate. We used three cuts to represent disease severity ranges in this mortality predictor scores. For the Glasgow Coma Scale, the cutoff was made at a score of 0–9, indicating severe trauma, 9–13 moderate, and 13–15 minor (McDougal, 2009). For the SOFA, we used for cutoff a score of 0–7 indicating a mortality rate of 37 %, a score of 7–10 a mortality rate of 60 %, and 10–24 greater than 90 % (Ferreira et al., 2001). In the SAPS-III score the cutoff was made a score of 30–46, indicating mortality of < 3 % and 46–101 with a mortality rate of greater than 70 % (Sakr et al., 2008).

To identify clinic confounders in the genetic association study, we performed a phenotypic analysis of the clinical data in the outcome of mechanical ventilation (Table S1) and death (Table S2) using Cox proportional hazard models. Based on that, we found that the faster progression to use MVS was associated with age ranges of 60–80 years (aHR 3.213; P = 0.001) and 80–90 years (aHR 2.921; P = 0.011); having the comorbidity COPD (aHR = 2.619; P < 0.001), and cough (aHR = 1.509; P = 0.009). Meanwhile dyspnea was associated with a slower progression to use MVS. The mortality predictor score, Glasgow scale cat 13–15 (aHR = 0.279; P = 0.001) was associated with a slower progression to use MVS. The SOFA cat 7–10 (aHR = 4.085; P < 0.001) and 10–24 (aHR = 10.481; P = 0.001) were associated with a risk for a faster progression to use MVS, as well the SAPS-III cat 46–101 (aHR = 3.134; P < 0.001). Similarly, faster progression to death was associated with age ranges of 60–80 years (aHR = 3.569; P = 0.001) and 80–90 years (aHR 4.029; P = 0.001), active cancer (aHR = 5.033; P = 0.024) and transplant (aHR = 15.726; P = 0.007). The mortality predictor score, SOFA cat 7–10 (aHR = 2.073; P < 0.001) and 10–24 (aHR = 2.249; P = 0.013) were associated with a risk for a faster progression to death, as well the SAPS-III cat 46–101 (aHR = 2.673; P < 0.001).

For the genetic analyses, we selected confounder variables, given they were at least suggestively associated with progression to the outcomes, such as age, diabetes mellitus, COPD, active cancer, current smoking, transplant, Glasgow Scale, SOFA, and SAPS-III, for progression

Table 1
Sociodemographic and clinical features for all individuals included in the study categorized according to mechanical ventilation support (MVS) use. (N = 451).

Features			Discharge (N = 277)		Death (N = 174)		[a]p-value
		Overall N = 451	**Without MVS (N = 239)**	**With MVS (N = 38)**	**Without MVS (N = 37)**	**With MVS (N = 137)**	
Gender; n (%)	Female	211 (46.8 %)	116 (48.5 %)	23 (60.5 %)	18 (48.6 %)	54 (39.4 %)	0.102
	Male	240 (53.2 %)	240 (53.2 %)	123 (51.5 %)	15 (39.5 %)	19 (51.4 %)	
Skin Color; n (%)	White	75 (16.6 %)	75 (16.6 %)	37 (15.5 %)	7 (18.4 %)	5 (13.5 %)	0.155
	Brown	274 (60.8 %)	274 (60.8 %)	144 (60.3 %)	25 (65.8 %)	18 (48.6 %)	
	Other	35 (7.8 %)	26 (10.8 %)	3 (7.9 %)	2 (5.4 %)	4 (2.9 %)	
Age; n (%)		60.25 (IQR = 21.84)	55.19 (IQR = 18.83)	55.45 (IQR = 20.83)	69.76 (IQR = 18.88)	67.48 (IQR = 17.05)	< **0.001**
	(18–40)	45 (10.9 %)	33 (15.1 %)	5 (13.9 %)	3 (10.7 %)	4 (3.1 %)	
	(40–60)	159 (38.6 %)	104 (47.5 %)	18 (50 %)	3 (10.7 %)	34 (26.4 %)	
	(60–80)	176 (42.7 %)	72 (32.9 %)	12 (33.3 %)	15 (53.6 %)	77 (59.7 %)	
	(80–90)	32 (7.8 %)	10 (4.6 %)	1 (2.8 %)	7 (25 %)	14 (10.9 %)	
Schooling; n (%)	University education	51 (11.3 %)	51 (11.3 %)	34 (14.2 %)	6 (15.8 %)	2 (5.4 %)	0.127
	High school	140 (31 %)	75 (31.4 %)	14 (36.8 %)	10 (27 %)	41 (29.9 %)	
	Low Education	205 (45.4 %)	108 (45.2 %)	14 (36.8 %)	16 (43.2 %)	63 (48.9 %)	
	Illiterate	27 (6 %)	13 (5.4 %)	0 (0 %)	4 (10.8 %)	10 (7.3 %)	
Diabetes Mellitus; n (%)	No	313 (69.4 %)	177 (74.1 %)	27 (71.1 %)	25 (67.6 %)	84 (61.3 %)	0.08
	Yes	138 (30.6 %)	62 (25.9 %)	11 (28.9 %)	12 (32.4 %)	53 (38.7 %)	
Coronary Artery Disease; n (%)	No	440 (97.6 %)	234 (97.9 %)	37 (97.4 %)	34 (91.9 %)	135 (98.5 %)	0.128
	Yes	11 (2.4 %)	5 (2.1 %)	1 (2.6 %)	3 (8.1 %)	2 (1.5 %)	
Systemic arterialhypertension; n (%)	No	231 (51.2 %)	135 (56.5 %)	19 (50 %)	18 (48.6 %)	59 (43.1 %)	0.093
	Yes	220 (48.8 %)	104 (43.5 %)	19 (50 %)	19 (51.4 %)	78 (56.9 %)	
COPD; n (%)	No	422 (93.6 %)	232 (97.1 %)	34 (89.5 %)	35 (94.6 %)	121 (88.3 %)	**0.007**
	Yes	29 (6.4 %)	7 (2.9 %)	4 (10.5 %)	2 (5.4 %)	16 (11.7 %)	
HIV; n (%)	Negative	406 (90 %)	210 (87.9 %)	38 (100 %)	26 (70.3 %)	132 (96.4 %)	< **0.001**
	Positive	24 (5.3 %)	16 (6.7 %)	0 (0 %)	3 (8.1 %)	5 (3.6 %)	
Hepatical Cirrhosis; n (%)	No	449 (99.6 %)	239 (100 %)	38 (100 %)	35 (94.6 %)	137 (100 %)	< **0.001**
	Yes	2 (0.4 %)	0 (0 %)	0 (0 %)	2 (5.4 %)	0 (0 %)	
Transplant; n (%)	No	450 (99.8 %)	239 (100 %)	38 (100 %)	36 (97.3 %)	137 (100 %)	**0.011**
	Yes	1 (0.2 %)	0 (0 %)	0 (0 %)	1 (2.7 %)	0 (0 %)	
Oxygen supplementation or ventilatory support	Yes	374 (82.9 %)	177 (74.1 %)	38 (100 %)	22 (59.5 %)	137 (100 %)	< **0.001**
	No	77 (17.1 %)	62 (25.9 %)	0 (0 %)	15 (40.5 %)	0 (0 %)	
Fever; n (%)	Yes	243 (53.9 %)	141 (59 %)	23 (60.5 %)	14 (37.8 %)	65 (47.4 %)	**0.025**
	No	208 (46.1 %)	98 (41 %)	15 (39.5 %)	23 (62.2 %)	72 (52.6 %)	
Cough; n (%)	Yes	275 (61 %)	156 (65.3 %)	24 (63.2 %)	20 (54.1 %)	75 (54.7 %)	0.178
	No	176 (39 %)	83 (34.7 %)	14 (36.8 %)	17 (45.9 %)	62 (45.3 %)	
Chest Pain; n (%)	No	411 (91.1 %)	217 (90.8 %)	36 (94.7 %)	36 (97.3 %)	122 (89.1 %)	0.374
	Yes	40 (8.9 %)	22 (9.2 %)	2 (5.3 %)	1 (2.7 %)	15 (10.9 %)	
Coryza; n (%)	No	415 (92 %)	215 (90 %)	35 (92.1 %)	35 (94.6 %)	130 (94.9 %)	0.354
	Yes	36 (8 %)	24 (10 %)	3 (7.9 %)	2 (5.4 %)	7 (5.1 %)	
Dyspneia; n (%)	Yes	312 (69.2 %)	158 (66.1 %)	33 (86.8 %)	19 (51.4 %)	102 (74.5 %)	**0.003**
	No	139 (30.8 %)	81 (33.9 %)	5 (13.2 %)	18 (48.6 %)	35 (25.5 %)	
Odynophagy; n (%)	No	435 (96.5 %)	226 (94.6 %)	37 (97.4 %)	37 (100 %)	135 (98.5 %)	0.127
	Yes	16 (3.5 %)	13 (5.4 %)	1 (2.6 %)	0 (0 %)	2 (1.5 %)	
Anosmia; n (%)	No	403 (89.4 %)	209 (87.4 %)	34 (89.5 %)	37 (100 %)	123 (89.8 %)	0.148
	Yes	48 (10.6 %)	30 (12.6 %)	4 (10.5 %)	0 (0 %)	14 (10.2 %)	
Loss Of Taste; n (%)	No	411 (91.1 %)	211 (88.3 %)	34 (89.5 %)	37 (100 %)	129 (94.2 %)	**0.053**
	Yes	40 (8.9 %)	28 (11.7 %)	4 (10.5 %)	0 (0 %)	8 (5.8 %)	
Diarrhea; n (%)	No	411 (91.1 %)	210 (87.9 %)	36 (94.7 %)	36 (97.3 %)	129 (94.2 %)	0.07
	Yes	40 (8.9 %)	29 (12.1 %)	2 (5.3 %)	1 (2.7 %)	8 (5.8 %)	
Abdominal Pain; n (%)	No	438 (97.1 %)	232 (97.1 %)	37 (97.4 %)	37 (100 %)	132 (96.4 %)	0.706
	Yes	13 (2.9 %)	7 (2.9 %)	1 (2.6 %)	0 (0 %)	5 (3.6 %)	
Nausea; n (%)	No	427 (94.7 %)	225 (94.1 %)	36 (94.7 %)	36 (97.3 %)	130 (94.9 %)	0.884
	Yes	24 (5.3 %)	14 (5.9 %)	2 (5.3 %)	1 (2.7 %)	7 (5.1 %)	
Headache; n (%)	Yes	70 (15.5 %)	45 (18.8 %)	7 (18.4 %)	1 (2.7 %)	17 (12.4 %)	**0.048**
	No	381 (84.5 %)	194 (81.2 %)	31 (81.6 %)	36 (97.3 %)	120 (87.6 %)	
Myalgia; n (%)	No	354 (78.5 %)	180 (75.3 %)	27 (71.1 %)	31 (83.8 %)	116 (84.7 %)	0.094
	Yes	97 (21.5 %)	59 (24.7 %)	11 (28.9 %)	6 (16.2 %)	21 (15.3 %)	
Some thrombosis	Yes	1 (0.2 %)	1 (0.2 %)	0 (0 %)	0 (0 %)	0 (0 %)	NC
	No	450 (99.8 %)	239 (100 %)	38 (100 %)	37 (100 %)	136 (99.3 %)	
Glasgow Scale		15 (IQR = 0)	15 (IQR = 0)	15 (IQR = 0)	15 (IQR = 0)	15 (IQR = 1)	< **0.001**
Glasgow scale cat	(0,9]	11 (2.9 %)	2 (0.9 %)	1 (3.2 %)	1 (3.2 %)	7 (6.9 %)	< **0.001**
	(9,13]	23 (6.1 %)	2 (0.9 %)	2 (6.5 %)	2 (6.5 %)	17 (16.7 %)	

(continued on next page)

Table 1 (*continued*)

Features			Discharge (N = 277)		Death (N = 174)		[a]*P*-value
		Overall N = 451	**Without MVS (N = 239)**	**With MVS (N = 38)**	**Without MVS (N = 37)**	**With MVS (N = 137)**	
	(13,15]	345 (91 %)	211 (98.1 %)	28 (90.3 %)	28 (90.3 %)	78 (76.5 %)	
SOFA		3 (IQR = 4)	2 (IQR = 1)	3 (IQR = 4)	4 (IQR = 4)	6 (IQR = 5)	**< 0.001**
SOFA cat	(0,7]	379 (85 %)	229 (97 %)	30 (81.1 %)	29 (78.4 %)	91 (66.9 %)	**< 0.001**
	(7,10]	53 (11.9 %)	6 (2.5 %)	6 (16.2 %)	8 (21.6 %)	33 (24.3 %)	
	(10,24.1]	14 (3.1 %)	1 (0.4 %)	1 (2.7 %)	0 (0 %)	12 (8.8 %)	
SAPS-III		46 (IQR = 14.75)	43 (IQR = 9)	43 (IQR = 13)	51 (IQR = 14)	57 (IQR = 21)	**< 0.001**
SAPS-III cat	(30,46]	230 (51.6 %)	164 (69.5 %)	22 (59.5 %)	14 (37.8 %)	30 (22.1 %)	**< 0.001**
	(46,101]	216 (48.4 %)	72 (30.5 %)	15 (40.5 %)	23 (62.2 %)	106 (77.9 %)	

[a] *P*-value were calculated using the unconditional logistic regression model. Associations were considered significant with a value of $P < 0.05$. N: number of individuals in each group. MVS: Mechanical Ventilation Support. NC: not calculated. COPD: Chronic obstructive pulmonary disease. SOFA: Sequential Organ Failure Assessment. SAPS III: Simplified Acute Physiology Score III. Cat: Category of small categories. Glasgow scale cat (0,9): severe trauma; (9,13): moderate trauma; (13,15): mild trauma/normal. SOFA cat (0,7): corresponded to mortality rate of 37 %; (7,10): corresponded to mortality rate of 60 %; (10,24): corresponded to mortality rate greater than 90 %. SAPS3 cat (30,46): corresponded to mortality of < 3 %; (46,101): corresponded to mortality rate of greater than 70 %.

to death, and age, systemic arterial hypertension, COPD, active cancer, obesity or previous bariatric surgery, HIV, Glasgow Scale Cat, SOFA, and SAPS-III for progression to use of mechanical ventilation in phenotypic analyses. We believe that including these variables in the genetic analysis was sufficient to eliminate potential sample biases.

3.2. Alleles, genotypes, and haplotypes associated with time to the event of mechanical ventilatory support (MVS)

Genotype frequencies of the 11 SNPs analyzed in the present study are in Hardy-Weinberg equilibrium (Table S3). The genotypes, alleles, carrier frequencies and haplotypes of the studied SNPs among the individuals who were submitted to MVS (N = 175) which had statistically significant results are shown in Table 2. The remaining ones are shown in Table S4.

The time of progression in weeks from the beginning of the symptoms until the use of MVS is described in Table S5. In this study, we observed that carrying the G allele (aHR = 0.391; P = 0.006), or the G/G genotype (aHR = 0.66; P = 0.005) in the NLRP3 rs10754558 variant or carrying the G allele in the IL1β rs1143634 variant (aHR = 0.309; P = 0.004), were associated with a slower progression to the use of MVS. We also performed the haplotype analysis and, considering the genetic variants of NLRP3 (rs1539019 - rs4612666 - rs3806268 - rs35829419 - rs10754558), carrying the A-T-G-A-G haplotype (aHR = 10.241; P < 0.001) was associated with a risk for faster progression to MVS use while carrying the C-T-G-C-G haplotype (aHR = 0.206; P = 0.024) was associated with a slower time to progression to use MVS. These analyzes of haplotypes were performed considering the most frequent haplotype of the NLRP3 (C-T-G-C–C) gene as references. All haplotype analyses are shown in Table S6.

Assuming the observed rates of use of ventilatory support in our cohort of approximately 83 %, and the minor allele frequencies among controls, i.e., those who did not required ventilatory support, of 0.02 to 0.4, for the n-sample of 451 patients and significance level (alpha of

Table 2
Analysis of alleles, genotypes, and haplotypes of COVID-19 individuals from the beginning of symptoms to the progression to mechanical ventilation outcome that showed significant results, using Cox proportional hazard models or time-to-event analyses.

Genes	SNP	Alleles /Genotype/Haplotypes	[a]pY	Crude Incidence/pY (CI95 %)	Mechanical Ventilation aHR (CI95 %)	[b]*P*-value
NLRP3	rs10754558	C/C	7.88	10.28 (8.16–12.77)	Reference	
		C/G	9.4	7.98 (6.28–10.01)	0.748 (0.51–1.096)	0.136
		G/G	3.55	5.35 (3.22–8.36)	**0.391 (0.2–0.763)**	**0.006**
		C	25.16	9.42 (8.26–10.7)	Reference	
		G	16.5	6.85 (5.64–8.23)	**0.66 (0.443–0.983)**	**0.005**
		No carrier C	3.55	5.35 (3.22–8.36)	Reference	
		Carrier C	17.28	9.03 (7.67–10.56)	2.202 (1.16–4.182)	0.16
IL1β	rs1143634	G/G	14.6	7.4 (6.07–8.93)	Reference	
		A/A	0.33	17.96 (6.59–39.1)	3.213 (1.358–7.603)	0.088
		G/A	5.86	10.24 (7.81–13.18)	1.02 (0.683–1.523)	0.922
		No carrier G	0.37	18.94 (7.61–39.02)	Reference	
		Carrier G	20.46	8.21 (7.02–9.55)	**0.309 (0.14–0.683)**	**0.004**
		No carrier A	14.63	7.45 (6.12–8.99)	Reference	
		Carrier A	6.2	10.65 (8.24–13.55)	1.113 (0.76–1.63)	0.583
NLRP3	rs1539019	**ATGAG**	0.02	40.58 (1.03–226.12)	**10.241 (1.375–76.25)**	**< 0.001**
	rs4612666	**CTGCG**	2.04	1.96 (0.54–5.03)	**0.206 (0.049–0.856)**	**0.024**
	rs3806268					
	rs35829419					
	rs10754558					

[a] pY:person-years; [b]P-value were calculated using the Cox's proportional risk model. Associations were considered significant with a value of $P < 0.05$. N: number of individuals in each group; aHR: adjusted Hazard ratio; CI95 %: 95 % confidence interval; NC: not calculated. Adjusted Hazard ratio were adjusted by age, systemic arterial hypertension, COPD, active cancer, obesity or previous bariatric surgery, HIV, Glasgow Scale Cat, SOFA, and SAPS3. A, T, G, and C = each allele count, irrespective of the genotype. Carrier-A = total of genotypes with the A allele; Carrier-T = total of genotypes with T allele; Carrier-C = total of genotypes with the C allele; Carrier-G = total of genotypes with the G allele; No carrier-A = total of genotypes without the A allele; No carrier-T = total of genotypes without the T allele; No carrier-C = total of genotypes without the C allele; No carrier-G = total of genotypes without the G allele.

0.05) we obtained statistical powers (1 - beta) greater than 99 % for the Cox proportional hazard analyses for the events of use of ventilatory support (data not shown).

3.3. Alleles, genotypes, and haplotypes associated with time to the event of death

Genotypes, alleles, and carrier frequencies of all the studied SNPs comparing COVID-19 individuals who evolve to death (N = 174) are shown in Table 3. In addition, the median time of weeks to progression to death is described in Table S7.

We observed that carrying the C allele of the NLRP3 rs10754558 variant increases the progression to death (aHR = 2.957; P = 0.005). Carrying the C allele of the NLRP3 rs4612666 variant also increases the progression to death (aHR = 2.342; P = 0.006). Moreover, the following alleles and genotypes were associated with a slower progression to death, as follows: carry either the G/G genotype (aHR = 0.323; P = 0.005) or the G allele (aHR = 0.688; P = 0.014) of the NLRP3 rs10754558 variant; the T/T genotype (aHR = 0.394; P = 0.004) or the T allele (aHR = 0.68; P = 0.006) of NLRP3 rs4612666 variant; the A/G genotype (aHR = 0.537; P = 0.005) or the G allele of the CARD8 rs6509365 variant (aHR = 0.563; P = 0.006), and the A/C genotype of the IFI16 rs1101996 variant (aHR = 0.569; P = 0.033). SNPs in the CARD8 (rs2043211), CASP-1 (rs572687), AIM2 (rs2276405), and NLRP3 (rs3806268, rs35829419, and rs1539019) genes did not reveal any significant association with the studied outcomes. We also performed the haplotype analysis of inflammasome SNP associated with the time to death, as shown in Table 4.

Carry the haplotypes A-C-A-C–C (aHR = 2.344; P = 0.002), A-C-G-C–C (aHR = 2.174; P = 0.042), C–C-A-C–C (aHR = 2.026; P = 0.002), C-T-A-C–C (aHR = 2.068; P = 0.012) or A-T-G-A-G (aHR = 34.611; P < 0.001) of the NLRP3 genetic variants (rs1539019 - rs4612666 - rs3806268 - rs35829419 - rs10754558) was associated with a higher risk for faster progression to death. These analyzes of haplotypes were performed considering the most frequent haplotype of the NLRP3 (C-T-G-C–C) gene as references.

It is worth noting that carrying the NLRP3 haplotype A-T-G-A-G was associated with an expressive faster progression of participants to MVS and death. No haplotypes of the CARD8 gene were associated with progression to the analyzed outcomes.

Assuming the observed rates of death in our cohort of approximately 61 %, and the minor allele frequencies among controls, i.e., those who were discharged after COVID-19, of 0.02 to 0.4, for the n-sample of 451 patients and significance level (alpha of 0.05) we obtained statistical powers (1 - beta) greater than 97 % for the Cox proportional hazard analyses (data not shown).

4. Discussion

COVID-19 severity and death have been mainly associated with older ages, male sex, and the presence of comorbidities, such as obesity, smoking, type-2 diabetes mellitus, and others (Ferreira et al., 2001; Sakr et al., 2008). Host factors are key to determining disease severity and progression in a disease as heterogeneous as COVID-19 (Wiersinga et al., 2020). In the present study, we showed that carrying the allele C in selected NLRP3 inflammasome SNPs (rs10754558 or rs4612666 variants) was associated with faster progression to death among in-hospitalized COVID-19 individuals. Moreover, it was also highlighted in our study that carrying the NLRP3 haplotype A-T-G-A-G was associated with an expressive risk for a faster progression to disease severity, as measured in the use of MVS and death. The pivotal role of NLRP3 inflammasome activation in the SARS-CoV-2 infection and COVID-19 has already been described (Wu et al., 2020; Wiersinga et al., 2020; López-Reyes et al., 2020). Rodrigues et al (Rodrigues, 2020) showed that the NLRP3 inflammasomes are active in individuals with COVID-19 and that the magnitude of this activation was associated with COVID-19 outcome. Moreover, it is described that NLRP3 inflammasome formation and a consequent hyperreactive immune response is considered the main cause of dysregulated immune response against SARS-COV-2 infection, contributing to the cytokine storm and severity and worse clinical outcome of COVID-19 (Rodrigues, 2020). Thus, potential drugs for blocking NLRP3-mediated inflammation are under development and/or clinical testing (Amin et al., 2022).

We acknowledge that several studies on inflammasome activation and COVID-19 outcomes have been published. However, as of our knowledge, only two studies associated inflammasome genetic polymorphisms and COVID-19 clinical profiles showing a risk effect of the NLRP3 rs10754558 C/G genotype, increasing the risk towards severe COVID-19 and mortality (Maes et al., 2022). Likewise in a recent study published by our group we show that NLRP3 rs1539019 A/A genotype, allele A or carrier A and CARD8 rs2043211 A/T genotype, allele T or carrier T were associated with protection against disease severity (de Sá et al., 2022). Supporting our results, where NLRP3 rs10754558 carrying the C allele was associated with a faster progression to death, Maes et al (Maes et al., 2022) also found a risk effect of the NLRP3 rs10754558 C/G genotype in severe COVID (Maes et al., 2022). However, in our study, the NLRP3 rs10754558 G/G genotype or G allele was associated with a protective effect, increasing the time until both, the use of MVS and death. We hypothesized that the difference in ethnicity predominant between the two studies is contributing to the differences in the results. In our study the individuals are predominantly self-declared brown/biracial individuals, with people from Southeast and North regions, and although both studies include Brazilian individuals, their study is focused on people from the South of Brazil, with the predominance of whites. Besides that, Toro *et al.* (2021) observed that individuals with the C/C genotype were prevalent among the individuals with Hepatitis C Virus and had a 58 % chance higher of developing hepatitis due to the virus (Toro et al., 2021). Studies of viral pathogens corroborate these findings, where the G allele and the G/G genotype of the NLRP3 rs10754558 variant were associated with a protective role against HIV-1 infection susceptibility for example, which corroborates our findings (Pontillo et al., 2012; Amin et al., 2022).

Other inflammasomes genetic variants included in our study, such as NLRP3 rs4612666, CARD8 rs6509365, IFI16 rs1101996, and the IL1β gene rs1143634 variants, were associated with protection or risk for a faster or slower progression to the severe outcomes of use of mechanical ventilation support or death in SARS-CoV-2 infected individuals. Our data suggest that these SNPs might modulate inflammasome activation, contributing to a worse or better prognosis in disease progression to severe outcomes.

Our study found that the carrier C allele of the NLRP3 rs4612666 variant was associated with faster progression to death. Indeed, Ehtesham *et al.* (2021) found that the C allele in this variant of the gene NLRP3 is associated with clinical characteristics and severe disease activity of systemic lupus erythematosus, an inflammatory autoimmune disease (Ehtesham et al., 2021). Also, Hitomi et al. (Hitomi, 2009) found the rs4612666 variant was significantly associated with susceptibility to food-induced anaphylaxis and found that the C allele showed higher transcriptional enhancer activity and mRNA stability. Therefore, we hypothesize, that one of the possible reasons for the C allele of the NLRP3 rs4612666 variant to be associated with faster progression to death, is that this variant could increase NLRP3 mRNA stability and enhance NLRP3 activity, which subsequently led to a series of inflammatory reactions, contributing to a faster progression to this outcome. However, further studies need to be carried out to confirm this hypothesis (Ehtesham et al., 2021; Hitomi, 2009). We also found in this same variant of NLRP3 that T/T genotype or allele T is associated with protection in time to progression to death. However, in the literature, most studies show a negative association of the T/T genotype and allele T of NLRP3 rs4612666 in several diseases (Perri et al., 2021; Zhao et al., 2017; Cheng et al., 2018). We assume that the association with protection in our study is attributed to the geographical/ethnic variation

Table 3
Analysis of alleles and genotypes of COVID-19 individuals from the beginning of symptoms to the progression to death outcome using Cox proportional hazard models or time-to-event analyses.

Genes	SNP	Alleles /Genotype	[a]pY	Crude Incidence/pY (CI95 %)	Death aHR (CI95 %)	[b]*P*-value
NLRP3	rs10754558	C/C	12.56	5.97 (4.7–7.48)	Reference	
		C/G	13.72	5.68 (4.49–7.09)	0.924 (0.616–1.386)	0.703
		G/G	5.04	4.17 (2.58–6.37)	**0.323 (0.147–0.709)**	**0.005**
		C	38.85	5.87 (5.13–6.68)	Reference	
		G	23.81	5.04 (4.18–6.03)	**0.688 (0.453–1.044)**	**0.014**
		No carrier C	5.04	4.17 (2.58–6.37)	Reference	
		Carrier C	26.29	5.82 (4.93–6.82)	**2.957 (1.397–6.26)**	**0.005**
NLRP3	rs4612666	C/C	12.42	6.28 (4.96–7.84)	Reference	
		C/T	13.73	5.32 (4.17–6.68)	0.856 (0.557–1.317)	0.48
		T/T	5.17	4.45 (2.82–6.67)	**0.394 (0.207–0.749)**	**0.004**
		C	38.58	5.94 (5.19–6.76)	Reference	
		T	24.08	4.94 (4.09–5.91)	**0.68 (0.457–1.012)**	**0.006**
		No carrier C	5.17	4.45 (2.82–6.67)	Reference	
		Carrier C	26.15	5.77 (4.89–6.77)	**2.342 (1.279–4.288)**	**0.006**
NLRP3	rs1539019	C/C	12.95	5.48 (4.28–6.92)	Reference	
		A/A	4.46	5.16 (3.27–7.74)	0.643 (0.337–1.223)	0.178
		C/A	13.92	5.75 (4.56–7.15)	0.796 (0.523–1.209)	0.284
		C	39.82	5.58 (4.87–6.36)	Reference	
		A	22.83	5.52 (4.6–6.57)	0.817 (0.554–1.206)	0.231
		Non carrier C	4.46	5.16 (3.27–7.74)	Reference	
		Carrier C	26.87	5.62 (4.76–6.59)	1.381 (0.752–2.535)	0.298
NLRP3	rs3806268	G/G	11.56	5.1 (3.89–6.58)	Reference	
		A/A	4.8	5.63 (3.71–8.19)	1.23 (0.658–2.3)	0.517
		G/A	14.97	5.88 (4.72–7.24)	1.363 (0.894–2.078)	0.15
		G	38.09	5.41 (4.7–6.2)	Reference	
		A	24.57	5.78 (4.87–6.81)	1.155 (0.779–1.711)	0.314
		No carrier G	4.8	5.63 (3.71–8.19)	Reference	
		Carrier G	26.53	5.54 (4.68–6.51)	0.979 (0.555–1.728)	0.941
NLRP3	rs35829419	C/C	29.81	5.6 (4.79–6.52)	Reference	
		A/A	0.03	33.2 (0.84–185)	NC	
		C/A	1.49	4.03 (1.48–8.77)	0.955 (0.382–2.385)	0.922
		C	61.1	5.56 (4.99–6.19)	Reference	
		A	1.55	5.16 (2.23–10.17)	0.956 (0.27–3.393)	0.932
		No carrier C	0.03	33.2 (0.84–185)	Reference	
		Carrier C	31.3	5.53 (4.73–6.42)	NC	
CARD8	rs6509365	A/A	15.43	5.57 (4.46–6.88)	Reference	
		A/G	13.32	5.48 (4.3–6.89)	**0.537 (0.346–0.832)**	**0.005**
		G/G	2.58	5.82 (3.26–9.6)	0.707 (0.333–1.501)	0.367
		A	44.18	5.55 (4.87–6.28)	Reference	
		G	18.47	5.58 (4.55–6.76)	0.74 (0.486–1.127)	0.11
		No carrier G	15.43	5.57 (4.46–6.88)	Reference	
		Carrier G	15.89	5.54 (4.44–6.82)	**0.563 (0.373–0.85)**	**0.006**
CARD8	rs2043211	A/A	17.65	5.67 (4.61–6.89)	Reference	
		A/T	11.82	5.24 (4.02–6.72)	0.759 (0.49–1.177)	0.218
		T/T	1.86	6.46 (3.34–11.29)	0.886 (0.377–2.081)	0.781
		A	47.12	5.56 (4.91–6.28)	Reference	
		T	15.54	5.54 (4.43–6.84)	0.859 (0.541–1.364)	0.315
		No carrier A	1.86	6.46 (3.34–11.29)	Reference	
		Carrier A	29.47	5.5 (4.68–6.41)	1.01 (0.437–2.334)	0.982
AIM2	rs2276405	C/C	30.18	5.57 (4.76–6.47)	Reference	
		C/T	1.14	5.26 (1.93–11.44)	1.171 (0.424–3.235)	0.761
		C	61.51	5.56 (4.99–6.18)	Reference	
		T	1.14	5.26 (1.93–11.44)	1.167 (0.284–4.785)	0.607
		No carrier T	30.18	5.57 (4.76–6.47)	Reference	
		Carrier T	1.14	5.26 (1.93–11.44)	1.171 (0.424–3.235)	0.761
CASP1	rs572687	G/G	21.23	5.79 (4.82–6.91)	Reference	
		A/A	0.94	6.37 (2.34–13.87)	1.069 (0.367–3.112)	0.903
		G/A	9.16	4.92 (3.59–6.58)	0.865 (0.549–1.362)	0.531
		G	51.61	5.64 (5.01–6.32)	Reference	
		A	11.04	5.16 (3.91–6.69)	0.925 (0.547–1.564)	0.666
		No carrier G	0.94	6.37 (2.34–13.87)	Reference	
		Carrier G	30.38	5.53 (4.72–6.43)	0.908 (0.312–2.643)	0.859
IFI16	rs1101996	C/C	13.51	6 (4.76–7.45)	Reference	
		A/A	3.74	6.15 (3.9–9.22)	1.136 (0.65–1.986)	0.655
		A/C	14.08	4.97 (3.88–6.28)	**0.569 (0.369–0.88)**	**0.011**
		C	41.09	5.65 (4.94–6.42)	Reference	
		A	21.56	5.38 (4.45–6.45)	0.911 (0.616–1.347)	0.59
		No carrier C	3.74	6.15 (3.9–9.22)	Reference	
		Carrier C	27.58	5.47 (4.64–6.42)	0.684 (0.402–1.162)	0.16

(*continued on next page*)

Table 3 (*continued*)

Genes	SNP	Alleles /Genotype	[a]pY	Crude Incidence/pY (CI95 %)	Death aHR (CI95 %)	[b]*P*-value
IL1β	rs1143634	G/G	20.27	5.18 (4.24–6.27)	Reference	
		A/A	0.74	6.74 (2.19–15.73)	2.055 (0.818–5.166)	0.126
		G/A	10.23	6.16 (4.73–7.88)	1.073 (0.704–1.636)	0.743
		No carrier G	0.84	7.19 (2.64–15.64)	Reference	
		Carrier G	30.49	5.51 (4.71–6.41)	0.5 (0.217–1.151)	0.103
		No carrier A	20.36	5.21 (4.26–6.3)	Reference	
		Carrier A	10.97	6.2 (4.81–7.86)	1.135 (0.759–1.698)	0.538

[a] pY:person-years; [b]P-value were calculated using the Cox's proportional risk model. Associations were considered significant with a value of $P < 0.05$. N: number of individuals in each group; aHR: adjusted Hazard ratio; CI95 %: 95 % confidence interval; N: number of individuals in each group; aHR: were adjusted by age, diabetes mellitus, COPD, active cancer, current smoking, transplant, Glasgow Scale, SOFA, and SAPS3. A, T, G, and C = each allele count, irrespective of the genotype. Carrier-A = total of genotypes with the A allele; Carrier-T = total of genotypes with T allele; Carrier-C = total of genotypes with the C allele; Carrier-G = total of genotypes with the G allele; No carrier-A = total of genotypes without the A allele; No carrier-T = total of genotypes without the T allele; No carrier-C = total of genotypes without the C allele; No carrier-G = total of genotypes without the G allele.

Table 4
Analyses among NLRP3 and CARD8 inflammasome haplotypes frequencies in Cox proportional hazard models for time to progression of death due to COVID-19.

Genes	Haplotypes	[a]pY	Crude Incidence/pY (IC95 %)	Death aHR (CI95 %)	[b]*P*-value
NLRP3	CTGCC	16.61	5.3 (4.25–6.53)	Reference	
rs1539019	**ACACC**	2.98	7.71 (4.89–11.58)	**2.344 (1.282–4.285)**	**0.002**
rs4612666	ACACG	11.59	5.52 (4.25–7.05)	0.827 (0.516–1.325)	0.458
rs3806268	**ACGCC**	2.21	6.33 (3.46–10.62)	**2.174 (0.918–5.152)**	**0.042**
rs35829419	ACGCG	2.45	4.49 (2.24–8.04)	0.92 (0.351–2.413)	0.775
rs10754558	**ATGAG**	0.04	28.1 (0.71–156.54)	**34.611 (4.502–266.058)**	**<0.001**
	ATGCC	2.16	4.62 (2.22–8.5)	0.424 (0.186–0.966)	0.234
	ATGCG	0.85	3.55 (0.73–10.36)	0.394 (0.091–1.714)	0.122
	CCACC	7.06	5.95 (4.29–8.04)	**2.026 (1.215–3.378)**	**0.002**
	CCACG	2.37	4.22 (2.02–7.76)	1.036 (0.419–2.564)	0.926
	CCGAC	0.03	33.2 (0.84–185)	NC	
	CCGCC	6.77	6.79 (4.97–9.06)	**2.074 (1.289–3.336)**	**<0.001**
	CCGCG	2.81	6.41 (3.8–10.14)	0.895 (0.341–2.347)	0.802
	CTACC	0.37	8.06 (1.66–23.55)	**2.068 (0.616–6.942)**	**0.012**
	CTACG	0.05	NC	NC	
	CTGAC	0.09	11.41 (0.29–63.6)	NC	
	CTGAG	0.88	5.71 (1.85–13.32)	1.562 (0.537–4.543)	0.505
	CTGCG	2.22	3.6 (1.56–7.1)	0.851 (0.321–2.252)	0.708
CARD8	AA	44.11	5.55 (4.88–6.3)	Reference	
rs2043211	AG	3.01	5.66 (3.29–9.05)	0.521 (0.286–0.948)	0.225
rs6509365	TA	0.07	NC	NC	NC
	TG	15.46	5.56 (4.45–6.87)	0.827 (0.595–1.147)	0.213

[a] pY:person-years; [b]P-value were calculated using the Cox's proportional risk model. Associations were considered significant with a value of $P < 0.05$. aHazard ratio: adjusted Hazard ratio. CI95 %: 95 % confidence interval; aHR: were adjusted by age, diabetes mellitus, COPD, active cancer, current smoking, transplant, Glasgow Scale, SOFA, and SAPS3; NC: not calculated.

between our study and all others. Most existing studies with this NLRP3 variant are in Chinese populations. Further validation is needed to clarify this question.

The A/G genotype or carrier G of CARD8 rs6509365 was associated with slower progression to death in our study. Da Silva *et al.* (2019) also showed that A/G genotype and carrier allele G of this variant in the gene CARD8 were significantly more common in healthy volunteers than in sporadic melanoma malignancy patients, suggesting a protective effect of this variant on melanoma development (da Silva et al., 2016). Furthermore, it has already been shown that rs6509365 A > G could reduce CARD8 gene expression (Ko et al., 2009). So, we can hypothesize that genotype of rs6509365 could represent a protective factor in the progression to death, due to the dual role exerted by CARD8 in the NLRP3 inflammasome apoptosis suppression (Pathan et al., 2001).

IFI16 gene is a key DNA sensor that triggers downstream type I interferon (IFN-I) production and antiviral immunity (Li et al., 2019) IFI16 rs1101996 is an intron polymorphism, and after extensive research on associations of this polymorphism with other diseases, we found no data. Therefore, for the first time, we report the association of the A/C genotype of the IFI16 rs1101996 variant with a slow progression to death in individuals with COVID-19.

IL-1β is a potent proinflammatory cytokine crucial for host-defense responses to infection (Lopez-Castejon and Brough, 2011). Our study found that the carrier G allele of the IL1β rs1143634 polymorphism is associated with a slower progression to MVS. Song et al. (Song, 2021) found that the genotype G/A is associated with a decreased risk of gastric cancer and may be a protective factor (Song, 2021). Also, Kotsa *et al.* (2021) found that the genotype G/A predisposes men to lower total fat mass and body mass index (BMI) (Mikhailova and Ivanoshchuk, 2021). Although we did not observe any association in our study with progression to the outcomes with obesity, it is already widely known that obese patients can have worse outcomes with COVID-19 infection, including respiratory failure, needs for mechanical ventilation, and higher mortality (Mikhailova and Ivanoshchuk, 2021; Sanchis-Gomar et al., 2020; Zhou et al., 2019). Based on this we hypothesize that the fact the G/A genotype of the IL1β rs1143634 polymorphism is associated with non-obese individuals may be linked with our finding that carrying the G allele is associated with slower progression to MVS outcome in individuals with COVID-19.

5. Conclusion

The present study reports that genetic polymorphisms of inflammasomes are associated with the progression to the use of MVS or death. Thus, we show that NLRP3 rs10754558 and rs4612666 variants, the CARD8 rs6509365 variant, the IFI16 rs1101996 variant, and the IL1β

rs1143634 variant were associated with a slower/faster progression to the use of MVS or death outcomes. The haplotypes of the NLRP3 gene variants included in this study was also associated with the progression to MVS or death events.

6. Funding

The study was supported by Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro - FAPERJ (Grant number SEI-260003/002689/2020; SEI- 260003/013002/2021 and SEI-260003/019710/2022) and INOVA FIOCRUZ/Fundação Oswaldo Cruz (Grant number 48401996705881). Hugo Perazzo is recipient of FAPERJ (E-26/201.351/2021), Mariza Gonçalves Morgado is recipient of CNPQ (314064/2018-4) and FAPERJ (E-26/201.177/2021), and Beatriz Grinsztejn is recipient of CNPQ (305789/2019-8) and FAPERJ (E-26/202.915/2018). The funding agencies played no role in the design of the study, data collection, analysis, or interpretation, nor in writing the manuscript.

7. Institutional Review Board Statement

The study protocol was approved by the Ethics Committee of the National Institute of Infectology Evandro Chagas (INI)/Oswaldo Cruz Foundation (FIOCRUZ), Rio de Janeiro, Brazil, under approval number CAAE: 32449420.4.1001.5262. All procedures were performed in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national).

8. Informed Consent Statement

All patients in the study were aware of and agreed to participate in the research and signed an informed consent form.

9. Data Availability Statement

The data that support the findings of this study are available upon request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

CRediT authorship contribution statement

Milena Neira-Goulart: Conceptualization, Methodology, Validation, Investigation, Data curation, Writing – original draft. **Nathalia Beatriz Ramos de Sá:** Conceptualization, Methodology, Validation, Investigation, Data curation, Writing – original draft, Supervision, Project administration, Funding acquisition. **Marcelo Ribeiro-Alves:** Methodology, Software, Formal analysis, Data curation, Writing – review & editing. **Hugo Perazzo:** Investigation, Data curation, Writing – review & editing. **Kim Mattos Geraldo:** Investigation, Data curation, Writing – review & editing. **Maria Pia Diniz Ribeiro:** Investigation, Data curation, Writing – review & editing. **Sandra Wagner Cardoso:** Investigation, Data curation, Writing – review & editing. **Beatriz Grinsztejn:** Investigation, Data curation, Writing – review & editing, Funding acquisition. **Valdiléa G. Veloso:** Investigation, Data curation, Writing – review & editing, Funding acquisition. **Larissa Rodrigues Gomes:** Investigation, Methodology, Writing – review & editing, Funding acquisition. **Andressa da Silva Cazote:** Investigation, Methodology, Writing – review & editing, Funding acquisition. **Dalziza Victalina de Almeida:** Investigation, Methodology, Writing – review & editing, Funding acquisition. **Carmem Beatriz Wagner Giacoia-Gripp:** Investigation, Methodology, Writing – review & editing, Funding acquisition. **Fernanda Heloise Cortes:** Investigation, Methodology, Writing – review & editing, Funding acquisition. **Mariza Gonçalves Morgado:** Conceptualization, Methodology, Validation, Data curation, Writing – original draft, Supervision, Project administration, Funding acquisition.

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

Data will be made available on request.

Acknowledgments

The authors are thankful to all patients who agreed to participate in this study and their families, the frontline health care workers of the INI/Fiocruz Hospital, and the RECOVER study team in Rio de Janeiro.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2023.147325.

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Supplementary Files

Table S1: Sociodemographic and clinical features for the individuals included in the outcome of mechanical ventilation using Cox proportional hazard models (N=175).

Features		Outcome (MVS)	[a]pY	Crude Incidence/pY (IC95%)	HR (IC95%)	[b]P-value
Overall		175	20.83	8.4 (7.2-9.74)		
Gender	Male	98	11.45	8.56 (6.95-10.43)	Reference	Reference
	Female	77	9.38	8.21 (6.48-10.26)	0.923 (0.678-1.255)	0.608
Skin Color	Brown	112	12.99	8.62 (7.1-10.37)	Reference	Reference
	White	33	3.81	8.67 (5.97-12.18)	1.061 (0.707-1.593)	0.774
	Other	30	4.03	7.44 (5.02-10.62)	0.815 (0.543-1.224)	0.324
Age	(18–40)	9	3.18	2.83 (1.29-5.37)	Reference	Reference
	(40–60)	52	7.78	6.68 (4.99-8.76)	1.925 (0.948-3.907)	0.070
	(60–80)	89	8.26	10.77 (8.65-13.26)	3.213 (1.618-6.381)	0.001
	(80-90)	15	1.61	9.32 (5.21-15.37)	2.921 (1.278-6.676)	0.011
Schooling	High school	55	6.64	8.28 (6.24-10.78)	Reference	Reference
	University Education	15	2.38	6.29 (3.52-10.37)	0.819 (0.46-1.46)	0.499
	Low Education	74	7.55	9.8 (7.7-12.31)	1.235 (0.86-1.774)	0.252
Diabetes Mellitus	No	111	14.13	7.86 (6.46-9.46)	Reference	Reference
	Yes	64	6.7	9.55 (7.36-12.2)	1.225 (0.892-1.683)	0.210
Coronary Artery Disease	No	172	20.19	8.52 (7.29-9.89)	Reference	Reference
	Yes	3	0.64	4.7 (0.97-13.74)	0.649 (0.207-2.036)	0.459
Systemic arterial hypertension	No	78	10.77	7.24 (5.73-9.04)	Reference	Reference
	Yes	97	10.06	9.64 (7.82-11.76)	1.284 (0.944-1.747)	0.112

COPD	No	155	19.9	7.79 (6.61-9.12)	Reference	Reference
	Yes	20	0.93	21.49 (13.12-33.18)	2.619 (1.637-4.188)	<0.001
HIV	Negative	170	18.82	9.03 (7.73-10.5)	Reference	Reference
	Positive	5	1.91	2.62 (0.85-6.11)	0.411 (0.169-1.003)	0.051
Hepatical Cirrhosis	No	175	20.67	8.47 (7.26-9.82)	Reference	Reference
	Yes	0	0.16	NC	NC	NC
Transplant	No	175	20.79	8.42 (7.22-9.76)	Reference	Reference
	Yes	0	0.04	NC	NC	NC
Active Cancer	No	173	20.74	8.34 (7.14-9.68)	Reference	Reference
	Yes	2	0.08	23.56 (2.85-85.12)	2.527 (0.626-10.198)	0.193
Obesity or previous bariatric surgery	No	137	17.16	7.98 (6.7-9.44)	Reference	Reference
	Yes	38	3.67	10.37 (7.34-14.23)	1.341 (0.93-1.934)	0.116
Current smoking	No	166	19.62	8.46 (7.22-9.85)	Reference	Reference
	Yes	9	1.2	7.47 (3.42-14.18)	0.989 (0.505-1.938)	0.975
Fever	Yes	88	12.49	7.04 (5.65-8.68)	Reference	Reference
	No	87	8.34	10.44 (8.36-12.87)	1.411 (1.04-1.916)	0.027
Cough	Yes	99	14.54	6.81 (5.53-8.29)	Reference	Reference
	No	76	6.29	12.08 (9.52-15.12)	1.509 (1.107-2.056)	0.009
Chest Pain	No	158	19.15	8.25 (7.02-9.64)	Reference	Reference
	Yes	17	1.68	10.1 (5.88-16.17)	1.21 (0.722-2.027)	0.469
Coryza	No	165	18.98	8.69 (7.42-10.13)	Reference	Reference
	Yes	10	1.85	5.4 (2.59-9.94)	0.663 (0.35-1.256)	0.207
Dyspneia	Yes	135	15.69	8.6 (7.21-10.18)	Reference	Reference
	No	40	5.14	7.79 (5.56-10.6)	0.681 (0.465-0.997)	0.048
Odynophagy	No	172	19.93	8.63 (7.39-10.02)	Reference	Reference
	Yes	3	0.9	3.33 (0.69-9.73)	0.379 (0.121-1.189)	0.096

Anosmia	No	157	18.26	8.6 (7.3-10.05)	Reference	Reference
	Yes	18	2.57	7.02 (4.16-11.09)	0.812 (0.491-1.341)	0.416
Loss Of Taste	No	163	18.58	8.77 (7.48-10.23)	Reference	Reference
	Yes	12	2.25	5.35 (2.76-9.34)	0.596 (0.323-1.1)	0.098
Diarrhea	No	165	18.57	8.88 (7.58-10.35)	Reference	Reference
	Yes	10	2.26	4.43 (2.13-8.15)	0.537 (0.283-1.018)	0.057
Abdominal Pain	No	169	20.32	8.32 (7.11-9.67)	Reference	Reference
	Yes	6	0.51	11.78 (4.32-25.65)	1.271 (0.562-2.873)	0.565
Nausea	No	166	19.79	8.39 (7.16-9.77)	Reference	Reference
	Yes	9	1.04	8.65 (3.96-16.42)	1.023 (0.522-2.004)	0.947
Headache	Yes	151	17.36	8.7 (7.37-10.2)	Reference	Reference
	No	24	3.47	6.92 (4.43-10.29)	0.775 (0.499-1.205)	0.257
Myalgia	No	143	15.81	9.05 (7.63-10.66)	Reference	Reference
	Yes	32	5.02	6.37 (4.36-8.99)	0.717 (0.487-1.055)	0.091
Outcome	Discharge	38	14.33	2.65 (1.88-3.64)	Reference	Reference
	Death	137	6.5	21.07 (17.69-24.91)	8.675 (5.984-12.577)	<0.001
Glasgow scale cat	(0,9]	8	0.18	43.61 (18.83-85.93)	Reference	Reference
	(9,13]	19	0.97	19.66 (11.84-30.7)	0.887 (0.378-2.082)	0.783
	(13,15]	106	16.99	6.24 (5.11-7.55)	0.279 (0.136-0.574)	0.001
SOFA cat	(0,7]	121	19.04	6.35 (5.27-7.59)	Reference	Reference
	(7,10]	39	1.46	26.68 (18.97-36.47)	4.085 (2.8-5.961)	<0.001
	(10,24]	13	0.18	71.94 (38.31-123.03)	10.481 (5.704-19.26)	<0.001
SAPS-III cat	(30,46]	52	12.03	4.32 (3.23-5.67)	Reference	Reference
	(46,101]	121	8.66	13.98 (11.6-16.7)	3.134 (2.245-4.374)	<0.001

[a]pY:person-years; [b]P-value were calculated using the Cox's proportional risk model. Associations were considered significant with a value of $P < 0.05$; HR: Hazard ratio; CI95%: 95% confidence interval; NC: not calculated. COPD: Chronic obstructive pulmonary disease. MVS: Mechanical Ventilation Support. SOFA: Sequential Organ Failure

Assessment. SAPS III: Simplified Acute Physiology Score III. Cat: Category of small categories. Glasgow scale cat (0,9]: severe trauma; (9,13]: moderate trauma; (13,15]: mild trauma/normal. SOFA cat (0,7]: corresponded to mortality rate of 37%; (7,10]: corresponded to mortality rate of 60%; (10,24]: corresponded to mortality rate >90%. SAPS3 cat (30,46]: corresponded to mortality of <3%; (46,101]: corresponded to mortality rate of >70%.

Table S2: Sociodemographic and clinical features for the individuals included in the outcome of death using Cox proportional hazard models (N=174).

Features		Outcome (Death)	[a]pY	Crude Incidence/pY (IC95%)	HR (IC95%)	[b]P-value
Overall		174	31.33	5.55 (4.76-6.44)		
Gender	Male	102	16.97	6.01 (4.9-7.3)	Reference	Reference
	Female	72	14.35	5.02 (3.92-6.32)	0.83 (0.602-1.144)	0.256
Skin Color	Brown	105	20.18	5.2 (4.26-6.3)	Reference	Reference
	White	31	5.26	5.89 (4-8.36)	1.082 (0.706-1.658)	0.717
	Other	38	5.88	6.46 (4.57-8.86)	1.217 (0.809-1.83)	0.346
Age	(18–40)	7	3.62	1.93 (0.78-3.98)	Reference	Reference
	(40–60)	37	11.27	3.28 (2.31-4.53)	1.738 (0.773-3.907)	0.181
	(60–80)	92	13.72	6.71 (5.41-8.23)	3.569 (1.652-7.708)	0.001
	(80-90)	21	2.72	7.71 (4.77-11.78)	4.029 (1.707-9.508)	0.001
Schooling	High school	51	9.44	5.4 (4.02-7.1)	Reference	Reference
	University Education	11	3.56	3.09 (1.54-5.52)	0.638 (0.319-1.277)	0.204
	Low Education	76	12.6	6.03 (4.75-7.55)	1.123 (0.767-1.644)	0.550
Diabetes Mellitus	No	109	21.13	5.16 (4.24-6.22)	Reference	Reference
	Yes	65	10.19	6.38 (4.92-8.13)	1.297 (0.939-1.791)	0.115
Coronary Artery Disease	No	169	30.57	5.53 (4.73-6.43)	Reference	Reference
	Yes	5	0.76	6.57 (2.13-15.33)	1.323 (0.541-3.236)	0.540
Systemic arterial hypertension	No	77	15.01	5.13 (4.05-6.41)	Reference	Reference
	Yes	97	16.32	5.94 (4.82-7.25)	1.333 (0.969-1.834)	0.077
COPD	No	156	29.01	5.38 (4.57-6.29)	Reference	Reference

	Yes	18	2.32	7.76 (4.6-12.27)	1.542 (0.941-2.528)	0.086
HIV	Negative	158	29.11	5.43 (4.61-6.34)	Reference	Reference
	Positive	8	2.11	3.78 (1.63-7.46)	0.647 (0.316-1.323)	0.233
Hepatical Cirrhosis	No	172	31.17	5.52 (4.72-6.41)	Reference	Reference
	Yes	2	0.16	12.59 (1.53-45.5)	2.209 (0.544-8.976)	0.268
Transplant	No	173	31.29	5.53 (4.74-6.42)	Reference	Reference
	Yes	1	0.04	28.1 (0.71-156.54)	15.726 (2.131-116.067)	0.007
Active Cancer	No	172	31.23	5.51 (4.72-6.4)	Reference	Reference
	Yes	2	0.1	19.74 (2.39-71.32)	5.033 (1.239-20.444)	0.024
Obesity or previous bariatric surgery	No	143	24.88	5.75 (4.84-6.77)	Reference	Reference
	Yes	31	6.44	4.81 (3.27-6.83)	0.857 (0.572-1.284)	0.454
Current smoking	No	166	28.85	5.75 (4.91-6.7)	Reference	Reference
	Yes	8	2.48	3.23 (1.39-6.35)	0.533 (0.249-1.143)	0.106
Fever	Yes	79	17.42	4.53 (3.59-5.65)	Reference	Reference
	No	95	13.9	6.83 (5.53-8.35)	1.253 (0.915-1.718)	0.160
Cough	Yes	95	20.63	4.6 (3.73-5.63)	Reference	Reference
	No	79	10.69	7.39 (5.85-9.21)	1.292 (0.937-1.78)	0.118
Chest Pain	No	158	28.79	5.49 (4.67-6.41)	Reference	Reference
	Yes	16	2.54	6.3 (3.6-10.23)	1.315 (0.771-2.244)	0.315
Coryza	No	165	29.02	5.69 (4.85-6.62)	Reference	Reference
	Yes	9	2.31	3.9 (1.79-7.41)	0.811 (0.413-1.591)	0.542
Dyspneia	Yes	121	23.8	5.08 (4.22-6.07)	Reference	Reference
	No	53	7.53	7.04 (5.27-9.21)	0.98 (0.674-1.426)	0.918
Odynophagy	No	172	30.32	5.67 (4.86-6.59)	Reference	Reference

	Yes	2	1.01	1.99 (0.24-7.17)	0.416 (0.103-1.685)	0.219
Anosmia	No	160	28.22	5.67 (4.83-6.62)	Reference	Reference
	Yes	14	3.11	4.51 (2.46-7.56)	0.949 (0.547-1.649)	0.854
Loss Of Taste	No	166	28.5	5.82 (4.97-6.78)	Reference	Reference
	Yes	8	2.82	2.83 (1.22-5.58)	0.553 (0.271-1.128)	0.103
Diarrhea	No	165	28.51	5.79 (4.94-6.74)	Reference	Reference
	Yes	9	2.82	3.19 (1.46-6.06)	0.62 (0.316-1.216)	0.164
Abdominal Pain	No	169	30.62	5.52 (4.72-6.42)	Reference	Reference
	Yes	5	0.71	7.05 (2.29-16.46)	1.733 (0.706-4.254)	0.230
Nausea	No	166	29.75	5.58 (4.76-6.5)	Reference	Reference
	Yes	8	1.58	5.06 (2.19-9.98)	1.061 (0.52-2.166)	0.870
Headache	No	156	26.88	5.8 (4.93-6.79)	Reference	Reference
	Yes	18	4.45	4.05 (2.4-6.4)	0.836 (0.511-1.368)	0.476
Myalgia	No	147	24.83	5.92 (5-6.96)	Reference	Reference
	Yes	27	6.49	4.16 (2.74-6.05)	0.803 (0.527-1.222)	0.305
Glasgow scale cat	(0,9]	8	0.94	8.52 (3.68-16.79)	Reference	Reference
	(9,13]	19	1.58	12.05 (7.25-18.81)	1.353 (0.569-3.217)	0.494
	(13,15]	106	22.45	4.72 (3.87-5.71)	0.604 (0.289-1.263)	0.181
SOFA cat	(0,7]	120	25.94	4.63 (3.83-5.53)	Reference	Reference
	(7,10]	41	3.98	10.31 (7.4-13.99)	2.073 (1.406-3.056)	<0.001
	(10,24]	12	1.2	9.96 (5.15-17.4)	2.249 (1.186-4.265)	0.013
SAPS-III cat	(30,46]	44	15.15	2.9 (2.11-3.9)	Reference	Reference
	(46,101]	129	15.98	8.07 (6.74-9.59)	2.673 (1.861-3.841)	<0.001

[a]pY:person-years; [b]P-value were calculated using the Cox's proportional risk model. Associations were considered significant with a value of $P < 0.05$; HR: Hazard ratio; CI95%: 95% confidence interval; NC: not calculated. COPD: Chronic obstructive pulmonary disease. MVS: Mechanical Ventilation Support. SOFA: Sequential Organ Failure Assessment. SAPS III: Simplified Acute Physiology Score III. Cat: Category of small categories. Glasgow scale cat (0,9]:

severe trauma; (9,13]: moderate trauma; (13,15]: mild trauma/normal. SOFA cat (0,7]: corresponded to mortality rate of 37%; (7,10]: corresponded to mortality rate of 60%; (10,24]: corresponded to mortality rate >90%. SAPS3 cat (30,46]: corresponded to mortality of <3%; (46,101]: corresponded to mortality rate of >70%.

Table S3: Characteristics of inflammasome SNPs included in the study.

Genes	SNP ID	Chromosome	Position	Ancestral allele	Variation allele	P-value HWEa
NLRP3	rs10754558	1	247448734	G	C,T	0.341
NLRP3	rs4612666	1	247435768	T	C	1
NLRP3	rs15390193	1	247436999	A	C,T	1
NLRP3	rs3806268	1	247424175	G	A,T	1
NLRP3	rs35829419	1	247425556	C	A,T	0.197
CARD8	rs6509365	19	48240212	A	G	0.906
CARD8	rs2043211	19	48234449	A	T	0.906
AIM2	rs2276405	1	159073406	C	A,T	1
CASP1	rs572687	11	105032992	G	A,T	0.577
IFI16	rs1101996	1	159028236	A	C,T	1
IL-1β	rs1143634	2	112832813	G	A	0.668

SNP: single nucleotide polymorphism; CARD8: Caspase Recruitment Domain-Containing Protein 8; AIM2: Absent In Melanoma 2; IFI16: Interferon Gamma Inducible Protein 16; CASP1: Caspase 1; IL-1β: Interleukin 1 Beta; NLRP3: NLR family Pyrin Domain Containing 3. P-value of the Hardy- Weinberg equilibrium.

Table S4: Analysis of alleles and genotypes of COVID-19 individuals from the beginning of symptoms to the progression to mechanical ventilation outcome using Cox proportional hazard models or time-to-event analyses.

Genes	SNP	Alleles /Genotype	[a]pY	Crude Incidence/pY (CI95%)	Mechanical Ventilation aHR (CI95%)	[b]*P*-value
		C/C	7.88	10.28 (8.16-12.77)	Reference	
		C/G	9.4	7.98 (6.28-10.01)	0.748 (0.51-1.096)	0.136
		G/G	3.55	5.35 (3.22-8.36)	**0.391 (0.2-0.763)**	**0.006**
NLRP3	rs10754558	C	25.16	9.42 (8.26-10.7)	Reference	
		G	16.5	6.85 (5.64-8.23)	**0.66 (0.443-0.983)**	**0.005**
		No carrier C	3.55	5.35 (3.22-8.36)	Reference	
		Carrier C	17.28	9.03 (7.67-10.56)	2.202 (1.16-4.182)	0.16
		C/C	7.74	10.08 (7.97-12.58)	Reference	
		C/T	9.33	7.71 (6.04-9.71)	0.882 (0.581-1.338)	0.554
		T/T	3.76	6.66 (4.31-9.82)	0.899 (0.53-1.525)	0.693
NLRP3	rs4612666	C	24.81	9.19 (8.03-10.46)	Reference	
		T	16.85	7.24 (6.01-8.65)	0.934 (0.647-1.35)	0.607
		No carrier C	3.76	6.66 (4.31-9.82)	Reference	
		Carrier C	17.07	8.79 (7.44-10.31)	1.047 (0.64-1.712)	0.855
		C/C	8.25	9.22 (7.26-11.54)	Reference	
		A/A	2.49	9.62 (6.17-14.32)	0.644 (0.351-1.179)	0.154
		C/A	10.09	7.43 (5.85-9.32)	0.916 (0.622-1.349)	0.657
NLRP3	rs1539019	C	26.58	8.54 (7.46-9.73)	Reference	
		A	15.08	8.16 (6.78-9.73)	0.831 (0.569-1.214)	0.181
		Non carrier C	2.49	9.62 (6.17-14.32)	Reference	
		Carrier C	18.34	8.24 (6.97-9.66)	1.491 (0.835-2.664)	0.177
		G/G	8.27	7.98 (6.17-10.16)	Reference	
NLRP3	rs3806268	A/A	2.36	12.73 (8.59-18.17)	1.06 (0.613-1.833)	0.836

		G/A	10.2	7.74 (6.13-9.65)	0.942 (0.632-1.403)	0.767
		G	26.74	7.89 (6.86-9.03)	Reference	
		A	14.92	9.32 (7.83-11)	1.008 (0.694-1.465)	0.951
		No carrier G	2.36	12.73 (8.59-18.17)	Reference	
		Carrier G	18.47	7.85 (6.62-9.24)	0.914 (0.551-1.516);	0.727
		C/C	19.56	8.54 (7.29-9.93)	Reference	
		A/A	0.02	52.18 (1.32-290.72)	NC	
		C/A	1.25	5.62 (2.26-11.58)	1.221 (0.531-2.808)	0.639
NLRP3	rs35829419	C	40.38	8.45 (7.57-9.39)	Reference	
		A	1.28	7.01 (3.2-13.31)	1.216 (0.384-3.857)	0.643
		No carrier C	0.02	52.18 (1.32-290.72)	Reference	
		Carrier C	20.81	8.36 (7.16-9.7)	NC	
		A/A	10.34	8.22 (6.56-10.16)	Reference	
		A/G	8.4	8.81 (6.92-11.06)	0.865 (0.589-1.27)	0.459
		G/G	2.08	7.68 (4.39-12.47)	0.533 (0.251-1.13)	0.101
CARD8	rs6509365	A	29.09	8.39 (7.37-9.51)	Reference	
		G	12.57	8.43 (6.9-10.2)	0.789 (0.524-1.187)	0.106
		No carrier G	10.34	8.22 (6.56-10.16)	Reference	
		Carrier G	10.49	8.58 (6.9-10.55)	0.797 (0.55-1.154)	0.23
		A/A	20.83	8.4 (7.2-9.74)	Reference	
		A/T	11.71	8.29 (6.72-10.11)	0.937 (0.627-1.4)	0.751
		T/T	7.56	8.86 (6.86-11.25)	0.457 (0.182-1.15)	0.096
CARD8	rs2043211	A	1.56	7.06 (3.52-12.63)	Reference	
		T	30.98	8.43 (7.43-9.51)	0.798 (0.514-1.239)	0.147
		No carrier A	10.68	8.33 (6.69-10.25)	Reference	
		Carrier A	1.56	7.06 (3.52-12.63)	2.134 (0.859-5.304)	0.103
AIM2	rs2276405	C/C	19.78	8.7 (7.45-10.1)	Reference	

		C/T	1.05	2.85 (0.59-8.32)	0.354 (0.087-1.447)	0.148
		C	40.61	8.55 (7.67-9.49)	Reference	
		T	1.05	2.85 (0.59-8.32)	0.364 (0.05-2.625)	0.112
		No carrier T	19.78	8.7 (7.45-10.1)	Reference	
		Carrier T	1.05	2.85 (0.59-8.32)	0.354 (0.087-1.447)	0.148
		G/G	14.27	8.13 (6.72-9.75)	Reference	
		A/A	0.79	6.32 (2.05-14.75)	0.629 (0.22-1.798)	0.387
		G/A	5.77	9.36 (7.03-12.21)	1.225 (0.814-1.844)	0.331
CASP1	rs572687	G	34.31	8.34 (7.4-9.36)	Reference	
		A	7.35	8.7 (6.7-11.11)	1.016 (0.637-1.62)	0.923
		No carrier G	0.79	6.32 (2.05-14.75)	Reference	
		Carrier G	20.04	8.48 (7.26-9.86)	1.638 (0.577-4.652)	0.354
		C/C	9.27	9.06 (7.23-11.22)	Reference	
		A/A	2.52	9.94 (6.43-14.67)	1.163 (0.68-1.99)	0.581
		A/C	9.04	7.3 (5.64-9.29)	0.722 (0.477-1.095)	0.125
IFI16	rs1101996	C	27.58	8.48 (7.43-9.64)	Reference	
		A	14.08	8.24 (6.81-9.88)	0.983 (0.668-1.446)	0.9
		No carrier C	2.52	9.94 (6.43-14.67)	Reference	
		Carrier C	18.31	8.19 (6.93-9.61)	0.731 (0.444-1.202)	0.217
		G/G	14.6	7.4 (6.07-8.93)	Reference	
		A/A	0.33	17.96 (6.59-39.1)	3.213 (1.358-7.603)	0.088
		G/A	5.86	10.24 (7.81-13.18)	1.02 (0.683-1.523)	0.922
IL1β	rs1143634	No carrier G	0.37	18.94 (7.61-39.02)	Reference	
		Carrier G	20.46	8.21 (7.02-9.55)	**0.309 (0.14-0.683)**	**0.004**
		No carrier A	14.63	7.45 (6.12-8.99)	Reference	
		Carrier A	6.2	10.65 (8.24-13.55)	1.113 (0.76-1.63)	0.583

[a]pY:person-years; [b]P-value were calculated using the Cox's proportional risk model. Associations were considered significant with a value of $P < 0.05$. N: number of individuals in each group; aHR: adjusted Hazard ratio; CI95%: 95%

confidence interval; NC: not calculated. Adjusted Hazard ratio were adjusted by age, systemic arterial hypertension, COPD, active cancer, obesity or previous bariatric surgery, HIV, Glasgow Scale Cat, SOFA, and SAPS3. A, T, G, and C = each allele count, irrespective of the genotype. Carrier-A = total of genotypes with the A allele; Carrier-T = total of genotypes with T allele; Carrier-C = total of genotypes with the C allele; Carrier-G = total of genotypes with the G allele; No carrier-A = total of genotypes without the A allele; No carrier-T = total of genotypes without the T allele; No carrier-C = total of genotypes without the C allele; No carrier-G = total of genotypes without the G allele.

Table S5: Progression in weeks until the outcome of mechanical ventilation (MVS) according to the inflammasome SNP, using Kaplan-Meier analyses.

Genes SNP	Genotypes	Records	Events	Median (weeks)	CI95% IL	CI95% SL
NLRP3 rs10754558	C/G	190	75	4.997	3.712	8.566
	C/C	174	65	4.854	3.712	9.565
	G/G	48	17	8.708	3.855	N/D
	No Carrier G	190	75	4.997	3.712	8.566
	Carrier G	222	82	5.568	3.855	9.565
NLRP3 rs4612666	C/T	176	69	5.282	3.569	7.566
	C/C	167	68	4.426	3.712	10.421
	T/T	69	20	9.565	3.712	N/D
	No Carrier T	176	69	5.282	3.569	7.566
	Carrier T	236	88	4.997	3.855	9.565
NLRP3 rs15390193	A/C	182	73	4.997	3.855	8.708
	C/C	175	62	5.282	3.712	9.565
	A/A	55	22	10.421	1.999	N/D
	No Carrier A	182	73	4.997	3.855	8.708
	Carrier A	230	84	5.282	3.712	9.565
NLRP3 rs3806268	A/G	200	82	4.711	3.712	5.710
	G/G	158	53	7.566	5.282	8.708
	A/A	54	22	N/D	1.999	N/D
	No Carrier A	200	82	4.711	3.712	5.710
	Carrier A	212	75	7.566	4.283	8.708
NLRP3 rs35829419	C/C	393	150	4.997	4.283	7.423
	A/C	18	6	8.566	1.428	N/D
	A/A	1	1	0.999	N/D	N/D
	No Carrier A	393	150	4.997	4.283	7.423
	Carrier A	19	7	8.566	1.428	N/D
CARD8 rs6509365	A/A	204	78	4.854	3.712	15.561
	A/G	168	67	4.854	3.569	7.423
	G/G	40	12	8.708	4.997	N/D
	No Carrier G	204	78	4.854	3.712	15.561
	Carrier G	208	79	5.568	4.283	8.566
CARD8 rs2043211	A/A	228	92	4.283	3.569	10.421
	A/T	155	56	5.568	4.426	8.566
	T/T	29	9	8.708	1.999	N/D
	No Carrier T	228	92	4.283	3.569	10.421

	Carrier T	184	65	5.710	4.854	8.566
AIM2	C/C	395	152	5.282	4.283	8.566
	C/T	17	5	7.566	2.141	N/D
rs2276405	No Carrier T	395	152	5.282	4.283	8.566
	Carrier T	17	5	7.566	2.141	N/D
CASP1	G/G	280	113	4.854	3.712	8.566
	A/G	116	39	4.997	4.283	N/D
rs572687	A/A	16	5	N/D	1.428	N/D
	No Carrier A	280	113	4.854	3.712	8.566
	Carrier A	132	44	4.997	4.283	N/D
IFI16	C/C	193	75	5.282	3.569	7.566
	A/C	169	60	5.568	4.283	10.421
rs1101996	AA	50	22	4.711	2.284	8.708
	No Carrier C	193	75	5.282	3.569	7.566
	Carrier C	219	82	4.997	4.283	8.708
	G/G	263	92	8.566	4.997	10.421
IL-1β	G/A	138	59	3.855	2.998	4.854
rs1143634	A/A	10	5	2.284	0.143	N/D
	No Carrier A	263	92	8.566	4.997	10.421
	Carrier A	148	64	3.712	2.998	4.854

CI95%: 95% confidence interval; N/D: not determined; Records: total of individuals; Events: individuals who entered in the outcome; IL: inferior limit; SL: superior limit; A, T, G, and C = each allele count, irrespective of the genotype. Carrier-A = total of genotypes with the A allele; Carrier T = total of genotypes with T allele; Carrier C = total of genotypes with the C allele; Carrier G = total of genotypes with the G allele; No Carrier A = total of genotypes without the A allele; No Carrier T = total of genotypes without the T allele; No Carrier C = total of genotypes without the C allele; No Carrier G = total of genotypes without the G allele.

Table S6: Analyses among NLRP3 and CARD8 inflammasome haplotypes frequencies in Cox proportional hazard models for time to progression to use mechanical ventilation due to COVID-19.

Genes	Haplotypes	[a]pY	Crude Incidence/pY (IC95%)	Mechanical Ventilation aHR (CI95%)	[b]*P*-value
	CTGCC	11.11	8.01 (6.43-9.86)	Reference	
	ACACC	1.75	11.4 (6.96-17.6)	1.413 (0.752-2.656)	0.283
	ACACG	7.59	7.64 (5.8-9.87)	0.74 (0.475-1.152)	0.15
	ACGCC	1.38	10.13 (5.54-16.99)	1.084 (0.469-2.504)	0.849
	ACGCG	1.89	6.33 (3.27-11.06)	0.444 (0.176-1.122)	0.068
	ATGAG	0.02	40.58 (1.03-226.12)	**10.241 (1.375-76.25)**	**< 0.001**
NLRP3	ATGCC	1.45	8.98 (4.78-15.35)	0.914 (0.432-1.936)	0.79
rs1539019	ATGCG	0.48	8.25 (2.25-21.13)	0.511 (0.139-1.885)	0.187
rs4612666	CCACC	4.01	11.21 (8.18-15)	1.119 (0.7-1.79)	0.626
rs3806268	CCACG	1.21	9.94 (5.14-17.36)	0.957 (0.422-2.167)	0.91
rs35829419	CCGAC	0.02	52.18 (1.32-290.72)	NC	
rs10754558	CCGCC	4.71	10.4 (7.69-13.75)	1.404 (0.907-2.172)	0.083
	CCGCG	2	8.02 (4.58-13.02)	0.794 (0.333-1.895)	0.586
	CTACC	0.15	26.09 (7.11-66.8)	2.269 (0.8-6.438)	0.082
	CTACG	0.05	0 (NaN-67.37)	NC	
	CTGAC	0.08	13.04 (0.33-72.68)	NC	
	CTGAG	0.71	7.02 (2.28-16.39)	1.329 (0.461-3.828)	0.558
	CTGCG	2.04	1.96 (0.54-5.03)	**0.206 (0.049-0.856)**	**0.024**
	AA	29.02	8.41 (7.39-9.53)	Reference	
CARD8	AG	1.96	8.66 (5.04-13.87)	0.796 (0.438-1.445)	0.453
rs2043211	TA	0.07	0 (NaN-49.9)	NC	NC
rs6509365	TG	10.61	8.39 (6.74-10.33)	0.787 (0.575-1.076)	0.127

[a]pY:person-years; [b]P-value were calculated using the Cox's proportional risk model. Associations were considered significant with a value of $P < 0.05$. aHazard ratio: adjusted Hazard ratio. CI95%: 95%

confidence interval; aHR: were adjusted by age, systemic arterial hypertension, COPD, active cancer, obesity or previous bariatric surgery, HIV, Glasgow Scale Cat, SOFA, and SAPS3 e; NC: not calculated; NaN: not a number.

Table S7: Progression in weeks until the outcome of death according to the inflammasome SNP, using Kaplan-Meier analyses.

Genes SNP	Genotypes	Records	Events	Median (weeks)	CI95% IL	CI95% SL
NLRP3 rs10754558	C/G	1	1	5.282	4.568	7.566
	C/C	190	75	5.710	4.711	7.281
	G/G	174	65	8.708	3.855	25.268
	No Carrier G	190	75	5.282	4.568	7.566
	Carrier G	222	82	5.853	4.854	8.708
NLRP3 rs4612666	C/T	176	69	5.282	4.711	7.281
	C/C	167	68	5.710	4.283	7.423
	T/T	69	20	9.565	4.283	N/D
	No Carrier T	176	69	5.282	4.711	7.281
	Carrier T	236	88	6.852	4.711	8.708
NLRP3 rs15390193	A/C	182	73	6.567	4.711	7.423
	C/C	175	62	5.710	4.854	8.851
	A/A	55	22	6.995	3.569	10.421
	No Carrier A	182	73	6.567	4.711	7.423
	Carrier A	230	84	5.853	5.139	8.851
NLRP3 rs3806268	A/G	176	69	4.854	4.140	6.852
	G/G	200	82	7.281	5.139	8.851
	A/A	158	53	6.995	4.711	25.268
	No Carrier A	200	82	4.854	4.140	6.852
	Carrier A	212	75	7.281	5.139	8.708
NLRP3 rs35829419	C/C	54	22	5.710	4.854	7.281
	A/C	393	150	8.851	8.851	N/D
	A/A	18	6	1.570	N/D	N/D
	No Carrier A	393	150	5.710	4.854	7.281
	Carrier A	19	7	8.851	2.427	N/D
CARD8 rs6509365	A/A	204	78	5.139	4.711	7.281
	A/G	168	67	6.995	4.283	8.280
	G/G	40	12	8.708	3.569	N/D
	No Carrier G	204	78	5.139	4.711	7.281
	Carrier G	208	79	6.995	4.711	8.708
CARD8 rs2043211	A/A	228	92	5.139	4.568	6.995
	A/T	155	56	6.995	4.711	8.708
	T/T	29	9	8.708	3.426	N/D
	No Carrier T	228	92	5.139	4.568	6.995

	Carrier T	184	65	7.281	4.854	8.708
AIM2	C/C	395	152	5.710	4.854	7.281
	C/T	17	5	5.853	3.855	N/D
rs2276405	No Carrier T	395	152	5.710	4.854	7.281
	Carrier T	17	5	5.853	3.855	N/D
CASP1	G/G	280	113	4.997	4.568	7.566
	A/G	116	39	6.852	5.710	8.280
rs572687	A/A	16	5	5.139	1.999	N/D
	No Carrier A	280	113	4.997	4.568	7.566
	Carrier A	132	44	6.852	5.282	7.423
IFI16	C/C	193	75	5.282	4.711	7.281
	A/C	169	60	8.280	5.139	11.564
rs1101996	AA	50	22	4.711	3.855	8.708
	No Carrier C	193	75	5.282	4.711	7.281
	Carrier C	219	82	6.852	4.711	9.565
	G/G	263	92	6.995	5.710	8.851
IL-1β	G/A	138	59	4.854	3.855	7.281
rs1143634	A/A	10	5	3.855	1.713	N/D
	No Carrier A	263	92	6.995	5.710	8.851
	Carrier A	148	64	4.854	3.855	5.710

CI95%: 95% confidence interval; N/D: not determined; Records: total of individuals; Events: individuals who entered in the outcome; IL: inferior limit; SL: superior limit; A, T, G, and C = each allele count, irrespective of the genotype. Carrier-A = total of genotypes with the A allele; Carrier T = total of genotypes with T allele; Carrier C = total of genotypes with the C allele; Carrier G = total of genotypes with the G allele; No Carrier A = total of genotypes without the A allele; No Carrier T = total of genotypes without the T allele; No Carrier C = total of genotypes without the C allele; No Carrier G = total of genotypes without the G allele.

3.3. DADOS NÃO PUBLICADOS

Este capítulo apresenta resultados que ainda não foram submetidos à publicação, que visam analisar a expressão gênica de *NLRP3* e *CASP1* em pacientes com as formas moderadas e graves/críticas da COVID-19 e avaliar a relação com os respectivos perfis genéticos.

3.3.1 Casuística

Foram incluídos 77 indivíduos maiores de 18 anos com diagnóstico confirmado de infecção pelo SARS-CoV-2 recrutados entre julho de 2020 e 31 de março de 2021. Dentro destes 77 indivíduos, 17 foram classificados como moderados (OMS < 6) e 60 como graves e críticos (OMS ≥ 6). Estes pacientes são um recorte dos 451 pacientes incluídos no Artigo 2, que foram internados com COVID-19 e fazem parte da coorte do estudo RECOVER-SUS (Rede Colaborativa para Geração de Evidências Científicas COVID-19 para o Sistema Único de Saúde), do Instituto Nacional de Infectologia Evandro Chagas da Fundação Oswaldo Cruz (INI/FIOCRUZ). Dentro dos critérios de inclusão estavam ser maior de 18 anos, assinar o termo de consentimento livre e esclarecido e confirmação de infecção por SARS-CoV-2 pelo teste de RT-PCR e como critérios de exclusão a não conformidade com as especificações citadas anteriormente (PERAZZO et al., 2022).

Todos os métodos foram realizados de acordo com as diretrizes e regulamentos relevantes. Os estudos foram aprovados pelo Comitê de Ética local (CAAE: 32449420.4.1001.5262).

3.3.2 Apresentação clínica.

Os pacientes incluídos foram definidos de acordo com a classificação de gravidade da OMS como COVID-19 moderado, grave e crítico nas primeiras 24h de internação (MARSHALL et al., 2020). O grupo moderado (OMS 4-5) incluiu pacientes sintomáticos hospitalizados sem oxigenoterapia ou oxigênio por máscara ou cateter nasal. O grupo grave (OMS 6-8) incluiu pacientes internados com oxigênio por VNI (ventilação não invasiva) ou alto fluxo, intubação e ventilação mecânica, pO2/FiO2 ≥

150 ou SpO2/FiO2 ≥ 200 e ventilação mecânica pO2/FiO2 < 150 (SpO2 /FiO2 < 200) ou vasopressores. O grupo crítico (OMS 9-10) incluiu pacientes com ventilação mecânica pO2/FiO2 < 150 e vasopressores, diálise ou ECMO e pacientes que morreram durante as primeiras 24h.

3.3.3 Análise da expressão gênica

O ensaio quantitativo dos RNAm relacionados aos genes *CASP-1 e NLRP3* foi realizado a partir do PBMC total isolado do sangue de pacientes com as formas moderadas, graves e críticas da COVID-19 no dia 0. A classificação clínica foi feita baseada na classificação de gravidade da OMS (MARSHALL et al., 2020). As amostras foram analisadas levando em consideração a correlação entre os genes alvos, as diferentes classificações clínicas com moderados (OMS < 6) e casos graves e críticos (OMS >= 6) e a correlação com os polimorfismos dos genes *CARD8* (rs2043211 e rs6509365) e *NLPR3* (rs1539019, rs107545558 e 4612666) que tiveram associação na análise genética. Utilizamos o guia MIQE como base para a realização dos experimentos (BUSTIN et al., 2009).

3.3.4 Extração de RNA total.

A extração foi feita a partir de PBMCs criopreservados (contagem de células entre 5.10^6 e 1.10^7) descongelado a 37ºC, posteriormente sendo lavadas com 10mL de meio RPMI com 20% de soro fetal bovino (SFB) e centrifugadas a 400 xg por 8 min. As células foram ressuspensas em 5mL de RPMI com 10% de SFB para contagem e avaliação da recuperação obtida. Após esta etapa as células foram novamente centrifugadas a 400xg por 8 min, prosseguindo assim para a fase de extração. As etapas subsequentes foram realizadas seguindo o protocolo do Mini Kit em colunas *Pure Link RNA Mini Kit (Invitrogen™)* com uma adaptação, incluindo mais uma centrifugação a 2.600 xg por 5 minutos para secagem da sílica, assegurando que nenhum resto de etanol contaminasse as amostras. As amostras foram tratadas com *DNase (Invitrogen™)* durante a etapa de extração para evitar a contaminação por gDNA (DNA genômico). Por fim, foi obtido o volume de 35 uL de RNA, que foram armazenados no freezer a -80°C.

A avaliação da integridade do RNA foi realizada através da plataforma RNA *TapeStation*®, como representado na figura 10 e foram selecionadas para prosseguir para a expressão aquelas com RIN acima de 6.0.

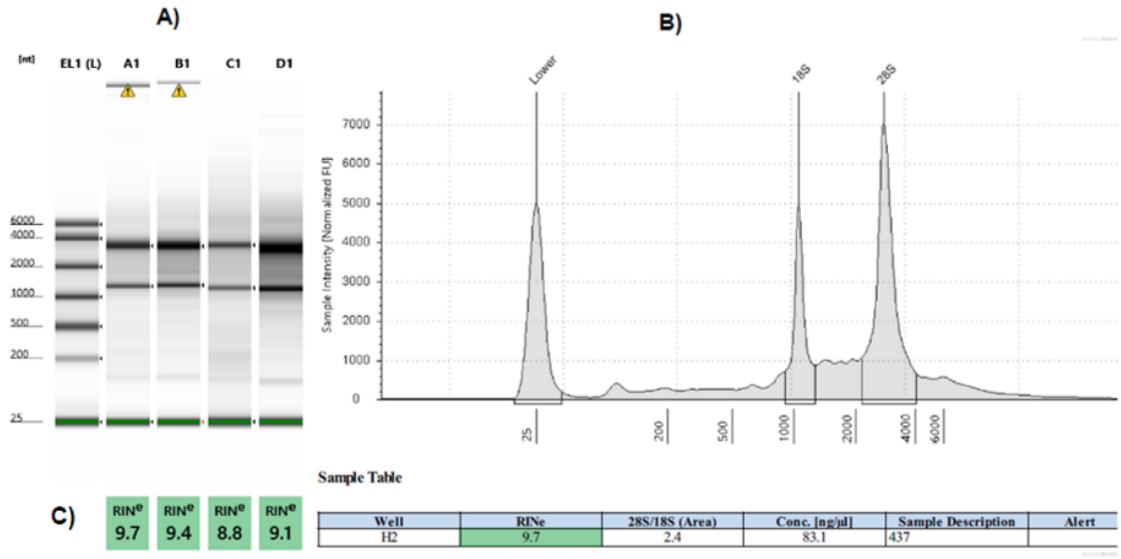

Figura 10: Avaliação da integridade do RNA realizada através da plataforma RNA *TapeStation*®. A: Gel de eletroforese gerado pelo software de avaliação de integridade e embaixo o respectivo RIN (número de integridade do RNA) da amostra. B: Eletroferograma individual de uma das amostras gerado pelo *software*, demonstrando os picos da região 18S e 28S. C: Destaca-se o RIN, relação 28S/18S, quantificação e o identificador de amostras, respectivamente.

Para a obtenção da quantificação dos ácidos nucléicos, utilizamos o Espectrofotômetro *Thermo Scientific™ NanoDrop* 2000 e consideramos a razão 260/280 e 230/280 para avaliar o nível de pureza do material.

3.3.5 Padronização da concentração de RNA

Foi feita a padronização da concentração de RNA de todas as amostras utilizadas antes da reação de transcriptase reversa, assegurando uma concentração de 40ng/µL, que foi o corte que utilizamos para a validação das amostras e padronização das mesmas. Água DEPC foi utilizada para a diluição das amostras que estavam acima dessa concentração pré-determinada.

3.3.6 Reação de Transcriptase Reversa

Foi utilizado o sistema de síntese de cDNA *SuperScript® III (Invitrogen™)* para RT-PCR para a realização desta etapa e assim as amostras foram submetidas a uma primeira etapa de desnaturação a 65°C por 5 minutos, deixando após no gelo por pelo menos 1 minuto para evitar a formação de estruturas secundárias. Em seguida, a reação foi incubada a 25ºC por 5 minutos para anelamento do *random primer*, posteriormente foi incubada novamente a 50º por 60 min e inativada a 70º por 15 min. O cDNA obtido foi armazenado a -20°C.

Amostras de controle No-RT foram feitas para comprovação de que nenhum DNA genômico estaria interferindo na expressão dos resultados. Essas amostras de controle No-RT foram feitas apenas retirando a enzima *SuperScript® III (Invitrogen™)* da reação, seguindo os passos restantes normais do protocolo.

3.3.7 Expressão Gênica através da PCR quantitativa (qPCR)

A técnica PCR em tempo real (qPCR), vem sendo empregada em numerosos estudos de análises de expressão gênica e nossos resultados foram analisados pelo método da quantificação relativa para avaliar a variação da expressão de 2 genes alvo (Tabela 1). O ensaio de qPCR foi realizado utilizando o kit comercial *TaqMan Gene Expression Assays (Applied Biosystems, Foster City, Cal*.) para cada alvo. Os genes endógenos utilizados como normalizadores nos ensaios de expressão gênica foram a *β*-actina (ACTB), o Gliceraldeído-3-fosfato desidrogenase (GAPDH) e o 18S ribosomal RNA (18S).

Tabela 2: Características dos genes incluídos na análise de expressão gênica.

Alvo gênico	Alvos e controles	Nome do gene	Função biológica	Cromossomo
CASP1	Alvo	Caspase-1	Enzima conversora de proteínas precursoras de inflamação	11
NLRP3	Alvo	Família NLR contendo domínio de pirina da proteína 3	Sensor intracelular responsável pela ativação de respostas inflamatórias	1
ACTB	Endógeno	Beta actina	Proteína do citoesqueleto	7
GAPDH	Endógeno	Glyceraldeído-3-fosfato desidrogenase	Envolvido na glicólise	12
18S	Endógeno	18S ribosomal RNA	Fundamental para a síntese protéica celular	18

Fonte: https://www.ncbi.nlm.nih.gov/

Para a realização do ensaio de qPCR, o cDNA foi utilizado em duplicata em um volume final de 20µL (já contendo o cDNA). Foram adicionados a cada poço (*ABI Prism optical plates, Applied Biosystems*), 10 µL de 2X *TaqMan Universal Master Mix* (1X) contendo: *AmpliTaq Gold* 250U, *AmpErase* UNG, 10X *Taqman Buffer* A e dNTPs, e 1µL 20x ensaio TaqMan contendo iniciadores e sondas específicas, em junções exon-exon, que impedem que o ensaio detecte cDNA, para as moléculas de interesse: *CASP-1* e *NLRP3* e para os controles endógenos: *β*-ACTINA, GAPDH e 18S (*Applied*). Todo material foi amplificado, em um total de quatro etapas: 1 ciclo a 50°C por 2 min, para a ativação da enzima *AmpEraser* UNG, 1 ciclo a 95°C por 10min para a ativação da *AmpliTaqGold* DNA polimerase e 40 ciclos contendo etapas de desnaturação a 95°C por 15 segundos, seguidos pelas etapas de hibridização e a extensão a 60°C por 1 min.

As amostras de controle No-RT foram incluídas nas placas realizadas em todos os alvos e endógenos, assim como um controle negativo. Um outro controle realizado foi um pool de cDNA constituído pela mistura de vários cDNAs produzidos a partir do RNA das próprias amostras incluídas no estudo para um controle da técnica empregada, eliminando mais um possível viés.

As amplificações foram realizadas no equipamento 7500 *Real-Time PCR System (Life Technologies/EUA).*

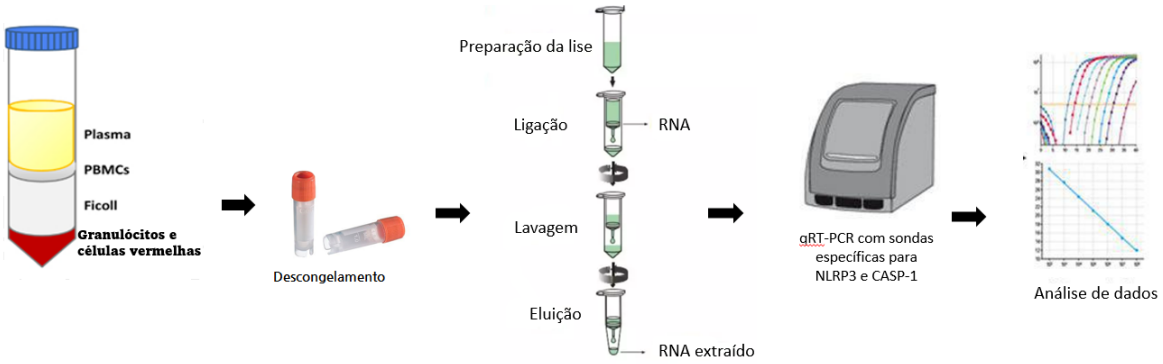

Figura 11: Ilustração da metodologia aplicada. A figura descreve a metodologia realizada para os resultados da expressão. Extração do PBMC, descongelamento dos *vials* criopreservados, extração do RNA, realização do ensaio RT-PCR e análise dos dados obtidos.

3.3.8 Análise estatística

Na avaliação das características sociodemográficas, clínicas e laboratoriais entre os diferentes grupos moderado e grave/crítico, para as variáveis nominais categóricas, foram utilizados testes chi-quadrados na avaliação das frequências entre os diferentes grupos para avaliar a hipótese de independência entre esses e essas variáveis de contingência. Comparações de médias entre os grupos de interesse, ou entre esses divididos pelo perfil de carreamento do alelo de menor frequência (MAF) dos SNPs avaliados, foram realizadas por contrastes obtidos de modelos lineares multivariados inferidos pelo método ordinário de mínimos quadrados, estimados entre os valores marginais médios obtidos desses modelos. Para o segundo caso, dado o número de comparações múltiplas, os valores de P foram corrigidos pelo método *Tukey Honest Significant Difference (HSD)*. As variáveis de confusão incluídas nos modelos multi-variados foram selecionadas para eliminar o viés amostral (DE SÁ et al., 2022b). Transformações logarítmicas (base 2) foram utilizadas para normalizar os níveis de expressão relativa gênica. Coeficiente de correlação de *Pearson* foram calculadas entre essas variáveis. Todas as análises estatísticas foram realizadas no *software* R v. 4.2.0. Para quantificação relativa de expressão, dados de acúmulo de fluorescência das reações de PCR em tempo real de cada amostra foram usados no ajuste de curvas sigmóides de quatro parâmetros de (ciclo × Rn) para representar cada curva de amplificação usando a biblioteca qPCR para o pacote estatístico R versão 4.0.2. O ciclo de quantificação (Cq) foi determinado para cada amplificação pelo máximo da segunda derivada da curva sigmóide ajustada. A eficiência de cada reação de amplificação foi calculada como a razão entre a fluorescência (Rn) do ciclo de quantificação (Cq) e a fluorescência do ciclo imediatamente anterior a essa (Cq-1). Para cada gene, a eficiência foi estimada pela média de todas as eficiências de cada reação de amplificação para aquele gene. Uma vez que tivemos a eficiência média dos genes alvo (ET) e de normalização ou *housekeeping* (EH) com os ciclos de quantificação (CqT e CqH) para todas as reações de amplificação, usamos a seguinte equação para calcular os valores de expressão normalizados (Rq) de cada gene alvo para cada amostra: $Rq = ET^{\Delta CqT}/EH^{\Delta CqH}$, onde o denominador é calculado pela média geométrica dos valores indicados entre o conjunto de genes de normalização.

3.3.9 Características clínicas e sociodemográficas

Um total de 77 indivíduos foram incluídos no estudo, dentro destes 17 eram do grupo moderado e 60 do grupo grave/crítico, suas características sociodemográficas e clínicas estão descritas na Tabela 3.

No geral, a maioria dos participantes foram do sexo masculino (57,1%) e a idade média de 60 anos (IQR=17,68). Dentro do grupo moderado a média de idade foi 67 anos (IQR=14,86) e do grupo grave/crítico 57 anos (IQR=19,24). Dos 77 indivíduos incluídos, 70 (90,9%) precisaram de alguma oxigenação complementar ou utilização de suporte ventilatório, dentro do grupo moderado 16 (94,1%) pacientes precisaram de oxigenação e no grupo grave/crítico 54 (90%). O sintoma mais apresentado foi a tosse, com 53 indivíduos (68,8%). Apenas a diferença na média de idade demonstrou ser significativa entre os grupos.

Tabela 3: Análise descritiva das características clínicas e sociodemográficas de acordo com o perfil clínico moderado e grave/crítico.

Características		Total (N=77)	Moderado (N=17)	Grave/crítico (N=60)	P-valor
Gênero; n (%)	Homem	44 (57,1%)	10 (58,8%)	34 (56,7%)	1
	Mulher	33 (42,9%)	7 (41,2%)	26 (43,3%)	
Cor da pele; n (%)	Pardo	50 (64,9%)	13 (76,5%)	37 (61,7%)	NC
	Branco	12 (15,6%)	1 (5,9%)	11 (18,3%)	
	Negro	7 (9,1%)	3 (17,6%)	4 (6,7%)	
	Amarelo	2 (2,6%)	0 (0%)	2 (3,3%)	
Escolaridade; n (%)	Ensino médio	25 (32,5%)	5 (29,4%)	20 (33,3%)	0,947
	Ensino fundamental	23 (29,9%)	6 (35,3%)	17 (28,3%)	
	Ensino superior	3 (3,9%)	0 (0%)	3 (5%)	
	Ensino fundamental incompleto	17 (22,1%)	4 (23,5%)	13 (21,7%)	
	Analfabeto	5 (6,5%)	1 (5,9%)	4 (6,7%)	
HAS; n (%)	Não	36 (46,8%)	7 (41,2%)	29 (48,3%)	0,805
	Sim	41 (53,2%)	10 (58,8%)	31 (51,7%)	
DM; n (%)	Não	48 (62,3%)	12 (70,6%)	36 (60%)	0,609
	Sim	29 (37,7%)	5 (29,4%)	24 (40%)	
DPOC; n (%)	Não	75 (97,4%)	16 (94,1%)	59 (98,3%)	0,92
	Sim	2 (2,6%)	1 (5,9%)	1 (1,7%)	
AVC prévio; n (%)	Não	73 (94,8%)	15 (88,2%)	58 (96,7%)	0,445
	Sim	4 (5,2%)	2 (11,8%)	2 (3,3%)	

Insuficiência cardíaca; n (%)	Não	74 (96,1%)	15 (88,2%)	59 (98,3%)	0,234
	Sim	3 (3,9%)	2 (11,8%)	1 (1,7%)	
Doença arterial coronariana; n (%)	Não	76 (98,7%)	16 (94,1%)	60 (100%)	0,498
	Sim	1 (1,3%)	1 (5,9%)	0 (0%)	
Obesidade ou cirurgia bariátrica prévia; n (%)	Não	64 (83,1%)	13 (76,5%)	51 (85%)	0,644
	Sim	13 (16,9%)	4 (23,5%)	9 (15%)	
Fumante; n (%)	Não	70 (90,9%)	16 (94,1%)	54 (90%)	0,965
	Sim	7 (9,1%)	1 (5,9%)	6 (10%)	
Cirrose hepática; n (%)	Não	76 (98,7%)	16 (94,1%)	60 (100%)	0,498
	Sim	1 (1,3%)	1 (5,9%)	0 (0%)	
Outras comorbidades; n (%)	Não	58 (75,3%)	12 (70,6%)	46 (76,7%)	0,846
	Sim	19 (24,7%)	5 (29,4%)	14 (23,3%)	
Oxigênio suplementar ou suporte ventilatório; n (%)	Sim	70 (90,9%)	16 (94,1%)	54 (90%)	0,965
	Não	7 (9,1%)	1 (5,9%)	6 (10%)	
Febre; n (%)	Sim	40 (51,9%)	7 (41,2%)	33 (55%)	0,464
	Não	37 (48,1%)	10 (58,8%)	27 (45%)	
Saturação abaixo de 95%; n (%)	Não	37 (48,1%)	11 (64,7%)	26 (43,3%)	0,2
	Sim	40 (51,9%)	6 (35,3%)	34 (56,7%)	
Tosse; n (%)	Sim	53 (68,8%)	12 (70,6%)	41 (68,3%)	1
	Não	24 (31,2%)	5 (29,4%)	19 (31,7%)	
Dor no peito; n (%)	Não	64 (83,1%)	15 (88,2%)	49 (81,7%)	0,786
	Sim	13 (16,9%)	2 (11,8%)	11 (18,3%)	
Anosmia; n (%)	Não	67 (87%)	16 (94,1%)	51 (85%)	0,563
	Sim	10 (13%)	1 (5,9%)	9 (15%)	
Perda do paladar; n (%)	Não	67 (87%)	17 (100%)	50 (83,3%)	0,163
	Sim	10 (13%)	0 (0%)	10 (16,7%)	
Diarréia; n (%)	Não	69 (89,6%)	14 (82,4%)	55 (91,7%)	0,509
	Sim	8 (10,4%)	3 (17,6%)	5 (8,3%)	
Dor abdominal; n (%)	Não	74 (96,1%)	17 (100%)	57 (95%)	0,818
	Sim	3 (3,9%)	0 (0%)	3 (5%)	
Náusea; n (%)	Não	74 (96,1%)	15 (88,2%)	59 (98,3%)	0,234
	Sim	3 (3,9%)	2 (11,8%)	1 (1,7%)	
Dor de cabeça; n (%)	Não	66 (85,7%)	16 (94,1%)	50 (83,3%)	0,466
	Sim	11 (14,3%)	1 (5,9%)	10 (16,7%)	
Mialgia; n (%)	Não	65 (84,4%)	16 (94,1%)	49 (81,7%)	0,384
	Sim	12 (15,6%)	1 (5,9%)	11 (18,3%)	
Idade; n (%)		60,26 (IQR=17,68)	67,81 (IQR=14,86)	57,22 (IQR=19,24)	**0,025**

Faixa etária; n (%)	(18,40]	3 (3,9%)	0 (0%)	3 (5%)	0,33
	(40,60]	34 (44,7%)	5 (31,2%)	29 (48,3%)	
	(60,80]	37 (48,7%)	10 (62,5%)	27 (45%)	
	(80,90]	2 (2,6%)	1 (6,2%)	1 (1,7%)	

Foram utilizados testes chi-quadrados na avaliação das frequências absolutas (relativas) entre os diferentes grupos para avaliar a hipótese de independência entre esses e essas variáveis de contingência. n: número de indivíduos em cada grupo. NC: não calculado. DM: diabetes mellitus, DPOC: doença pulmonar obstrutiva crônica ?,

3.3.10. Análise dos resultados de expressão gênica

A seguir, avaliamos a expressão dos genes alvo *NLRP3* e *CASP-1* frente às diferentes classificações clínicas (moderado *vs* grave/crítico). Como apresentado na Figura 12, tanto a expressão do gene *CASP-1* (LogFC=-0,098; P= 0,664), como do *NLRP3* (LogFC= 0,0206; P= 0,266) não apresentaram diferenças significativas entre os grupos.

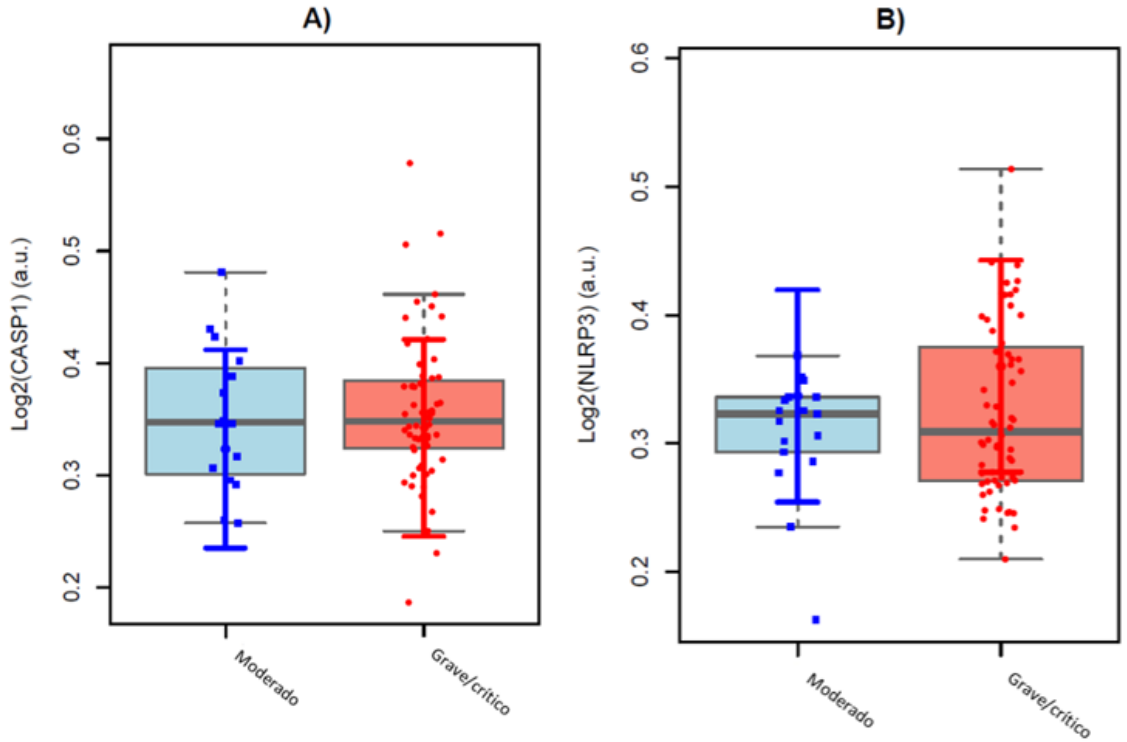

Figura 12: Expressão diferencial dos genes *NLRP3* e *CASP-1* entre os grupos moderado e grave/crítico. Em azul temos a representação amostral (*boxplot + strip-plot*) dos níveis de expressão relativa do grupo moderado e, em vermelho do grupo grave/crítico para (A) *CASP-1* e (B) *NLRP3*. Os níveis de expressão relativa foram log-transformados (base 2). Os strip-plots apresentam os valores amostrais, com distribuição apresentada na forma de *box-plots*, onde as caixas de cores esmaecidas representam o IQR e as barras cinzas horizontais centrais representam as medianas amostrais. Contrastes/diferenças foram estimadas entre os valores marginais médios obtidos de modelos lineares multi-variados inferidos pelo método ordinário de mínimos quadrados onde idade, gênero, cor da pele, escolaridade, hipertensão arterial sistêmica, diabetes mellitus, doença arterial coronariana, obesidade ou cirurgia bariátrica prévia e outras comorbidades foram consideradas como variáveis de confusão para eliminar o viés amostral. Linhas linhas verticais coloridas representam os

intervalos de confiança de 95% (IC 95%) dos valores marginais médios estimados a partir dos modelos ajustados (círculo colorido central). Foram consideradas significativas as expressões com um P-valor < 0.05. A.U.= unidade arbitrária.

Analisamos a expressão dos genes alvo de acordo com as diferentes classificações clínicas associados à presença ou ausência de carreadores MAF (alelo de menor frequência) dos diferentes polimorfismos incluídos no estudo (rs57268, rs1101996, rs1143634, rs1539019, rs2043211, rs3806268, rs4612666, rs6509365, rs10754558) (DE SÁ et al., 2022b). Dentre estes, apenas os indivíduos carreadores do polimorfismo rs10754558 *NLRP3* apresentaram uma tendência de significância na expressão da *CASP-1* entre os grupos clínicos estudados. Na Tabela 4, observamos uma tendência de diminuição da expressão de *CASP-1* (LogFC= -0,07866; P=0,080) no grupo moderado frente ao grupo grave/crítico, ambos carreadores do MAF (alelo G) do referido polimorfismo (Figura 13). Essa tendência foi também observada quando comparamos a expressão da *CASP-1* entre indivíduos não carreadores do MAF do polimorfismo do rs10754558 do gene *NLRP3* do grupo grave/crítico obtivemos uma diminuição da expressão da *CASP-1* (LogFC= -0,04590; P=0,091). No entanto, todas as comparações não deram diferenças significativas.

Tabela 4: Comparações de médias dos níveis de expressão relativa log-transformados (base 2) entre os grupos de interesse divididos pelo perfil de carreamento do MAF dos SNPs avaliados.

Alvo gênico	Carreadores do SNP rs10754558 do *NLRP3* de acordo com o perfil clínico	LogFC (base 2)	EP	P-valor
CASP-1	(Não Carreador/ Moderado) *vs* (Carreador/ Moderado)	0,07895	0,037	0,164
	(Não carreador Moderado) *vs* (Não Carreador Grave/Crítico)	0,04619	0,028	0,359
	(Não Carreador Moderado) *vs* (Carreador Grave/Crítico)	0,00029	0,027	1
	(Carreador Moderado) *vs* (Não Carreador Grave/Crítico)	-0,03276	0,032	0,738
	(Carreador Moderado) *vs* (Carreador Grave/Crítico)	-0,07866	0,032	0,080
	(Não Carreador Grave/Crítico) *vs* (Carreador Grave/Crítico)	-0,04590	0,019	0,091

Contrastes foram obtidos de modelos lineares multivariados inferidos pelo método ordinário de mínimos quadrados, estimados entre os valores marginais médios obtidos desses modelos, onde idade, gênero, cor da pele, escolaridade, hipertensão arterial sistêmica, diabetes mellitus, doença arterial coronariana, obesidade ou cirurgia bariátrica prévia e outras comorbidades foram consideradas como variáveis de confundimento para eliminar o viés amostral. Valores de P foram corrigidos pelo método *Tukey Honest*

Significant Difference (HSD). Foram consideradas significativas as expressões com um P-valor ajustado < 0.05, como tendência foi aceito um P-valor < 0.1. EP: erro padrão; LogFC: Log de *Fold Change.*

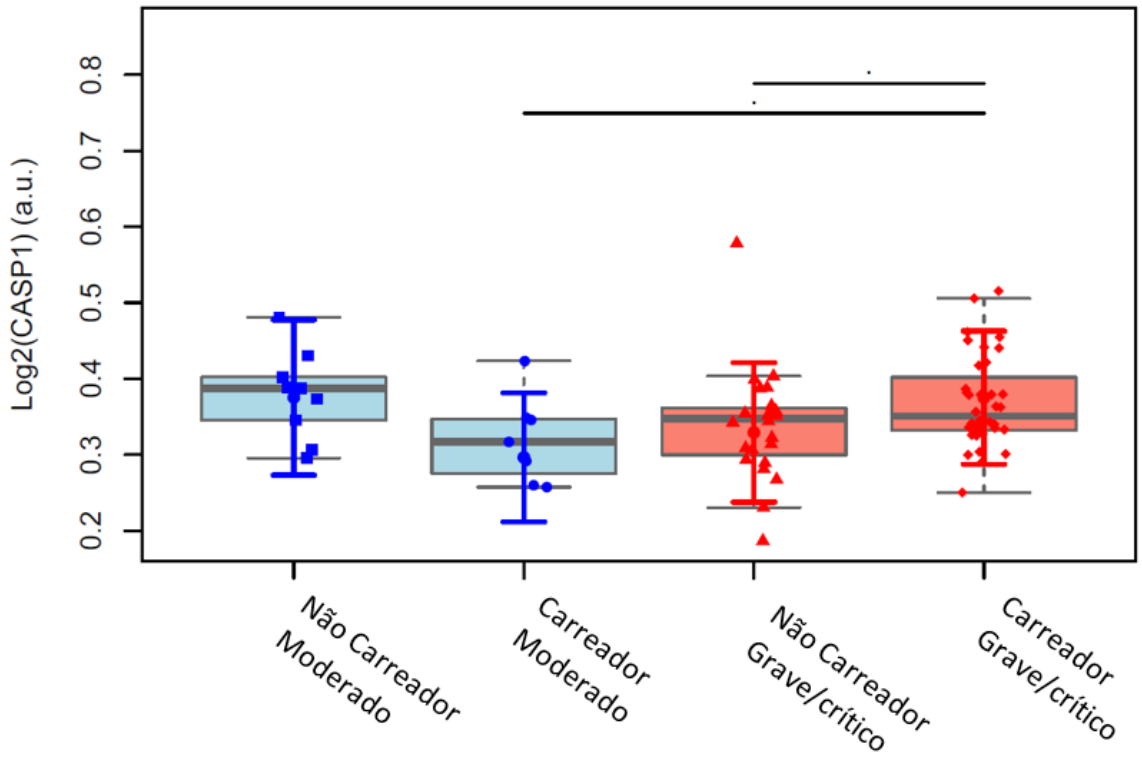

Figura 13: Expressão diferencial dos genes *NLRP3* e *CASP-1* entre os grupos moderado e grave/crítico divididos pelo perfil de carreamento do MAF do polimorfismo rs10754558 do gene *NLRP3*. Em azul temos a representação amostral (*boxplot* + *strip-plot*) dos níveis de expressão relativa do grupo Moderado e, em vermelho do grupo grave/crítico. Os níveis de expressão relativa foram log-transformados (base 2). Os *strip-plots* apresentam os valores amostrais, com distribuição apresentada na forma de *box-plots*, onde as caixas de cores esmaecidas representam o IQR e as barras cinzas horizontais centrais representam as medianas amostrais. Contrastes/diferenças foram estimadas par-a-par entre os valores marginais médios obtidos de modelos lineares multi-variados inferidos pelo método ordinário de mínimos quadrados onde idade, gênero, cor da pele, escolaridade, hipertensão arterial sistêmica, diabetes mellitus, doença arterial coronariana, obesidade ou cirurgia bariátrica prévia e outras comorbidades foram consideradas como variáveis de confusão para eliminar o viés amostral. Linhas linhas verticais coloridas representam os intervalos de confiança de 95% (IC 95%) dos valores marginais médios estimados a partir dos modelos ajustados (círculo colorido central). Valores de P foram corrigidos pelo método *Tukey Honest Significant Difference* (HSD). Foram consideradas significativas as expressões com um P-valor ajustado < 0.05, como tendência foi aceito um P-valor < 0.1. A.U.= unidade arbitrária.

A análise de correlação da expressão dos genes *CASP-1* e *NLRP3* com todos os indivíduos incluídos neste estudo, independentemente de seu perfil clínico, apresentou uma correlação positiva fraca (rho= 0,249; P=0,0298), como podemos observar na Figura 14A.

Posteriormente, analisamos a correlação entre *CASP-1* e *NLRP3* dentro do grupo moderado, observamos uma tendência de correlação positiva (rho= 0,492; P=0,063), como demonstrado na Figura 14B. No entanto, não foi observada

correlação quando esta análise foi feita entre os indivíduos do grupo grave/crítico (rho= 0,213; P= 0,103), como observado na Figura 14C.

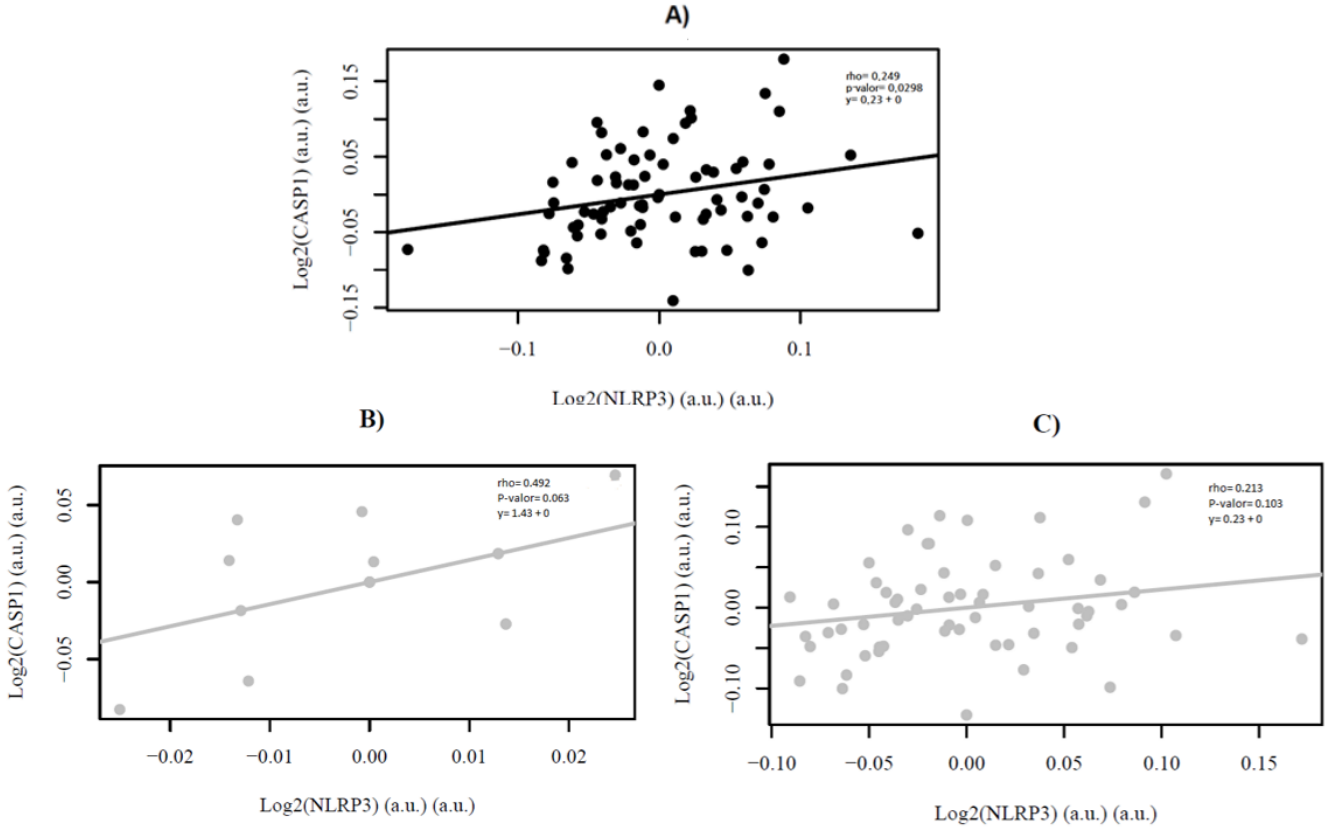

Figura14: Análise de correlação de Pearson entre *CASP-1* e *NLRP3* frente aos perfis clínicos. Correlação de Pearson ajustadas (obtidas entre os resíduos dos modelos onde idade, gênero, cor da pele, escolaridade, hipertensão arterial sistêmica, diabetes mellitus, doença arterial coronariana, obesidade ou cirurgia bariátrica prévia e outras comorbidades foram consideradas como variáveis de confusão para eliminar o viés amostral) entre os níveis de expressão relativa log-transformados (base 2) de CASP-1 e NLRP3, em (A) incluindo todos os pacientes; em (B) incluindo apenas pacientes do grupo moderado; e, em (C) incluindo apenas os pacientes do grupo grave/crítico. Correlações foram consideradas significativas com um P-valor < 0.05, como tendência foi aceito um P-valor < 0.1. A.U. = unidade arbitrária.

4. DISCUSSÃO

Dado o amplo espectro do curso clínico e das complicações da COVID-19, a identificação de fatores de risco poderia prever a gravidade da doença, dentre estes podemos destacar idade, hipertensão, diabetes mellitus e outros fatores já conhecidos (LIU et al., 2020). No entanto, essas condições não explicam completamente a gravidade da doença, portanto, variações genéticas, que influenciam o resultado clínico podem ser consideradas de relevância (FRICKE-GALINDO; FALFÁN-VALENCIA, 2021). Muitos estudos recentes relataram que mediadores inflamatórios como IL-1β, IL-6 e LDH estão intimamente associados a casos graves de COVID-19 (TERPOS et al., 2020). A sinalização do inflamassoma é necessária para a defesa contra a infecção viral, no entanto, ela contribui para a resposta hiperinflamatória e a ativação do inflamassoma NLRP3 provou desempenhar um papel fundamental na gravidade da infecção por SARS-CoV-2 (RODRIGUES et al., 2020). Como resultado, dado o grande número de pessoas afetadas pela COVID-19, uma consideração completa do envolvimento dos inflamassomas no curso da doença pode fornecer uma melhor compreensão da patogênese e ajuda no desenvolvimento terapêutico.

No presente estudo, avaliamos biomarcadores inflamatórios, através da análise molecular de polimorfismos dos inflamassomas e de sua expressão gênica, em pacientes infectados pelo SARS-CoV-2 e sua associação com diferentes desfechos clínicos. Como demostrado no Artigo 1, observamos a associação do polimorfismo rs1539019 do gene NLRP3 e o polimorfismo rs2043211 do gene *CARD8* com proteção contra a severidade da doença em indivíduos infectados com SARS-CoV-2. O artigo 2 reporta que polimorfismos genéticos do inflamassoma estão associados com o tempo de progressão para desfechos graves (uso de ventilação mecânica ou óbito), mostrando que os polimorfismos rs10754558 e rs4612666 do gene *NLRP3*, rs6509365 do gene *CARD8*, rs1101996 do gene IFI16 e rs1143634 do gene *IL-1β* foram associados com a progressão mais lenta/rápida para o uso de suporte ventilatório mecânico (SVM) ou óbito. Os haplótipos das variantes do gene *NLRP3* incluídos neste estudo também foram associadas à progressão para os desfechos graves descritos.

Como esperado, não observamos um mesmo polimorfismo que tenha tido associação em ambos os estudos. No entanto, foi possível observar que o gene

NLRP3 e o gene *CARD8* apresentaram polimorfismos que, mesmo diferentes, foram associados aos desfechos em ambas as análises. Tanto o polimorfismo rs2043211, quanto o rs6509365 do gene *CARD8* obtiveram associações de proteção contra severidade e progressão mais lenta para o desfecho óbito, respectivamente. Já foi observado em estudos anteriores a associação desses polimorfismos com risco/proteção de outras doenças, como na síndrome da resposta inflamatória sistêmica (KO et al., 2009), doenças cardiovasculares (HUANG et al., 2018), melanoma (DA SILVA et al., 2016), dentre outras.

Já foi visto que o polimorfismo rs6509365 pode reduzir a expressão do gene *CARD8*, levando a uma inibição reduzida do inflamassoma NLRP3 e, consequentemente, um aumento da sua ativação. Além disso, outras funções já foram relatadas para o *CARD8*, que incluem a regulação da apoptose e propriedades inibidoras de NF-κB (BOUCHIER-HAYES et al., 2001; PATHAN et al., 2001; RAZMARA et al., 2002). Levando em consideração o exposto, propomos que a hipótese para a progressão mais lenta em indivíduos carreando o polimorfismo rs6509365, poderia ser através da expressão reduzida de *CARD8*. Essa redução, consequentemente, poderia levar a uma ativação maior do inflamassoma NLRP3, permitindo uma resposta imunológica mais eficiente frente ao vírus, ao mesmo tempo que regula e suprime a morte excessiva por apoptose, o que poderia acarretar uma resposta hiperinflamatória.

O polimorfismo rs2043211 por sua vez introduz um códon de parada (Cys10Stop), que resulta em uma expressão truncada do CARD8, tornando a regulação da inibição da atividade do NF-κB e da CASP-1 inviável, levando a altos níveis de pró-IL-1β e apoptose (BAGNALL et al., 2008). No entanto, já foi visto que a associação com polimorfismos do gene *NLRP3* previne a produção excessiva de IL-1β e por sua vez altos níveis plasmáticos de IL-1β já foram correlacionados a casos severos de COVID-19 (GIRIJA; SHANKAR; LARSSON, 2020), o que poderia explicar a associação de proteção que obtivemos.

O polimorfismo rs1539019, rs10754558 e o rs4612666 do gene *NLRP3* apresentaram genótipos, alelos ou carreadores envolvidos na proteção contra severidade ou progressão mais lenta para desfechos graves da COVID-19. Seus papéis individualmente já foram descritos e discutidos nos Artigos 1 e 2. No artigo 2 observamos que carrear o alelo G ou o genótipo G/G do polimorfismo rs10754558 do

gene *NLRP3* foi envolvido tanto na progressão mais lenta para o óbito, quanto no uso de ventilação mecânica. Esse resultado é corroborado por outros trabalhos, onde carrear o alelo G deste mesmo polimorfismo foi associado a um papel protetivo contra a infecção pelo HIV (PONTILLO et al., 2012b), assim como este mesmo alelo exibiu um efeito protetor na redução dos riscos de doenças autoimunes, principalmente em populações latino-americanas (WU; WU; LIANG, 2021). O polimorfismo rs10754558 do gene *NLRP3* é um SNP que está envolvido em uma maior estabilidade do mRNA e expressão mais estável do *NLRP3* (SHEN et al., 2019). Já foi visto que mutações na região 3'-UTR, onde o mesmo está localizado, podem alterar a atividade da via do inflamassoma (HITOMI et al., 2009). Por esse motivo, hipotetizamos que esse efeito protetor que encontramos, juntamente com uma progressão mais lenta para óbito, pode ser explicado por uma expressão mais estável do *NLRP3*, regulando a produção de fatores inflamatórios, incluindo IL-1β e IL-18 e resultando em uma resposta imune mais eficaz contra o vírus.

Por outro lado, esse mesmo polimorfismo quando carreando o alelo C exibiu uma progressão mais rápida para o óbito. Um outro estudo evidenciou a associação do polimorfismo rs10754558 do gene *NLRP3* com risco para quadros mais severos da COVID-19, corroborando este nosso achado (MAES et al., 2022). Até onde sabemos estes são os únicos trabalhos a envolveram o polimorfismo rs10754558 do *NLRP3* com a severidade de quadros clínicos na COVID-19.

Em relação as análises de expressão gênica das moléculas *CASP-1* e *NLRP3*, observamos uma tendência de redução da expressão da *CASP-1* em indivíduos carreadores do alelo G do polimorfismo rs10754558 *NLRP3* apresentando a forma moderada da COVID-19, quando comparados aos da forma grave/crítica. Esse resultado está de acordo com o nosso achado de que carrear o alelo G do referido polimorfismo está associado a uma proteção para desfechos graves na COVID-19. A CASP-1 desencadeia a morte celular por piroptose pela clivagem de GSDMD e produção das citocinas IL-1β e IL-18, já tendo sido associada a dano vascular (KAHLENBERG et al., 2014). Foi observado, que pacientes em remissão completa ou parcial de nefrite lúpica, uma doença inflamatória, tiveram uma redução da expressão de *CASP-1* e que tratamentos que suprimiam a expressão de *CASP-1* tiveram uma boa resposta (CAO et al., 2021). Diante do exposto, acreditamos que essa tendência de diminuição de expressão da *CASP-1* em pacientes carreadores do alelo G do

polimorfismo rs10754558 *NLRP3* possa estar contribuindo para casos menos graves da doença.

Uma tendência de associação também foi observada quando comparamos indivíduos não carreadores do MAF do polimorfismo rs10754558 *NLRP3* do grupo grave/crítico frente aos que são carreadores neste mesmo grupo, demonstrando uma diminuição da expressão da *CASP-1*. Já foi observado na literatura a associação do polimorfismo rs10754558 *NLRP3* com o risco de desenvolvimento de artrite reumatóide e um aumento da expressão da CASP-1 nestes indivíduos, quando comparado a indivíduos saudáveis, assim como foi visto no lúpus eritematoso sistêmico em outro estudo (ADDOBBATI et al., 2018; DA CRUZ et al., 2020). Ao nosso conhecimento não foi observada a diminuição da expressão da CASP-1 associada à proteção em qualquer outra doença na literatura. Não foi observado na literatura outros estudos que analisavam alterações na expressão gênica de alguma molécula do inflamassoma frente ao MAF de polimorfismos do próprio inflamassoma. No entanto, nossos resultados não foram estatisticamente significativos, apenas indicaram tendências.

Uma correlação positiva foi observada entre os níveis de expressão de *CASP-1* e *NLRP3*, em todos os indivíduos incluídos no estudo, demonstrando que os níveis de expressão gênica dessas moléculas estão diretamente relacionados entre si. Esse resultado é o esperado, já que a ativação do NLRP3 ocorre quando ele se oligomeriza, levando ao acoplamento da proteína adaptadora ASC, que irá se ligar a pró-caspase-1, sofrendo uma ativação autoproteolítica, que na sua forma ativa será CASP-1 (SCHRODER; TSCHOPP, 2010). Portanto, os mecanismos estão intrínsecos e diretamente relacionados normalmente no organismo. No entanto, na nossa casuística, obtivemos uma correlação positiva fraca.

Quando dividimos o grupo entre moderado e grave/crítico, obtivemos uma tendência de correlação positiva forte entre *CASP-1* e *NLRP3* dentro do grupo moderado. Apesar disso, quando analisamos os níveis de expressão destes genes individualmente em indivíduos deste grupo não observamos resultados significativos. No grupo grave/crítico não observamos nenhuma correlação entre a expressão dos genes avaliados.

Foi feito um levantamento da literatura e até onde sabemos, não existe nenhuma análise de correlação entre os níveis de expressão da *CASP-1* e *NLRP3* para

corroborar os nossos achados. Nossos resultados demonstram uma correlação positiva entre a expressão de *CASP-1* e *NLRP3* em pacientes com COVID-19.

As limitações das análises realizadas para os Artigos 1 e 2 estão incluídas nas respectivas discussões. Em relação à análise de expressão gênica, reconhecemos que a disparidade de tamanho amostral entre os grupos moderado e grave/crítico pode ser um dos fatores limitantes dos resultados obtidos nesta análise. Essa escolha se baseou na classificação clínica dos pacientes no momento de internação. No entanto, os resultados obtidos foram corrigidos na análise estatística de acordo com idade, gênero, cor da pele, escolaridade e algumas comorbidades. Outros critérios como presença de comorbidade, gênero, idade e evolução clínica para alta ou óbito podem ser usados em análises futuras, além de análises avaliando a expressão proteica e regulação pós-transcricional, que acrescentariam e dariam maior robustez aos dados apresentados na expressão gênica. Uma outra limitação seria a dificuldade de acesso a dados referentes ao uso de medicamentos pré-hospitalização, já que não sabemos que tipo de medicação o paciente incluído tomou antes de dar entrada no hospital. Nossas análises são referentes somente ao D0 e todos no primeiro ano da pandemia. Há possibilidade de alguns pacientes estarem sob efeito de corticoides, que são medicamentos que possuem ação durante o curso da infecção (LIU et al., 2020) e que poderiam inibir a expressão desses genes *in vivo,* mascarando uma possível diferença de expressão entre os grupos analisados. No entanto, nossos pacientes são um recorte de um estudo maior chamado RECOVER e nesse estudo os pacientes foram submetidos a utilização de diferentes medicações, inclusive corticoides, desta forma mesmo que amostras de outro momento do paciente fossem utilizadas a medicação entraria como limitação nesse quesito para as análises de expressão gênica.

5. CONCLUSÕES

Diante do exposto, o presente estudo reporta que polimorfismos genéticos dos inflamassomas estão associados com a proteção contra o desenvolvimento de doença severa da COVID-19, assim como está envolvido no tempo de evolução para o uso de suporte ventilatório mecânico ou óbito. Assim, mostramos que a variante rs1539019 do *NLRP3* e a variante rs2043211 do *CARD8* está associada com proteção contra doença severa em indivíduos infectados pelo SARS-CoV-2. Além disso, as variantes rs10754558 e rs4612666 do gene *NLRP3*, a variante rs6509365 do gene *CARD8*, a variante rs1101996 do gene *IFI16* e a variante rs1143634 do gene *IL1β*, foram associadas a uma progressão mais lenta/rápida para os desfechos óbito ou SVM. Os haplótipos das variantes do gene *NLRP3* incluídas no estudo também demonstraram associação tanto para proteção contra doença severa, quanto para tempo para os eventos óbito ou SVM. Chamou a atenção o haplótipo ATGAG, que apresentou um elevado risco para uma progressão mais rápida tanto para o SVM, quanto para óbito.

A expressão gênica de *CASP-1* e *NLRP3* não apresentou nenhuma associação significativa com os desfechos clínicos estudados. No entanto, a correlação da expressão desses genes alvo é positiva em pacientes com COVID-19. Nosso trabalho ressalta a importância da análise de variações genéticas nos genes do inflamassoma como fatores de risco na evolução clínica da COVID-19.

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7. ANEXO

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 20 September 2022
DOI 10.3389/fcimb.2022.962059

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Clinical Microbiology, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 05 June 2022
ACCEPTED 30 August 2022
PUBLISHED 20 September 2022

CITATION
de Sá NBR, de Souza NCS, Neira-Goulart M, Ribeiro-Alves M, Da Silva TP, Pilotto JH, Rolla VC, Giacoia-Gripp CBW, de Oliveira Pinto LM, Scott-Algara D, Morgado MG and Teixeira SLM (2022) Inflammasome genetic variants are associated with tuberculosis, HIV-1 infection, and TB/HIV-immune reconstitution inflammatory syndrome outcomes.
Front. Cell. Infect. Microbiol. 12:962059. doi: 10.3389/fcimb.2022.962059

Inflammasome genetic variants are associated with tuberculosis, HIV-1 infection, and TB/HIV-immune reconstitution inflammatory syndrome outcomes

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Background: Tuberculosis (TB) and AIDS are the leading causes of infectious diseases death worldwide. Here, we investigated the relationship between from single nucleotide polymorphisms (SNPs) of the NLRP3, CARD8, AIM2, CASP-1, IFI16, and IL-1β inflammasome genes, as well as the profiles of secreted proinflammatory cytokines (e.g., IL-1β, IL-18, IL-33, and IL-6) with the TB clinical profiles, TB-HIV coinfection, and IRIS onset.

Methods: The individuals were divided into four groups: TB-HIV group (n=88; 11 of them with IRIS), HIV-1 group (n=20), TB group (n=24) and healthy volunteers (HC) group (n=10), and were followed up at INI/FIOCRUZ and HGNI (Rio de Janeiro/Brazil) from 2006 to 2016. Real-time PCR was used to determine the genotypes of the Single Nucleotide Polymorphism (SNPs), and ELISA was used to measure the plasma cytokine levels. Unconditional logistic regression models were used to perform risk estimations.

Results: A higher risk for extrapulmonary TB was associated with the TT genotype (aOR=6.76; P=0.026) in the NLRP3 rs4612666 Single Nucleotide Polymorphism (SNP) and the C-C-T-G-C haplotype (aOR=4.99; P= 0.017) in the NLRP3 variants. This same Single Nucleotide Polymorphism (SNP) was associated with lower risk against extrapulmonary TB when the carrier allele C (aOR=0.15; P=0.021) was present. Among those with HIV-1 infections, a higher risk for TB onset was associated with the GA genotype (aOR=5.5; P=0.044) in

the IL1-β rs1143634 Single Nucleotide Polymorphism (SNP). In contrast, lower risk against TB onset was associated with the A-G haplotype (aOR=0.17; P= 0.026) in the CARD8 variants. Higher IL-6 and IL-33 levels were observed in individuals with TB. A higher risk for IRIS onset was associated with CD8 counts ≤ 500 cells/mm^3 (aOR=12.32; P=0.010), the presence of extrapulmonary TB (aOR=6.6; P=0.038), and the CT genotype (aOR=61.06; P=0.026) or carrier allele T (aOR=61.06; P=0.026) in the AIM2 rs2276405 Single Nucleotide Polymorphism (SNP), whereas lower risk against IRIS onset was associated with the AT genotype (aOR=0.02; P=0.033) or carrier allele T (aOR=0.02; P=0.029) in the CARD8 rs2043211 Single Nucleotide Polymorphism (SNP) and the T-G haplotype (aOR=0.07; P= 0.033) in the CARD8 variants. No other significant associations were observed.

Conclusions: Our results depict the involvement of genetic polymorphisms of crucial innate immunity genes and proinflammatory cytokines in the clinical outcomes related to TB-HIV coinfection.

KEYWORDS

tuberculosis, HIV-1, TB-HIV/IRIS, inflammasome Single Nucleotide Polymorphism (SNP), proinflammatory cytokines

Introduction

Tuberculosis (TB) and AIDS are the major causes of death from infectious diseases worldwide. In 2020, 10 million TB cases were estimated globally, including 815,000 cases among people living with HIV (PLWH) (World Health Organization, 2020), making TB the most common comorbidity leading to death among PLWH (World Health Organization, 2020). In 2019, 16% of all cases of TB that were reported to the World Health Organization (WHO) were extrapulmonary TB (EPTB) (World Health Organization, 2020). Combined antiretroviral therapy (cART) during TB treatment improves survival by restoring immune functions (Müller et al., 2010). However, treatment with anti-TB drugs followed by cART initiation can lead to a paradoxical immune reconstitution inflammatory syndrome (IRIS) (Shelburne et al., 2005). Current research has established some pathological mechanisms that are related to IRIS development, such as high viral loads, low baseline CD4+ T-cell counts (<50–100 cells/mm3) (Antonelli et al., 2010; Luetkemeyer et al., 2014) with high levels of CD4 activation and replication (Tibúrcio et al., 2021), and short time intervals between TB treatment and cART (French et al., 2004; Chang et al., 2014; Tan et al., 2016). The genetic basis of host susceptibility to infectious diseases has received enormous attention (Fellay et al., 2009; Seaby et al., 2016; Wu et al., 2017). Highly polymorphic class I and II human leukocyte antigens (HLAs), killer-cell immunoglobulin-like receptor (KIR), cytokine genes, and genes involved in inflammation (inflammasome genes) are actively contributing factors that are associated with susceptibility and/or resistance to TB and HIV-1 infection (Kulkarni et al., 2008; Levy, 2009; Martin and Carrington, 2013; Pontillo et al., 2013; De Lima et al., 2016; Naranbhai and Carrington, 2017; Tsiara et al., 2018; de Sá et al., 2020). However, studies linking host genetics to the pathogenesis of IRIS are still scarce (Narendran et al., 2016; de Sá et al., 2020).

Inflammasomes are cytosolic multiprotein oligomers of the innate immune system that are responsible for the activation of inflammatory responses, including toll-like receptors (TLRs) and nod-like receptors (NLRs) that interact with several adaptor proteins, which leads to the activation of caspase-1 and induces the release of the proinflammatory cytokines such as IL-1ß and IL-18 (Rathinam and Fitzgerald, 2016). Different pattern-recognizing receptors (e.g., NLRP1 and NLRP3) can activate inflammasome assembly in response to specific stimuli, which leads to inflammation and the innate immune response (Man and Kanneganti, 2015). Dysregulation of the inflammasome has been associated with susceptibility to TB-HIV coinfection and TB-HIV/IRIS (Lai et al., 2015; Tan et al., 2015; Tan et al., 2016). In this regard, some investigations have found that TB-HIV/IRIS is associated with changes in the expression of cytokines that are related to the inflammasome activation pathway and other proinflammatory cytokines, such as IL-1β, IL-18, IL-33, IL-6, IL-17, IL-22, TNF, and IFN-γ, which suggests a key role in the development of TB-HIV/IRIS

(Tadokera et al., 2011; Conesa-Botella et al., 2012; Tan et al., 2015; Tan et al., 2016; Ravimohan et al., 2018).

Although some studies have already observed the relationships among inflammasome coding genes and inflammasome activation-related cytokines with TB-HIV coinfected individuals, these studies are still scarce, especially for TB-HIV/IRIS individuals (Tan et al., 2016; Marais et al., 2017; Ravimohan et al., 2018; Ravimohan et al., 2020). In a previous study, we evaluated the role of host genetic markers (e.g., HLA-B, HLA-C, and KIR) in the risk and/or protection of TB-HIV coinfection outcomes, including the increased risk for TB-HIV/IRIS (de Sá et al., 2020). Considering the highly inflammatory profiles observed in TB-HIV coinfections, which increase during TB-HIV/IRIS, in the present study, we investigated the distributions of 11 single nucleotide polymorphisms (SNPs) of the major inflammasome pathway genes (e.g., NLRP3, CARD8, AIM2, CASP-1, IFI16, and IL-1β), cytokine levels (e.g., IL-1β, IL-6, IL-18, and IL-33), and their potential influence on the susceptibility to TB and/or HIV-1 as well as on the occurrence of TB-HIV/IRIS.

Materials and methods

Patient enrollment and study design

This study nested two clinical and immunological follow-up studies conducted in the Laboratory of AIDS & Molecular Immunology (IOC/FIOCRUZ) from 2006 to 2016, as previously described (da Silva et al., 2013; da Silva et al., 2017; Giacoia-Gripp et al., 2019). All participants signed an informed consent form, and the local ethics committee approved the studies. The study participants consisted of 142 individuals, who were divided into four groups as follows: individuals with TB and infected with HIV-1 (TB-HIV group, n=88; 11 of them with paradoxical TB-HIV/IRIS); individuals infected with HIV-1 without a diagnosis of TB (HIV-1 group, n=20); individuals with TB and seronegative for HIV-1 infection (TB group, n=24); and healthy controls with neither HIV-1 infection nor TB (HC, group, n=10).

The individuals were enrolled and followed up at the Clinical Research Laboratory on Mycobacteria (LAPCLINTB) of the National Institute of Infectious Diseases Evandro Chagas, Oswaldo Cruz Foundation (INI/FIOCRUZ), Rio de Janeiro, Brazil (2006-2011/2014-2016) and at the Nova Iguaçu General Hospital (HGNI), Rio de Janeiro, Brazil (2014-2016). The details regarding patient eligibility, enrollment, inclusion/exclusion criteria, anti-TB and cART treatments, study design, demographic and clinical data at the study entry visit, and availability of blood samples were previously described (de Sá et al., 2020). All TB-HIV coinfected individuals were investigated for the identification of IRIS development in both clinical centers. All IRIS cases observed in the study were classified as paradoxical, tuberculosis-associated IRIS, described as a worsening of TB signs and symptoms starting after cART initiation during TB treatment, mainly presenting enlargement of lymph nodes and inflammatory signs, not explained by any other diseases or by an adverse effect of drug therapy (Robertson et al., 2006; Meintjes et al., 2008), as previously detailed by our group (Demitto et al., 2019). In general, the IRIS cases included in the present study were self-resolving, or, if necessary, the patients were treated with corticoid-based therapy, such as Prednisone.

Skin color was self-declared following the classification system used by the Brazilian Institute of Geography and Statistics (IBGE) (Instituto Brasileiro de Geografia e Estatística, 2013) (which is an entity linked to the Brazilian Federal Government that is responsible for the official collection of statistical, geographic, cartographic, geodetic, and environmental information in Brazil).

Genomic DNA extraction

DNA was extracted from whole blood using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Nordrhein-Westfalen, Germany) according to the manufacturer's instructions. The DNA concentrations were determined using a Thermo Scientific NanoDrop 2000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and the filtrates containing the isolated DNA were stored at -20°C until genomic analyses.

Single nucleotide polymorphism selection and genotyping

We selected 11 Single Nucleotide Polymorphism (SNPs) in six inflammasome genes by considering the relevance of each gene in the inflammasome pathway: CARD8 (rs2043211, rs6509365); AIM2 (rs2276405); IFI16 (rs1101996); CASP-1 (rs572687); IL-1β (rs1143634); and NLRP3 (rs10754558, rs1539019, rs4612666, rs3806268, and rs35829419). The Single Nucleotide Polymorphism (SNPs) were selected based on previous studies associating polymorphisms in inflammasome genes with HIV, tuberculosis, and HIV-TB (Pontillo et al., 2010; Pontillo et al., 2012; Pontillo et al., 2013). Single Nucleotide Polymorphism (SNP) genotyping was performed using commercially available TaqMan assays (Applied Biosystems/AB and Life Technologies) on the ABI7500 Real-Time platform (Applied Biosystems/AB and Life Technologies). Allelic discrimination was carried out employing Thermo Fisher Connect Software (Waltham, Massachusetts, EUA). The haplotype analyses were conducted by considering the most frequent haplotype of the NLRP3 (C-C-C-G-C haplotype) and CARD8 (AA) genes as the references. Detailed Single Nucleotide Polymorphism (SNP) information is provided in Table S1.

Inflammatory cytokine plasma levels

The plasma concentrations of the proinflammatory cytokines included in the study were measured at study entry (baseline) before anti-TB and cART therapies, as follows: IL-1β/IL-1F2 (DuoSet ELISA Kit, R & D Systems, #DY201); IL-18 (Human Instant ELISA Kit, Thermo Fisher, BMS267INST); and IL-6 and IL-33 (Human Mini ABTS ELISA Development Kit, PeproTech, Inc., Rocky Hill, NJ) according to the manufacturer's instructions. Standard curves were prepared by preparing serial dilutions of the aliquots that corresponded to the cytokine standards supplied by the manufacturers. Determination of the optical densities of samples and standards was performed using the BioTek ELx800TM absorbance microplate reader (BioTek® Instruments Inc., Vermont, USA) at wavelengths of either 405 or 450 nm, according to each protocol.

Statistical analyses

For the descriptions of the patient samples included in the study, according to the sociodemographic, clinical, and laboratory characteristics among the individuals of the four groups, nonparametric Kruskal–Wallis rank-sum tests were used for continuous numerical variables, while Fisher's exact tests were used for comparing the relative frequencies of the different levels of nominal/categorical variables. In the Single Nucleotide Polymorphism (SNP) analyses, the genotype frequencies were determined by direct count. The relative risks were described as adjusted odds ratios (aORs) with 95% CIs estimated through multiple unconditional logistic regression models. The log-transformed (base 10) least-squares mean differences of the plasma levels of cytokines that were measured by ELISA among the groups were estimated by fixed effects multiple linear regression models. The homozygous genotypes of the minor frequency allele (carriers) were compared with other genotypes (noncarriers) to observe better the differences caused by the variations. Adjustments to the confidence levels were made using Sidak's method, and P-value adjustments for multiple comparisons were made using Tukey's method whenever necessary. For both the cytokine serum levels and for the relative risk analysis, we included any clinical phenotypic marker that was associated with different outcomes as confounders in the modeling to eliminate any possible bias. All statistical analyses were performed using R version 4.1.3 (R Core Team, 2022).

Results

Sociodemographic, clinical, and laboratory characteristics

The sociodemographic, clinical, and laboratory characteristics of the 142 individuals included in the present study, which were categorized according to the presence or absence of TB, are listed in Table 1. Among the 88 TB-HIV coinfected individuals, 11 had paradoxical TB-HIV/IRIS. Most of the participants were males (73.9%). The overall proportions of individuals with white or brown skin color were equivalent (39.4% and 40.1%, respectively), which indicated that ethnicity was not dependent on group arrangement, which could influence the genetic analyses discussed here. Regarding educational levels, 43% of the individuals had lower secondary education, and 27.5% had an upper secondary education. No significant differences were observed among the groups (Table 1).

We further analyzed the sociodemographic, clinical, and laboratory characteristics according to the clinical TB presentations of the individuals included in this study [pulmonary (PTB) *vs.* extrapulmonary TB (EPTB)] and according to the occurrence or absence of TB in PLWH. As depicted in Tables S2 and S3, there were no statistically significant differences among the groups in either analysis.

The sociodemographic, clinical, and laboratory characteristics of TB-HIV coinfected individuals with and without IRIS are listed in Table S4. It is noteworthy that TB-HIV individuals with EPTB (ORadj=6.6; P=0.038) or CD8 ≤ 500 cells/mm3 (ORadj=12.32; P=0.010) values presented an increased risk for IRIS.

Alleles, genotypes, and haplotypes of inflammasome genes

The genotype frequencies of the 11 Single Nucleotide Polymorphism (SNPs) analyzed in the present study were in Hardy-Weinberg equilibrium among the groups (Table S1). An unconditional logistic multiple regression model that compared the genotypes, alleles, carriers, or haplotype frequencies of the 11 Single Nucleotide Polymorphism (SNPs) between the TB and without TB groups did not show any statistical significance (data not shown).

Among PLWH with and without TB, the unconditional logistic multiple regression model that compared the genotypes, alleles, carriers, or haplotype frequencies of the 11 Single Nucleotide Polymorphism (SNPs) showed an increased risk for TB onset only for individuals with the G/A genotype (ORadj=5.5; P=0.044) in the IL-1β rs1143634 polymorphism (Table 2). On the other hand, lower risk of TB onset among PLWH was associated with the CARD8 A-G haplotype (ORadj=0.17; P= 0.026) (Table 2). Similar analyses were also conducted according to the different clinical TB presentations (PTB and EPTB) regardless of HIV-1 coinfection (Table 2), and an increased risk for EPTB was associated with carrying the T/T genotype (ORadj=6.76; P=0.026) in the NLPR3 rs4612666 polymorphism or the NLRP3 C-C-T-G-C haplotype (ORadj=4.99; P= 0.017). On the other hand, protection against EPTB was associated with carrier C (ORadj=0.15; P=0.021) in

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TABLE 1 Sociodemographic, clinical, and laboratory data for individuals included in the study categorized according to the presence or absence of TB.

Features	Overall N=142	All the groups		aOR[a] (95%CI)	*P*-value[b]
		With TB N=112	Without TB N=30		
Gender; n (%)					
Male	105 (73.9%)	86 (76.79%)	19 (63.33%)	Reference	Reference
Female	37 (26.1%)	26 (23.21%)	11 (36.67%)	0.53 (0.22-1.3)	0.166
SkinColor[c]; n (%)					
Brown	57 (40.1%)	41 (36.61%)	16 (53.33%)	Reference	Reference
Black	29 (20.4%)	25 (22.32%)	4 (13.33%)	2.45 (0.71-8.43)	0.313
White	56 (39.4%)	46 (41.07%)	10 (33.33%)	1.88 (0.74-4.78)	0.313
Education[d]; n (%)					
Bachelor	9 (6.3%)	6 (5.36%)	3 (10%)	1.11 (0.22-5.67)	1
Upper-secondary	39 (27.5%)	27 (24.11%)	12 (40%)	0.72 (0.28-1.85)	1
Lower-secondary	61 (43%)	46 (41.07%)	15 (50%)	Reference	Reference
Primary	26 (18.3%)	26 (23.21%)	0 (0%)	NC	NC
Unknown	7 (4.9%)	7 (6.25%)	0 (0%)	NC	NC
HIV status; n (%)					
Yes	108 (76.1%)	88 (78.57%)	20 (66.67%)	Reference	Reference
No	34 (24.9%)	24 (21.43%)	10 (33.33%)	1.74 (0.19-15.67)	0.621
CD4 count (cells/μL); n (%)					
≤ 200 cells/μL	87 (63%)	67 (62.04%)	20 (66.67%)	Reference	Reference
> 200 cells/μL	51 (37%)	41 (37.96%)	10 (33.33%)	11.37 (0.86-150.77)	0.065
CD8 count (cells/μL); n (%)					
≤ 500 cells/μL	60 (45.1%)	44 (42.72%)	16 (53.33%)	Reference	Reference
> 500 cells/μL	73 (54.9%)	59 (57.28%)	14 (46.67%)	1.42 (0.61-3.3)	0.418
CD4/CD8 ratio; n (%)					
≤ 1	106 (79.7%)	85 (82.52%)	21 (70%)	Reference	Reference
> 1	27 (20.3%)	18 (17.48%)	9 (30%)	0.31 (0.03-3.01)	0.311

[a]Odds ratios were adjusted by skin color, education, site of tuberculosis, HIV transmission route, and CD8 count. [b]P-values were calculated using the unconditional logistic regression model. Associations were considered significant with a value of $P < 0.05$. [c]Skin color categorization followed the classificatory system employed by the Brazilian Institute of Geography and Statistics (IBGE) (Instituto Brasileiro de Geografia e Estatística, 2013). [d]Classification, according to the International Standard Classification of Education (ISCED) maintained by the United Nations Educational, Scientific and Cultural Organization (UNESCO). N, number of individuals in each group; TB, tuberculosis; %, Frequencies; aOR, adjusted odds ratio; 95% CI, 95% confidence interval. NC, not calculated.

the NLPR3 rs4612666 polymorphism (Table 2). No significant associations were observed for other polymorphisms and outcomes.

TB-HIV/IRIS and inflammasome-related markers

By comparing the TB-HIV coinfected individuals with and without IRIS in relation to the allelic frequencies of the 11 Single Nucleotide Polymorphism (SNPs) analyzed in the present study, an increased risk for IRIS was associated with the C/T genotype (ORadj=61.06; P=0.026) and carrier-T (ORadj=61.06; P=0.026) in the AIM2 rs2276405 polymorphism. Nevertheless, a trend of increased risk for IRIS was also associated with bearing the T allele (ORadj= 1.49; P= 0.050) in the same polymorphism. Otherwise, lower risk IRIS onset was associated with the A/T genotype (ORadj=0.02; P=0.033) or carrier-T (ORadj=0.02; P=0.029) in the CARD8 rs2043211 polymorphism and with the CARD8 T-G haplotype (ORadj=0.07; P= 0.033) (Table 3). No significant associations were observed for the other polymorphisms.

Cytokines and inflammasome-related markers

By comparing the plasma cytokine levels (IL-1β, IL-6, IL-18, and IL-33) among the groups and outcomes, we observed that the plasma levels of IL-6 and IL-33 were higher among individuals with TB than those without TB (P <0.0001 for both comparisons) (Figure 1A). Similarly, higher levels of IL-6 and IL-33 were observed in PLWH with TB than in those without TB (P <0.0001, for both comparisons) (Figure 1B). No differences in the IL-1β and IL-18 levels were observed among

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TABLE 2 Unconditional logistic multiple regression model of risk and protection factors for TB and for distinct TB clinical presentations according to selected inflammasome SNP genetic profiles.

Gene SNP (rs)	Genotypes, alleles and haplotypes	PLWH					Site of TB				
		With TB N=88	Without TB N=24	aOR[a]	95% CI	P-value[b]	PTB N=63	EPTB N=49	aOR[a]	95% CI	P-value[b]
IL-1β rs1143634	G/G	62 (70.45%)	17 (85%)	Ref			43 (68.25%)	35 (71.43%)	Ref		
	A/A	2 (2.27%)	1 (5%)	1.08	0.05-24.34	0.961	1 (1.59%)	1 (2.04%)	1.3	0.08-22.29	0.857
	G/A	24 (27.27%)	2 (10%)	5.5	1.04-29.02	**0.044**	19 (30.16%)	13 (26.53%)	0.64	0.26-1.61	0.346
	G	148 (84.09%)	36 (90%)	Ref		0.094	105 (83.33%)	83 (84.69%)			
	A	28 (15.91%)	4 (10%)	1.14	0.98-1.33		21 (16.67%)	15 (15.31%)	0.94	0.78-1.12	0.490
	Non-Carrier-G	2 (2.27%)	1 (5%)	Ref			1 (1.59%)	1 (2.04%)			
	Carrier-G	86 (97.73%)	19 (95%)	1.28	0.07-23.73	0.870	62 (98.41%)	48 (97.96%)	0.68	0.04-11.57	0.791
	Non-Carrier-A	62 (70.45%)	17 (85%)	Ref			43 (68.25%)	35 (71.43%)			
	Carrier-A	26 (29.55%)	3 (15%)	4.22	0.97-18.42	0.055	20 (31.75%)	14 (28.57%)	0.68	0.28-1.65	0.388
NLPR3 rs4612666	C/C	38 (45.24%)	6 (30%)	Ref			31 (51.67%)	19 (41.3%)	Ref		
	C/T	35 (41.67%)	12 (60%)	0.31	0.09-1.04	0.057	26 (43.33%)	18 (39.13%)	0.98	0.4-2.39	0.965
	T/T	11 (13.1%)	2 (10%)	0.63	0.1-3.94	0.618	3 (5%)	9 (19.57%)	6.76	1.26-36.23	**0.026**
	C	111 (66.07%)	24 (60%)	Ref			88 (73.33%)	56 (60.87%)	Ref		
	T	57 (33.93%)	16 (40%)	0.93	0.83-1.05	0.223	32 (26.67%)	36 (39.13%)	1.15	0.99-1.34	0.061
	Non-Carrier-C	11 (13.1%)	2 (10%)	Ref			3 (5%)	9 (19.57%)	Ref		
	Carrier-C	73 (86.9%)	18 (90%)	0.89	0.16-4.94	0.897	57 (95%)	37 (80.43%)	0.15	0.03-0.75	**0.021**
	Non-Carrier-T	38 (45.24%)	6 (30%)	Ref			31 (51.67%)	19 (41.3%)	Ref		
	Carrier-T	46 (54.76%)	14 (70%)	0.36	0.11-1.14	0.082	29 (48.33%)	27 (58.7%)	1.44	0.63-3.3	0.383
NLRP3 rs10754558 rs1539019 rs4612666 rs3806268 rs35829419	CCTGC	25 (14.88%)	9 (22.5%)	0.58	0.16-2.19	0.423	6 (5.26%)	15 (17.05%)	4.99	1.33-18.71	**0.017**
CARD8 rs2043211 rs6509365	AG	4 (2.27%)	4 (10%)	0.17	0.03-0.81	**0.026**	4 (3.17%)	2 (2.04%)	0.52	0.09-3.05	0.467

[a]Odds ratios were adjusted by skin color, education, site of tuberculosis, HIV transmission route, and CD8 count. [b]P-values were calculated using the unconditional logistic regression model. Associations were considered significant with a value of $P < 0.05$. N, number of individuals in each group; TB, tuberculosis; PLWH, people living with HIV; aOR, adjusted odds ratio; 95% CI, 95% confidence interval; Ref, Reference; PTB, Pulmonary TB; EPTB, Extrapulmonary TB, A, T, G, and C = each allele count, irrespective of the genotype. Carrier-A = total of genotypes with the A allele, Carrier-T = total of genotypes with T allele, Carrier-C = total of genotypes with the C allele, Carrier-G = total of genotypes with the G allele, Non-Carrier-A = total of genotypes without the A allele, Non-Carrier-T = total of genotypes without the T allele, Non-Carrier-C = total of genotypes without the C allele, Non-Carrier-G = total of genotypes without the G allele. Bold indicate statistically significant results.

the analyzed groups. Similar analyses were also conducted according to the different clinical TB presentations (PTB and EPTB), and no statistically significant differences were observed among the groups (Figure 1C). Moreover, analyses of the IL-1β, IL-6, IL-18, and IL-33 plasma levels between individuals with and without IRIS showed that the mean IL-33 plasma levels were slightly higher among individuals with IRIS than among those without IRIS (P=0.073), indicating a trend for associating IL-33

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TABLE 3 Unconditional logistic multiple regression model of risk and protection factors for TB-HIV/IRIS among TB-HIV individuals.

GeneSNP (rs)	Genotypes, alleles and haplotypes	TB-HIV individuals		aOR[a] (CI95%)	*P*-value[b]
		Without IRIS (N =77)	With IRIS (N = 11)		
CARD8 rs2043211	A/A	43 (56.58%)	10 (90.91%)	Reference	
	A/T	25 (32.89%)	1 (9.09%)	0.02 (0-0.73)	**0.033**
	T/T	8 (10.53%)	0 (0%)	NC	NC
	A	111 (73.03%)	21 (95.45%)	Reference	
	T	41 (26.97%)	1 (4.55%)	0.9 (0.81-1)	0.060
	Non Carrier-A	8 (10.53%)	0 (0%)	Reference	
	Carrier-A	68 (89.47%)	11 (100%)	NC	NC
	Non Carrier-T	43 (56.58%)	10 (90.91%)	Reference	
	Carrier-T	33 (43.42%)	1 (9.09%)	0.02 (0-0.67)	**0.029**
CARD8 rs6509365	A/A	40 (51.95%)	9 (81.82%)	Reference	
	A/G	29 (37.66%)	2 (18.18%)	0.13 (0.01-1.21)	0.073
	G/G	8 (10.39%)	0 (0%)	NC	NC
	A	109 (70.78%)	20 (90.91%)	Reference	
	G	45 (29.22%)	2 (9.09%)	0.93 (0.84-1.03)	0.175
	Non Carrier-A	8 (10.39%)	0 (0%)	Reference	
	Carrier-A	69 (89.61%)	11 (100%)	NC	NC
	Non Carrier-G	40 (51.95%)	9 (81.82%)	Reference	
	Carrier-G	37 (48.05%)	2 (18.18%)	0.12 (0.01-1.08)	0.058
AIM2 rs2276405	C/C	72 (97.3%)	9 (90%)	Reference	
	C/T	2 (2.7%)	1 (10%)	61.06 (1.62-2294.92)	**0.026**
	C	146 (98.65%)	19 (95%)	Reference	
	T	2 (1.35%)	1 (5%)	1.49 (1-2.22)	**0.050**
	Non Carrier-C	74 (100%)	74 (100%)	Reference	
	Carrier-C	10 (100%)	10 (100%)	NC	NC
	Non Carrier-T	72 (97.3%)	9 (90%)	Reference	
	Carrier-T	2 (2.7%)	1 (10%)	61.06 (1.62-2294.92)	**0.026**
CARD8 rs2043211 rs6509365	TG	42 (27.27%)	1 (4.55%)	0.07 (0.01-0.81)	**0.033**

[a]Odds ratios were adjusted by skin color, education, site of tuberculosis, HIV transmission route, and CD8 count. [b]P-values were calculated using the unconditional logistic regression model. Associations were considered significant with a value of $P < 0.05$. N, number of individuals in each group; TB, tuberculosis; aOR, adjusted odds ratio; 95% CI, 95% confidence interval. A, T, G, and C = each allele count, irrespective of the genotype. Carrier-A = total of genotypes with the A allele, Carrier-T = total of genotypes with T allele, Carrier-C = total of genotypes with the C allele, Carrier-G = total of genotypes with the G allele, Non-Carrier-A = total of genotypes without the A allele, Non-Carrier-T = total of genotypes without the T allele, Non-Carrier-C = total of genotypes without the C allele, Non-Carrier-G = total of genotypes without the G allele. NC, not calcuated. Bold indicate statistically significant results.

plasma levels with TB-HIV/IRIS (Figure 1D). No statistical significance was observed for the plasma levels of the other cytokines (Figure 1D).

We next explored the relationships among the 11 Single Nucleotide Polymorphism (SNPs) and the plasma levels of the studied cytokines according to the carriers of the minor frequency allele (MFA) in the studied outcomes (with TB *vs.* without TB, PLWH with *vs.* without TB, PTB *vs.* EPTB, and with TB-HIV/IRIS *vs.* without IRIS) (Table 4). By comparing the carriers of the minor frequency allele in individuals with and without TB, CARD8 (rs2043211 and rs6509365), CASP-1 (rs572687), IFI16 (rs1101996), and NLRP3 (rs3806268, rs4612666, rs1539019, and rs10754558) had significant associations with the differences in IL-6 plasma levels; while IFI16 (rs1101996), and NLRP3 (rs3806268, rs1539019, and rs10754558) were significantly associated with differences in IL-33 plasma levels (Table 4). Among PLWH with and without TB, CARD8 (rs2043211 and rs6509365), CASP-1 (rs572687), IFI16 (rs1101996), IL-1β (rs1143634), and NLRP3 (rs3806268, rs4612666, rs1539019, and rs10754558) were significantly associated with differences in IL-6 plasma levels; while CARD8 (rs2043211 and rs6509365), IFI16 (rs1101996), and NLRP3 (rs3806268, rs4612666, rs1539019, and rs10754558) were significantly associated with differences in IL-33 plasma levels (Table 4). Among PTB and EPTB individuals, CARD8 (rs2043211 and rs6509365) was significantly associated with differences in IL-1β plasma levels (Table 4). Among individuals with and without IRIS, no statistically significant association was observed between the plasma cytokine levels and the minor frequency allele (Table S5).

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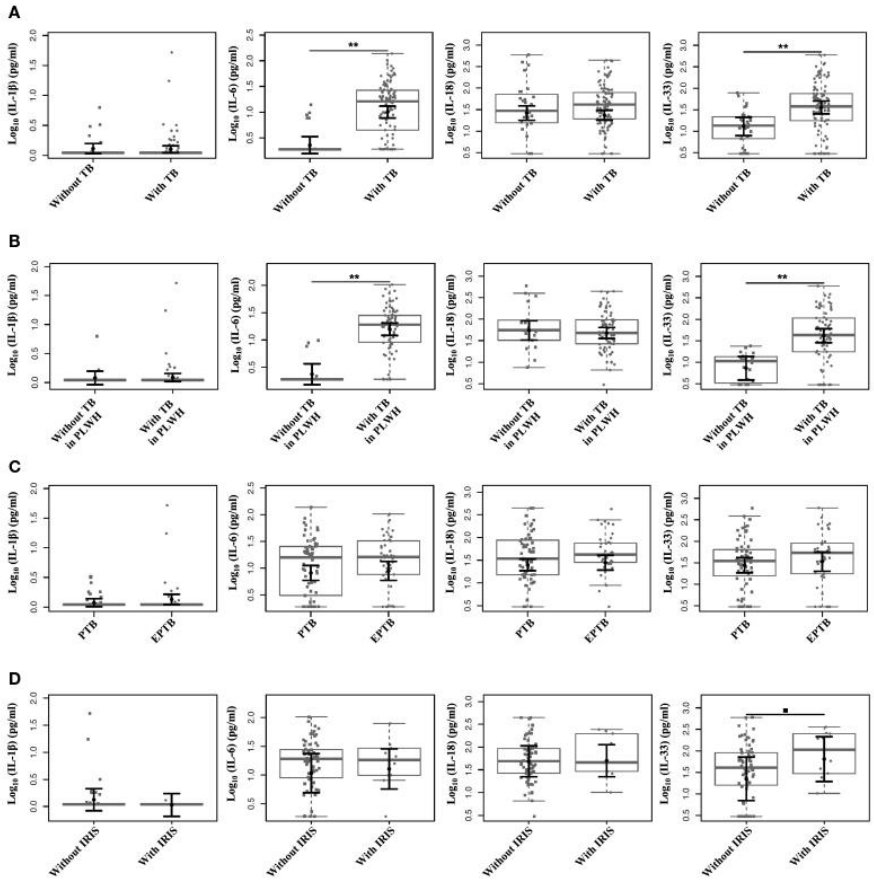

FIGURE 1
Comparison of plasma levels among the individuals included in the study categorized according to the outcomes. Plasma levels of IL-1β, IL-6, IL-18, and IL-33 measured by distinct ELISAs. **(A)** Comparison of plasma cytokine levels according to the presence or absence of TB. **(B)** Comparison of plasma cytokine levels according to the presence or absence of TB among PLWH. **(C)** Comparison of plasma cytokine levels according to different TB clinical presentations (PTB and EPTB). **(D)** Comparison of plasma cytokine levels according to the presence or absence of IRIS. Boxplots show the IQRs and sample medians (central solid gray line). Least-square means of log-transformed (base 10) levels of cytokines were pairwise compared among groups with T tests. Adjustments to the confidence levels were made by Sidak's method, and P value adjustments were made by multiple comparisons using Tukey's method. **P <0.0001; *P=0.073.

Discussion

Innate immunity and inflammation are biological mechanisms with important roles in susceptibility to or protection from HIV infection and/or TB-related outcomes (Salie et al., 2015; Ravimohan et al., 2018; Zhao et al., 2019). Aberrantly high inflammasome activation and its signaling in different cells and tissues lead to several inflammatory pathologies, including IRIS (Chang et al., 2014; Marais et al., 2017). TB-associated IRIS (TB-IRIS) incidence ranges from 4% to 54% in different populations (Bana et al., 2016). In the studies conducted by our group, the incidence of TB-HIV/IRIS was determined to be approximately 12% (Serra et al., 2007). The low incidence of TB-HIV/IRIS may be due to the introduction of antiretroviral therapy for newly diagnosed TB-HIV individuals in Brazil, who still had higher CD4 levels when at the time when they were recruited and included in this study. Beyond the very low CD4 counts (<100/mm3) and short time intervals between the initiation of anti-TB and antiretroviral therapies (Laureillard et al., 2013), the discrepancies in the IRIS frequencies could also be attributed to difficulties in clinical diagnosis (no specificity of symptoms) or differences in the genetic backgrounds among the populations included in the studies (Bourgarit et al., 2006; Wilkinson et al., 2015). Indeed, a previous study conducted by

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TABLE 4 Plasma levels of cytokines according to the evaluated SNPs of the individuals included in the study.

Cytokines	Gene SNP (rs)	Carrier	Mean (CI95%)		P-value	Mean (CI95%)		P-value	Mean (CI95%)		P-value
			With TB	Without TB		With TB among HIV	Without TB among HIV		PTB	EPTB	
IL-1β	CARD8 rs2043211	A	0.142 (0.061 - 0.222)	0.202 (0.077 - 0.327)	0.842	0.151 (0.048 - 0.255)	0.129 (-0.063 - 0.321)	0.997	0.069 (-0.022 - 0.159)	0.319 (0.181 - 0.458)	**0.009**
	CARD8 rs6509365	A	0.139 (0.061 - 0.217)	0.165 (0.055 - 0.274)	0.981	0.141 (0.039 - 0.242)	0.102 (-0.046 - 0.251)	0.975	0.065 (-0.021 - 0.151)	0.301 (0.174 - 0.429)	**0.007**
	CASP-1 rs572687	G	0.055 (-0.039 - 0.149)	0.056 (-0.104 - 0.215)	1.000	0.039 (-0.082 - 0.159)	0.038 (-0.189 - 0.264)	1.000	0.025 (-0.114 - 0.163)	0.048 (-0.078 - 0.173)	0.994
	IFI16 rs1101996	C	0.083 (0.013 - 0.154)	0.081 (-0.032 - 0.194)	1.000	0.058 (-0.03 - 0.147)	-0.009 (-0.165 - 0.147)	0.864	0.06 (-0.025 - 0.145)	0.087 (-0.026 - 0.2)	0.977
	IL-1β rs1143634	G	0.1 (0.009 - 0.19)	0.042 (-0.125 - 0.208)	0.931	0.1 (-0.019 - 0.218)	0.058 (-0.235 - 0.352)	0.994	0.054 (-0.054 - 0.163)	0.176 (0.017 - 0.335)	0.573
	NLRP3 rs3806268	G	0.117 (0.044 - 0.191)	0.132 (0.003 - 0.26)	0.997	0.127 (0.039 - 0.214)	0.153 (-0.021 - 0.326)	0.993	0.052 (-0.039 - 0.143)	0.165 (0.059 - 0.271)	0.314
	NLRP3 rs35829419	C	0.048 (-0.135 - 0.232)	0.117 (-0.202 - 0.435)	0.982	0.06 (-0.238 - 0.357)	0.07 (-0.292 - 0.431)	1.000	0.032 (-0.201 - 0.264)	0.062 (-0.269 - 0.392)	0.999
	NLRP3 rs4612666	C	0.065 (-0.013 - 0.143)	0.091 (-0.016 - 0.198)	0.976	0.039 (-0.052 - 0.129)	0.047 (-0.091 - 0.186)	1.000	0.048 (-0.046 - 0.143)	0.053 (-0.064 - 0.17)	1.000
	NLRP3 rs1539019	C	0.092 (0.015 - 0.17)	0.068 (-0.062 - 0.197)	0.985	0.093 (0.001 - 0.185)	0.012 (-0.148 - 0.173)	0.798	0.073 (-0.029 - 0.174)	0.098 (-0.024 - 0.22)	0.987
	NLRP3 rs10754558	C	0.123 (0.054 - 0.193)	0.042 (-0.088 - 0.172)	0.652	0.109 (0.032 - 0.185)	0.039 (-0.118 - 0.197)	0.86	0.09 (-0.001 - 0.181)	0.158 (0.057 - 0.259)	0.684
IL-6	CARD8 rs2043211	A	0.953 (0.793 - 1.114)	0.353 (0.102 - 0.603)	**<0.001**	1.136 (0.966 - 1.307)	0.342 (0.024 - 0.659)	**<0.001**	0.84 (0.646 - 1.035)	1 (0.703 - 1.298)	0.775
	CARD8 rs6509365	A	0.899 (0.744 - 1.053)	0.385 (0.166 - 0.605)	**0.001**	1.071 (0.905 - 1.237)	0.45 (0.204 - 0.697)	**<0.001**	0.829 (0.641 - 1.018)	0.827 (0.555 - 1.1)	1.000
	CASP-1 rs572687	G	0.946 (0.756 - 1.135)	0.29 (-0.033 - 0.614)	**0.003**	1.15 (0.948 - 1.352)	0.289 (-0.094 - 0.671)	**0.001**	0.615 (0.329 - 0.901)	1.063 (0.803 - 1.322)	0.074
	IFI16 rs1101996	C	1.01 (0.869 - 1.15)	0.357 (0.133 - 0.581)	**<0.001**	1.185 (1.035 - 1.334)	0.354 (0.093 - 0.615)	**<0.001**	0.892 (0.715 - 1.069)	1.023 (0.788 - 1.258)	0.778
	IL-1β rs1143634	G	1.003 (0.827 - 1.178)	0.528 (0.197 - 0.858)	0.067	1.202 (1.012 - 1.392)	0.49 (-0.002 - 0.982)	**0.042**	0.978 (0.757 - 1.199)	0.867 (0.551 - 1.182)	0.934
	NLRP3 rs3806268	G	0.917 (0.774 - 1.061)	0.319 (0.073 - 0.565)	**<0.001**	1.095 (0.954 - 1.236)	0.391 (0.118 - 0.663)	**<0.001**	0.754 (0.571 - 0.938)	0.932 (0.72 - 1.145)	0.516
	NLRP3 rs35829419	C	0.726 (0.36 - 1.091)	0.155 (-0.478 - 0.789)	0.406	0.931 (0.434 - 1.429)	0.339 (-0.264 - 0.941)	0.432	0.578 (0.093 - 1.064)	0.857 (0.169 - 1.546)	0.916
	NLRP3 rs4612666	C	1.01 (0.855 - 1.166)	0.282 (0.07 - 0.494)	**<0.001**	1.226 (1.073 - 1.378)	0.386 (0.155 - 0.617)	**<0.001**	0.985 (0.789 - 1.182)	0.83 (0.591 - 1.069)	0.703
	NLRP3 rs1539019	C	0.913 (0.759 - 1.066)	0.317 (0.067 - 0.568)	**<0.001**	1.153 (1 - 1.307)	0.383 (0.12 - 0.646)	**<0.001**	0.838 (0.63 - 1.047)	0.86 (0.609 - 1.11)	0.999
	NLRP3 rs10754558	C	1.075 (0.939 - 1.211)	0.231 (-0.017 - 0.479)	**<0.001**	1.279 (1.156 - 1.402)	0.316 (0.072 - 0.56)	**<0.001**	1.03 (0.847 - 1.214)	0.987 (0.781 - 1.192)	0.985
IL-18	CARD8 rs2043211	A	1.363 (1.201 - 1.524)	1.333 (1.07 - 1.595)	0.997	1.591 (1.395 - 1.788)	1.684 (1.293 - 2.076)	0.975	1.329 (1.149 - 1.509)	1.588 (1.313 - 1.864)	0.345
	CARD8 rs6509365	A	1.31 (1.155 - 1.465)	1.417 (1.191 - 1.643)	0.857	1.516 (1.33 - 1.703)	1.773 (1.483 - 2.062)	0.467	1.28 (1.109 - 1.45)	1.523 (1.277 - 1.77)	0.303
	CASP-1 rs572687	G	1.333 (1.146 - 1.519)	1.43 (1.112 - 1.748)	0.947	1.69 (1.463 - 1.917)	1.667 (1.241 - 2.094)	1.000	1.303 (1.036 - 1.571)	1.458 (1.215 - 1.701)	0.801
	IFI16 rs1101996	C	1.361 (1.224 - 1.498)	1.413 (1.185 - 1.641)	0.976	1.695 (1.53 - 1.86)	1.636 (1.332 - 1.941)	0.986	1.418 (1.259 - 1.577)	1.381 (1.17 - 1.593)	0.991
	IL-1β rs1143634	G	1.548 (1.379 - 1.718)	1.354 (1.034 - 1.673)	0.716	1.826 (1.615 - 2.036)	1.668 (1.125 - 2.211)	0.95	1.617 (1.423 - 1.811)	1.522 (1.244 - 1.799)	0.939
		G			0.999		1.72 (1.365 - 2.076)	0.998			0.82

(Continued)

TABLE 4 Continued

Cytokines	Gene SNP (rs)	Carrier	Mean (CI95%)		P-value	Mean (CI95%)		P-value	Mean (CI95%)		P-value
			With TB	Without TB		With TB among HIV	Without TB among HIV		PTB	EPTB	
	NLRP3 rs3806268		1.353 (1.21 - 1.496)	1.336 (1.072 - 1.6)		1.684 (1.52 - 1.847)			1.36 (1.188 - 1.533)	1.466 (1.266 - 1.665)	
	NLRP3 rs35829419	C	1.301 (0.939 - 1.664)	1.37 (0.742 - 1.999)	0.998	1.338 (0.787 - 1.889)	1.77 (1.1 - 2.439)	0.751	1.497 (1.059 - 1.936)	1.014 (0.391 - 1.636)	0.6
	NLRP3 rs4612666	C	1.424 (1.273 - 1.576)	1.458 (1.243 - 1.674)	0.993	1.734 (1.565 - 1.903)	1.768 (1.493 - 2.042)	0.997	1.468 (1.289 - 1.648)	1.44 (1.222 - 1.657)	0.996
	NLRP3 rs1539019	C	1.345 (1.193 - 1.498)	1.39 (1.126 - 1.654)	0.989	1.611 (1.441 - 1.782)	1.725 (1.415 - 2.035)	0.91	1.345 (1.155 - 1.536)	1.459 (1.23 - 1.688)	0.846
	NLRP3 rs10754558	C	1.43 (1.294 - 1.565)	1.606 (1.341 - 1.871)	0.602	1.718 (1.577 - 1.859)	1.949 (1.642 - 2.257)	0.531	1.478 (1.308 - 1.648)	1.471 (1.281 - 1.662)	1.000
IL-33	CARD8 rs2043211	A	1.346 (1.142 - 1.55)	1.076 (0.757 - 1.394)	0.476	1.419 (1.172 - 1.665)	0.706 (0.248 - 1.164)	**0.038**	1.13 (0.896 - 1.365)	1.605 (1.246 - 1.964)	0.094
	CARD8 rs6509365	A	1.38 (1.184 - 1.577)	1.024 (0.745 - 1.303)	0.146	1.447 (1.208 - 1.685)	0.796 (0.441 - 1.151)	**0.018**	1.167 (0.941 - 1.393)	1.592 (1.265 - 1.919)	0.105
	CASP-1 rs572687	G	1.616 (1.372 - 1.861)	1.194 (0.777 - 1.61)	0.275	1.635 (1.346 - 1.925)	0.89 (0.344 - 1.437)	0.07	1.431 (1.057 - 1.805)	1.606 (1.267 - 1.946)	0.883
	IFI16 rs1101996	C	1.585 (1.406 - 1.764)	1.075 (0.789 - 1.361)	**0.008**	1.628 (1.415 - 1.84)	0.806 (0.436 - 1.176)	**0.001**	1.517 (1.294 - 1.74)	1.513 (1.217 - 1.808)	1.000
	IL-1β rs1143634	G	1.501 (1.274 - 1.728)	1.266 (0.838 - 1.694)	0.778	1.574 (1.302 - 1.847)	0.85 (0.143 - 1.556)	0.234	1.36 (1.08 - 1.641)	1.6 (1.199 - 2.001)	0.748
	NLRP3 rs3806268	G	1.544 (1.357 - 1.731)	1.035 (0.714 - 1.356)	**0.03**	1.608 (1.399 - 1.816)	0.863 (0.46 - 1.265)	**0.008**	1.35 (1.111 - 1.588)	1.583 (1.306 - 1.859)	0.51
	NLRP3 rs35829419	C	1.472 (0.995 - 1.95)	1.257 (0.429 - 2.085)	0.969	1.56 (0.844 - 2.276)	1.274 (0.406 - 2.141)	0.957	1.41 (0.786 - 2.034)	1.517 (0.632 - 2.403)	0.997
	NLRP3 rs4612666	C	1.514 (1.313 - 1.715)	1.097 (0.824 - 1.371)	0.051	1.576 (1.361 - 1.792)	0.969 (0.642 - 1.295)	**0.013**	1.393 (1.14 - 1.647)	1.475 (1.167 - 1.783)	0.971
	NLRP3 rs1539019	C	1.572 (1.372 - 1.772)	1.057 (0.731 - 1.383)	**0.022**	1.673 (1.454 - 1.892)	0.893 (0.517 - 1.268)	**0.002**	1.457 (1.19 - 1.724)	1.555 (1.235 - 1.875)	0.958
	NLRP3 rs10754558	C	1.662 (1.488 - 1.837)	0.96 (0.641 - 1.279)	**<0.001**	1.709 (1.528 - 1.889)	0.858 (0.5 - 1.216)	**<0.001**	1.602 (1.37 - 1.835)	1.572 (1.312 - 1.832)	0.997

P-values were calculated using the unconditional logistic regression model. Associations were considered significant with a value of $P < 0.05$. TB, tuberculosis; aOR, adjusted odds ratio; 95% CI, 95% confidence interval; PTB, Pulmonary TB; EPTB, Extrapulmonary TB. The AIM2 rs2276405 polymorphisms had insufficient observations and/or no way to calculate the standard error (all observations from one or more groups were equal to the lower detection limit of the assay) for the analyses between the groups with vs. without TB, with TB vs. without TB among HIV and PTB vs. EPTB. A, A allele; C, C allele; G, G allele. Bold indicate statistically significant results.

our group showed that the HLA-B*41 allele, KIR2DS2, and the combination of KIR/HLA-C pairs were associated with an increased risk of TB-HIV/IRIS onset (de Sá et al., 2020).

Here, we showed that the C/T genotype (ORadj=61.06; P=0.026) or carrier-T (ORadj=61.06; P=0.026) in the AIM2 rs2276405 polymorphism was associated with an increased risk of TB-HIV/IRIS in TB-HIV individuals, whereas lower risk IRIS onset was associated with the A/T genotype (ORadj=0.02; P=0.033) or carrier-T (ORadj=0.02; P=0.029) in the CARD8 rs2043211 polymorphism and with the CARD8 T-G haplotype (ORadj=0.07; P= 0.033).

Absent in melanoma 2 (AIM2) is a cytosolic sensor for double-stranded DNA (dsDNA) and tumor suppressor that is responsible for inflammasome activation and is involved in the host immune response to viruses and intracellular bacteria (Saiga et al., 2012). AIM2 binds to HIV dsDNA and may trigger acute inflammation and pyroptosis (Ekabe et al., 2021). Regarding the AIM2 rs2276405 polymorphism, to our knowledge, only one study showed a significant difference in the genotype frequencies of this Single Nucleotide Polymorphism (SNP) between individuals with and without TB in a Taiwanese population (Liu et al., 2020). In our study, no association between AIM2 polymorphisms and the occurrence of TB or its clinical presentations or inflammasome-related cytokines was observed.

CARD8 negatively regulates the expression of the NLRP3 inflammasome by inhibiting the oligomerization of this receptor in unstimulated cells (Tangi et al., 2012; Ito et al., 2014). The CARD8 gene rs2043211 polymorphism is an A to T transversion on the template strand (Ko et al., 2009). A Brazilian study found

an association between the CARD8 rs6509365 polymorphism and susceptibility to TB-HIV coinfection (Pontillo et al., 2013). This effect was stronger when this Single Nucleotide Polymorphism (SNP) was combined with the CARD8 rs2043211 polymorphism, supporting a novel association between the CARD8 gene and TB-HIV coinfection (Pontillo et al., 2013). However, in our study we did not observe any association of both CARD8 polymorphisms with TBHIV coinfection. However, when analyzing the CARD8 haplotypes a lower risk of TB onset among PLWH was observed. Moreover, a lower risk of IRIS associated with the CARD8 rs2043211 polymorphism and CARD8 haplotype was detected in our study. We also observed that carrying the MAF of both CARD8 polymorphisms was associated with increased levels of IL-6 in TB individuals compared to those without TB, IL-1β for those with EPTB clinical presentations, and IL-33 for TB-HIV cases.

Concerning the NLRP3 polymorphisms analyzed here, an increased risk for EPTB was associated with the TT genotype in the NLPR3 rs4612666 polymorphism or the C-C-T-G-C NLRP3 haplotype, whereas carrier-C in the NLRP3 rs4612666 polymorphism was associated with protection against EPTB. Increased levels of IL-6 or IL-33 and IL-18 or IL-33 were found in TB individuals both without and with HIV carrying the MFA of some selected NLRP3 polymorphisms. In addition, the G/A genotype in the IL-1β rs1143634 polymorphism was associated with TB risk among PLWH. Increased levels of IL-33 were found in TB individuals without and with HIV who were carrying the IL-1β rs1143634 MFA.

The NLPR3 rs4612666 polymorphism has already been associated with rheumatoid arthritis (Cheng et al., 2021) and cardiovascular diseases (Mahendra et al., 2021). The IL-1β rs1143634 polymorphism is associated with susceptibility to myocardial infarction (Fang et al., 2018), an aggressive phenotype of breast cancer (Wang and Yuan, [[NoYear]]), and is a predictive factor for a severe course of chronic periodontitis (Brodzikowska et al., 2019). To the best of our knowledge, this is the first study to report the association of these polymorphisms with the studied TB and TB-HIV outcomes. More studies are needed to confirm these findings.

It is evident that studying only one gene polymorphism is insufficient to explain the complexity of TB-HIV inflammatory outcomes. However, descriptions of genetic associations, even if at the Single Nucleotide Polymorphism (SNP) level, help understand the complex mechanisms that are involved in infectious diseases. It must be considered that other components of inflammasomes may regulate inflammation, in addition to other host genetic factors that are linked to TB-HIV immunopathogenesis. HLA and KIR alleles associations were previously described by our group (de Sá et al., 2020) and others, that should be considered in the search for genetic biomarkers of inflammatory diseases, including TB-HIV/IRIS. The importance of the selected inflammasome genes justifies the research conducted in the present study and the results obtained, which were generated using a suitable statistical approach, to adequately demonstrate the relationships among inflammasome-mediated innate immunity Single Nucleotide Polymorphism (SNPs) and TB-HIV/IRIS, as well as the occurrence of TB and its clinical presentations.

Several studies have related potential biomarkers to cytokine production as predictors of TB-HIV/IRIS onset. Tan et al. (2015) showed that individuals with TB-IRIS have higher levels of plasma IL-18 both in the pre-cART phase and during TB-HIV/IRIS (Tan et al., 2015). Similarly, Conesa-Botella et al. (2012) reported that in individuals without corticosteroid therapy, the levels of tumor necrosis factor (TNF), interferon-gamma (IFN-γ), and plasma levels of IL-6 and IL-18 were significantly higher in TB-HIV individuals with TB-IRIS than in those without IRIS at week two after starting cART. In contrast only the IFN-γ levels were higher in IRIS individuals at baseline (Conesa-Botella et al., 2012). In the present study, possibly due to the low number of subjects with TB-HIV/IRIS, no increase in the IL-1β and IL-18 cytokines, the typical inflammasome stimulation products, as well as IL-6 was detected for this group, but a trend toward increased IL-33 plasma levels was observed.

IL-6 is a known downstream target of IL-1β that is consistently higher in serum samples from individuals with NLRP3 inflammasome-mediated conditions (Brydges et al., [[NoYear]]; Tanaka et al., 2014). IL-6 is a proinflammatory cytokine with a pleiotropic effect on inflammation, immune response, and hematopoiesis (Tanaka et al., 2014). High levels of IL-6 have been described as a potential biomarker for TB and are associated with higher plasma viral loads and faster progression to AIDS in several studies (Boulware et al., 2011; Singh and Goyal, 2013; Joshi et al., 2015). In this study, IL-6 levels were higher in individuals with TB than those without TB and among PLWH with TB than those without TB.

Regarding IL-33, several studies report that this cytokine acts as an "alarm" that can be released upon tissue damage, stress, or infection, which acts as a danger signal for the immune system (Andreone et al., [[NoYear]]; Cayrol and Girard, [[NoYear]]; Neumann et al., 2018). In this study, the IL-33 levels were higher in individuals with TB than in those without TB and in PLWH with TB than those without TB. The role of this cytokine in HIV-1 infection and TB has already been described (Xuan et al., 2014; Wu et al., 2018), which shows potential therapeutic effects on established MTB infections, which might represent a novel therapy for PTB (Piñeros et al., 2017). Zhao et al., 2021 showed that the plasma IL-33 levels were significantly higher in individuals with PTB than in healthy individuals (Zhao et al., 2021).

As mentioned earlier, in addition to inflammasome stimulation and cytokine release in TB-HIV coinfection, these

processes can lead to extensive inflammation with cell damage and, consequently, overproduction of IL-33, which increases in those who progress to TB-HIV/IRIS, as suggested by our study. Therefore, additional studies are needed to investigate the roles of CARD8 and AIM2 gene variations in the modulation of inflammasome and cytokine secretion, mainly IL-33, in the context of TB-HIV/IRIS.

In conclusion, our study contributes to the generation of knowledge on the role of inflammasome Single Nucleotide Polymorphism (SNPs) and inflammatory cytokines in TB-HIV outcomes and the evolution toward TB-HIV/IRIS. Nevertheless, it is relevant to note that some limitations of the current study should be considered, mainly concerning the limited sample size and low frequency of HIV/TB-IRIS cases. Therefore, additional studies with larger populations are needed to understand better the importance and roles of inflammasome Single Nucleotide Polymorphism (SNPs) and inflammatory cytokines in TB-HIV/IRIS.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by IOC/FIOCRUZ (CAAE 51959215.5.0000.5248), INI/FIOCRUZ (CAAE 51959215.5.3002.5262), and HGNI (CAAE 51959215.5.3001.5254) Ethical Boards. The individuals/participants provided their written informed consent to participate in this study. The patients/participants provided their written informed consent to participate in this study.

Author contributions

NSá: conceptualization, methodology, validation, investigation, writing - original draft and visualization. NSo and MN-G: methodology, validation, and investigation. MR-A: software, formal analysis and resources, and writing - original draft. TS, JP, VR, CG-G, LdOP, and DS-A: resources and writing - review & editing. MM and ST: conceptualization, methodology, writing - original draft, and supervision. All authors read and approved the manuscript.

Funding

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq (Grants numbers 404573/2012-6; 311345/2014-0; 435002/2018-0; 314064/2018-4), Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro- FAPERJ (Grant number E-26/010.001673/2019), and the France Recherche Nord & Sud Sida-HIV Hépatites - ANRS (Grant number ANRS12274). NBRS is recipient of INOVA FIOCRUZ/ Fundação Oswaldo Cruz postdoctoral fellowship. MGM is recipient of CNPQ (314064/2018-4) and FAPERJ (E-26/201.177/2021) research fellowships.

Acknowledgments

The authors are thankful to all individuals who agreed to participate in this study as volunteers and permitted the analysis of their biological material. We are in debt to Iury Amâncio Paiva and Jéssica Badolato Corrêa da Silva for technical support.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.962059/full#supplementary-material

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