MARSILENI PELISSON

COVID-19 E O POLIMORFISMO DO GENE *P2RX7*

Londrina
2023

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Tese de Doutorado apresentada à Universidade Estadual de Londrina - UEL, Programa de Pós-Graduação em Fisiopatologia Clínica e Laboratorial, como requisito para a obtenção do título de Doutor.

Orientadora: Profª. Drª Eliana Carolina Vespero

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RESUMO

PELISSON, Marsileni. **COVID-19 e o polimorfismo do gene *p2rx7*.** 2023. 178 f. Tese (Doutorado em Fisiopatologia Clínica e Laboratorial) – Universidade Estadual de Londrina, Londrina, 2023.

Na COVID-19, o agravamento de grupos com determinadas comorbidades e características demográficas, especialmente para sexo e idade, encontram explicações na fisiologia. No entanto, o estadiamento e a patogênese da doença - em parte resumida como “síndrome imunológica” - e suas bases genéticas, ainda carecem de total esclarecimento. Várias doenças com a imunopatogênese vinculadas de alguma forma à atividade dos receptores purinérgicos - em especial P2X7, o inflamassoma NLRP3 e a morte celular induzida – apresentam correlação com a COVID-19, quer seja como fator de risco ao agravamento ou desfecho, quer seja como condição pós-COVID-19. Assim, bases genéticas que qualificam a funcionalidade do receptor P2X7 podem auxiliar no entendimento da patogênese da doença. Desta forma, são objetivos deste trabalho avaliar o perfil epidemiológico e laboratorial de pacientes hospitalizados com a infecção pelo SARS-CoV-2 em um serviço de referência para a COVID-19 e determinar a associação dos polimorfismos do gene *p2rx7* com os fatores epidemiológicos, a severidade da COVID-19 pré-vacinação e com o desfecho da doença severa-crítica após a hospitalização. Dados epidemiológicos, clínicos, laboratoriais e tomográficos foram relacionados ao desfecho da COVID-19 em até 180 dias após o início dos sintomas. Amostras de sangue foram obtidas para a análise de parâmetros laboratoriais e polimorfismos de nucleotídeo único (SNP) do gene *p2rx7* (rs3751143 e rs2393799), os quais podem ter relação com a funcionalidade do receptor transmembrana. Os exames foram realizados segundo metodologias normatizadas em laboratórios clínicos e a análise genética por meio de qPCR e sondas Taqman®VIC™/FAN™ - rs3751143A>C e rs2393799C>T. Haplótiplos dos SNP foram utilizados para a comparação das demais variáveis estudadas: Haplótipo I [AA] e [CC]; II [AC;CC] e [CC]; III [AA] e [CT;TT]; IV [AC;CC] e [CT;TT]. A análise estatística das variáveis categóricas utilizou teste Qui-Quadrado e das variáveis contínuas a ANOVA de uma via (W e F*), após 1000 reamostragens. Kaplan-Meier e os modelos de regressão de Cox foram utilizados para análise de sobrevivência e a regressão bivariada para variáveis de risco de agravamento e óbito. IBM SPSS Statistics 20.0 e GraphPad Prism 8.0.0 foram utilizados para as análises estatísticas e geração de gráficos. Todas as análises foram realizadas ao nível de significância inicial de $p<0,05$. Os resultados indicaram a idade superior a 60 anos, cardiopatia e o diabetes como fatores de risco para o agravamento e óbito na COVID-19; que exames como PCR, relação neutrófilo/linfócito, desidrogenase lática e interleucina 6 são biomarcadores associados à severidade da COVID-19 e, que pacientes com genótipo mutante da rs3751143 têm três vezes mais risco de óbito que pacientes com a mutação na rs2393799 ($p<0,001$, HR=3,002 [1,939 - 4,647]). Assim, os resultados confirmam que idosos perfazem o grupo mais susceptível à gravidade e sugerem que mutações do gene *p2rx7* podem influenciar significativamente o óbito em pacientes com COVID-19 severa-crítica.

Palavras-chave: P2RX7; polimorfismo genético; COVID-19; severidade; mortalidade.

ABSTRACT

PELISSON, Marsileni. **COVID-19 and the *p2rx7* gene polymorphism**. 2023. 178 pages. Thesis (Doctorate in Clinical and Laboratory Pathophysiology) – State University of Londrina, Londrina, 2023.

In COVID-19, the greater susceptibility of groups with certain comorbidities and demographic characteristics, especially sex and age, to deterioration finds physiological explanations. However, the staging of COVID-19 and the pathogenesis of the disease - partly summarized as "immune syndrome" - and its genetic basis remain to be fully elucidated. Several diseases with immunopathogenesis somehow related to the activity of purinergic receptors - especially P2X7, the NLRP3 inflammasome and induced cell death - are correlated with COVID-19, either as a risk factor for exacerbation or outcome, or as a post-COVID-19 condition, suggesting that genetic bases qualifying P2X7 receptor function may help in understanding disease pathogenesis. Therefore, the objectives of this work are to evaluate the epidemiologic and laboratory profile of hospitalized patients with SARS-CoV-2 infection in a reference service for COVID-19 and to determine the presence of an association of p2rx7 gene polymorphisms with epidemiologic factors, symptoms, COVID-19 severity and outcome of severe critical illness after hospitalization in a non-vaccination period. Epidemiologic, clinical, laboratory, and tomographic data related to the outcome of COVID-19 within 180 days of symptom onset were collected. Blood samples were taken for the analysis of laboratory parameters and single nucleotide polymorphisms (SNP) of the p2rx7 gene - rs3751143 and rs2393799 - which may be related to the functionality of the transmembrane receptor of the same name. The studies were performed using standard clinical laboratory methods and genetic analysis using qPCR with Taqman®VICTM/FANTM probes - rs3751143A>C and rs2393799C>T. Haplotypes containing SNP genotype clusters were used to compare the other variables studied: haplotype I [AA] and [CC]; II [AC;CC] and [CC]; III [AA] and [CT;TT]; IV [AC;CC] and [CT;TT]. Statistical analysis of categorical variables was performed with the chi-square test and continuous variables with one-way ANOVA (W) after 1000 resamples. Kaplan-Meier curve and Cox regression models were used for survival analysis, and bivariate regression was used to evaluate risk variables for worsening and death. IBM SPSS Statistics 20.0 and GraphPad Prism 8.0.0 were used for statistical analysis and graph generation. All analyses were performed at an initial significance level of $p<0.05$. The results indicated age over 60 years, heart disease and diabetes as risk factors for worsening and death in COVID-19; that tests such as CRP, neutrophil/lymphocyte ratio, lactic dehydrogenase and interleukin 6 are biomarkers associated with the severity of COVID-19 and that patients with the rs3751143 mutant genotype have three times the risk of death than patients with the rs2393799 mutation ($p <0.001$, HR=3.002 [1.939 - 4.647]). Thus, the results confirm that the elderly are the most susceptible group and suggest that mutations in the *p2rx7* gene may significantly influence death in patients with severe COVID-19.

.**Keywords:** P2X7; genetic polymorphism; COVID-19; severity; mortality.

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LISTA DE ABREVIATURAS E SIGLAS

ABNT	Associação Brasileira de Normas Técnicas
ACE2	*Angiotensin-converting enzyme 2 (*Enzima Conversora da Angiotensina 2)
AD	Doença de Alzheimer
ADP	Adenosina Difosfato
Al	*Allele*
ALT	Alanina Aminotransferase
AMP	Adenosina Monofosfato
ANPE	Alanina Aminopeptidase
APC	Célula Apresentadora de Antígeno
ASC	Proteína Adaptadora Apoptótica Contendo Domínio de Recrutamento e Ativação de Caspase
ASGr	Receptor de Asialoglicoproteína
AST	Aspartato Aminotransferase
AT1	Pneumócitos tipo 1
AT2	Pneumócitos tipo 2
ATPe	Adenosina Trifosfato Extracelular
AXL	Receptor Tirosina Proteína Quinase UFO
Aβ	Proteína Amilóide Beta
CASP	Caspase
CCCE	Centro de Ciência e Engenharia
95CI	Intervalo de Confiança 95%
COVID-19	*Coronavirus Disease 2019*
CPK	Creatina Fosfoquinase
CPK-MB	Creatina Fosfoquinase Fração Miocárdio e Cérebro
CRP	*C Reactive Protein* (Proteína C Reativa)

Da	Daltons
DAMP	*Damage-Associated Molecular Patterns* (Padrões Moleculares Associados ao Dano)
DENV	Vírus da Dengue
df	*Degrees of Freedom* (Graus de Liberdade)
DM	Diabetes Mellitus
DM2	Diabetes Mellitus tipo 2
DNA	Ácido Desoxirribonucléico
DPB	Proteína Ligante de Vitamina D
DPP4	Dipeptidil Peptidase 4
E	Envelope
eATP	*Extracellular Adenosine Triphosphate*
EDTA	Ácido Etileno-Diamino-Tetracético
GABA	Ácido Gama-Amino-Butírico
G-CSF	Fator Estimulador de Colônias Granulocitárias
GSDMD	Gasdermina D
Gr	*Group* (Grupo)
Gt	*Genotype* (Genótipo)
HA	Hipertensão Arterial
HCV	Vírus da Hepatite C
HDL-SRB1	Receptor de Lipoproteína de Alta Densidade 1
HIV	Vírus da Imunodeficiência Humana
HR	Hazard Ratio
HSP	Proteína do Choque Térmico
HT	Hipotireoidismo
Ht	*Haplotype* (Haplótipo)
IBGE	Instituto Brasileiro de Geografia e Estatística

ICVT	Comitê Internacional para Taxonomia de Virus
IFN	Interferon
IL	Interleucina
IQ25	Interquartil 25%
IQ75	Interquartil 75%
LB	Linfócitos B
LIBC	*Latent Iron Binding Capacity* (Capacidade latente de Ligação de Ferro)
LDH	Lactato Desidrogenase
LLC	Leucemia Linfocítica de Células B
LPS	Lipopolissacarídeo
LT	Linfócitos T
LTh	Lintócitos T Auxiliares
LT*Naive*	Linfócitos T Originários
LTreg	Linfócitos T Regulatórios
MAPK	Proteína Quinase Ativada por Mitógeno
MCP-1	Proteína Quimio-Atraente de Monócitos
MDA-5	Sensor Associado à Diferenciação de Melanoma
MIS-C	Síndrome Inflamatória Multissistêmica
N	Nucleocapsídeo
NAD	Nicotinamida Dinucleotídeo
NET	*Neutrophil Extracellular Trap* (Armadilha Extracelular de Neutrófilo)
NF-kB	Fator Nuclear Kappa B
NK	*Natural Killer*
NLR	Razão Neutrófilos/Linfócitos
NLRP3	Domínio de Ligação e Oligomerização de Nucleotídeo, com Domínio Rico em Repetições de Leucina e Domínio Pirina 3

NO	Óxido Nítrico
NRP-1	Neuropilina 1
NS1	Proteína Não-Estrutural 1 do DENV
OR	Odds Ratio
OMS	Organização Mundial da Saúde
p2rx7	Gene codificador do receptor P2X7
P2RX7	Receptor P2X7
PAMP	*Molecular Pattern Associated with Pathogens* (Padão Molecular Associado a Patógenos)
PCR	Proteína C Reativa
PCT	Procalcitonina
PK	Doença de Parkinson
Pmild	Pacientes com COVID-19 leve
Pmod	Pacientes com COVID-19 moderada
PRP	Receptor Plasmático de Padrões Moleculares
Psev	Pacientes com COVID-19 severa ou crítica
qPCR	Reação em Cadeia da Polimerase quantitativa
RDW	Amplitude de Distribuição de Glóbulos Vermelhos
RIG-1	Receptor Iduzível pelo Ácido Retinóico
RNA	Ácido Ribonucléico
RNAds	RNA Dupla Fita
RNAm	RNA Mensageiro
RNAss	RNA Simples Fita
ROS	Radicais Oxigênio Reativos
S	*Spike*
SARS-CoV-2	*Severe Acute Respiratory Syndrome Coronavirus 2*
SASP	Fenótipo Secretório Associado à Senescência

SD	*Standard Deviation* (Desvio Padrão)
SDRA	Síndrome do Desconforto Respiratório Agudo
SI	Sistema Imunológico
SN	Sistema Nervoso
SNC	Sistema Nervoso Central
SNP	*Single Nucleotide Polymorphism* (Polimorfismo de Nucleotídeo Único)
SpO_2	Saturação periférica de O_2
SRAG	Síndrome Respiratória Aguda Grave
TARV	Terapia Antirretroviral
TC	Tomografia Computadorizada
TDE	Tamanho do Efeito
TB	Tuberculose
TIBC	*Total Iron Binding Capacity* (Capacidade Total de Ligação de Ferro)
TBL	Tuberculose Linfática
TBP	Tuberculose Pulmonar
Tfh	Linfócitos T Foliculares
TLR	*Toll Like Receptors* (Receptores Semelhantes a Toll)
TMPRSS2	Serina Protease Transmembrana 2
TNF	Fator de Necrose Tumoral
TR	Trato Respiratório
TRI	Trato Respiratório Inferior
TRS	Trato Respiratório Superior
VCAM-1	Proteína de Adesão Vascular 1
VCC	Microvesículas
VE	Vesículas Extracelulares

SUMÁRIO

1 INTRODUÇÃO

1.1 COVID-19

Em 30 de dezembro de 2019, uma comunicação ao site ProMED da Sociedade Internacional para Doenças Infecciosas (*International Society for Infectious Disease*) com o relato de casos de uma pneumonia de causa desconhecida na China (“ProMED Posts - ProMED-mail”, 2019) colocou o mundo em alerta.

Os casos iniciais da infecção respiratória com aspectos clínicos e radiológicos (SONG *et al.*, 2020) de pneumonia viral extensa se concentravam em Wuhan, Hubei, China, envolvendo pessoas que haviam frequentado um mercado conhecido como Huanan (Huang *et al*., 2020) e posteriormente familiares, contactantes e profissionais de saúde (Worobey *et al.*, 2022).

Em fevereiro de 2020, o Comitê Internacional para Taxonomia de Virus - *International Committee on Taxonomy of Viruses* (ICTV) anunciava a denominação para o novo coronavírus isolado, como SARS-CoV-2 (S*evere Acute Respiratory Syndrome Coronavirus* 2) (Gorbalenya *et al.*, 2020) sob intensa investigação acerca da origem transicional do vírus e de seu antecessor (SARS-CoV) a partir de morcegos e outros animais (Andersen *et al*., 2020; W. Li *et al*., 2005; Zhou & Shi, 2021). Mais tarde esta origem foi sedimentada em bases genéticas e epidemiológicas (Jiang; Wang, 2022; Lytras *et al.*, 2021a; Lytras *et al.*, 2021b; Pekar *et al.*, 2022; Worobey *et al*., 2022).

Em meio à discussão sobre as origens do vírus, a COVID-19 (*Coronavirus Disease* 2019), foi assim denominada em fevereiro de 2020 pela Organização Mundial da Saúde (OMS) (Cucinotta; Vanelli, 2020) e conduzida a *status* de pandemia em meados de março de 2020, em razão da rápida disseminação geográfica global do vírus.

Apesar de sua homologia estrutural com seu antecessor epidêmico e outros betacoronavírus, SARS-CoV-2 demonstra características próprias que puderam determinar além do *spillover (*salto entre diferentes espéciens*)*, sua eficiente transmissão entre humanos, sua patogenicidade, além da capacidade de imunoevasão (Li *et al.*, 2005; Quan *et al.*, 2020; Zhou *et al.*, 2020a).

Centenas de alterações genômicas, algumas delas cruciais para o estabelecimento da infectividade das variantes de SARS-CoV-2 - Alfa, Beta, Gama,

Delta, Kappa, Epsilon, Eta, Iota, Lambda, Mu e Omicron - foram documentadas, denotando a seleção de mutantes mais adaptados ao hospedeiro humano durante o período pandêmico (Candido *et al.*, 2022; Delshad *et al.*, 2022; GISAID: RE3DATA, 2023; Magazine *et al.*, 2022; Pachetti *et al.*, 2020), o que pode justificar em parte as ondas de casos que se sucederam no período.

1.1.1 Epidemiologia da COVID-19

Já à época de sua caracterização, SARS-CoV-2 foi responsabilizado por milhares de casos de doença respiratória, hospitalizações e centenas de óbitos (Bi *et al*., 2020; Chen, G. *et al*., 2020b).

A doença causada pelo novo coronavírus (COVID-19) foi determinante para 7.023.127 óbitos e mais de 773 milhões de casos notificados ao redor de mais de 200 países, segundo o painel global COVID-19 do Centro de Ciência e Engenharia *(*CCCE) da Universidade Johns Hopkins (JHU) (JOHNS HOPKINS UNIVERSITY, 2022), (Dong; Du; Gardner, 2020). De acordo com dados publicados pelo painel e referendados pela OMS, no pico de número de casos notificados, foram 102.064 mortes diárias, em 18 de janeiro de 2021 (WHO, 2023).

Em pouco mais de três anos de pandemia, foram 704.488 mortes e 37.704.598 casos notificados no Brasil, com letalidade de 1,87% (MATHIEU *et al.*, 2020). No país, o primeiro semestre de 2021 chegou a apresentar a triste marca de 1.308 óbitos notificados em 05 de fevereiro de 2021 (Brasil, 2023).

Para além dos números oficiais, evidências epidemiológicas recentes indicam que os óbitos relacionados à COVID-19 podem ser 2,74 vezes maiores que os números estimados por órgãos oficiais (Msemburi *et al.*, 2023), sendo influenciados pelas condições socioeconômicas locais e globais e, pelas políticas e estratégias de atenção à saúde e controles adotadas pelas diversas nações (Aquino *et al.*, 2020; Chung *et al.*, 2021; Cordato *et al.*, 2023; Hsiang *et al.*, 2020; Paital; Das; Parida, 2020; Prem *et al.*, 2020; Yamaka *et al.*, 2022).

O grande avanço no número de infectados, e o surgimento de variantes do SARS-CoV-2 (Alpha, Beta, Delta e Omicron) acabaram por estabelecer perfis de transmissão e diferentes períodos de incubação para a doença. De forma geral, a incubação varia de 1,8 a 18,9 dias com média de 6,6 dias, mas com períodos mais longos em crianças que em outros pacientes e, também, maiores que em outras

infecções respiratórias virais (Wu *et al*., 2022).

Curiosamente, o período de incubação foi se estreitando frente às subsequentes variantes do SARS-CoV-2, de medianas de 5,0/4,8 para 4,5;4,4 e 3,4 com dados obtidos de 2020 a 2023 (Bi *et al.*, 2020; Lauer *et al.*, 2020; Mefsin *et al.*, 2022; Ogata *et al.*, 2022; Ogata; Tanaka, 2023; Wu *et al.*, 2022). Essas variantes, foram também responsabilizadas por menor ou maior infectividade do vírus e por quadros clínicos mais brandos durante a pandemia (Nyberg *et al.*, 2022).

De forma geral, em dados compilados pela OMS, a maioria dos casos de COVID-19 foram identificados em adultos entre 25 e 64 anos, em todos os momentos da pandemia. No entanto, menos de um terço dos óbitos ocorreram entre essa população e se concentraram em pessoas com mais de 65 anos (WHO, 2023).

De fato, observou-se que a mortalidade se expande com o avançar da idade. E, com o acúmulo de comorbidades, essa se amplifica ainda 1,7 vezes, se o paciente for do sexo masculino (Deng *et al.*, 2020; Gold *et al.*, 2020).

Desta forma, pôde-se observar ao longo do tempo que, assim como a necessidade de hospitalização, a chance de óbito em pacientes severos e críticos durante a internação foi extremamente influenciada pela idade, pelo gênero e pela presença de comorbidades (Benítez *et al.*, 2022; Chen *et al.*, 2021a, 2021b; Gold *et al.*, 2020; Justino *et al.*, 2022; Pennington *et al.*, 2021; Takahashi *et al.*, 2020; Viveiros *et al.*, 2022).

Dentre as condições predisponentes ao agravamento estão a hipertensão arterial, o diabetes mellitus (DM), as doenças cardíacas e respiratórias, a obesidade, a presença de imunossupressão ou imunodeficiência, o uso de tabaco, desordens neurológicas, entre outras (Carethers, 2021; Chatterjee *et al.*, 2023; Chenchula *et al.*, 2023; July; PranatA, 2021; Mahamat-Saleh *et al.*, 2021).

Por outro lado, dados iniciais que direcionavam grupos étnicos ou raciais à maior gravidade e mortalidade da COVID-19 no Brasil (Baqui *et al.*, 2020) e no mundo (Adegunsoye; Ventura; Liarski, 2020; Golestaneh *et al.*, 2020; Pennington *et al.*, 2021; Price-Haywood *et al.*, 2020), em sua maioria, foram revistos. Uma vez que essa relação de vulnerabilidade é complexa e multifatorial, é importante considerar que diferenças podem não ser inerentes à raça ou etnia em si, mas sim podem refletir fatores genéticos, sociais, econômicos, culturais que afetam ou são próprias dessas populações (Carethers, 2021; Khanijahani *et al.*, 2021; Rentsch *et al.*, 2020).

Ainda, fatores genéticos individuais ou compartilhados por populações

específicas, presumivelmente, poderiam influenciar a suscetibilidade à infecção por SARS-CoV-2 e sua evolução. Assim, variantes genéticas foram associadas aos processos patológicos da COVID-19, incluindo aqueles implicados na entrada de vírus nas células tais como polimorfismos da enzima conversora da angiotensina 2 (*angiotensin-converting enzyme 2* - ACE2), serina protease transmembrana 2 (*transmembrane serine protease* 2 -TMPRSS2), da glicoproteína CD26 (Adli *et al.*, 2022; Dos Santos *et al.*, 2021) e os alelos do lócus do sistema sanguíneo ABO (Ellinghaus *et al.*, 2020; Ray *et al.*, 2021; Wu *et al.*, 2020a; Zhang *et al.*, 2021), entre outros.

Também, podem ter envolvimento genes que codificam a resposta imune, tais como interleucina (IL) 6, variante 174C e polimorfismos de outras interleucinas como IL-10, IL-17A (Karcioglu Batur; Hekim, 2021; Ulhaq; Soraya, 2020); assim como da proteína ligante de vitamina D (DBP) (Karcioglu Batur; Hekim, 2021); apolipoproteína E (APOE4) (Kurki *et al.*, 2021; Ostendorf *et al.*, 2022); oligoadenilato sintetase (OAS1) (Magusali *et al.*, 2021) que ligam inflamação-neurodegeneração-COVID-19 e inúmeros outros polimorfismos em genes que codificam receptores, antígenos celulares e moléculas envolvidas na ligação COVID-19 e comorbidades, e em diversas vias de sinalização inter e intracelulares (Dos Santos *et al.*, 2021; Pairo-Castineira *et al.*, 2021; Ramos-Lopez *et al.*, 2020) as quais estão implicadas nos processos brevemente descritos a seguir.

1.1.2 Manifestações Clínicas da COVID-19

Na COVID-19, a maioria dos pacientes sintomáticos deixam de transmitir o SARS-CoV-2 após em média 10 dias do início dos sintomas (Jang *et al.*, 2021) e a carga viral se torna indetectável após aproximadamente duas semanas, com vistas à produção dos anticorpos neutralizantes (Garcia-Beltran *et al.*, 2021; Puhach; Meyer; Eckerle, 2023).

No entanto, desafios foram se sucedendo com o avanço da pandemia e o conhecimento acerca da dinâmica das infecções. Evidências laboratoriais demonstraram que a infecção pelo SARS-CoV-2 não se manifesta como doença em todos os pacientes, uma vez que a presença do ácido ribonucleico (RNA) viral, bem como a soro conversão são observadas em indivíduos sem doença diagnosticada clinicamente (Kronbichler *et al.*, 2020; Long *et al.*, 2020).

Neste sentido, a despeito da variabilidade entre as faixas etárias e dentro da perspectiva de indivíduos testados como positivos para SARS-CoV-2, de 30 a 40,5% dos infectados não desenvolvem sintomas (Ma *et al*., 2021b; Rasmussen & Popescu, 2021).

Assim, o espectro clínico da COVID-19 se caracteriza por assintomáticos; quadros considerados leves, cujas manifestações autolimitadas se assemelham às demais síndromes gripais de outras etiologias; quadros moderados que necessitam de hospitalização, os quais implicam em pneumonia viral e exacerbação de doenças pré-existentes; e casos severos ou críticos que necessitam de hospitalização com cuidados intensivos, em razão de falha respiratória e de outros órgãos (Marshall *et al.*, 2020).

Nos casos leves, a sintomatologia mais frequente envolve tosse seca, febre, mialgia, fadiga, dispneia, cefaleia, disfagia, diarreia e vômitos, congestão nasal desordens de olfato e paladar, manifestações cutâneas e oculares (Da Costa *et al.*, 2020; Farid *et al.*, 2022; Gandhi; Lynch; Del Rio, 2020; Lechien *et al.*, 2020; Sharma *et al.*, 2022; Wadman *et al.*, 2020). As desordens sensitivas – hiposmia, anosmia, disgeusia e ageusia – tornaram-se prevalentes e persistentes marcadores da COVID-19 (Cardoso *et al.*, 2022; Lechien *et al.*, 2020).

Durante o período pandêmico, sem vacinação, cerca de 80% dos casos diagnosticados apresentavam-se como leves ou moderados (Verity *et al.*, 2020). No entanto, já nos primeiros casos notificados da doença se observou a ocorrência de pneumonia viral e síndrome mediada por altos níveis de citocinas que permeiam os casos moderados e graves (Huang *et al*., 2020).

Alterações pulmonares em imagem, a exemplo de consolidações e opacificações “em vidro fosco”, e manifestações clínicas de pneumonia e síndrome do desconforto respiratório agudo (SDRA) são encontradas nos casos moderados e severos (Chen *et al*., 2020; Xu *et al*., 2020b).

Para a maioria dos pacientes com COVID-19, a ampla resposta antiviral exerce pressão seletiva sob os vírus e resulta no controle da infecção e da sintomatologia nas duas primeiras semanas após o início dos sintomas (Jang *et al.*, 2021; Puhach; Meyer; Eckerle, 2023).

No entanto, essa resposta não se mostra uniformemente eficiente para o bloqueio do dano causado pela replicação viral em parte dos doentes (Blanco-Melo *et al.*, 2020). Nestes, a COVID-19 pode se manifestar como um quadro já inicialmente

grave, mas mais comumente, a doença aguda se mostra bifásica, com sintomas leves ou moderados seguidos de uma fase de piora respiratória que ocorre entre 9 a 12 dias do início dos sintomas (Huang *et al*., 2020).

Em pacientes que desfavoravelmente acumulam as variáveis preditoras de gravidade, o que se observa é o desequilíbrio da resposta imunológica frente à infecção, direcionado à hiper inflamação (Gustine; Jones, 2021; Mishra; Singh; Singh, 2020). Os moderados demonstram quadro clínico e tomográfico de pneumopatia, necessitam de monitoramento, porém sem apresentar hipóxia ao ar ambiente, isto é, oximetria com saturação periférica de oxigênio (SpO_2) $\geq$94% (CDC, 2023; Chen *et al.*, 2020a; Gandhi; Lynch; Del Rio, 2020).

A exacerbação dos sintomas respiratórios como dispneia e hipóxia (SpO_2<94%) se segue às anormalidades que comprometem grande parte dos pulmões (CDC, 2023; KANNE *et al.*, 2020; PAN *et al.*, 2020). Posteriormente, pacientes criticamente enfermos têm alta probabilidade de óbito em razão da somatória das disfunções adquiridas no curso da COVID-19 (CDC, 2023; Maslove *et al.*, 2022; Vasquez *et al.*, 2021).

Nesses últimos, a necessidade de suporte respiratório prossegue com coagulopatia (Conway *et al.*, 2022; Perico *et al.*, 2021), insuficiência renal (Copur *et al.*, 2022; Shetty *et al*., 2021), lesão cardíaca (Calvo-Fernández *et al*., 2021; Nasab *et al.*, 2023), acidentes vasculares cerebrais e infartos cerebrais (Luo *et al.*, 2022; Nannoni *et al.*, 2021) e, por fim, disfunção orgânica múltipla (Guan *et al*., 2020; Huang *et al*., 2020; Vasquez *et al*., 2021; . Zhao *et al*., 2022).

Desta forma, boa parte dos pacientes com quadros mais graves percorrem um *continuum* da doença, porém, essencialmente, a COVID-19 é doença aguda bifásica, com a fase inicial no trato respiratório superior (TRS) que evolui para lesões pulmonares e hipoxemia em sua segunda fase (Casadevall; Pirofski, 2020), na qual há pouca ou nenhuma evidência da multiplicação de vírus no trato respiratório inferior (TRI) (Wu *et al*., 2020).

Ainda que alguns pacientes apresentem alterações mais precoces, aproximadamente 50% têm anormalidades pulmonares tomográficas próximo ao décimo dia do início dos sintomas (Kanne *et al.*, 2020; Pan *et al.*, 2020) e a evolução dos quadros leves/moderados para severos ocorre entre 7 e 14 dias, em especial em pacientes com mais de 60 anos (Hu *et al.*, 2021).

Posteriormente, a despeito da relação com a evolução da doença dentro da

fase aguda, muitos pacientes sobreviventes permanecem doentes após os iniciais 30 dias sintomáticos, caracterizando as fases sub-aguda e crônica da COVID-19 (Carfì; Bernabei; Landi, 2020; Nalbandian *et al.*, 2021; Raman *et al.*, 2022), as quais podem impactar em morbidade e também mortalidade mais tardias.

Ainda nesse período pós-COVID-19, afora pacientes que adquiriram sequelas pós-doença severa, mesmo pacientes que não foram hospitalizados têm risco 1,59 vezes maior de óbito (Hazard Ratio: 1,46-1,73) que os demais pacientes adultos não acometidos por essa doença (Al-Aly; Xie; Bowe, 2021).

Então, ao grande número de indivíduos afetados pela COVID-19 passaram a se somar aqueles com síndrome pós-aguda (condição pós-COVID-19), definida a princípio como sintomas persistentes e/ou tardios, ou ainda complicações a longo prazo para além de quatro semanas após o início dos sintomas (Nalbandian *et al.*, 2021; Nalbandian; Desai; Wan, 2023).

Em conjunto, a condição pós-COVID-19 está correlacionada às sequelas de quadros severos, complicações dos quadros leves e moderados, além de à maior chance de novos diagnósticos, recorrências, de alterações laboratoriais e de polimedicação (Al-Aly; Xie; Bowe, 2021; DeueL *et al*., 2022; Lippi; Sanchis-Gomar; Henry, 2023).

Obviamente, as principais manifestações que impactam significativamente a qualidade de vida do paciente pós-COVID-19 são as pulmonares, com dispnéia, hipóxia e redução da capacidade pulmonar (Blanco *et al.*, 2021; Maslove *et al*., 2022; Mo *et al.*, 2020).

No entanto, também as manifestações cardiovasculares (XIE *et al.*, 2022) – dor, taquicardia, arritmias, miocardite e pericardite, fibrose miocárdica e disfunção autonômica - são resultado da lesão miocárdica, de eventos trombóticos e isquêmicos e da resposta autoimune (Raman *et al.*, 2022) ou ainda, da toxicidade medicamentosa de terapias instituídas durante a hospitalização (Joshee; Vatti; Chang, 2022).

Na COVID-19, a desregulação glicêmica parece resultar do ataque direto do SARS-CoV-2 às células beta do pâncreas via receptor ACE2 (Fignani *et al.*, 2020) e da hiperinflamação (Coate *et al.*, 2020; He *et al.*, 2021a), além da repercussão infecciosa e/ou inflamatória também no tecido adiposo e menor disponibilidade de adiponectina (Reiterer *et al.*, 2021). Todos os fatores em conjunto determinam, principalmente, a resistência à insulina ou a insuficiência na sua produção (Coate *et al.*, 2020; Fignani *et al.*, 2020; He *et al.*, 2021a; Reiterer *et al.*, 2021).

As lesões renais adquiridas durante a fase crítica da doença podem repercutir em semanas ou meses de tratamento dialítico. Na COVID-19, a injúria glomerular é multifatorial, onde os danos diretos do SARS-CoV-2 se somam ao quadro inflamatório-séptico, às disfunções pulmonar e miocárdica, à hipercoagulabilidade plasmática e à desidratação ancorados na fisiopatologia da doença viral, assim como à nefrotoxidade de medicamentos administrados durante a hospitalização (BOWE *et al.*, 2021; BRUCHFELD, 2021; RAY; REDDY, 2023).

Variações quimiossensoriais, depressão, ansiedade, distúrbios do sono, confusão mental, perda de memória e concentração, encefalomielite miálgica são algumas das alterações neurológicas e/ou psiquiátricas, dentro do maior espectro clínico na condição pós-COVID-19. As causas são multifatoriais, isto é, decorrem da resposta inflamatória, de derrames, do uso de benzodiazepínicos, da imobilização/ bloqueio neuromuscular e sedação prolongados (Joshee; Vatti; Chang, 2022; Meinhardt *et al.*, 2021; Nalbandian; Desai; Wan, 2023; Premraj *et al.*, 2022; Sankar; Gould; Prescott, 2023; Sukocheva *et al.*, 2022; Zeng *et al.*, 2023) e de outras interações de caráter psicossocial (Chen *et al*., 2022; Rogers *et al*., 2020; Schou *et al*., 2021).

Ainda, foi observado risco significativamente aumentado após infecção pelo SARS-CoV-2 do desenvolvimento de uma série de doenças autoimunes – espondilite anquilosante, lúpus eritematoso sistêmico, doença mista de tecido conjuntivo, artrite reumatóide, psoríase, DM tipo 1, doença celíaca; síndrome de Sjögren, entre outras – e também, maior mortalidade entre pacientes quando comparados a controles (Chang *et al.*, 2023).

Autores ainda relatam anemia hemolítica, púrpura trombocitopênica, tireoidite, doença de Kawasaki e síndrome de Guillain-Barré, como doenças pós-COVID-19 cuja gênese envolve a autoimunidade (Yazdanpanah; Rezaei, 2022).

Eventos tromboembólicos - embolismo pulmonar, trombose venosa profunda, e trombose periférica - também afetam significativamente mais os pacientes recuperados de COVID-19 que outros pacientes (Hazard Ratio: 2,39) (Xie *et al.*, 2022) até doze meses depois dos sintomas iniciais, em especial naqueles do sexo feminino (Zuin *et al.*, 2023).

Ainda, diversas desordens podem coexistir na condição pós-COVID-19 tais como as a diminuição da função renal (Bowe *et al.*, 2021), DM tipo 2 ou seu descontrole (Sathish *et al.*, 2021; Wander *et al.*, 2022; Watson, 2022; Xie; Al-Aly,

2022), dislipidemia (He *et al.*, 2021a; Li *et al.*, 2021), alteração do microbioma intestinal (disbiose) e translocação (Bernard-Raichon *et al.*, 2022), perda capilar (Raman *et al.*, 2022), e síndrome inflamatória múltissistêmica (MIS-C) que afeta jovens e crianças (Graciano-Machuca *et al.*, 2021).

Em resumo, dentre as ondas de casos e variantes do SARS-CoV-2, a COVID-19 se comporta como uma doença multissistêmica, com um extensivo espectro clínico, e três fases distintas: de infecção e doença clássica, cujas manifestações são advindas da agressão do patógeno (inflamação canônica), doença crítica ancorada na resposta do hospedeiro (inflamação não canônica) e, por fim, doença crônica de amplitude, patogênese e impacto socioeconômico ainda a serem totalmente desvendados (Augustin *et al.*, 2021; CAsadevall; Pirofski, 1999, 2020; Gusev *et al.*, 2021, 2022).

A revisitação de conceitos básicos da interação entre patógenos e os hospedeiros nos induz a encaixar as infecções por SARS-CoV-2 em modelos padronizados de lesão tecidual e resposta imunológica. Nestes, a proximidade se dá em modelo compartilhado com outros vírus como Influenza e Vírus da Hepatite B (HVB), bactérias como *Streptococcus* pyogenes e *Mycobacterium tuberculosis* e fungos como *Coccidioides immitis/posadazi*, onde os processos patológicos ocorrem para além da lesão direta do patógeno (Casadevall; Pirofski, 1999), com a resposta inflamatória canônica clássica inicial e controle do vírus, recrudescimento com inflamação sistêmica aguda e posterior deterioração imunológica e/ou inflamação crônica de intensidade variável, mesmo após a eliminação do patógeno (Casadevall; Pirofski, 2020; Gusev *et al.*, 2022).

Desta forma, tanto a COVID-19 em sua fase aguda como os quadros mais tardios se mostram cientificamente desafiadores em muitos aspectos, mas em especial no entendimento de sua fisiopatogenia e de sua interação com os fatores que isoladamente ou em conjunto predispõem os pacientes a quadros graves e ao óbito.

1.1.3 Biomarcadores na COVID-19

Biomarcadores são parâmetros que se prestam ao diagnóstico e prognóstico de doenças de maneira a auxiliar na estratificação de risco precoce e na instituição de intervenções terapêuticas rápidas para o paciente (Strimbu; Tavel, 2010). E, no contexto da COVID-19, foram pertinentemente identificados marcadores envolvidos

no processo inflamatório e aqueles que refletem alterações orgânicas múltiplas.

Neste sentido, várias metanálises foram realizadas de maneira a sumarizar marcadores com significativa evidência de estarem relacionados à severidade, ao prognóstico e ao desfecho de hospitalizações. Dentre eles estão os de origem inflamatória e imunológica como proteína C reativa (PCR), procalcitonina (PCT) e interleucina-6 (IL-6), os hematológicos (contagem de linfócitos, a razão neutrófilos linfócitos (NLR), dímero-D, ferritina, fração de amplitude de distribuição de glóbulos vermelhos (RDW), os cardíacos (troponina, creatina fosfoquinase (CPK) e sua fração miocárdica e cerebral (CK-MB) e mioglobina) e os hepáticos (aspartato aminotransferase (AST), alanina aminotransferase (ALT), bilirrubina total e albumina) (Abers *et al.*, 2021; Battaglini *et al.*, 2022; Kermali *et al.*, 2020; Malik *et al.*, 2021; Semiz, 2022; Xu; Ilyas; Weng, 2023).

Características de gênero, faixa etária, assim como as comorbidades associadas, os procedimentos terapêuticos e o momento da coleta da amostra podem influenciar resultados isolados, a exemplo da ferritinemia (Kaushal *et al.*, 2022) e dos marcadores imunológicos (Abers *et al.*, 2021).

Desta forma, muitas associações de parâmetros e escores podem se tornar auxiliares no estabelecimento do prognóstico da COVID-19 e da detecção de determinadas complicações, como NLR (Asghar *et al.*, 2022; Regolo *et al.*, 2022; Sejópoles *et al.*, 2023; Simadibrata *et al.*, 2021), fibrinogênio/albumina e uréia/albumina (Ulloque-Badaracco *et al.*, 2022), troponina/hipóxia/idade (Manocha *et al.*, 2021), entre outras estratégias que acumulam parâmetros laboratoriais, de imagem e clínicos (Halmaciu *et al.*, 2022; Rahman *et al.*, 2022; Zhou *et al.*, 2020b).

Muitos dos exames utilizados como biomarcadores na COVID-19 são já aplicados em outras condições clínicas. Desta forma, as alterações na COVID-19 são observadas na sepse e em síndromes respiratórias agudas de outras etiologias, porém de forma menos pronunciada (Leisman *et al.*, 2020).

No Quadro **1** estão alguns marcadores sanguíneos elencados em revisões sistemáticas (Battaglini *et al.*, 2022; Malik *et al.*, 2021; Semiz, 2022) que sinalizam seu significado clínico na COVID-19 e a sua relação com a severidade e/ou a mortalidade dos pacientes acometidos pela doença.

QUADRO 1 – Biomarcadores sanguíneos na COVID-19

BIOMARCADORES	RESULTADO	SIGNIFICADO CLÍNICO	SEVERIDADE	MORTALIDADE
PCR, IL-6, PCT	↑	Inflamação	+	+
IL-10, IL-8, TNF-α	↑		+	
Neutrófilo (N)	↑		+	+
Linfócito (L)	↓		+	+
Relação N/L	↑		+	+
Ferritina	↑		+	+
Hemoglobina	↓	Anemia hemolítica	+	
RDW	↑		+	
Plaquetas	↓	Hipercoagulação	+	+
D-dímero	↑		+	+
Fibrinogênio	↑	Inflamação	+	
AST	↑	Inflamação, citólise, lesão hepática	+	+
ALT	↑	Lesão hepática	+	+
LDH	↑	Inflamação, lesão celular, apoptose de leucócitos	+	+
CPK	↑	Disfunção miocárdica, rabdomiólise	+	+
CPK-MB	↑	Disfunção miocárdica	+	+
Troponina	↑		+	+
Albumina	↓	Inflamação, lesão renal	+	+
Uréia	↑	Lesão renal	+	
Creatinina	↑		+	+

PCR: Proteína C Reativa; IL: interleucina); PCT: Procalcitonina; TNF: Fator de Necrose Tumoral; RDW: *Red Cell Distribution Width* (amplitude de distribuição dos eritrócitos); AST: Aspartato Transaminase; ALT: AlaninaTransaminase; LDH: Lactado Desidrogenase; CPK: Creatina Fosfoquinase; MB: M*iocardic and Brain fraction* (fração cardíaca e cerebral).
Fonte: Adaptado de Semiz, 2022 e Battaglini *et al*., 2022.

1.1.4 Fisiopatologia da COVID-19

1.1.4.1 *A ınvasão do trato respiratório e o início da resposta imune inata*

No TRS, as células ciliadas são o primeiro alvo dos SARS-CoV-2. Para o acesso ao citoplasma, os vírus se utilizam da interação da glicoproteína *Spike* (S) da sua coroa com a ACE2 presente na membrana das células receptoras (Ashraf *et al.*, 2021; Hoffmann *et al.*, 2020; Zhang; Li; Niu, 2020).

Após o reconhecimento pela ACE2, para a fusão do vírus com a membrana citoplasmática (MC) é necessária a clivagem proteolítica da subunidade S2 da *Spike*

pelas serina proteases TMPRSS2 ou TMPRSS4 (Hoffmann *et al.*, 2020; Wruck; Adjaye, 2020), que assim como ACE2, são também variavelmente expressas nos diferentes tecidos (Bertram *et al.*, 2012). A subunidade S1 se ancora firmemente à ACE2, enquanto S2 facilita a fusão do envelope do vírus com a célula receptora e desta com as células adjacentes (Jackson *et al.*, 2022).

Mediante as evidências, ACE2 é indiscutivelmente o principal receptor funcional para o acesso do SARS-CoV-2 às células consideradas alvo diretos, assim como para sua replicação em diferentes células (Ashraf *et al*., 2021; Hoffmann *et al*., 2020; Xu *et al*., 2020; Zhang; Li; Niu, 2020) e não somente ACE2 nativos, mas aqueles solubilizados nos líquidos corpóreos (Allison, 2021; Batlle *et al.*, 2022; Yeung *et al.*, 2021; Yeung; Teng; Yuen, 2022) ou expressos em vesículas extracelulares (VE) (Couch *et al.*, 2021). Estas últimas capazes de transferir o receptor a outras células, tornando-as também susceptíveis aos vírus (Xia *et al.*, 2021).

Sendo assim, mediante a funcionalidade da enzima ACE2, é plausível que as interações moleculares com o receptor possam convergir para a associação com variáveis ou fatores de risco para a COVID-19.

Neste contexto, pesquisas denotam a maior prevalência de ACE2 em homens idosos que em mulheres de mesma idade em todos os tecidos investigados, e mais significativamente nos pulmões. Essas vêm de encontro às evidências clínico-epidemiológicas acerca da discrepância de severidade da COVID-19 entre os sexos (Fernández-Atucha *et al.*, 2017; Viveiros *et al.*, 2022).

Mediante o contrassenso de que ACE2 seja minimamente expresso em células do TRI (Hamming *et al.*, 2004b; Han *et al.*, 2020; Hikmet *et al.*, 2020; Zou *et al.*, 2020), outros processos e componentes celulares foram relacionados à interação com moléculas virais para a facilitação da infecção, tais como o receptor tirosina proteína quinase UFO (AXL) (Wang *et al*., 2021); sulfato de condroitina; receptor de lipoproteína de alta densidade 1 (HDL-SRB1); proteína do choque térmico (HSP); alanilaminopeptidase (ANPE); dipeptidil peptidase (DPP4); receptor de asialoglicoproteína (ASGr); lectinas; neuropilina-1 (NRP-1); ligantes de efrina e receptores Eph; CD147; CD209; CD249; CD206; sulfato de heparano; P2X7; entre outros (Gusev *et al.*, 2022; Zalpoor *et al.*, 2022).

Também, interações da proteína S com ácidos siálicos (Sun, 2021), polissacarídeos (Watanabe *et al.*, 2020) e glicolipídeos sializados (Nguyen *et al.*, 2022; Pruimboom, 2021; Uraki; Kawaoka, 2022) já foram estudadas de forma produtiva.

Uma dessas interações se baseia na ligação do domínio de receptor (*receptor-binding domain* - RBD) da Spike ao antígeno A do grupo sanguíneo ABO (Breiman; Ruvën-Clouet; Le Pendu, 2020; Wu *et al.*, 2021). Ainda que provavelmente multifatorial, alguns estudos estabeleceram a associação de maior severidade da COVID-19 com indivíduos subgrupos A, B e AB quando comparado aos indivíduos do grupo O/Anti-A+/AntiB+ (Barnkob *et al.*, 2020; Goel *et al.*, 2021; Ray *et al.*, 2021; Wu *et al.*, 2020a; Zhang *et al.*, 2021).

Na sequência, após a fusão envelope-membrana, dá-se o início a replicação do SARS-CoV-2 (RNAss), gerando RNA dupla fita (RNAds) intermediários, os quais são reconhecidos por receptores citoplasmáticos de padrões moleculares (*plasmatic receptor pattern* - PRP) da célula infectada (Janeway; Medzhitov, 2002; Sampaio *et al.*, 2021) para o início das reações bioquímicas e interações celulares da imunidade inata.

As células infectadas, por meio dos PRP intracelulares como o receptor induzível pelo ácido retinóico (*retinoic acid-inducible* I - RIG-I) e sensor associado à diferenciação de melanoma (*melanoma differentiation-associated* - MDA-5), e os neutrófilos e macrófagos via sensores Toll-*like* (TLR) TLR3 e 4, reconhecem a invasão dos SARS-CoV-2 e produzem Interferon (IFN) I e III, os quais são moléculas centrais da imunidade inata, e que de forma direta ou indireta têm atividade antiviral (Janeway; Medzhitov, 2002; Khanmohammadi; Rezaei, 2021).

Complexos, intricados e coordenados processos de sinalização nas células infectadas culminam na transcrição (via fator transcricional NF-κB) e translação de receptores e de citocinas para compor a resposta imune inata contra o vírus. Ao final deste primeiro estágio, interferon I e III (IFN-I e IFN-III) promovem alterações em células não infectadas adjacentes de maneira a prevenir a replicação do SARS-CoV-2 nestas (Kim; Shin, 2021; Zhang *et al.*, 2020).

Indiretamente, os IFN também induzem a produção de quimiocinas que recrutam macrófagos, monócitos, neutrófilos e células n*atural killer* (NK) para o local (Blanco-Melo *et al.*, 2020). Nos estágios iniciais da COVID-19 e, em especial no TRS, neutrófilos e outras células macrofágicas são cruciais para a resposta antiviral e o controle da infecção (Hadjadj *et al.*, 2020).

Neste ínterim, é possível que a infecção e a replicação também possam ocorrer no trato gastrintestinal, determinadas pela expressão de receptores ACE2 para o vírus, em especial no intestino (Hamming *et al.*, 2004a; Hikmet *et al.*, 2020).

Também, a regulação positiva de ligantes para as glicoproteínas do vírus, assim como a produção de citocinas antivirais são influenciadas pelo microbioma intestinal e podem ter reflexos locais e sistêmicos, inclusive acredita-se, devolutivamente no trato respiratório (TR) (Sarkar *et al.*, 2021; Yeoh *et al.*, 2021).

Suspeita-se ainda que outras células à distância sejam infectadas por meio da viremia, como resultado da deterioração do estado geral do paciente mais tardiamente (Andersson *et al.*, 2020) e que partículas virais residuais vindas do intestino possam subsidiar manifestações clínicas tardias (Augustin *et al.*, 2021).

1.1.4.2 *O trato respiratório inferior e a síndrome inflamatória*

Assim, como doença bifásica, o progresso da COVID-19 leve/moderada para o quadro severo/crítico se dá mediante a infecção e dano pulmonar extremo, e o desequilíbrio entre respostas pró e anti-inflamatórias locais e sistêmicas.

Da mesma forma que nos tecidos iniciais, nos pulmões a produção de IFN se mostra necessária à limitação da infecção e, neste sentido, a produção limitada de INF-α ou a presença de anticorpos anti-IFN se concretiza em quadros permissivos e severos de COVID-19 (Hadjadj *et al.*, 2020). Assim, uma vez que a resposta imune inata ou adaptativa no TRS não seja suficiente, a seguir, as alterações promovidas pela infecção das células ciliadas e caliciformes secretoras da traqueia e dos brônquios alteram a dinâmica do *cleareance* mucociliar e facilitam o acesso do SARS-CoV-2 aos pulmões (Robinot *et al.*, 2021; Zhu *et al.*, 2020).

Então, o deslocamento de vírus até o trato respiratório inferior (TRI) leva à infecção e destruição de células alveolares, os pneumócitos tipo 1 (AT1) e, prioritariamente, pneumócitos tipo 2 (AT2) (Bridges *et al.*, 2022). Neste ambiente, AT1 são células envolvidas nas trocas gasosas e perfazem a maioria do revestimento alveolar, mas, AT2 secretam surfactante pulmonar e, acima de tudo, são células progenitoras de AT1 (Robinot *et al.*, 2021; Zhu *et al.*, 2020).

Nos pacientes com esses quadros, a injúria dos vírus nas células alveolares, com redução e não diferenciação celular em AT1, assim como nas células endoteliais (Xu *et al*., 2023) permite o extravasamento de fluido dos vasos para a luz alveolar (Bridges *et al.*, 2022).

Tal processo é alimentado pela quebra de barreira do glicocálix, pela vasodilatação (via sistema cinina-calicreína-ACE2) (Van de Veerdonk *et al.*, 2020) e

por uma avalanche de mediadores pró-inflamatórios como as citocinas IL-1, IL-6, fator de necrose tumoral alfa (TNF-α), IL-2, IL-8, IL-17, entre outras, e quimiocinas como a proteína quimio-atraente de monócitos (*monocyte chemoattractant protein 1* - MCP-1), CXCL2, CXCL8, CXCL9 e CXCL16, CXCL10, CCL-5 e fator estimulador de colônias granulocitárias (G-CSF) (Blanco-Melo *et al.*, 2020; Huang; Xu; Zhou, 2021); fatores pró-coagulantes (fator tissular ativado, colágeno, padrões moleculares associados a danos (*damage associated molecular patterns* - DAMP); fatores antifibrinolíticos (inibidor da ativação do plasminogênio 1 - PAI1); pró-agregantes plaquetários (armadilhas extracelulares dos neutrófilos - *neutrophil extracellular traps* - NETs); indutores da ativação de complemento; indutores da ativação celular citotóxica; assim como pela hiperativação plaquetária (Conway *et al.*, 2022; Lamers; Haagmans, 2022).

Como agravante, além da diversidade de citocinas e quimiocinas, e em decorrência destas, há o grande influxo de células imunes para os pulmões, em especial neutrófilos e monócitos (Chen *et al.*, 2020a, 2020b; Parasher, 2021; Vasquez *et al.*, 2021).

Notadamente, infiltrados mononucleares são achados frequentes em lesões pulmonares em necrópsias (Xu *et al*., 2020) e neutrófilos se perpetuam nos alvéolos e na circulação em razão da liberação de proteína de adesão vascular 1 (VCAM-1) pelas AT1 e da granulocitopoiese induzida por IL-17 (Pacheco; Faria, 2021).

Como consequência, a membrana hialina e o padrão intersticial difuso de lesão (vidro fosco) da COVID-19 visto em imagem radiológica pulmonar podem evoluir ao final para uma extensiva fibrose no interstício (Mo *et al.*, 2020; Pan *et al.*, 2020), uma vez que fibroblastos se proliferam para substituir o tecido lesionado. Isto irá impactar na perda progressiva e, por vezes, irreversível da capacidade ventilatória do paciente (Brandao-Rangel *et al*., 2021; Xu *et al*., 2020).

Então, nas pneumonias e na SDRA associadas à COVID-19, as unidades de trocas gasosas deixam de ser funcionais e se tornam ambientes cheios de exsudato, citocinas, muco, células inflamatórias, restos celulares e, por fim, fibrose (Quan *et al*., 2021; Xu *et al*., 2020).

A seguir, o que se observa originalmente nos pulmões adquire contornos sistêmicos, diretamente pelo efeito pró-inflamatório ainda mais amplo de citocinas e quimiocinas (Xiong *et al.*, 2020), pelo estresse oxidativo mediado por espécies oxigênio reativos (ROS) (Cecchini; Cecchini, 2020; Zhao *et al.*, 2022), pelas

consequências orgânicas funcionais determinadas pela hipóxia (Deng *et al*., 2020), pela possível desregulação do sistema renina-angiotensina via receptores ACE2 (kirtipal *et al.*, 2022; Zhao *et al.*, 2022), pelos eventos tromboembólicos mediados por NETs (Borges *et al*., 2020; Cesta *et al*., 2023; Tsai *et al*., 2021; Veras *et al*., 2020) e pela disfunção endotelial (Xu *et al*., 2023).

Evidências acerca de quadros trombóticos durante a fase aguda e pós-aguda da COVID-19, sugerem que a infecção viral afeta direta ou indiretamente o endotélio dos vasos, lesionando a seguir indistintamente múltiplos órgãos (Xu; Ilyas; Weng, 2023).

Posteriormente, um outro cenário se estabelece nos pacientes sobreviventes, onde há a exaustão da resposta imune celular e um estado disfuncional permanente desta (Alahdal; Elkord, 2022; Barnova *et al.*, 2021). A linfocitopenia, com diminuição dos níveis de linfócito T (LT) CD4+ e CD8+ e aumento de marcadores celulares de exaustão das células T (Diao *et al.*, 2020) se tornam presentes mediante a severidade da COVID-19 (Gil-Etayo *et al.*, 2021) e, possivelmente sejam resultado da atividade dos altos níveis de citocinas (TNF-α, IFN-γ) (Barnova *et al.*, 2021; KARKI *et al.*, 2020, 2021) e da morte celular induzida (Ferreira *et al.*, 2021; Karki *et al.*, 2021; Karki; Kanneganti, 2022).

Tais fenômenos podem ser determinantes ou decorrentes do direcionamento para resposta celular com fenótipo T auxiliar 17 (Th17) em detrimento da resposta celular regulatória (T*reg*) (De Biasi *et al.*, 2020; Martonik *et al*., 2021), ou ainda, da polarização da resposta senescente Th2 (De Biasi *et al*., 2020; Gil-Etayo *et al.*, 2021).

Observa-se que o fenótipo Th17 está envolvido na migração de neutrófilos e monócitos para o local da infecção, porém é característico também da inflamação crônica e da ocorrência de doenças autoimunes, o que denota sua participação no prolongamento do quadro clínico da doença (Martonik *et al*., 2021).

Aqueles efeitos esperados e coordenados para a resposta celular antiviral eficiente – 1) resposta Th1 e NK, ativação de macrófagos e efeito citotóxico sobre células infectadas; 2) reposta regulatória (Treg) para a limitação da inflamação; 3) resposta Th2 voltada à produção de anticorpos e memória imunológica - são minimizadas com o avançar da idade, num processo fisiológico reconhecido como imunossenescência (Wang *et al.*, 2022).

Adicionalmente, o envelhecimento contribui negativamente para a geração de nova memória imunológica, em razão da menor disponibilidade e desdiferenciação de

células T originárias (LT*naive*) (Baroja-Mazo *et al.*, 2014) com o avançar dos anos (Cañete; Vinuesa, 2020; Jarjour; Masopust; Jameson, 2021; Minato; Hattori; Hamazaki, 2020).

Desta forma, mesmo na ausência de infecção, são marcadores importantes da imunossenecência: o fenótipo secretório associado a senescência (SASP) (Aiello *et al.*, 2019), com aumento de MCP-1, IL-6, IL-8, IL-18, IL-29, IFN-γ e TNF-α e expressão de receptores celulares respectivos; a diminuição de LT efetoras e LT*naive*; o aumento de radicais oxigênio reativos (ROS), glicólise e disfunção mitocondrial (Liu *et al.*, 2023).

Possivelmente, a polarização imunológica divergente do esperado na COVID-19 seja fruto da concomitância da patogenicidade e da imuno-evasão do SARS-CoV-2 (Taefehshokr *et al.*, 2020), assim como do processamento imunológico insuficiente que acompanha a senilidade (Wang *et al.*, 2022).

Além da imunossenescência (Liu *et al*., 2023) e da influência do sexo (Gadi *et al.*, 2020; Takahashi *et al.*, 2020) sobre a evolução da COVID-19, possivelmente fatores genéticos possam influenciar o risco individual de severidade e óbito dos pacientes (Dos Santos *et al.*, 2021).

Em suma, a inflamação é um processo protetivo para o organismo. Este tem por objetivo bloquear infecções e impedir o dano tecidual. Entretanto, na COVID-19, este se amplifica e desregula de tal forma que, sem intervenção, a injúria que se inicia nos pulmões torna-se multissistêmica e direciona pacientes mais susceptíveis a uma evolução desastrosa e, por vezes, ao óbito (Casadevall; Pirofski, 2020).

Desta forma, os mecanismos dessa amplificação desordenada e sustentada se mostram um frutífero campo de estudo para elucidar a fisiologia da resposta orgânica ao SARS-CoV-2, bem como para estabelecer estratégias terapêuticas mais eficientes para a doença.

1.1.4.3 *O inflamassoma NLRP3 e a COVID-19*

Para as células, sinais exógenos ou endógenos vindos da injúria tecidual, da hipóxia ou da invasão por microrganismos indicam a necessidade de resposta imediata que irá suscitar inúmeras alterações moleculares capazes de minimizar o dano e/ou limitar a dispersão de patógenos (Da Silva *et al.*, 2022; Riteau *et al.*, 2012).

A resposta imediata é dada pela ativação de sistemas conhecidos como

inflamassomas, capazes de amplificar a resposta inflamatória em minutos, liberando citocinas pró-inflamatórias e ou determinando a morte de células infectadas (Abdul-Sater; Philpott, 2016; Paerewijck; lamkanfi, 2022).

Dentre os vários inflamassomas, aquele que se utiliza do sensor com domínio de ligação e oligomerização de nucleotídeo, com domínio rico em repetições de leucina e domínio pirina 3 (*nucleotide-binding oligomerization, leucine rich repeat and pyrin domain containing 3* - NLRP3) é o mais estudado (Huang; Xu; Zhou, 2021; Wang; Hauenstein, 2020; Yang *et al.*, 2019; Zhang *et al.*, 2023; Zhong *et al.*, 2020).

A ativação do inflamassoma via NLRP3 na COVID-19 pode explicar a hiperinflamação advinda do microambiente pulmonar, suas consequências sistêmicas e, possivelmente as alterações imunológicas a longo prazo. Em células do sistema imune (SI), tal ativação tem a participação significativa de receptores purinérgicos transmembrana, os quais funcionam como detectores de alterações nos níveis de adenosina trifosfato extracelular (ATPe) respectivos ao dano celular (Baroja-Mazo *et al.*, 2014; Khakh; North, 2006; Rodrigues; Tomé; Cunha, 2015).

Entende-se que em macrófagos, a ativação clássica (canônica) requer duas etapas: *priming* e ativação. A etapa de *priming* (sinal 1) é fornecida por estímulos inflamatórios, como agonistas de receptores TLR4, que induzem a expressão de NLRP3 e pró-IL-1β mediada pelo fator de transcrição NF-κappa B (*nuclear factor* - NF-κB). A seguir, a etapa de ativação é desencadeada por padrões moleculares associados a patógenos (*pathogen associated molecular pattern* – PAMP) e DAMP (Zhang *et al.*, 2023), No entanto, a etapa de iniciação é suficiente para que em monócitos ocorra a ativação da caspase-1 (CASP-1) e a liberação de IL-1β (Yang *et al.*, 2019).

Supõe-se que, frente à expansiva infecção do SARS-CoV-2, o microambiente pulmonar se mostra extremamente rico em ATPe, seus derivados, restos celulares e antígenos virais, os quais são capazes em conjunto de impulsionar a amplificação do processo inflamatório (Da Silva *et al.*, 2022; Lee *et al.*, 2012).

Sensores intracelulares NLRP3 são ativados e a seguir se associam a moléculas da proteína adaptadora apoptótica contendo domínio de recrutamento e ativação de caspase (*apoptotic speck like protein containing a caspase recruitment activation domain* - ASC) e às pró-CASP-1 (Martinon; Burns; Tschopp, 2002). Estas últimas sofrerão autoclivagem e, ao final, diversas unidades de CASP-1 formarão o complexo multimérico NRLP3/ASC/CASP-1 apropriadamente denominado

inflamassoma NLRP3 (Abdul-Sater; Philpott, 2016; Paerewijck; Lamkanfi, 2022). Este amplifica a maturação proteolítica de IL-1β e IL-18 e, também, pode determinar a morte da célula infectada por piroptse (Y. Huang *et al*., 2021; L. Wang & Hauenstein, 2020; W. J. Zhang *et al*., 2023).

A IL-1β é um potente regulador pró-inflamatório nas células alvo para o recrutamento de neutrófilos, ativação de macrófagos e expansão de LT sensibilizadas (Ferrari *et al.*, 2006; Gabay; Lamacchia; Palmer, 2010). E, IL-18 induz a produção de IFN em outras células, medeia a resposta Th1 e aumenta a atividade citotóxica de LTCD8 e células NK (Tsutsui *et al.*, 2003; Yasuda; Nakanishi; Tsutsui, 2019).

A morte celular induzida e relacionada a patógenos (piroptose) é dependente da atividade da CASP-1 sobre a gasdermina D (GSDMD), uma proteína que após clivagem é capaz de se oligomerizar na membrana celular (MC) para a formação de poros, os quais determinam grande efluxo de citocinas e lise da célula parasitada (Burdette *et al*., 2021; X. Liu *et al*., 2016) (**Figura 1**).

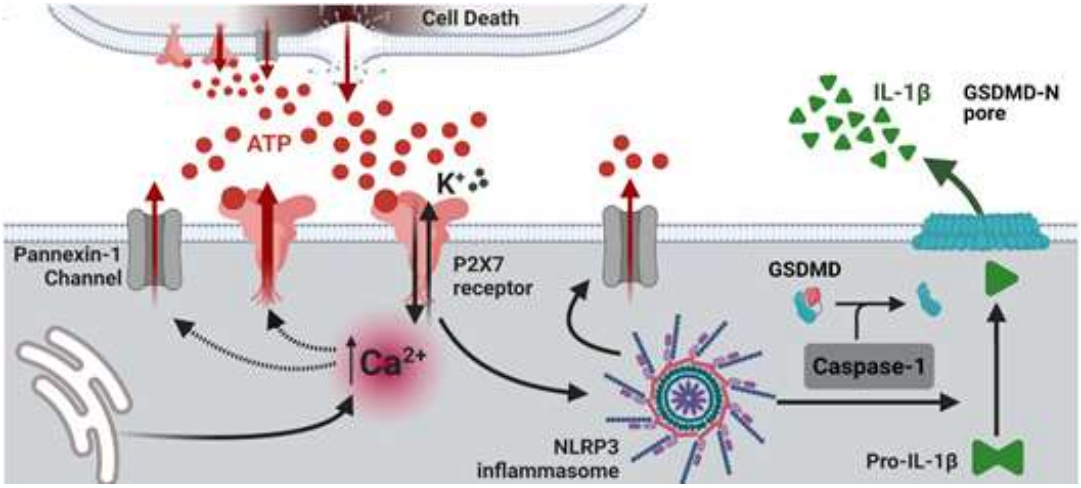
A seguir, em diferentes tipos celulares, a ativação via NLRP3 pode ser envolvida em outros processos, como a apoptose e a autofagia (Biasizzo; Kopitar-Jerala, 2020; Huang; Xu; Zhou, 2021).

Além do ATPe presente no microambiente sob agressão e hipóxia, a montagem de inflamassomas é induzida em macrófagos por PAMPs de SARS-CoV-2 como a proteína viral do nucleocapsídeo (N) (Pan *et al*., 2021), pela viroporina codificada em ORF3 (*open read frame 3*) (Siu *et al*., 2019; Xu *et al*., 2022), e pela proteína S (Theobald *et al.*, 2021), em um processo em que a resposta inflamatória excessiva obtida está associada à severidade da COVID-19.

O inflamassoma também foi relacionado à proteína do envelope (E), porém de forma ambígua, quer seja por meio da indução do complexo multiproteico via NRLP3, ou mitigando a expressão deste receptor em fases iniciais da infecção, como parte da evasão imunológica do SARS-CoV-2 (Yalcinkaya *et al.*, 2021).

Estudos demonstraram também que a severidade da COVID-19 está acompanhada da intensa morte de monócitos mediada por NLRP3 (Ferreira *et al*., 2021), da mesma forma que macrófagos pulmonares (Sefik *et al.*, 2022). Mas, por outro lado, supõe-se que a outra face que caracterizaria uma relação negativa inflamassoma/SARS-CoV-2 seria a supressão da piroptose e não liberação da IL-1β processada de monócitos como forma de imuno-evasão do vírus (Ma *et al*., 2021).

FIGURA 1 – A relação ATP-P2X7-NLRP3 e a secreção de IL-1β



As células que morrem liberam ATP. O ATP se liga e ativa os receptores P2X7, o aumento da concentração intracelular de Ca^{2+} induz a formação de grandes poros pelos P2X7 e pela panexina-1, ambos permitindo a liberação de mais ATP num processo de autoalimentação positiva. O efluxo de K+ induz a montagem do inflamassoma NLRP3 e a formação de mais canais de panexina-1. A montagem do inflamassoma NLRP3 ativa a caspase-1, que cliva a pró-IL-1β em IL-1β e promove sua liberação. A caspase-1 também cliva a gasdermina (GSDMD), que interage com os fosfolipídios da membrana para formar um poro que permite a liberação de IL-1β. Esta libertação também pode ocorrer através de microvesículas, exossomas ou endossomas (não mostrados).
Fonte: Adaptado de (Oliveira-Giacomelli *et al.*, 2021).

Também, além do efeito sobre monócitos circulantes e macrófagos pulmonares, a ativação do inflamassoma NRLP3 -via ACE2-NF-kB- foi demonstrada experimentalmente na micróglia, pelo efeito indutor da proteína S (Albornoz *et al.*, 2022), de maneira a conectar o NRLP3 à patogênese das alterações neurológicas e piora da progressão de doenças neurodegenerativas como Alzheimer (AD) e Parkinson (PK) após a COVID-19 (Huang *et al.*, 2023).

Desta forma, o processo inflamatório essencial ao controle infeccioso passa a ter contornos diferentes dos fisiologicamente esperados, quer seja pela ascensão do vírus sobre os processos moleculares de ativação da resposta imune, quer seja pela amplificação desta dentro de um contexto vicioso e que tem como desfecho possível a morte celular.

1.2 O RECEPTOR P2X7 (P2RX7), O INFLAMASSONA NLRP3 E O ATP EXTRACELULAR

O ATPe é um dos mais ubíquos DAMP já reconhecidos, soando como um imprescindível sinal de alarme para a injúria tecidual (Mortaz *et al.*, 2010; Riteau *et al.*, 2012). Assim, no microambiente, as células imunes - em especial macrófagos

(Pelegrin, 2021) – percebem a alteração nos níveis do ATPe via sensores transmembrana, os canais purinérgicos (Khakh; North, 2006; North, 2002).

Expressos em macrófagos, células dendríticas, neutrófilos e tipos celulares de diversos tecidos, esses canais são responsáveis pelo trânsito de prótons e ATP pela MC e capazes de responder rápida (milissegundos) e proporcionalmente à ativação dada pelo aumento substancial de ATPe (Burnstock; Kennedy, 2011).

Para todos os receptores, a presença abundante de altos níveis de ATP no microambiente extracelular determina a “abertura” dos canais e maior permeabilidade dos mesmos aos cátions (influxo de Na^+, Ca^{2+} e H^+ e efluxo de K^+). A alteração da concentração intracelular de cátions, em especial K^+, é o sinal iônico para que haja a ativação do inflamassoma mediado por NLRP3 em células do SI. Mas, respostas diferentes são obtidas em cada tipo celular e são, provavelmente, respectivas também à funcionalidade de cada variante do receptor purinérgico expresso (Burnstock, 2014; North, 2002).

Dentre esses, o receptor P2X7 (P2RX7) tem sido o mais frequentemente associado a condições patológicas (Burnstock; Kennedy, 2011) e, portanto, mais abordado experimentalmente em modelos funcionais, assim como por meio de análises genômica, proteica estrutural e espacial e da expressão gênica, principalmente ligadas à funcionalidade no SI e no sistema nervoso (SN) (Di Virgilio *et al.*, 2017; Khakh; ALAN North, 2006; Miras-Portugal *et al.*, 2021; Sluyter, 2017).

Os purinoreceptores P2X, em especial P2X7, estão amplamente distribuídos nos tecidos e em células do SI relacionados a diversas funções fisiológicas (Illes, 2020; Martínez-Cuesta *et al.*, 2020) (**Quadro 2**), dentre elas a modulação da liberação pré-sináptica de neurotransmissores no SN, especialmente glutamato, ácido gama-amino-butírico (GABA) e arginina-vasopressina (Miras-Portugal *et al.*, 2017, 2021).

Além das funções no SI e no SN, pesquisadores concluíram que a interação de receptores P2X7, P2Y13, TKR e NMDA e a ativação de cinases, algumas dependentes de Ca^{2+}, resultam na transcrição de genes envolvidos na sobrevivência de células e sua proliferação, com diferentes nuances em células de origem neural ou não (Miras-Portugal *et al.*, 2021; Ortega *et al.*, 2010).

No **Quadro 2** estão listadas as principais funções e processos para os quais existem evidências relacionadas a P2X7, assim como as alocações do receptor.

Acerca da funcionalidade do receptor, pesquisas demonstraram que a estimulação tônica de baixo nível por ATP liberado endogenamente gera um estímulo

trófico em P2X7, a qual aumenta a eficiência do metabolismo mitocondrial de forma contínua (Di Virgilio; Ferrari; ADinolfi, 2009; Orioli *et al.*, 2017). E, apenas em P2X7, a ativação de rápido fluxo transmembrana de cátions é desencadeada somente por altas concentrações de ATPe (de 0,05-1 mM) (Martinon; Burns; Tschopp, 2002).

QUADRO 2 – P2X7, suas funções fisiológicas e alocação celular

FUNÇÕES E PROCESSOS	ALOCAÇÃO CELULAR
Sinalização apoptótica	**Transmembrana (MC) em células de diversos tecidos**
Transporte e regulação da concentração de Ca^{2+}, Na^{+} e K^{+}	
Formação de vesículas a partir da MC (*blebbing*)	**Vesículas na MC**
Resposta ao ATP, NAD, RNAds, LPS	**Mitocôndria**
Ativação do inflamassoma NLRP3 e secreção de citocinas	**Junção neuromuscular**
Envolvido na cascata MAPK	**Pré-sinapse**
Homeostase, citotoxidade, proliferação e diferenciação de LT	**Pós-sinapse**
Envolvido no potencial excitatório pós-sináptico	**Corpúsculos neurais**
Secreção de GABA e glutamato	

MC: membrana celular; LT: linfócito T; MAPK: mitogen-activated protein kinase (proteína quinase ativada por mitógeno; GABA: ácido gama-aminobutírico
Fonte: Adaptado de NCBI (2023)

Assim, somente essa sinalização potente e o consequente decréscimo do K^{+} intracelular são determinantes para a ativação do fator transcricional NF-kB e a formação do complexo multiprotéico NLRP3 (Kahlenberg; Dubyak, 2004), em especial em células apresentadoras de antígenos (*antigen presenting cells* – APC) como monócitos, macrófagos teciduais e células dendríticas (Di Virgilio *et al.*, 2017; Liu; Cao, 2016) (**Figura 1**)

Desta forma, P2X7 participa ativamente de processos fisiológicos celulares regulando o transporte de ATP e íons pelas membranas (Sluyter, 2017), mas também da liberação de citocinas IL-1β, IL-18, além da piroptose, durante a resposta imune inata, de acordo com o estímulo de seu ligante externo (Adinolfi *et al.*, 2018).

A seguir, após longa ativação por ATPe, P2X7 pode induzir a formação der macroporos na MC (Di Virgilio; Schmalzing; Markwardt, 2018), o suficiente para

permitir a passagem de substâncias maiores e complexas como moléculas orgânicas de até 900Da e corantes fluorescentes (Alves *et al.*, 2014; Di Virgilio *et al.*, 2017; Di Virgilio; Schmalzing; Markwardt, 2018).

O receptor ainda responde a estímulos intracelulares como o lipopolissarídeo (LPS) (Gabarin *et al.*, 2021) junto ao domínio C terminal. Assim, tem seu limiar de ativação pelo ATP diminuído e, via CASP-11, responde de forma não canônica a componentes bacterianos na sepse, por exemplo (Di Virgilio *et al.*, 2017; Yang *et al.*, 2015).

Além disso, alterações na concentração de lipídios (Murrell-Lagnado, 2017) e defensinas (Semple *et al.*, 2019) podem se tornar gatilhos para alterações celulares promovidas por P2X7, a exemplo da dilatação de poros na MC (Coddou *et al.*, 2011). E, mecanismos independentes de NLRP3 são suficientes também para a secreção de, por exemplo, TNF-α (Suzuki *et al.*, 2004) e IL-6 (Magni *et al.*, 2021) em células não imunes.

Além das ACP, P2X7 está altamente expresso em LT periféricos e, no processo de morte celular via ATPe, os LT*naive* são mais susceptíveis à ativação deste receptor que os demais LT (Winzer *et al.*, 2022) de

Adicionalmente, P2X7 se mostra um canal não seletivo, uma vez que, em LT- especialmente T*reg* Foxp3^{+}P2X7^{+} - mesmo quantidade micromolar de nicotinamida dinucleotídeo (NAD^{+}) é suficiente para ribosilação de P2X7, e indução de apoptose, como função moduladora do SI (Hubert *et al.*, 2010).

E, ainda, mesmo que P2X7 seja reconhecido primariamente por sua sinalização imune e ativação do inflamassoma NLRP3, este também afeta a eliminação de detritos extra e intracelulares por meio de modificações da função lisossômica, fagocitose e pelo processo de autofagia (Campagno; Mitchell, 2021). Tais processos são necessários para reciclagem e remoção de proteínas e organelas danificadas no tecido, mas seu descontrole gera efeitos celulares deletérios (Biasizzo; Kopitar-Jerala, 2020; Campagno; Mitchell, 2021).

A seguir, durante a resposta imune adaptativa, P2X7 participa ainda da modulação da resposta Th1, entre Th17 e T*reg* (SAVIO *et al.*, 2018), assim como nas interações entre células foliculares (Tfh) e linfócitos B (LB) para a produção de anticorpos específicos (Proietti *et al.*, 2014).

Uma vez que esse receptor é multifacetado, fisiologicamente, a sua ativação deve ser multicontrolada, seja por mecanismos que alteram a expressão do receptor

purinérgico, isto é, regulação transcricional e pós-traducional - incluindo polimorfismos de nucleotídeo único (*Single Nucleotyde Polymorphisms* - SNP) - regulação do promotor via metilação do DNA, fatores de transcrição (por exemplo, Sp1 e HIF-1α), a geração de diferentes variantes de *splicing e* por microRNA (miRNA), além da fosforilação do receptor, sua glicosilação, ribosilação e palmitoilação (alterações lipídicas) e, ainda, por meio de chaperonas com a proteína de choque térmico 90 (*heat shock protein of* 90 kDa - HSP90) (Jimenez-Mateos *et al.*, 2015, 2019; Sluyter, 2017).

A modulação de P2X7 é possível ainda por meio de moléculas não próprias como peptídeos antimicrobianos derivados da catelicidina, polimixina B, ivermectina, tenidap, clemastina e ginsenosídeos (Sluyter, 2017).

Assim, a dinâmica de resposta a diferentes quantidades de estimulo exógeno qualifica a diferenciada funcionalidade do receptor em células de diferentes origens, assim como sua estrutura, expressão, modulação e associação com canais não seletivos como panexina-1 e outros receptores como P2X4 e TLR4 (Lemaire *et al.*, 2011; Martínez-Cuesta *et al.*, 2020; Semple *et al.*, 2019; Sluyter, 2017).

P2X7 está presente em inúmeros tipos celulares, inclusive nos pulmões, em células renais, exócrinas, endoteliais, do sistema nervoso, entre outras (Burnstock, 2014; Fagerberg *et al.*, 2014; Sluyter, 2017). E, também, foi determinada sua expressão em células progenitoras hematopoiéticas (Rossi *et al.*, 2012) e mesenquimais (Jiang *et al.*, 2017), assim como em diversos tipos celulares apresentando malignidade (Lara *et al.*, 2020; Qian *et al.*, 2017).

Mesmo multi-expresso, P2X7 acumula evidências de que o processo inflamatório seja a maior de suas funcionalidades, em especial pela sua alocação em células centrais da resposta imune inata e adaptativa (Adinolfi *et al.*, 2018; Burnstock; Boeynaems, 2014; Burnstock; Kennedy, 2011; Miras-Portugal *et al.*, 2021; Sluyter, 2017; Vargas-Martínez *et al.*, 2020).

No entanto, segundo Martínez-Cuesta *et al*. (2020), diversas perguntas ainda se mantém sem respostas concretas acerca de P2X7, dentre estes, qual a participação de seus polimorfismos, variantes e processos de modulação na biologia das células.

Além disso, muito ainda se discute sobre a ambiguidade do receptor e onde sua funcionalidade se cruza com sua participação em processos patológicos (Savio *et al.*, 2018).

1.2.1 A Relação P2X7- Doenças e o seu Contexto Genético Polimórfico

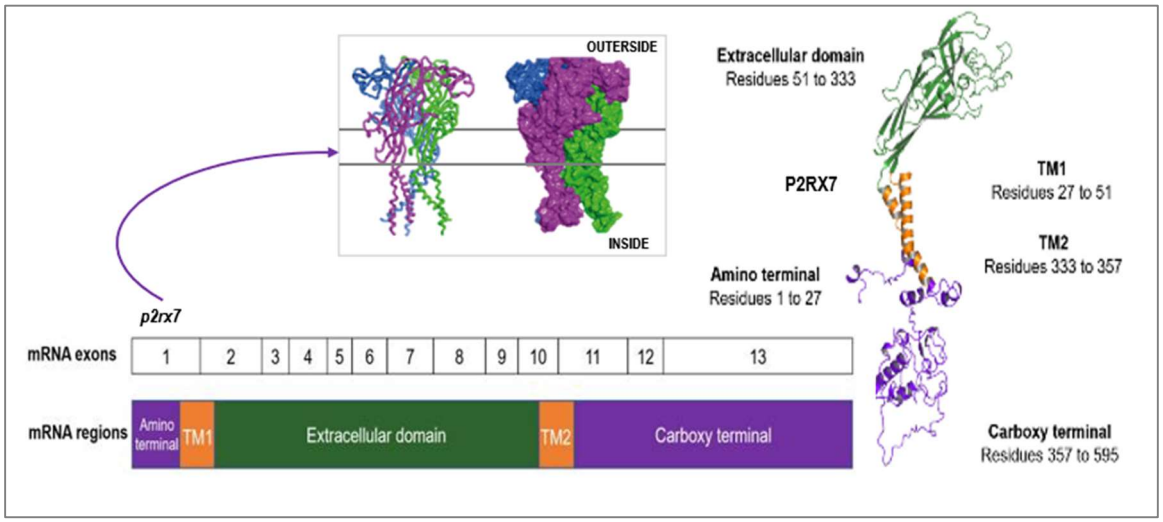
Alocado no cromossomo 12 humano, *p2rx7* tem 53kb e sequências genômicas organizadas em 13 exons para a codificação da proteína P2X7 de 595 aminoácidos (**Figura 2**). Este gene é altamente polimórfico, no qual foram identificadas mais de 13.000 SNP e pelo menos 150 apenas na alça extracelular que se liga ao ATPe e no domínio C-terminal intracitoplasmático (Sluyter, 2017). Este ainda também sofre *splicing* gerando variantes com diversificação no número de exons e na estrutura genômica a ser decodificada (De Salis *et al.*, 2022).

Dentre as muitas variantes genéticas já estudadas, a mais reportada e relacionada a condições patológicas é o polimorfismo não sinônimo na posição 1513A>C, designada rs3751143. A mutação determina a alteração do resíduo ácido glutâmico para alanina na posição 496 da proteína transmembrana P2X7 (Glu496Ala/E496A). O polimorfismo Glu496Ala confere uma característica incomum ao receptor em células nativas ou transinfectadas, uma vez que a captação de etídio induzida por ATP é abolida, mas a abertura imediata do canal seletivo para cátions não é afetada (Boldt *et al.*, 2003; Di Virgilio *et al.*, 2017).

Segundo Gu *et al.* (2001) e Saunders *et al.* (2003), a mutação 1513A→C no exon 13 altera a constituição citoplasmática do receptor (carboxiterminal), determina a perda da função apoptótica de linfócitos e macrófagos quando em homozigose e a perda de 50% de função quando em heterozigose, implicando, a princípio, na patogênese e evolução da tuberculose (TB) doença (Abhimanyu *et al.*, 2023; Azad; Sadee; Schlesinger, 2012; Fernando *et al.*, 2007; Keikha; Karbalaei, 2022; Niño-Moreno *et al.*, 2007; Placido *et al.*, 2006; Taheri *et al.*, 2019).

A seguir, Wiley *et al*. (2002) observaram que o efeito anti-apotótico da mutação 1513A→C contribui para a patogênese de leucemia linfocítica crônica de células B (LLC), abrindo campo para estudos de associação e debates acerca da participação de P2X7 na fisiopatogênese também de malignidades hematológicas (De Marchi; Pegoraro; Adinolfi, 2021; Nückel *et al.*, 2004; Thunberg *et al.*, 2002; Zhang; Zhu, 2020), bem como da modulação da resposta inflamatória e do efeito citotóxico desta mutação em neoplasias (Wesselius *et al.*, 2012).

FIGURA 2 – Sequências (exons) codificadoras da proteína P2X7 (P2RX7) organizada em homotrímeros para formar o receptor purinérgico transmembrana.



Fonte: Modificado de De Salis *et al.*, 2022.

Dentre outros SNP já estudados de P2X7, a mutação intrônica conhecida como rs2393799 na posição -762 de *p2rx7,* determina a mudança de citosina para timidina (-762C>T) na região promotora do gene. Seu polimorfismo está citado por autores como perda de função para o alelo C, porém apresenta resultados conflitantes na literatura quanto ao seu efeito sobre a susceptibilidade a doenças, em especial à TB (Duan *et al.*, 2016; Tekin *et al.*, 2010; Yi *et al.*, 2014), possivelmente em razão das diferentes populações envolvidas nos estudos de associação.

1.2.1.1 *P2X7 e as doenças do sistema nervoso*

Patologias inflamatórias crônicas, metabólicas e neurodegenerativas foram já relacionadas à expressão e ativação de P2X7 (Pelegrin, 2021b).

No sistema nervoso central (SNC), importantes linhas de investigações foram conduzidas acerca do papel da sinalização purigênica em desordens cerebrais. Nestas, a participação de P2X7 na fisiopatogênese já foi sedimentada ou existem evidências que sinalizam neste sentido, tais como nos processos hemorrágicos e isquêmicos cerebrais (Zhao *et al*., 2018a) e doenças neurodegenerativas (Illes, 2020; Illes *et al.*, 2020; Zelentsova *et al.*, 2022) , principalmente AD, PK e Huntington

(Andrejew *et al.*, 2020; Cherninskyi *et al.*, 2023; Francistiová *et al.*, 2020; Ollà *et al.*, 2020; RIBEIRO *et al.*, 2021; Thawkar; Kaur, 2019), assim como na esclerose múltipla (Cardona *et al.*, 2017), na epilepsia (Morgan *et al.*, 2020; Wong; Engel, 2023) e nas desordens neuropsiquiátricas como a depressão, a ansiedade e a esquizofrenia (Burnstock; Kennedy, 2011), além da adicção ao álcool e às drogas (Miras-Portugal *et al.*, 2021).

P2X7 tem sido descrito como intrínseco a todas as células do SNC, mas particularmente na micróglia (imunidade inata) e oligodendrócitos (Jimenez-Mateos *et al.*, 2019). Embora já exista consenso quanto à relação entre o receptor e a modulação da neurotransmissão e da dor de origem neural (Hua *et al.*, 2022; Miras-Portugal *et al.*, 2017; Sandy-Hindmarch *et al.*, 2022; Sluyter, 2017), outros pesquisadores sugerem que no SNC, P2X7 teria sua expressão relacionada somente a quadros patológicos (Cardona *et al.*, 2017; Morgan *et al.*, 2020), com evidências que sinalizam para a forte correlação entre neurodegeneração e neuroinflamação (Illes, 2020; Illes *et al.*, 2020; Illes; Khan; Rubini, 2017).

Assim, na neurodegeneração associada ao envelhecimento, observa-se excessivo acúmulo lisossomal na micróglia, possivelmente relacionado à disfunção do sistema P2X7-NLRP3 (Campagno; Mitchell, 2021).

Ainda, pesquisas associaram a funcionalidade do receptor à patogênese da AD por vias que determinam a deposição da proteína amilóide beta (Aβ) (Chiozzi *et al.*, 2019); indução da migração da micróglia, fagocitose e ativação do NLRP3 nesta; indução do estresse oxidativo mitocondrial, e consequente perda neuronal; direcionadas à manifestação e/ou pior evolução da doença (Francistiová *et al.*, 2020; Thawkar; Kaur, 2019).

Contraditoriamente, para pacientes com AD pré-clínico versus controles, a menor expressão de P2X7 na superfície de leucócitos foi proposta como um biomarcador para esta doença (Li *et al.*, 2022b). Ainda de forma discordante do contexto da maioria das evidências, estudos de associação indicam que o ganho de função do receptor poderia assumir um papel protetivo na evolução da AD (Sanz *et al.*, 2014).

Para além da AD, outros autores sugerem, por meio de evidências em modelos animais, que o antagonismo do receptor poderia ser útil como antipsicótico nos casos de esquizofrenia, por exemplo (Huang *et al.*, 2021a).

Assim, mediante a forte associação de P2X7 com diversas desordens

neurológicas e cérebro vasculares, estudos de polimorfismos genéticos e expressão do receptor também têm sido acompanhados pela descoberta e desenvolvimento de diversas substâncias com potencial antagonista terapêutico (Andrejew *et al.*, 2020; Bai *et al.*, 2021), no intuito de bloquear a evolução do estado demencial relacionado às condições clínicas desafiadoras que envolvem o SNC.

Assim, a DP já foi associada ao genótipo rs1513A>C e até mesmo à utilização de radioligantes para diagnósticos por imagem, os quais conseguem vislumbrar diferenças de expressão de P2X7 quando na presença da rs3751143 (Van Weehaeghe *et al.*, 2019).

Interessantemente, a associação de SNP de *p2rx7* com PK e AD e a frequência maior de mutantes com perda de função do receptor (1513CC) na população idosa da Europa e América do Norte direcionaram à hipótese de que indivíduos caucasianos com este fenótipo P2X7 antiinflamatório, e que vivem em países de alta renda, possam ter uma expectativa de vida mais longa (Sanz *et al.*, 2020) que idosos de outras origens. A comprovação desta estabeleceria a ligação definitiva entre P2X7 e os caminhos que nos direcionam à senescência.

1.2.1.2 *P2X7, a hipertensão arterial e as alterações metabólicas*

Para a hipertensão arterial (HA), desordem que afeta grande parte da população, há consenso que a disfunção resulta de uma interação múltipla de sistemas. Entretanto, todos os tecidos envolvidos são alocação de subunidades P2X, os quais recebem a influência da liberação de ATP por eritrócitos e plaquetas e sua degradação a adenosina difosfato (ADP), adenosina monofosfato (AMP) e adenosina (Burnstock, 2017; Burnstock; Kennedy, 2011).

A relação ATP, resposta inflamatória e HA foi estudada por Zhao *et al*. (2019) em modelo animal, concluindo que na fisiopatologia desta, diferentes concentrações de ATP interferem na relação APC e células efetoras, via P2X7.

A despeito da resposta inflamatória, Li *et al.* (2017) sugeriram que a atividade do ATPe sobre P2X7, possivelmente superexpresso em glomérulos de hipertensos, pode também contribuir para o desenvolvimento de HA.

No intuito de validar esta associação, estudo caso-controle com 248 famílias com HA demonstrou associação positiva da alteração na pressão diastólica noturna com a variação alélica intrônica de *p2rx7* (rs591874), o que poderia sugerir o receptor

como potencial alvo terapêutico para a HA (Palomino-Doza *et al.*, 2008).

Da mesma forma que na HA, o diabetes tipo 2 (DM2) e suas complicações microvasculares, ou a resistência à insulina são condições clínicas multifatoriais que envolvem tecidos ocupados por P2X7, e sofrem influência da ativação da resposta inflamatória, a qual pode ser a origem ou o desfecho do processo envolvendo o receptor (Solini; Novak, 2019).

Ainda no contexto metabólico, P2X7 está significativamente expresso também em osteoblastos e osteoclastos (Burnstock; Kennedy, 2011). Desta forma, foram obtidas evidências de que o receptor seria candidato a alvo terapêutico também para a osteoporose em mulheres pós-menopausa. No mesmo contexto, a inatividade de P2X7 foi associada também à maior prevalência de fraturas em pessoas sujeitas a grande estresse físico (Varley *et al.*, 2016).

Nessas pacientes, a perda funcional polimórfica do receptor (1513CC) está fortemente relacionada à perda óssea e à fratura vertebral em razão da ausência de modulação deste, e da sua influência na diferenciação e vida útil dos tipos celulares envolvidos na homeostase óssea (Huang *et al.*, 2021b; Wang *et al.*, 2018).

1.2.1.3 *P2X7 e as doenças do trato respiratório inferior (TRI)*

Também no TRI, entende-se que P2X7 seria importante fisiologicamente na eliminação de patógenos, uma vez que está expresso nos macrófagos alveolares (Di Virgilio *et al.*, 2017) e que de sua ativação pelo ATPe também depende a regulação das funções autócrinas e parácrinas das células alveolares via ACE2 e, em parte, o movimento mucociliar (Barth; Kasper, 2009).

Neste sentido, estudos indicam que a ativação dos receptores traria benefícios terapêuticos aos pacientes com fibrose cística (Burnstock, 2014; Burnstock; Kennedy, 2011) ou ainda, que P2X7 seria possível alvo terapêutico alternativo também para doenças como o enfisema pulmonar (Jiao *et al.*, 2022).

Mas, por outro lado, evidências demonstram que há acúmulo de ATPe nas vias aéreas de pacientes com doença pulmonar obstrutiva crônica (DPOC), determinante para a ativação do inflamassoma pulmonar via P2X7 e sinalização entre células que expressam o receptor neste microambiente, isto é, células AT1, neutrófilos, macrófagos e células dendríticas (Mortaz *et al.*, 2010).

Assim, uma vez que as principais fontes de ATPe são a hipóxia e a citólise

encontrados no microambiente pulmonar de doentes, Cantin (2022) validou experimentalmente em monócitos a hipótese de que possa haver participação de NLRP3 - P2X7 na fisiopatologia também da fibrose cística.

Ainda, autores observaram a diminuição de chance do fenótipo eosinofílico em pacientes asmáticos pediátricos na presença do polimorfismo genético homozigótico minor de *p2rx7* – *rs1183296*, *rs208290*, *rs2393799* e *rs656612* (Ren *et al.*, 2023), abrindo portas para estudos incluindo as patologias que envolvem a hipersensibilidade e sua relação com receptores purinérgicos.

1.2.1.4 *P2X7 e as neoplasias*

Diversos estudos foram conduzidos no intuito de correlacionar P2X7 também a distúrbios neoplásicos e a vislumbrar marcadores para estas condições clínicas (Lara *et al.*, 2020; Lili *et al.*, 2019; Qian *et al.*, 2017; Yang *et al.*, 2016).

De forma apropriada, P2X7 é designado por autores como "*chef d'orchestre*" na resposta imune antitumoral onde a relação ATP/P2RX&/NLRP3/IL-18 tem papel crucial (Janho Dit Hreich *et al.*, 2021).

A estimulação de P2X7 por pequenas quantidades e curto período suscita a resposta ao ATPe dentro de limites fisiológicos. No entanto, o microambiente tumoral se mostra continuamente rico em ATPe, determinando a alta estimulação de receptores e consequente abertura de macroporos na MC, com sua progressiva dilatação e posterior lise de células neoplásicas (Pippel *et al.*, 2017).

Porém, a alta expressão de P2X7 em quase todas as neoplasias até agora investigadas pode não ser coincidência, mas, ao contrário, causalmente relacionada ao estímulo que o ATP liberado por células tumorais fornece. Neste contexto, em células de glioma C6, Matyśniak *et al.* (2022) evidenciaram que a grande quantidade de ATPe induz a expressão de P2X7 nas células deste tumor, sem que haja apoptose e, ainda, com aumento da viabilidade e adesão destas ao colágeno após a estimulação do receptor P2X7.

Também, recentemente, pesquisadores comprovaram que a progressão de osteossarcoma está vinculada a P2X7, onde este induz o crescimento do tumor e metástases por meio da reprogramação do metabolismo da glicose nas células tumorais, esta mediada por pró-oncogenes c-Myc (Sheng *et al.*, 2023), e acompanhada de altos níveis de catepsinas (Jelassi *et al.*, 2011), assim como de

ativação de vias fosforilativas em outras neoplasias (Qiao *et al.*, 2022).

Por outro lado, contraditoriamente, pesquisadores observaram que animais *p2rx7* - têm tumores mais agressivos que os *p2rx7* + (Hofman *et al.*, 2015).

Em resumo, a depender da intensidade do estímulo (ATPe), é provável que os receptores P2X contribuam para a antiproliferação celular por meio da resposta inflamatória e da geração de poros citolíticos em conjunto com outros receptores celulares (Burnstock, 2014), mas por outro lado, de forma dúbia, participam dos mecanismos moleculares que intensificam a atividade metabólica, a proliferação e a disseminação metastática de tumores (Di Virgilio *et al.*, 2017; Di Virgilio; Schmalzing; Markwardt, 2018), via ATPe (He *et al.*, 2021b).

SNP já foram estudados, alguns exaustivamente (rs3751143), na tentativa de estabelecer associação alélica de *p2rx7* com a progressão de tumores sem grande sucesso para a maioria (Wang *et al.*, 2021a), à exceção de rs3751143 e algumas neoplasias hematológicas (Zhang; Zhu, 2020). No entanto, as variantes *splice* - P2X7B e nfP2X7 - demostraram forte evidência de correlação com a perda da capacidade citolítica das células e, provavelmente, pior progressão neoplásica (De Salis *et al.*, 2022; Pegoraro; De Marchi; Adinolfi, 2021).

1.2.1.5 *P2X7 e as infecções*

Em infecções, a participação de P2X7 pode ser produto da interação direta ou indireta deste com patógenos clássicos bacterianos como *M. tuberculosis*; protozoários como *Leishmania amazonensis e Toxoplasma gondii*; vírus Influenza, Vírus da Imunodeficiência Humana (HIV), Vírus da Dengue (DENV) e fungos (Soare *et al.*, 2021).

Naquelas causadas por microrganismos intracelulares, o reconhecimento e ativação da resposta imune inata mediada por macrófagos é crucial para limitar a viabilidade de patógenos, infecções de curso crônico ou sua reativação. Tais células atuam de forma bimodal na manifestação de doenças, por ser a principal célula efetora para o controle do patógeno e inflamação, ao mesmo tempo que funciona como reservatório deste.

Neste contexto, a exemplo de outras doenças infecciosas, diversos genes e seus polimorfismos podem se associar à qualidade da resposta imune inata ao *M. tuberculosis* na TB (Azad; Sadee; Schlesinger, 2012). Mas, experimentalmente,

Fairbairn *et al.* (2001) demonstraram que a maturação do fagolisossoma em macrófagos é dependente da ativação de P2X7 por ATP. Esta é relacionada à fosfolipase D para que haja a fusão, e independentemente de radicais oxigênio reativos no processo, culmina com a atividade bactericida do macrófago sobre *M. tuberculosis*.

Assim, há indícios de que a perfeita atuação de P2X7 implica na menor chance de desenvolvimento de TB após infecção, em razão da maior efetividade para a eliminação de reservatórios a longo prazo (Placido *et al.*, 2006).

À diversidade de células fagocíticas ou não que expressam P2X7 se soma o alto polimorfismo genético do receptor, condição que provavelmente impõe respostas diferentes ao tipo de estímulo, infecciosos ou não (Fuller *et al.*, 2009; Sluyter, 2017). Dessa forma, as variantes genéticas e seus alelos têm sido estudados e associados não somente à TB, mas a outras infecções cujo agente tem sobrevivência intracelular (Di Virgilio *et al.*, 2017).

Estudos sumarizados em metanálise por Taheri *et al.* (2019) tiveram como objetivo associar os polimorfismos de *p2rx7* – rs3751143, rs2393799, rs208294, rs2230911, rs7958311 e rs1718119 – com o risco de desenvolvimento de TB doença em populações caucasianas, africanas e asiáticas, e alcançaram o intuito na maioria das observações com os SNP rs3751143, rs2393799 e rs208294. Todavia, Yi *et al* (2014), também em metanálise, excluiram a participação da rs2393799 no risco de evolução da TB doença.

Entretanto, experimentos e estudos de associação conseguiram demonstrar a participação da variante genética rs3751143 na incapacidade de macrófagos eliminarem *M. tuberculosis* (Niño-Moreno *et al.*, 2007; Placido *et al.*, 2006) e no desenvolvimento de TB extrapulmonar (Fernando *et al.*, 2007), em razão da perda da função do receptor. Mas, por outro lado, na metanálise envolvendo 10.544 pacientes, Keikha e Karbalaei (2022) sustentam que o SNP se mostra determinante para a susceptibilidade à TB, porém, isoladamente, esta variante alélica não deva ser considerada como biomarcador previsor para desenvolvimento da doença.

De forma diversa de estudos anteriores, mais recentemente, Abhimanyu *et al.* (2023) relacionaram a rs2393799 (alelo C) com 1,5 e 7 vezes mais chance de desenvolvimento de TB pulmonar (TBP) e de linfonodos (TBL), respectivamente, sem que houvesse a associação semelhante com a rs3751143 (alelo C). Neste estudo, dentre os diversos genes estudados, entre interleucinas, mediadores e receptores, o

maior risco isolado para TBL foi obtido com a variante rs2393799.

Para a hanseníase, um estudo caso-controle com sequenciamento de RNAm (RNAseq) demonstrou a significante correlação da perda – homozigoto rs3751143 [CC] - e ganho de função de P2X7 - rs1718119 [GG] - com a manifestação ou não da doença, respectivamente (Souza *et al.*, 2021).

Na mesma linha de pesquisa, Pereira (2019) avaliou por meio de análise *in sílico* a expressão gênica do sistema purinérgico em monócitos infectados por *Mycobacterium leprae*, determinando que pacientes multibacilares têm menor expressão do receptor, o que contribuiria para a evolução da forma paucibacilar para a multibacilar da hanseníase. Outro achado importante deste pesquisador foi a participação da não expressão de P2X7 no acúmulo induzido de lipídios intracelulares em células não infectadas de modelo animal, e mais expressivamente, em infectadas, observando a vantagem nutricional de *M. leprae* em células sem o receptor.

A variante 1513A>C de *p2rx7*, também foi associada à falha terapêutica em pacientes com infecção crônica por *Coxiella burnetii* (febre Q) adquirida durante uma epidemia (Buijs *et al.*, 2021), possivelmente relacionada à falha no reconhecimento do microrganismo pelos receptores intracelulares de função compartilhada P2X7 e TLR (Ammerdorffer *et al.*, 2015).

Por fim, a insuficiência de macrófagos frente a outros patógenos intracelulares como a *Chlamydia* spp. (Coutinho-Silva *et al.*, 2001, 2003; Darville *et al.*, 2007), *Leishmania* spp. (Chaves *et al.*, 2009) e *Toxoplasma gondii* (Lees *et al.*, 2010) foram também evidenciadas mediante diferentes mutações em *p2rx7*.

Em infecções respiratórias, poucos estudos tentaram correlacionar a etiologia viral à resposta inflamatória pela ativação de P2X7, ainda que seja consenso que a inflamação induzida por vírus respiratórios seja determinante para a doença crítica (Flerlage *et al.*, 2021). Mas, experimentalmente, a expressão de P2X7 foi ligada à imunopatogênese da gripe (Leyva-Grado *et al.*, 2017), assim como à menor severidade da infecção pelo Influenza mediante o uso de antagonistas de P2X7 (Rosli *et al.*, 2019).

Em infecções não respiratórias, a presença do Vírus da Hepatite C (HCV) ou Epstein Barr (HBV), ou seus antígenos, concorrem com o aumento da expressão do receptor (Ferrari; Rubini; Burns, 2022). Autores ainda, levantaram a hipótese de que P2X7 possa ter um papel pró-viral, por exemplo na hepatite por HCV, pois encontraram aumento da expressão deste como consequência da superexpressão

das proteínas E1/E2 do HCV nas células (Manzoor *et al.*, 2016).

E, na infecção por DENV, a estimulação do receptor aumenta a liberação de INF e previne a infecção de células fagocitárias e, de forma inversa, sua inibição torna o processo permissivo (Ferrari; Rubini; BUrns, 2022). Também, na infecção aguda (NS1+), P2X7 induz a liberação de óxido nítrico (NO) e modula fatores como TNF, CXCL8, CCL2 (MCP-1) e CXCL10 associados à gravidade da dengue (Corrêa *et al.*, 2016).

Diferentemente das tentativas de se correlacionar P2X7 com a fusão do HIV-1 com células, as quais não foram em todo frutíferas, resultados recentes demonstram a significativa participação de P2X7-ATPe como mediadores da liberação de vírions dos compartimentos celulares por meio de microvesículas (VCC). Estes funcionam como reservatórios do HIV-1 em macrófagos teciduais, protegem os vírions da terapia antirretroviral (TARV) e sustentam a infecção. Desta forma, bloqueadores de P2X7 e/ou da liberação das VCC seriam a interferência farmacológica plausível para findar tais reservatórios virais (Graziano; Vicenzi; Poli, 2019).

Muitas das pesquisas citadas, nos leva a pensar se o papel convencional do P2X7 como mediador da resposta pró-inflamatória possa ser ainda mais amplo, ligado à interação direta com patógenos, à tolerância a esses ou à imunomodulação necessárias às relações hospedeiro-patógeno para a minimização do dano durante infecções.

1.2.1.6 *P2X7 e a COVID-19*

Dentro e fora do contexto inflamatório, a participação de receptores que respondem aos níveis de purinas extracelulares mediante lesão tecidual é consenso em inúmeras condições patológicas (Gusev *et al.*, 2022; Huang *et al.*, 2021b). E, de acordo com Cekic e Linden (2016), a sinalização purigênica relacionada à injúria ocorre em fases distintas que determinam respostas variadas e proporcionais aos estímulos e sua duração.

Pacientes infectados com SARS-CoV-2 que evoluem severamente permanecem num estado de estimulação contínua pelo ATPe, em razão do infiltrado pulmonar e da hipóxia ou, ainda, vivenciam o recrudescimento do processo inflamatório inicial (Chen *et al.*, 2020a; Gusev *et al.*, 2022; Karki; Kanneganti, 2022; Lamers; Haagmans, 2022; Quan *et al.*, 2020).

Nos pacientes que adentram fases mais tardias da infecção ainda doentes, a estimulação crônica contínua de P2X7 se sobrepõe às complicações da COVID-19 a longo prazo (Al-Aly; Xie; Bowe, 2021; Nalbandian *et al.*, 2021).

Desta forma, a exemplo de outras patologias infecciosas ou não, diversos são os indícios de que os padrões de resposta de receptores purinérgicos, em especial P2X7, se cruzam com a patogênese e evolução da COVID-19 (Illes, 2021).

Neste sentido, na tentativa de estabelecer marcadores prognósticos para a severidade da doença, Vultaggio-Poma *et al.* (2023) e García-Villalba *et al*. (2022) conseguiram correlacionar apropriadamente os níveis circulantes da proteína P2X7 com a severidade da COVID-19.

Assim, uma vez que o receptor está amplamente expresso, o infiltrado pulmonar e a hipóxia tecidual na COVID-19 caracterizam o ambiente propício à franca ativação do receptor, direcionado à constituição do inflamassoma NRLP3 (Howrylak; Nakahira, 2017) de células imunes, hiperativação da resposta inflamatória com a secreção de citocinas direta ou indiretamente (IL-6, IL-1β, IL-18, TNF-α) e quimiocinas (Barth; Kasper, 2009; Chang *et al.*, 2020; Conti; Gallenga; Frydas, 2020; Howrylak; Nakahira, 2017; Leyva-Grado *et al.*, 2017; Riteau *et al.*, 2012).

A seguir, se estabelecem alterações celulares que vão desde mudanças no metabolismo glicídico à morte celular induzida em células do SI (piroptose) e apoptose de células alveolares, mediadas por P2X7 (Ferreira *et al.*, 2021; Freeman; Swartz, 2020).

Tal contexto em que P2X7 está inserido se estende à diminuição de LT, disfunção de LTCD8 (Bergamaschi *et al.*, 2021) e desvio de resposta a Th17 em detrimento das células T*reg*, - altamente susceptíveis à morte por ativação de P2X7 na presença de grande quantidade de ATPe (Winzer *et al.*, 2022) - caracterizando o processo de descontrole e deterioração imunológica da COVID-19 (De Biasi *et al.*, 2020; Martonik *et al.*, 2021).

Portanto, P2X7 está inserido em vias de sinalização intra e intercelulares que contribuem amplamente para a homeostase metabólica mas, especialmente, para a resposta imunológica à agressão.

Na COVID-19, é plausível que as lesões teciduais suscitem a resposta padronizada mediada por diversos receptores celulares, porém, as variações individualizadas que direcionam para a síndrome imunológica indicam a necessidade de estudos aprofundados sobre a relação dos SARS-CoV-2 com tais receptores,

assim como sobre a variabilidade genética dos pacientes que possa contribuir para os diferentes desfechos da doença.

2 JUSTIFICATIVA

Ainda que o exposto seja um mínimo rascunho da intersecção COVID-19 e sinalização purinérgica, os receptores ATP-sensíveis possivelmente tenham influência na intensidade de resposta que será dada à infecção nas fases aguda e subaguda (Franciosi *et al*., 2021) e estes, em conjunto com receptores para outras purinas, sejam também moderadores da condição pós-COVID-19 (Nalbandian *et al.*, 2021).

Por sua vez, P2X7 é o receptor ATPe-sensível mais estudado frente às condições clínicas que envolvem o processo inflamatório a longo prazo, tais como doenças neurológicas e neurodegenerativas (Miras-Portugal *et al.*, 2017; Zalpoor *et al.*, 2022), autoimunes (Cao *et al.*, 2019; Di Virgilio; Giuliani, 2016), infecções por microrganismos intracelulares (Fairbairn *et al.*, 2001; Placido *et al.*, 2006), neoplasias (Matyśniak *et al.*, 2022) vasculopatias (Ribeiro *et al.*, 2021; Zhao; Chen; Feng, 2018), doenças dermatológicas (Geraghty *et al.*, 2016), entre outras.

Assim, a amplitude de órgãos e sistemas inseridos no escopo “P2X7 e doença” reflete a sua distribuição em células imunes e não imunes, e essa ubiquidade se compara à dispersão orgânica das complicações da infecção pelo SARS-CoV-2, a exemplo das cardiovasculares (Nasab *et al.*, 2023; Xie *et al.*, 2022), pulmonares (Blanco *et al.*, 2021; Mo *et al.*, 2020), metabólicas (Govender *et al.*, 2021; Kazakou *et al.*, 2022; Rizvi *et al.*, 2022; Watson, 2022; Xie; Al-Aly, 2022) e doenças neurodegenerativas (Huang *et al.*, 2023; Li *et al.*, 2022a), as quais guardam de antemão alguma relação com o processo inflamatório.

Entende-se que inúmeras variáveis interferem no processo infeccioso/inflamatório da COVID-19, desde as formas de interação do SARS-CoV-2 com o hospedeiro (Gupta, 2022; Jackson *et al.*, 2022; Lamers; Haagmans, 2022; Pruimboom, 2021) até a variabilidade genética/epigenética (Luiza *et al.*, 2021; Ozturkler; Kalkan, 2022) de ambos.

Porém, em conjunto, a busca por evidências que expliquem a razão da equação inflamação/SARS-CoV-2 justifica a pesquisa de marcadores

epidemiológicos, laboratoriais e genéticos que se associem às evoluções da COVID-19, na tentativa de melhor elucidar o contexto imunológico amplo da doença e a participação de P2X7 neste.

Desta forma, procuramos caracterizar os pacientes com COVID-19 que foram atendidos no Hospital Universitário de Londrina, de maneira a confrontar os dados epidemiológicos e laboratoriais deste hospital de referência para COVID-19 com dados nacionais e internacionais acerca da doença e, normalizar fatores que possam vir a interferir na análise de associação alélica à COVID-19 na população estudada.

Uma vez que não estão disponíveis dados na literatura que comprovem a ligação de polimorfismos genéticos de *p2rx7* com a COVID-19, neste estudo transversal, buscamos evidências da associação de SNP do receptor com as comorbidades pré-existentes, com a sintomatologia inicial, com a severidade da COVID-19 e com os desfechos clínicos de pacientes após a hospitalização em razão de complicações da doença.

Foram investigadas mutações genéticas funcionais do gene *p2rx7*, as quais já têm estudos em outras doenças, como a rs3751143/(1513A>C; Glu496Ala) relacionada à perda de funcionalidades do receptor e a rs2393799/(-762C>T), cujo alelo C suspeita-se estar ligado à menor expressão do receptor.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar o perfil epidemiológico, laboratorial de pacientes hospitalizados com a infecção pelo SARS-CoV-2 em um serviço de referência para a COVID-19 no Paraná, Brasil, e determinar a associação de polimorfismos de *p2rx7* com os fatores epidemiológicos, a severidade da COVID-19 no período pré-vacinação e com o desfecho da doença severa/crítica após a hospitalização.

3.2 OBJETIVOS ESPECÍFICOS

I. Caracterizar parâmetros demográficos, clínicos e laboratoriais dos pacientes com COVID-19 moderada ou severa-crítica hospitalizados no HU-UEL;
II. Relacionar a presença de comorbidades com a severidade da COVID-19 (leve, moderada e severa-crítica);
III. Avaliar a associação de parâmetros laboratoriais de pacientes hospitalizados com o desfecho óbito em até 180 dias após início dos sintomas da COVID-19;
IV. Avaliar a frequência dos polimorfismos (SNP) rs3751143 e rs2393799 de *p2rx7* em pacientes com COVID-19 leve, moderada ou severa-crítica;
V. Determinar se há associação das rs3751143 e rs2393799 com as comorbidades de pacientes com a COVID-19;
VI. Avaliar se os haplótipos que contemplam agrupamentos alélicos das mutações diferem quanto à sintomatologia da COVID-19, quanto às comorbidades e aos parâmetros laboratoriais nos pacientes hospitalizados com COVID-19;
VII. Determinar se os genótipos e haplótipos dos SNP diferem quanto ao risco de óbito a curto e longo prazo após o início dos sintomas da COVID-19.

4 METODOLOGIA

4.1 ASPECTOS ÉTICOS

Este protocolo de pesquisa foi aprovado pelo Comitê de Ética em Pesquisa Institucional da Universidade Estadual de Londrina, Paraná, Brasil (CAAE:31656420.0.0000.5231) (**ANEXO A**) e todos os participantes e seus responsáveis foram informados detalhadamente sobre a pesquisa e assinaram o termo de consentimento livre e esclarecido (**APÊNDICE A**).

4.2 DELINEAMENTO DO ESTUDO

Este estudo descritivo transversal foi conduzido com o objetivo de descrever e avaliar aspectos demográficos, clínicos, laboratoriais e genéticos de pacientes infectados com SARS-CoV-2 no período pré-vacinação da pandemia de COVID-19, com dados coletados entre março e setembro de 2020 no Hospital Universitário de Londrina, Paraná, Brasil.

4.3 PACIENTES

Foram inseridos no estudo 434 pacientes adultos ($\geq$18 anos), de ambos os sexos, sequencialmente internados no Hospital Universitário de Londrina, Paraná, Brasil, com diagnóstico clínico e laboratorial de infecção pelo SARS-CoV-2. Sessenta e nove pacientes foram caracterizados como casos moderados (Pmod) e 365 como severos-críticos (Psev) para a COVID-19.

Também, 167 indivíduos adultos, recuperados da doença, e caracterizados como sintomáticos leves (Pmild), foram inseridos no estudo, após pelo menos trinta dias do início dos sintomas. Destes, 41 foram pacientes provenientes de busca espontânea por atendimento médico, mas em sua maioria (126), foram selecionados entre trabalhadores da saúde e do círculo de convivência dos pesquisadores, com no máximo 3 pessoas da mesma família.

4.3.1 Critérios de Exclusão

A presença de neoplasias; infecções crônicas laboratorialmente diagnosticadas como hepatite B ou C, infecção pelo HIV e sífilis; doenças autoimunes e/ou a utilização contínua prévia de medicamentos anti-inflamatórios ou imunomoduladores foram utilizados como critérios de inelegibilidade dos casos.

4.3.2 Amostras e Variáveis Analisadas

Para a coleta de informações dos pacientes, foi construído um instrumento para a anotação dos resultados pertinentes como sim ou não (**ANEXO B**). Para as medicações, sua classificação foi realizada de acordo com a indicação/atividade principal do medicamento reportado.

Para pacientes com déficit cognitivo ou sem condições clínicas para resposta aos questionamentos, foram inseridas as informações obtidas com os cuidadores.

Desta forma, foram coletadas informações acerca da idade, etnia autodeclarada, sexo e a presença de comorbidades: DM tipo 1 ou 2, hipotireoidismo (HT), HA, insuficiência cardíaca, renal dialítica ou não, hepática, doença pulmonar crônica, obesidade (Índice de Massa Corpórea - IMC$\geq$30Kg/m^2), dislipidemia, doença neurocognitiva - neurológica em tratamento, quadro demencial ou consequente a acidentes vasculares cerebrais -, uso de álcool ou tabaco. Foi observado o uso de medicação contínua pelos pacientes (hipolipemiantes, anti-hipertensivos/cardiotônicos, hipoglicemiantes orais e insulina, levotiroxina, medicamentos com atividade no sistema nervoso central e anti-agregantes plaquetários), assim como questionados o período decorrido desde o início dos sintomas (dias) e as manifestações clínicas até o momento da admissão (febre, cefaleia, odinofagia, coriza e espirros, sintomas gastrintestinais, mal estar/fadiga/fraqueza, mialgia, tosse e dificuldade respiratória).

Na admissão hospitalar, foram mensurados os dados antropométricos, os sinais vitais e a saturação de oxigênio capilar (SpO_2 [%]), estes últimos utilizados em conjunto com a avaliação clínica como critérios para o atendimento ambulatorial ou hospitalização, definição do caso como síndrome respiratória aguda grave (SRAG) e classificação do caso de COVID-19 na escala de gravidade (**Quadro 3**).

Mediante suspeita clínica de pneumonia, os pacientes hospitalizados foram

submetidos à tomografia computadorizada (TC) pulmonar, em média até 24 horas após a admissão.

Para os pacientes hospitalizados, foram observados o desfecho (alta, óbito ou transferência/evasão), a presença de evento tromboembólico, a necessidade de intubação orotraqueal e de terapia de substituição renal no período de internação. Entre os casos cujo desfecho foi de alta ou transferência/evasão, a ocorrência de óbitos foi observada por 30, 60 e até 180 dias após o início dos sintomas da doença.

Ao final do período de hospitalização, os casos foram classificados por médico especialista segundo a gravidade da evolução como moderados (Pmod) e severos/críticos (Psev), após terem seus dados da evolução clínica obtidos do prontuário eletrônico da instituição e confrontados com os critérios estabelecidos pela OMS em 2020 (WHO, 2020) (**Quadro 3**).

QUADRO 3 – Resumo dos critérios clínicos utilizados para a classificação de severidade da COVID-19 segundo a Organização Mundial da Saúde (2020)

SEVERIDADE	DOENÇA PULMONAR	CRITÉRIOS	DOENÇA SISTÊMICA
LEVE	-	Sintomáticos sem evidência de pneumonia viral ou hipóxia	-
MODERADA	Pneumonia	Sinais clínicos de pneumonia sem sinais de gravidade, incluindo $SpO_2 \geq 90\%$ em ar ambiente. Se necessidade de O2, em máscara ou cateter nasal	-
SEVERA	Pneumonia Severa	Sinais clínicos de pneumonia com mais um dos seguintes: frequência respiratória > 30 respirações/min; dificuldade respiratória grave; ou SpO2 < 90% em ar ambiente. Necessidade de oxigenoterapia invasiva (VM), não invasiva (VNI)/alto fluxo	-
CRÍTICA	SRAG	Pneumonia com sintomas respiratórios novos ou agravados. Imagens de tórax com alterações não explicadas por outras causas. Comprometimento da oxigenação em adultos, com SRAG classificada como leve, moderada ou severa de acordo com a relação PaO_2/FiO_2.	Sepse Choque séptico

SRAG: Síndrome Respiratória Aguda Grave; SpO2: Saturação periférica de O_2; PaO_2/FiO_2 (Pressão de O_2 em mmHg)/(Fração inspirada de O_2 em mmHg); VNI: Ventilação não invasiva. VM: Ventilação mecânica

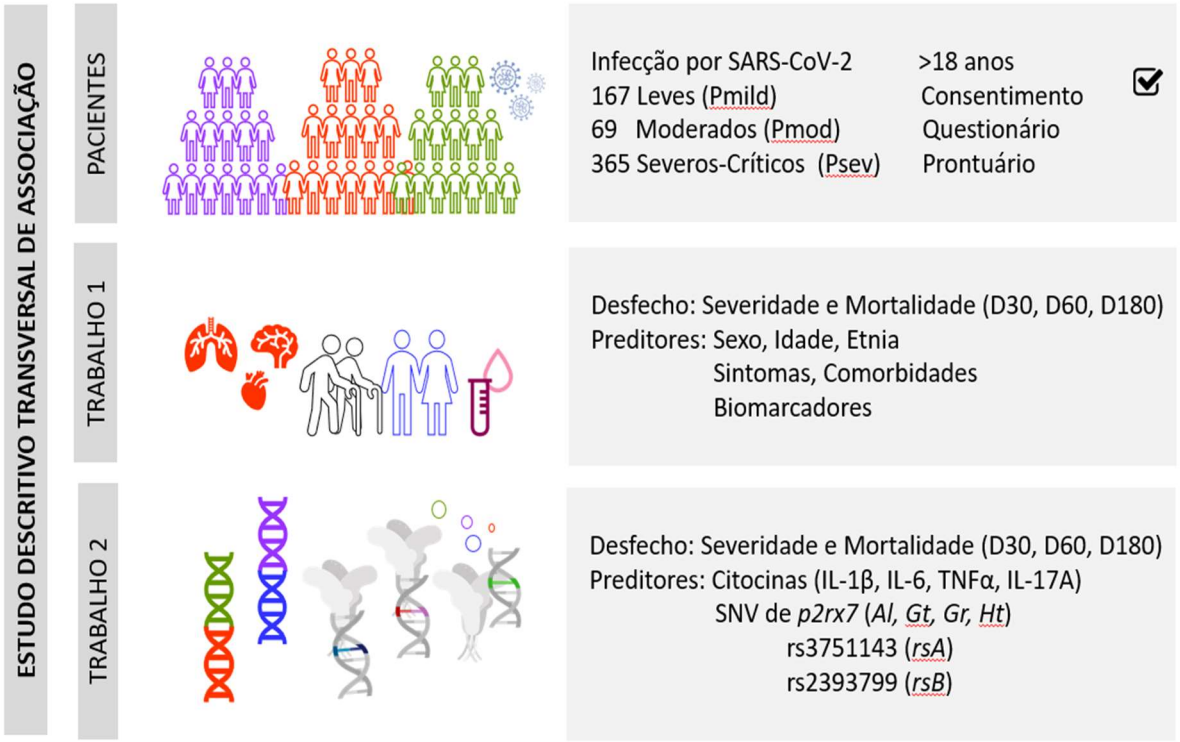
Fonte: Adaptado de WHO, 2020 (*Clinical Management of COVID-19: Interim Guidance*)

Todos os pacientes foram submetidos à obtenção de amostras de nasofaringe para a detecção de SARS-CoV-2 à época do diagnóstico. Os testes foram realizados com extração de RNA viral e protocolos de amplificação disponíveis, validados e aplicados à época seguindo orientações da OMS (WHO, 2020).

Amostras de sangue periférico - sem anticoagulante e com ácido etileno-diamino-tetracético (EDTA) - foram coletadas dos pacientes hospitalizados no momento da admissão para a realização dos exames laboratoriais e análise genética. E, da mesma forma, para os pacientes com doença leve que tiveram acompanhamento ambulatorial, a obtenção das amostras de sangue foi realizada no primeiro atendimento, porém, para os indivíduos com doença leve convocados a participar do estudo, as amostras foram obtidas após a entrevista e, somente para a análise genética. Para a análise genética prospectiva de todos os pacientes, os *buffy coats* das amostras foram mantidos a -80 °C até o momento do ensaio.

A **Figura 2** representa o fluxograma de resumo do estudo.

FIGURA 3 – Organograma do estudo



4.4 EXAMES LABORATORIAIS E DOSAGEM DE CITOCINAS

Os exames laboratoriais realizados dos pacientes admitidos para internação foram: hemograma, glicose, ureia, creatinina, sódio, potássio, gasometria, lactato, ALT, AST, CPK, troponina, ferritina, ferro sérico, transferrina e dímero-D. Foram utilizados os equipamentos BC-6800 (Mindray, Nanshan, China), ALINITY I (Abbott, Illinois, USA), Architect C8000 (Abbott, Illinois, USA), Dimension RxL (Siemens Healthcare Diagnostics, Norwood, Massachussets, USA), e reagentes seguindo protocolos específicos dos fabricantes para cada analito.

A razão entre os valores absolutos (/mm^3) de Neutrófilos/Linfócitos e Monócitos/Neutrófilos foram utilizadas para compor os índices NLR e MNR, respectivamente. Para a razão Hematócrito/Hemoglobina foram utilizados os valores obtidos em % e g/dL dos respectivos parâmetros.

Porções de plasma mantidos a -80 °C foram utilizados para a dosagem das interleucinas IL-1β, IL-6, IL-17 e TNF-α de 134 pacientes Psev distribuídos em grupos que contemplavam proporcionalmente os haplótipos dos SNP em estudo. O kit Human ProcartaPlex™ Multiplex Immunoassay (ThermoFisher Scientific - Waltham, Massachusetts, USA) foi utilizado para a determinação das concentrações das citocinas IL-1β, IL-6, IL-17 e TNF- α. A cada cavidade das microplacas de reação foram adicionados 50µL de *beads* magnéticas recobertas com anticorpo específico para a detecção de IL-1β, IL-6, IL-17 e TNF-α, das quais foi retirado o líquido excedente e procedida a lavagem. A cada poço correspondente foram inseridos 25 µL do branco, padrões e amostras, além de 25 µL do tampão universal. As placas foram seladas e incubadas à temperatura ambiente por 60 minutos, sob agitação. Após duas lavagens, foram adicionados 25 µL do anticorpo de detecção (biotinilado), seguido de 30 minutos de incubação e duas lavagens. Ao final, 50µL de estreptavidina/ficoeritrina foram inseridos nos poços e repetido o procedimento de incubação e lavagem anteriores. Após ressuspensão das *beads* em 120µL de tampão de leitura e incubação de 5 minutos sob agitação, as leituras foram realizadas no equipamento Luminex MAGPIX™ (Luminex Corporation, Austin, Texas, USA). Oito concentrações dos padrões foram utilizadas como calibradores. Resultados iguais ou inferiores ao limite de detecção da técnica para IL-1β, IL-6, IL-17 e TNF-α foram reportados como ≤4,76pg/mL, ≤15,09pg/mL, ≤3,50pg/mL e ≤11,89pg/mL, respectivamente.

4.5 TOMOGRAFIA COMPUTADORIZADA (TC)

Opacidades em vidro fosco, consolidações com sinal do halo, presença de nódulos, derrame pleural e linfadenopatia foram utilizados em conjunto para determinar o comprometimento pulmonar classificado em escala de 0–25%, 25–50%, 50–75% e mais de 75%, de acordo com resultados da tomografia computadorizada (TC) de tórax sem contraste realizada no equipamento BRYGHT SPEED/GE16 (General Eletric Healthcare - America: Milwaukee, USA).

As avaliações foram revisadas por médico especialista por meio das imagens digitalizadas e disponíveis no prontuário eletrônico dos pacientes.

4.6 POLIMORFISMOS DE NUCLEOTÍDEO ÚNICO (SNP) DO GENE *P2RX7*

Os pacientes Pmild, Pmod e Psev foram avaliados segundo o agrupamento alélico dos SNP rs3751143A > C e rs2393799C > T para o gene codificador do receptor citoplasmático transmembrana P2X7 (*p2rx7*).

O DNA genômico foi extraído e purificado a partir do *buffy coat* utilizando o método de resina em coluna, seguindo o protocolo do fabricante (Biopur, Mobius Life Science - Pinhais, Paraná, Brasil). Sua quantificação foi determinada através do equipamento Nanodrop 2000c™ Spectrophotometer (ThermoFisher Scientific - Watman, Massachusetts, USA) em 260nm. O DNA purificado e eluido em água ultrapura foi mantido em freezer -80 °C até o momento da genotipagem.

A tipagem dos SNP de *p2rx7* rs3751143A > C e *p2rx7* rs2393799C > T foi obtida por meio de PCR em tempo real (qPCR) com sondas Taqman®VIC™/FAN™ CCTGAGAGCCACAGGTGCCTGGAGG**[A/C]**GCTGTGCTGCCGGAAAAAGCCGGGG e TGGTGTCCCTCACTGAATAGGTCAA**[C/T]**AAACCTAACTTTGTTGGACTGCCAC, respectivamente, na concentração final de 200mM (ThermoFisher Scientific - Waltham, Massachusetts, USA). Para cada reação foram utilizados 1,25µL de TaqMan™ Genotyping Master Mix (ThermoFisher Scientific - Waltham, Massachusetts, USA) (4X); 0,125µL da sonda respectiva (40X); 1,125µL de água ultrapura e 2,5µL de DNA, para um volume final de reação de 5,0µL em microplacas de 384 poços. A amplificação e interpretação dos genótipos foram realizadas no equipamento QuantStudio™ 6 Pro Real-Time PCR System e Analysis Software version 1.6 (ThermoFisher Scientific - Waltham, Massachusetts, USA), onde foram discriminados

três genótipos para as variantes rs3751143 [AA, CC, AC] e rs2393799 [CC, TT, CT].

4.6.1 Agrupamentos dos SNP (Haplótipos)

As variações alélicas dos SNP de *p2rx7* foram estudadas isoladamente rs3751143 [AA;AC;CC], rs2393799 [CC;CT;TT] ou em agrupamentos de haplótipos respectivos de rs3751143/rs2393799 - Haplótipo I [AA]/[CC]; Haplótipo II [AC;CC]/[CC]; Haplótipo III [AA]/[CT;TT]; Haplótipo IV [AC;CC]/[CT;TT] - para a associação com a severidade da COVID-19, sintomas iniciais, dados demográficos, fatores de risco, alterações tomográficas (em escore), exames laboratoriais, e com o desfecho óbito em 30 (D_{30}), 60 (D_{60}) ou 180 (D_{180}) dias após o início dos sintomas.

4.7 Análise Estatística

Para o estudo foi utilizada amostragem não-probabilística com dados coletados no período compreendido entre março e setembro de 2020 para condução do estudo transversal.

A normalidade dos dados coletados foi verificada por meio dos testes de Komogorov-Smirnov ou Shapiro-Wilk, conforme adequado aos agrupamentos dos pacientes. A homogeneidade de variância foi avaliada por meio do teste de Levene.

Para a análise estatística descritiva foram mensuradas a frequência simples (n) e relativa (%) dos dados qualitativos dentro dos grupos de pacientes pré-definidos de acordo com a severidade da COVID-19 (Pmild, Pmod e Psev), aos grupos definidos pelos alelos dos SNP isoladamente e aos agrupamentos alélicos (Haplótipos I, II, III, IV). Para as variáveis contínuas com dados não-paramétricos, a mediana foi utilizada como medida de tendência central e os interquartis (IQ25% e IQ75%) como medida de dispersão e, para os dados cuja análise paramétrica foi pertinente, também em média e desvio-padrão (±SD).

Foram aplicados os testes de Qui-Quadrado de independência de Pearson (X^2) ou Exato de Fisher (EF), mediante o número de grupos e participantes nas tabelas de contingência, para a realização de análise bivariada da associação das variáveis categóricas (sexo, etnia autodeclarada, comorbidades e sintomas) com os agrupamentos pré-definidos de COVID-19 e variantes alélicas.

Os resultados foram analisados para a significância de alfa <0,05 com a

correção de Bonferroni para (k) amostras e a comparação entre pares (*post-hoc*) foi realizada utilizando residuais ajustados padronizados para múltiplas comparações (teste z). Quando z >2,57 para a análise de 5 grupos (df=4) e z>2,64 para a análise de 6 grupos (df=5), os resultados foram considerados significativos para associação (Macdonald; Gardner, 2000; Sharpe, 2015).

Para as variáveis contínuas, foi realizada a análise de variância (one way ANOVA - F) com procedimento de reamostragem (*bootstrap*) de 1.000 vezes para normalização dos dados e equilíbrio amostral entre os conjuntos de pacientes. A ANOVA de Brown-Forsythe (F*) ou correção de Welch (W) foi aplicada para as análises de dados sem homocedasticidade (Levene; p<0,05) e o *post-hoc* de Games-Howel às análises de variância com p<0,05. Os resultados da diferença das médias (ΔM) e seu intervalo de confiança (CI95%) corrigido para a assimetria das distribuições das estimativas após reamostragem (*Bias-corrected and acelerated* - BcaCI95%) foram utilizados para a interpretação quanto à diferença entre os grupos (Ferreira *et al.*, 2008). Também para as variáveis contínuas, o coeficiente de variação (CV%) foi utilizado para determinar a homogeneidade dos resultados com referência à média intragrupo.

Quando apropriado, foram aplicados também os testes de Kruskal-Wallis (*H*) e Mann-Whitney (*U*) para dados sem distribuição normal, no intuito de analisar também a diferença da variabilidade entre os grupos.

Para as curvas de sobrevivência foram utilizados os métodos de Kaplan-Meier-log-rank para a análise bivariada simples e, os modelos de regressão de Cox para a análise multivariada, com a obtenção da estimativa de risco proporcional de óbito ao longo do tempo - *Hazard Ratio* (HR) - frente às covariáveis.

O efeito das variáveis categóricas e contínuas sobre a classificação de severidade da COVID-19 foi mensurado por meio da regressão logística bivariada, com resultados expressos por meio de χ^2 para adequação do modelo e a *Odds Ratio* (OR) e seus intervalos de confiança (CI95%) como medida de associação das variáveis dicotômicas.

Os tamanhos de efeito (TDE) de cada variável independente categórica sobre a variável dependente de agrupamento foi determinada para os testes de Mann-Whitney por meio do coeficiente de contingência de Pearson (r^2), calculado como $r2 = z^2/N$. A proporção das variâncias que pode ser atribuída às variáveis independentes foi reportada como *Eta* ao quadrado ordinal (η^2) para a ANOVA e *Eta* quadrático para

teste de Kruskal-Wallis (η^2_H). Para as tabelas de contingência analisadas por meio de Qui-quadrado (χ^2), os coeficientes *Phi* (Φ) ou V^2 de Cramer (V^2) foram aplicados às tabelas 2x2 e maior que 2x2, respectivamente (Fritz; Morris; Richler, 2012; Tomczak; Tomczak, 2014). A interpretação dos resultados utilizou o coeficiente apropriado e o contexto dos agrupamentos, para os quais a proximidade dos valores a 1 indica forte TDE e a zero, um efeito desprezível.

Os dados laboratoriais foram estudados dentro dos agrupamentos de severidade da COVID-19 para estabelecer a correlação entre dois parâmetros. Para tanto, foi utilizada a correlação de Spearman (*r*) e os intervalos a seguir para a interpretação dos resultados: correlação fortemente positiva [0,71 – 1,0]; moderadamente positiva [0,501 - 0,70]; fracamente positiva; [0,50 - 0,30]; fortemente negativa: [-1,0 - -0,71]; moderadamente negativa: [-0,7 - -0,501]; fracamente negativa: [-0.5 - 0.3] e correlação nula: [-0.29 – 0.29]. Os resultados foram informados na matrix de correlação quando $p<0,05$ e CI95% com valores que não passam pelo 1.

Para as análises laboratoriais e demais variáveis contínuas, quando apropriado, foi aplicada a regressão linear múltipla para a determinação da influência de um ou mais parâmetros sobre os resultados encontrados para a variável dependente.

A tabulação de dados foi realizada no programa Windows Excel 2019 version 21.02. O IBM SPSS Statistics version 20.0 for Windows (IBM Corporation, Armonk, NY, USA) e o GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, California, USA) foram utilizados para as análises estatísticas e geração de gráficos. Todas as análises foram realizadas ao nível de significância inicial de $p<0,05$ ou menor quando apropriadas correções foram necessárias.

5 EPIDEMIOLOGIC ASPECTS AND BIOMARKERS OF SEVERITY AND MORTALITY IN PATIENTS WITH COVID-19 TREATED IN A REFERENCE HOSPITAL DURING THE PRE-VACCINATION PERIOD.

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ABSTRACT

Mass vaccination against SARS-CoV-2 has helped curb the COVID-19 pandemic, which resulted in 7 million fatalities over 4 years. Hospital referral units encountered several challenges while treating patients with moderate or severe illness, leading to multiple deaths. Thus, we conducted a retrospective observational association study with the aim of evaluating epidemiologic, laboratory diagnostic and imaging data related to the severity and outcome of patients admitted to a reference regional hospital for treatment of COVID-19 in the prevaccination period. A total of 601 COVID-19 cases were reviewed, including 167 mild, 69 moderate, and 365 severe cases, to survey demographic data and the incidence of comorbidities. The study evaluated chest tomographic exams and laboratory parameters for prognosis, utilizing Chi-square tests, one-way ANOVA, and Cox regression to establish risk estimates for severity and mortality ($p<0.05$). Age over 60 years, diabetes, and cardiac and neurocognitive disorders were linked to negative disease outcomes and, obesity was a risk factor for severe-critical COVID-19 among non-elderly patients. Additionally, laboratory parameters including NLR (neutrophil lymphocyte ratio), CRP (C-reactive protein), IL-6 (interleukin 6), LDH (lactic dehydrogenase), blood glucose, parameters related to iron metabolismo, except ferritin, were linked to disease severity. Hence, we posit that the accumulation of COVID-19 data gathered through both prospective and retrospective means in epidemiological research, along with investigations into the pathophysiology of the disease, will consistently aid in developing uniform protocols for enhancing the diagnostic and therapeutic capabilities of coronavirus and other emerging viral infections that may affect susceptible populations.

5.1 INTRODUCTION

Hospitals experienced periods of adaptation to the new reality and faced the worst face of the COVID-19 pandemic, especially reference units for the treatment of the disease. The administration of COVID-19 vaccines has dramatically reduced the infection rate, severity and mortality of this disease (WHO, 2023), but we believe that data on the disease should be reviewed, not only because of the fear of new pandemics (1–3) but as a way to understand the epidemiologic and pathophysiologic relationships of other infectious diseases and the usefulness of available tools for prognosis.

In this context, epidemiological data on demographics and the presence of comorbidities related to the disease prove to be far-reaching indicators in the conduct of health policies that support prevention and therapeutic intervention. However, in the direct approach to the patient, biomarkers that can predict the evolution of the disease are tools that can support rapid interventions and contribute to patient survival (4–6).

Therefore, we conducted a retrospective observational association study with the aim of evaluating epidemiologic, laboratory diagnostic and imaging data related to the severity and outcome of patients admitted to a reference regional hospital for treatment of COVID-19 in the prevaccination period.

5.2 MATERIAL AND METHODS

5.2.1 Patients

During the pre-vaccine COVID-19 pandemic period, from March through October 2020, this cross-sectional descriptive study selected 601 adult patients (over 18 years of age) of both sexes with laboratory-confirmed SARS-CoV-2. From these, a total of 434 sequentially hospitalized patients were studied, including 69 with moderate disease (Pmod) and 365 with severe to critical disease. These patients were admitted to the University Hospital of Londrina in Paraná, Brazil for the treatment of COVID-19. Additionally, 167 patients with mild symptoms (Pmild) who were not hospitalized were included: 41 Pmild patients received clinical and laboratory diagnostic procedures

alongside and 126 recovered individuals who were selected at least 30 days after their infection diagnosis and agreed to participate in the study.

Demographic data (gender, age and self-declared ethnicity), as well as the presence of comorbidities (respiratory, cardiovascular, urinary, metabolic and neurocognitive), tobacco and alcohol addiction and continuous use of medications data were assessed using a structured questionnaire. Family members or caregivers answered the questions instead of patients with neurological dementia or serious clinical conditions. Cancer with palliative care, chronic and serious infections (aids syphilis, hepatitis C and sepsis) and autoimmune diseases were considered ineligibility criteria for cases.

Anthropometric data, vital signs and capillary oxygen saturation [SPO2 (%)] were measured at upon hospital admission. These parameters and the clinical assessment were broad criteria for outpatient care or hospitalization, defining the case as severe acute respiratory syndrome (SARS) and classification of the COVID-19 case on the severity scale (7).

The outcome (discharge, death or transfer/evasion), presence of a thromboembolic event, orotracheal intubation and renal replacement therapy were observed in hospitalized groups. Among discharge or transfer/evasion outcomes, the occurrence of deaths was observed for 30, 60 and up to 180 days after COVID-19 symptoms onset.

In the end of the hospital stay, cases were classified as Pmod or Psev by a physician according to their clinical evolution, based on an analysis of data from the hospital's electronic medical records and compared with criteria established by the World Health Organization (7).

This research protocol was approved by the Institutional Research Ethics Committee of the State University of Londrina, Paraná, Brazil (CAAE:31656420.0.0000.5231), and all participants and their legal guardians were fully informed about the research and signed a free and informed consent form.

5.2.2 Laboratory analyses and chest tomography

Peripheral blood samples collected during the initial visit from 41, 69 and 365 patients with Pmild, Pmod and P sev conditions, respectively, underwent analysis using manufacturer-specific protocols for each test with appropriate reagents and equipment: BC-6800 (Mindray, Nanshan, China), ALINITY I (Abbott, Illinois, USA), Architect C8000 (Abbott, Illinois, USA), Dimension RxL (Siemens Healthcare Diagnostics, Norwood, Massachussets, USA), Luminex MAGPIXTM (Luminex Corporation, Austin, Texas, USA; ThermoFisher Scientific – Waltham, Massachusets, USA). Hematimetric parameters, total leukocytes, absolute number of neutrophils, lymphocytes and monocytes, platelets, glucose, creatinine, bun urea, C-reactive protein (CRP), lactic dehydrogenage (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine phosphokinase (CPK) and its cerebral and myocardial fraction (CPK-MB), troponin, D -dimer, ferritin, transferrin saturation, latent (LIBC) and total transferrin iron binding capacity (TIBC), serum iron, interleukin 6 (IL-6), interleukin 17A (IL-17A), interleukin 1 beta (IL-1β) and tumor necrosis fator (TNF) were evaluated. The NLR and MNR indices were constructed using the ratio of absolute values (/mm3) of Neutrophils/Lymphocytes and Monocytes/Neutrophils, respectively.

The main changes observed by specialist on non-contrast chest tomography (CT) scan - BRYGHT SPEED/GE16 equipment (General Electric Healthcare - America: Milwaukee, USA) - were ground-glass opacities, consolidations with halo sign, nodules, pleural effusion and lymphadenopathy. Taken together, the dregree of lung involvement of patients admitted to hospital was translated into a scale: 1 (<5%), 2 (5–25%), 3 (26–50%), 4 (51–75%), or 5 (>75%) adapted from Li and collaborators (8), from the lobar involvement scale for the lungs in general.

5.2.3 Stathistics

The retrospective cross-sectional study utilized non-probability sampling and data collection took place from March to September 2020. The normality of the data and homogeneity of variance were assessed via the Komogorov-Smirnov/Shapiro-Wilk and Levene tests, respectively. For descriptive statistical analysis, simple (n) and

relative (%) frequencies were measured. The Chi-squared test (χ^2) or Fisher's Fisher exact test (EF), when appropriate, was used to perform a bivariate analysis of the association of categorical variables (sex, self-reported ethnicity, comorbidities, and symptoms) with the predefined groupings of COVID-19. Results were analyzed for significance at alpha <0.05. And for pairwise comparison (post hoc), standardized adjusted residuals for multiple comparisons (z-test) were evaluated. If z > 2.57 (df = 4) and z > 2.64 (df = 5), the results were considered significant for the association of dichotomous and ordinal independent variables with the COVID-19 severity and the outcome (9).

For continuous variables with non-parametric data, the median was used as a measure of central tendency and the interquartiles (IQ25% and IQ75%) as a measure of dispersion, and for data where parametric analysis was appropriate, the mean and standard deviation (±SD) were also used.

One-way ANOVA (F) with a 1.000-fold resampling procedure (bootstrap) was used to normalize the data. Brown-Forsythe ANOVA (F*) or Welch correction (W) was used to analyze data without homoscedasticity (Levene; p<0,05) and Games-Howel post-hoc for analysis of variance with p<0,05. The results of the difference in means (∆M) and its 95% confidence interval (95CI) corrected for the asymmetry of the distribution of the estimates after resampling (Bias-corrected and accelerated – BcaCI) were used to interpret the difference between groups (10). Two-way ANOVA was applied to evaluate the frequencies of comorbidities distributed across age groups. The coefficient of variation (CV%) was also used for continuous variables to determine the homogeneity of the results compared to the within-group mean. When appropriate, Kruskal-Wallis (H) and Mann-Whitney (U) tests were also applied to non-normally distributed data to examine the difference in variability between groups.

For survival curves, Kaplan-Meier/Log-Rank test methods were used for simple bivariate analysis and Cox regression models for multivariate analysis to obtain an estimate of the proportional risk of death over time versus covariates - hazard ratio (HR). The effect of categorical and continuous variables on COVID-19 severity classification was measured using bivariate logistic regression, with results expressed as χ^2 to fit the model and odds ratio (OR) and its confidence intervals as the measure of association for dichotomous variables. The effect sizes (ES) of each categorical independent variable on the grouping dependent variable were determined for the

Mann-Whitney tests using Pearson's contingency coefficient (r^2), calculated as $r2 = z^2/N$ and, for the contingency tables 2x2 analyzed using Chi-squared test ($\chi 2$), Cramer's coefficient (V^2) was applied (11,12). The appropriate ES coefficient and the context of the clusters were used to interpret the results, with values close to 1 indicating a strong ES and zero indicating a negligible effect.

To determine the correlation between two parameters, laboratory data were evaluated within the COVID-19 severity groupings. The Spearman correlation matrix (*r*) was used for this purpose. Coefficient intervals approaching 1 and -1 indicate positive and negative correlations, respectively. Values between -0.29 and 0.29 indicate no correlation between the variables. Where appropriate, multiple linear regression was also used to determine the influence of one or more parameters on the results obtained for laboratory analyses and other continuous variables.

Data tabulation was performed in Windows Excel 2019 version 21.02. IBM SPSS Statistics version 20.0 for Windows (IBM Corporation, Armonk, NY, USA) and GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, California USA) were used for statistical analysis and graph generation. All analyses were performed at an initial significance level of $p<0.05$ or lower, when appropriate corrections were required.

5.3 RESULTS

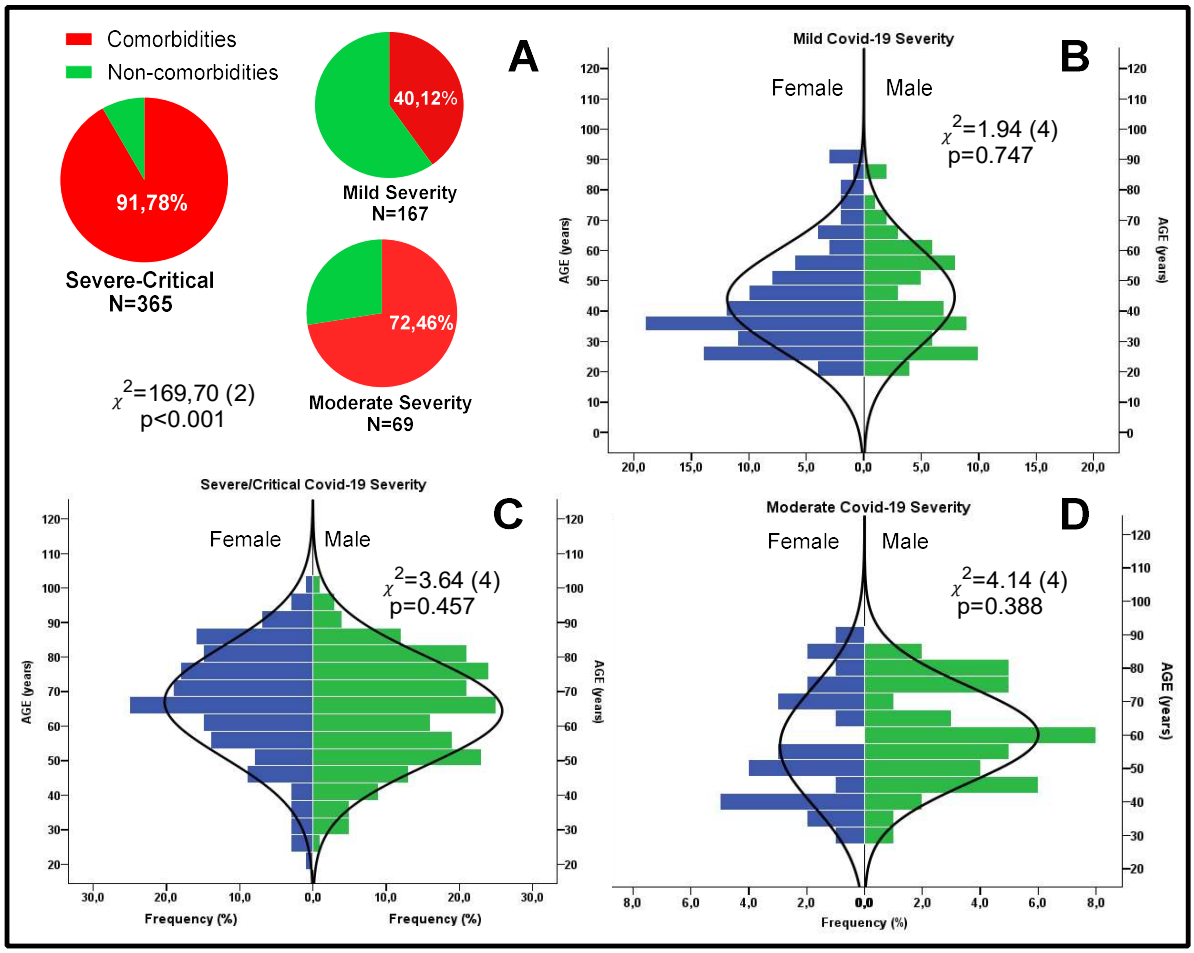
5.3.1 COVID-19 Patients Characterization

Based on the exclusion criteria, data from 48 patients were not evaluated in the study. Subsequently, data from 601 COVID-19 patients, classified into three severity levels: 167 as Pmild, 69 as Pmod, and 365 as Psev, were analyzed. Participants ranged in age from 21 to 100 years with a median age of 59.0 (IQ25=45.0; IQ75=73.5).The severity groups had notable distinctions in their age distribution, as detailed in **Table 1** and **Figure 1** [$\chi 2=146.46$ (2) $p<0.001$]. Specifically, 29 patients (17.36%) Pmild, 32 (46.38%) Pmod, and 239 (65.48%) Psev were older than 59 years.

The gender distribution showed variability [$\chi 2=14.97$ (2) $p<0.001$], with a higher prevalence of males among the hospitalized patients, Pmod (62.32%) and Psev

(53.34%), in contrast to Pmild (39.32%) treated on an outpatient basis. There was no significant difference in self-declared ethinicity responses among the three groups (non-caucasians: 34 Pmild (20.36%), 13 Pmod (18.84%), 59 Psev (16.16%) (χ2=0.14 (2) p=0.564).

FIGURE 1 – Distribuition histograms of comorbidities, age, and sex among COVID-19 patients classified by severity of the disease.



A: Frequency of having at least one comorbidity between mild, moderate, and severe COVID-19. **B, C** and **D**. Frequencies of age, in years, for female (represented by blue) and male (represented by green) patients in COVID-19 severity groups. The lines are the reference of normality distribution curve. Cathegorical variable were verified through the Pearson Chi-square Test (χ2) with statistical significance of p<0.05.

TABLE 1 - Distribution of demographic and pre-existing conditions among COVID-19 patients diagnosed with mild, moderate, or severe-critical illness between March and September 2020.

Conditions	COVID-19 Severity			Stathistics (df2)
	Pmild (n=167)	Pmod (n=69)	Psev (n=365)	
Age (years)				
Mean (±SD)	44.19 (16.78)	58.80 (15.65)	65.61 (15.84)	F=3.59 p<0.001
Median (IQ25-IQ75)	40 (31-55) [a]	57 (46-73) [b]	66 (55-77) [c]	χ2=106.49 p<0.001
Group n (%)				
18 – 49	82 (49.10) [a]	7 (10.14) [b]	22 (6.03) [b]	χ2=146.46 p<0.001
50 – 59	56 (33.53) [a]	30 (43.48) [a]	104 (28.49) [b]	
60 – 69	14 (8.38) [a]	12 (17.39) [ab]	77 (21.10) [b]	
70 – 79	7 (4.19) [a]	9 (13.04) [b]	86 (23.56) [b]	
≥80	8 (4.79) [a]	11 (15.94) [b]	76 (20.82) [b]	
Sex n(%)				χ2=14.97 p=0.001
Female	101 (60.48) [a]	26 (37.68) [b]	163 (44.66) [b]	
Male	66 (39.32) [a]	43 (62.32) [b]	202 (53.34) [b]	
Ethinicity n(%)				χ2=0.28 p=0.867
Caucasian	133 (79.64)	54 (78.26)	295 (80.82)	
Non Caucasian	34 (20.36)	15 (21.74)	70 (19.18)	
Underlying Conditions n(%)				
Neurocognitive Disorder	4 (2.40) [a]	6 (8.70) [ab]	55 (15.07) [b]	χ2=19.44 p<0.001
Pulmonary Disease	1 (0.60) [a]	4 (5.80) [ab]	37 (10.14) [b]	χ2=16.21 p<0.001
Kidney Disease	3 (1.80) [a]	11 (15.94) [b]	43 (11.78) [b]	χ2=17.09 p<0.001
Diabetes Mellitus	16 (9.58) [a]	19 (27.54) [b]	149 (40.82) [b]	χ2=52.99 p<0.001
Hypothyroidism	10 (5.99)	3 (4.35)	31 (8.49)	χ2=2.07 p=0.354
Heart Disease	5 (2.99) [a]	5 (7.25) [ab]	63 (17.26) [b]	χ2=23.61 p<0.001
Arterial Hypertension	29 (17.37) [a]	36 (52.17) [b]	220 (60.27) [b]	χ2=85.31 p<0.001
Dyslipidemia	3 (1.80) [a]	4 (5.80) [ab]	43 (11.78) [b]	χ2=15.62 p<0.001
Obesity	31 (18.56)	12 (17.39)	101 (27.67)	χ2=7.06 p=0.029
Continous medication	52 (31.14) [a]	35 (50.72) [b]	201 (55.07) [b]	χ2=26.34 p<0.001
Tobacco previous use	11 (6.59) [a]	14 (20.29) [b]	73 (20.0) [b]	χ^2=16.01 p<0.001

Pmild = COVID-19 mild severity; Pmod = COVID-19 moderate severity; Psev = COVID-19 severe and critical severity. df: degrees of freedom. Age was measured in years and reported as mean (±SD) and median with interquartile range (IQ25-IQ75). Dichotomous variables were presented as absolute numbers and percentages. A Kruskal-Wallis test was used to evaluate differences in age distribution, while a one-way ANOVA was utilized to determine mean differences (p<0.05). Categorical independent variables were tested using the Pearson Chi-square Test (χ2) and resulted in statistical significance at p<0.01 (Bonferroni correction). Post-hoc pairwise comparisons between groups were conducted and letters (a, b, c) were utilized to indicate differences at p<0.05 or z test>2.58. he same letter indicated no statistically significant difference, while distinct letters indicated a significant pairwise difference.

Part **A** of **Figure 1** illustrates the relative frequency of at least one comorbidity among the patients included in the study, separated by COVID-19 severity. In **B**, **C**, and **D**, the age distribution frequencies do not differ between genders in each clinical category of the disease.

The diagnosis of SARS upon hospitalization (38.52%; 230/365) had a significant association with the need for orotracheal intubation (OTI) (34,24%; 125/365) during their hospital stay (OR=3.21 (1) [2.02 – 5.11] p<0.001), according to Psev's findings.

The medians (IQ25 and IQ75) for the time from symptom onset to referral to medical care – Pmod = 7 days (4-8); Psev = 6 days (4-8) - and the means (±SD) – Pmod = 6,93 (±4.20); Psev= 6.58 days (±3.99) demonstrate no significant association with COVID-19 severity [F=0.424(1) p=0.515] [*U*=12.056.0 (1) p=0.573].

Table 1 lists the factors evaluated and the results found for the difference in distribution between the severity groups. The ES coefficients for sex (V^2=0.158 p=0.001), dyslipidemia (V^2=0.161 p<0.001), lung disease (V^2=0.164 p<0.001), and tobacco use (V^2=0.163, p<0.001) were considered small. Its was moderate for the presence of heart disease (V^2=0.198 p<0.001), neurocognitive disorder (V^2=0.180 p<0.001) and need for continuous medication (V^2=0.210 p<0.001). High ES values were identified for diabetes mellitus (DM) (V^2=0.297 p<0.001) and arterial hypertension (HA) (V^2=0.377 p<0.001).

The study reassessed data from COVID-19 patients who were hospitalized to determine the risk factors associated with deteriorating conditions after admission (**Table 2**). The patient groups were largely uniform in most parameters, and comorbidities had a minimal impact on the severity classification of the patients. Only the age distribution and means (p<0.001), along with the medians (p=0.010), indicate a difference between groups. However, the effects suggest that age has little influence on the severity of patients who are already hospitalized (r^2=0.02/insignificant; V^2=0.15/small).

In the same way, binary logistic regression modeling was applied to the presence or absence of risk factors, with the outcome being Psev in hospitalized patients. The results reveal that age is the single predictive factor for severe-critical disease in hospitalized cases (χ2=10.46 (1) p=0.001; OR=1.027 [1.010 - 1.044]).

Age was used as a predictive factor for Psev, and as a result, a two-way ANOVA was conducted to determine whether significant differences existed between comorbidities and their interactions, including those with heart disease, diabetes mellitus (DM), HA, obesity, and neurocognitive disorders across age groups. The results indicated that the level of significance was high concerning neurocognitive disorders (F (1) =10.092 p=0.002) and heart disease (F=7.032 (1) p=0.008), even though, there was no interaction among the factors.

The analysis of frequency percentages of clinical conditions indicates a significant difference in the occurrences of these conditions between different age groups (F=6.311 (5.20) p=0.003). (**Figure 2**). Hypertension was found to be the most prevalent comorbidity in hospitalized patients who were over 50 years old. And obesity held this position in patients between the ages of 18 and 49, showing a significant decrease of frequencies in older patients.

More specifically, in patients up to 59 years of age, obesity has a different frequency between Pmod (18.32%) and Psev (40.48%), OR=2.914 [1.202 - 7.630], p=0.0188. Though, this difference is not sustained in patients aged 60 years and older, where Pmod (16.62%) and Psev (20.92%), p=0.6410.

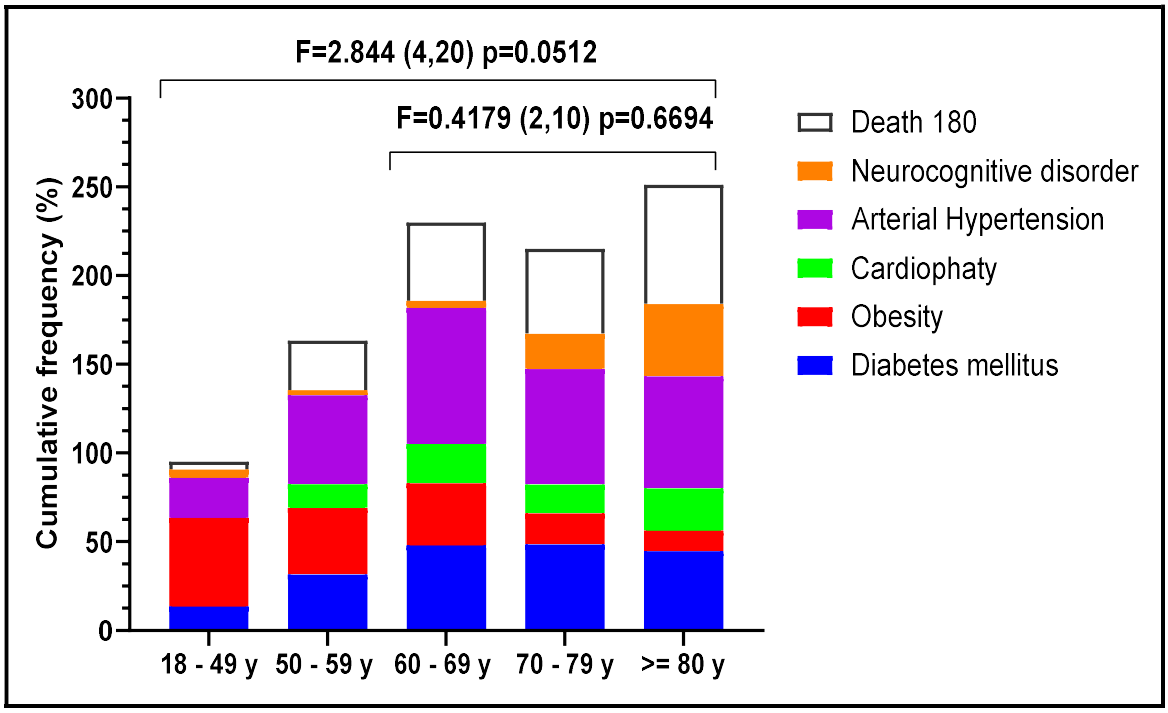
In terms of grouped comorbidities, however, the age groups in general do not differ significantly (p=0.0512) in Psev, especially in the elderly (p=0.6694) (**Figure 2**).

TABLE 2 - Risk estimates of demographic and clinical parameters for the worsening of the condition of patients hospitalized with COVID-19 of moderate and severe/critical severity.

	COVID-19 Severity	
	Psev x Pmod	
Conditions	**Stathistic (df1)**	**Risk and Effect size**
Age median	χ2=6.676 p=0,010	V^2=0.15
Age distribution	*U*=15717 p=0,001	r^2=0.02
Gender n (%)	χ2=1.15 p=0.284	
Female		
Male		
Ethinicity n (%)	χ2=0.24 p=0.623	
Caucasian		
Non Caucasian		
Underlying Conditions n (%)		
Neurocognitive Disorder	χ2=1.95 p=0.162	
Pulmonary Disease	χ2=1.28 p=0.258	
Kidney Disease	χ2=0.92 p=0.337	
Diabetes Mellitus	χ2=4.32 p=0.038	OR:1.09 [1.00 - 1.18] V^2=0.10
Hypothyroidism	χ2=2.07 p=0.354	
Heart Disease	χ2=4.40 p=0.036	OR:1.12 [1.03 - 1.22] V^2=0.10
Arterial Hypertension	χ2=1.57 p=0.210	
Dyslipidemia	χ2=2.15 p=0.142	
Obesity	χ2=3.18 p=0.074	
Continous medication	χ2=0.44 p=0.506	
Tobacco previous use	χ^2=0.03 p=0.956	

Df: degrees of freedom; Pmod = COVID-19 moderate severity; Psev = COVID-19 severe and critical severity. The statisical analysis was performed using Mann-Whitney Test (U) for age. Diference on distribuition of cathegorical independente variable were verified through the Pearson Chi-square Test (χ2) with statistical significance of p<0.05. OR:Odds Ratio risk estimate; r^2: Pearson's effect size; V^2= Phi and Cramer's effect size.

FIGURE 2 – Cumulative frequency of comorbidities and death outcome in 180 days after COVID-19 symptoms onset according to age patient groups with severe-critical illness.



Statistics: two-way ANOVA was used to assess the difference between the set of comorbidity frequencies in hospitalized patients age groups with severe or critical COVID-19. All tests were performed at p<0.01 significance level. Y: years.

5.3.2 Laboratory Analyses and Chest Tomography

Differences in means, medians, and variances of leukocytes (*W*=9.00 (2.475) p<0.001), monocytes (*F*=4.42 (2, 475) p=0.012), NL Ratio (*W*=13,97 (2,475) p<0.001), CRP (*W*=28.91 (2, 471) p<0.001), LDH (*W*=41.65 (2, 473) p<0.001), AST (*W*=18.76 (2, 474) <0.001), IL-6 (*W*=7.33 (2, 176) p=0.001) and bun urea (F=3.71 (2, 456) p=0.025) were observed between the Pmild, Pmod, and Psev groups during initial patient treatment, as part of several laboratory tests. Analyses were conducted using both non-parametric (Kruskal-Wallis) and parametric (ANOVA) statistical methods with one-way design and 1000 resamples. Results were similar and are presented comparatively through mean differences (ΔM) [95CI] between pairwise comparisons in **Table 3.**

Furthermore, parameters involved in iron metabolism were evaluated, with significant differences between the distributions of serum iron levels (W=4.49 (2, 463)

p=0.014), TIBC (*F*=6.46 (2, 463) p=0.002) and LIBC (F=5.31 (2, 463) p=0.005). However, no significant differences were found in ferritin levels (F=1.46 (2,465) p=0.233) or the percentage of transferrin saturation (F=0.56 (2, 463) p=0.573).

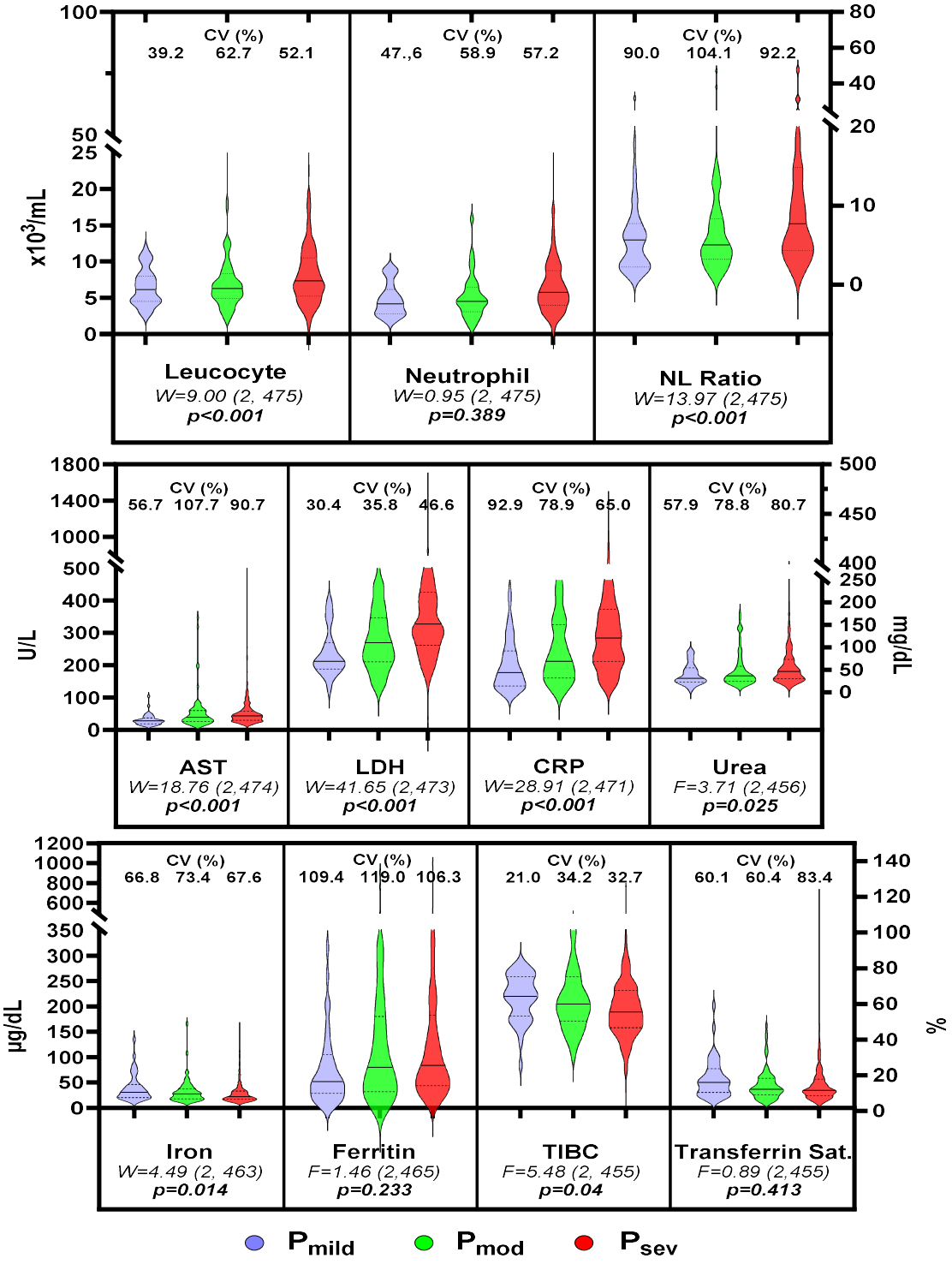
It was not possible to discern differences among COVID-19 severity groups in the other analyses performed. The laboratory parameters, which exhibited statistical significance in differences between the Pmild, Pmod, and Psev patient groups, with the exception of IL-6, are comparatively displayed in **Figure 3**. This allows for observation of data distribuition and the coefficients of variation (CV%) for each patient group's parameters.

TABLE 3 – Laboratory parameter differences among COVID-19 patients grouped according to disease severity.

Parameter	COVID-19 Severity			Stathistics ΔM [95BcaCI]
	Pmild N=41	Pmod N=69	Psev N=365	
	Mean (±SD)			
Leucocyte ($x10^3$/μL) RI: 4,0 - 11,0	6.43 (2.52)[b]	7.17 (4.50)	8.29 (4.29)[a]	[ab] 1.86 [0.77 – 2.94]
Neutrophil ($x10^3$/μL) RI: 2.0 - 7.5	4.64 (2.35)[b]	5.18 (3.05)	6.56 (3.75)[a]	[ab] 19.26 [1.05 – 2.83]
NLR	6.09 (5.63)[c]	6.96 (7.24)[b]	11.42(10.52)[a]	[ab] 4.53 [2.13 – 6.72] [ac] 5.33 [3.03 – 7.48]
Iron (μg/dL) RI: 50 - 175	39.87 (26.63)[b]	32.18 (23.63)	27.77 (18.77)[a]	[ab]12.04[- 22.56 – -3.71]
LIBC (μg/dL) RI: 140 - 280	177.1 (44.6)	189.1 (70.2) [b]	166.6 (57.5)[a]	[ab] 22.33 [- 41.91 – -4.08]
TIBC (μg/dL) RI: 250 - 450	216.9 (45.6) [c]	221.3 (75.6) [b]	196.2 (64.1)[a]	[ab] -25.10 [-45.82 – -6.99] [ac] -20.76 [-36.05 – -3.95]
CRP (mg/L) RI: <5.00	61.54 (57.15)[c]	98.39 (78.58)[b]	135.4 (88.01)[a]	[ab] 37.01 [17.32 – 57.81] [ac] 73.86 [54.56 – 93.64] [bc] 36.84 [10.79 – 64.48]
LDH (U/L) RI: 81 - 234	236.9 (72.0)[c]	284.7 (102.0)[b]	365.2 (170.1)[a]	[ab] 80.54 [52.8 – 110.90] [bc] 47.74 [15.93 – 79.91]
AST (U/L) RI:15 – 37	30.9 (17.4)[c]	55.1 (59.3)[b]	52.9 (48.0)[a]	[ac] 22.03 [15.1 – 28.55] [bc] 24.23 [11.68 – 39.48]
Urea (mg/dL) RI: 15 - 38	40 (23)[b]	50 (40)	59 (48)[a]	[ab] 19.05 [10.59 – 27.98]
IL-6 Φ (pg/mL) RI:1.5 -7.0	-	129.6(172.1)[b]	294.6(489.5)[a]	[ab] 165.09 [40.2 – 290.0]

Laboratory parameter differences among COVID-19 patients were grouped according to disease severity and paired using severely ill patients as a reference. RI: Reference Interval; NLR: Neutrophil-Lymphocyte Ratio; MNR: Monocyte-Neutrophil Ratio; Transferrin Sat.: Transferrin Saturation; LIBC: Latent Iron Binding Capacity; TIBC:Total Iron Binding Capacity CRP: C Reactive Protein; LDH: Lactate Dehydrogenase; AST: Aspartate Aminotransferase; IL-6: Interleukin 6; The results were expressed as mean and standard seviattion (±SD). Stathistic performed through one-way ANOVA with bootstrap 1000 times and post-hoc was interpreted by ΔM and Bias Corrected and Acelerated (Bca) 95% Confidence Interval (95CI). [a b c. 1. 2] reported to demonstrate the difference between groups when stathistics are significant.Φ N= 30 P mod and 134 Psev individuals.

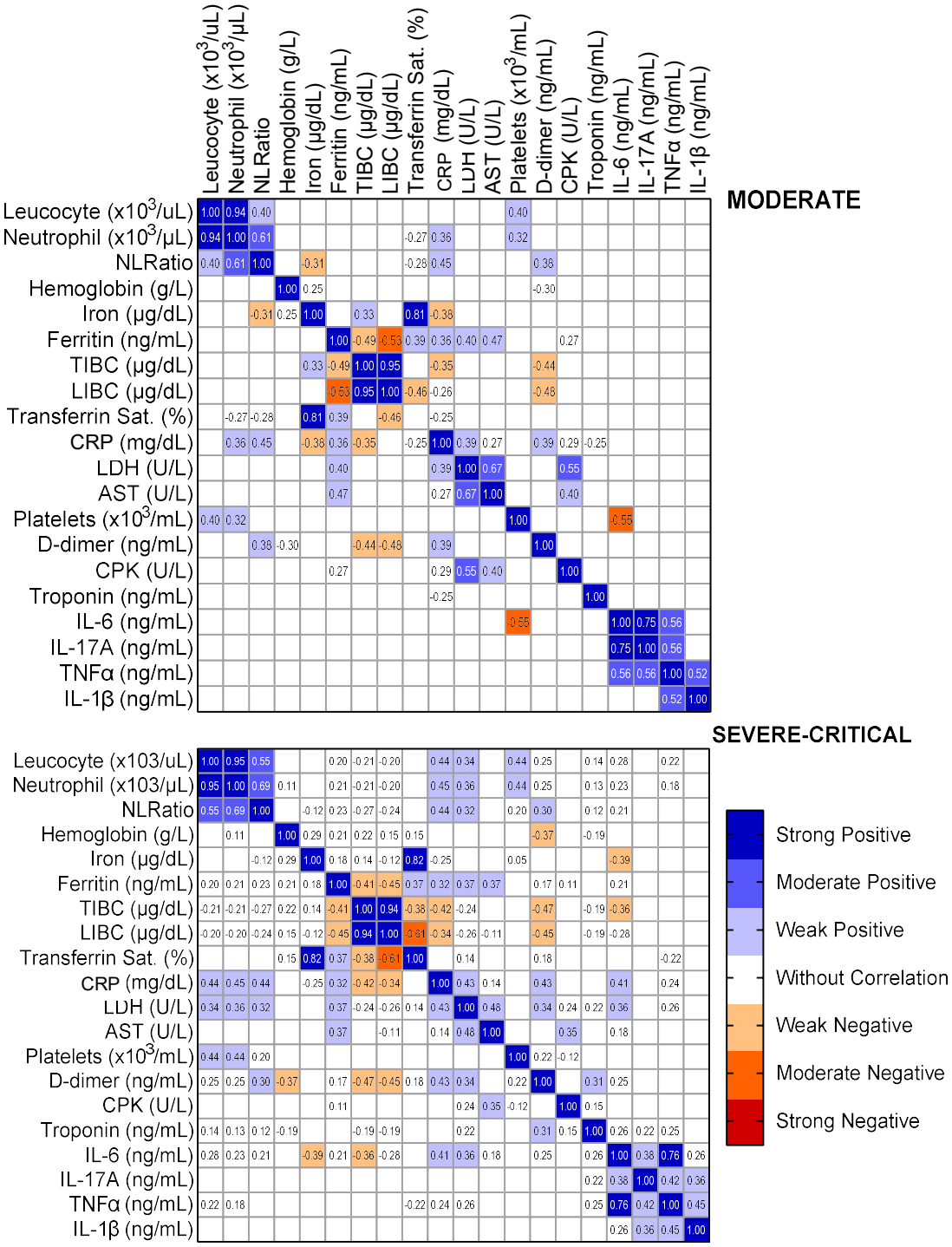
FIGURE 3. Distribution of laboratory parameter results and their variation coefficient (%) obtained from COVID-19 patients grouped by level of severity.



Pmild: Mild COVID-19 Severity; Pmod: Moderate Severity; Psev: Severe-Critical Severity. NLR: Neutrophil-Lynphocyte ratio; AST: Aspartate Aminotransferase; LDH: Lactate Dehydrogenase; CRP: C Ractive Proten; TIBC: Total Iron Binding Ccapacity. Stathistics: one way ANOVA test (F) with bootstrap (1000 times) and post-hoc by Bias Corrected and Acelerated (Bca) 95% Confidence Interval (95CI); One way ANOVA with Welch correction if the equal variance is not assumed; (df, N): degrees of freedom and amostral number; CV(%): Variation Coefficient. Solid and dotted lines in violins are median and interquartiles (IQ25- IQ75), respectively. All tests were performed at p<0,05 significance level.

Spearman's correlation was employed to analyze the Pmod and Psev laboratory test results interaction (**Figure 4**). Strong and moderately positive correlations are observed between cellular and molecular markers of the inflammatory process within the matrices. Additionally, variable interactions exist between inflammatory markers and indicators associated with iron metabolism, particularly.

FIGURE 4 – Spearman correlation matrix for laboratory analyze results obtained from patients with moderate and severe-critical COVID-19.



Cells numbers were interpreted by Spearman correlation coefficient: strong positive: [0.71 – 1.0]; moderate positive [0.501 -0.70]; weak positive; [0.5 – 0.30]; strong negative: [-1.0 - -0.71]; moderate negative: [-0.7 - -0.501]; weak negative: [-0.5 - 0.3]; null correlation: [-0.29 – 0.29]; The tests are performed at p<0.05 significance level and 95CI with range not goes through number 1. Blank cell without number are from insufficient results to determinate correlation. NLR: Neutrophil-Lymphocyte Ratio; TIBC:Total Iron Binding Capacity; LIBC: Latent Iron Binding Capacity; Transferrin Sat.: Transferrin Saturation; CRP: C Reactive Protein; LDH: Lactate Dehydrogenase; AST: Aspartate Aminotransferase; CPK: Creatinine Phosphokinase; IL:Interleukin; TNF: Tumoral Necrosis Factor.

Of those hospitalized, 375 patients (62 Pmod and 313 Psev) underwent chest CT at the beginning of hospitalization, with findings of pulmonary involvement on a scale of 0 to 5: 29 (7.7%) score 1, 68 (18.1%) score 2, 98 (26.1%) score 3, 114 (30.4%) score 4, and 66 (17.6%) score 5. Of the 180 Psev patients with an impairment score of 4 or 5, 68 (37.78%) received ventilatory support through OTI, whereas 50 (37.04%) of the 135 Psev patients with a CT-determined impairment score of less than 50% (scores 1, 2, and 3) were intubated.

There was no difference in the mean time from symptom onset to image analysis, ranging from 5.97 (score 2) to 6.93 days (score 4).

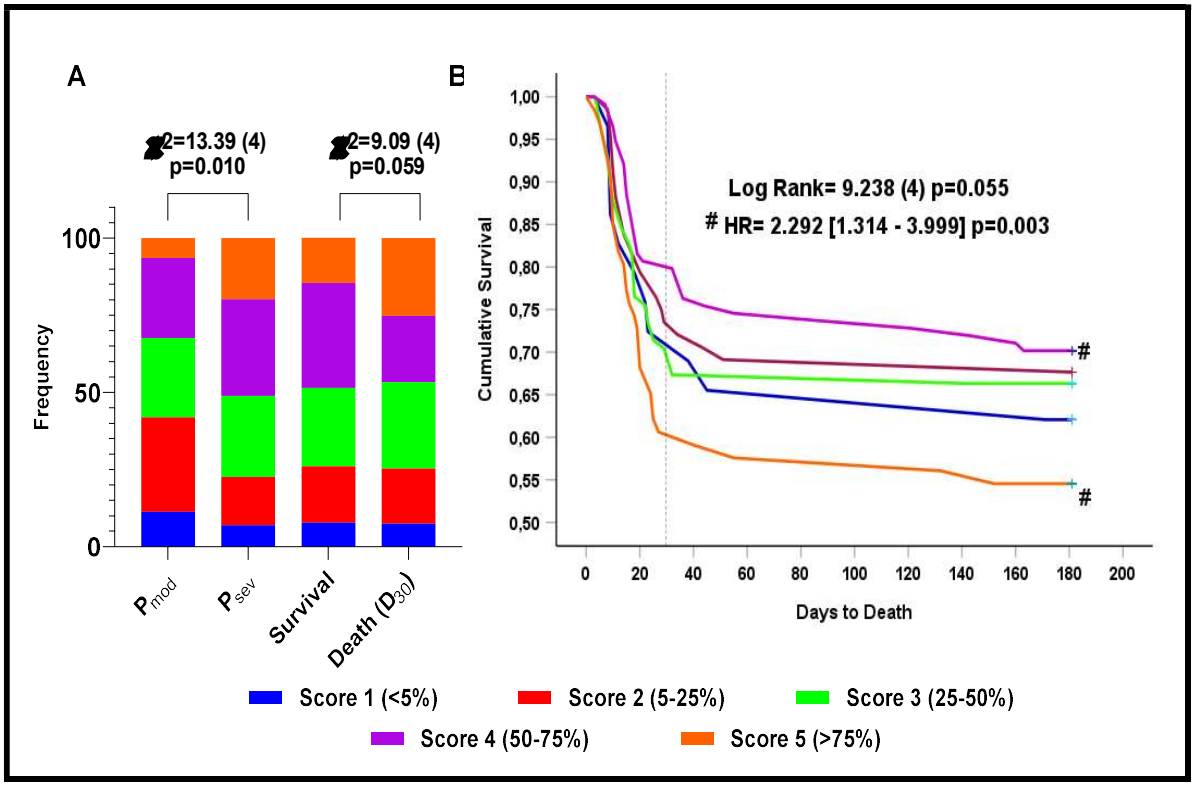
In the bivariate linear regression model, we introduced the variables SARS diagnosed upon admission and neurocognitive disease as predictors of changes in CT scores. Only 7.8% of the variance in the scores was explained by these variables in the model (R^2adj =0.078; F=15.70 p<0,001). The other comorbidities included as variables in the regression models were not significant for the risk of greater pulmonary involvement.

Nonetheless, the distribution of CT scores varied between the Pmod and Psev cohorts, and the scores had a moderate influence (V2=0.189) on the characterization of patients' clinical status as severe/critical (**Figure 5**). There was no association between radiologic scores and patients' scaled age (χ2=19.51 (16) p=0.243), the presence of previous lung disease (χ2=0.83 (4) p=0.934), heart disease (χ2=8.36 (4) p=0.079), or DM (χ2=3.67 (4) p=0.452). Although there were differences in the scores of patients with and without neurocognitive disorder (χ2=15.33 (4) p=0.004), no relationship was found between greater pulmonary involvement (scores 3, 4, and 5) and this clinical condition.

There was no difference in the time course of the disease between the CT score groups. In addition, the frequencies of tomographic scores for patients who survived or died during the acute phase of COVID-19 (D30) also did not differ (χ2=9.09 (4, 375) p=0.059). The observed numbers of deaths in D30 patients with scores 4 and 5 (27 and 23) differs from the expected values (18.8 and 32.5), indicating a slight disparity between deaths in the two groups (V^2=0.059). Thus, by utilizing only scores 4 and 5 on the survival function, there is evidence that patients with over 75% lung involvement face a 2-fold lower chance of survival than patients with 50 to 75% involvement (χ2=8.49(1) p=0.003, HR=2.292 [1,314 – 3,999]). Nevertheless, there was no proportionality in the risk of premature death for radiological scores below 4 (Log

Rank = 9.238 (4, 375) p=0.055) (**Figure 5**).

FIGURE 5 – Relationship between lung involvement observed by computed tomography and the disease severity or death within 30 days after COVID-19 symptoms onset.



Pmod= COVID-19 moderate severity; Psev = COVID-19 severe-critical disease. **A.**- Frequency of pulmonary radiological scores difference between disease severity or 30 days outcome, assessed by Chi-square test. **B** - Cumulative survival assessed by Kaplan-Meier for general survival curve (Log-Rank) and by Cox Regression to estimate death risk covariance of tomographic scores 4 and 5 (HR: Hazard Ratio [95%CI]). All tests were performed at p<0,05 significance level.

5.3.3 Outcome of COVID-19 Hospitalized Patients

Of the hospitalized patients, 69 Pmod were discharged cured or improved within a period of 2 to 29 days, 4 underwent hemodialysis due to pre-existing renal disease, and none required intervention for a thrombotic event during hospitalization. Among the Psev patients, 207 (56.71%) were discharged under the same conditions after a range of 3 to 103 days, but 19 were transferred or escaped, and 139 (38.08%) had a negative hospitalization outcome after a period of 1 to 132 days. Only one thromboembolic event was diagnosed among the latter group. Out of the 13 Psev

patients who underwent dialysis, 5 (1.37%) received renal replacement therapy without prior disease. Orotracheal intubation was a therapeutic approach used for 125 (34.25%) Psev.

After hospital discharge, 19 patients died between symptom onset and 180 days (D180): 2 (2.9%) Pmod and 17 (4.66%) Psev.

Patients with at least one comorbidity faced double the risk of dying from COVID-19 within 180 days (D180) compared to hospitalized patients without pre-existing conditions (χ^2=5.533 (1) p=0.021; OR=2.326 p=0.030 [1.086-4.986]. However, the binary regression model did not provide conclusive evidence on the potential impact of medication use to manage these risk factors on hospitalized COVID-19 patients' outcomes.

Being classified as Psev significantly increased the risk of death by 25 times in D180 (χ^2=39.35 (1) OR=25.00 [6.03-103.6], p<0.0001) - 155 out of 157 deaths (98.73%). In this group, 130 (35.62%) patients died within D30, with a cumulative 148 at D60 and 156 at D180. Age over 80 years old on D30 (45 Psev; 34.62%) was linked to death from COVID-19, while age under 60 years old (25 Psev; 19.3%) was associated with survival (χ^2=35.53 (4) p<0.001). This association persisted as patients approached D60 and D180, with 49 (33.11%) and 52 (32.91%) patients over 80 years of age experiencing death, respectively.

By the same way, the risk of mortality from SARS-CoV-2 infection significantly increases in patients aged 60-70 years (odds ratio [OR]=10.706 [1.363-84.077] p=0.024), 70-80 years (OR=13.731 [1.764-106.887] p=0.012), and over 80 years (OR=30.484 [3.895-238.599] p=0.001), as found through logistic regression analysis.

In addition, there was no significant difference in survival rates between women and men (HR=1.149 [0.836 - 1.580] p=0.391). Despite higher survival rates for women in all age groups, no variation was found in the relationship between the groups. Ethnicity also did not affect the survival of patients hospitalized with COVID-19 (HR= 1.094 [0.722 – 1.658] p=0.670) (**Table 2**).

Bivariate regression models were utilized and factors such as age, pre-existing diseases, radiological score, and laboratory tests - which revealed notable differences between Pmod and Psev patients (see **Table 2**) - were hierarchically introduced to determine their impact on patient mortality D180 (No and Yes) who had severe or critical COVID-19 (**Table 4**).

In the best logistic regression model (χ2=66.516 (3) p<0.001), it was

determined that age (OR=1.043 [1.026-1.059] p=0.001), neurocognitive disorders (OR=2.306 [1.237-4.299], p=0.009), and diabetes (OR=1.736 [1.131-2.664] p=0.012) predict D180 to some extent, but the interaction of these factors does not pose a greater risk of death to patients. Again, no significant contribution was made by other comorbidities, laboratory test results, or specific radiological scores to the models that incorporate covariates for death in D30, D60 and D180.

Table 5 shows the differences in laboratory test means for patients who either survived or did not to the disease. We evaluated those that were significant for increased COVID-19 severity among hospitalized patients, as well as ferritin, D-dimer, CPK, lactate, and glucose, which could potentially serve as outcome markers. In this case, most severity markers continue to be significantly different between survivors and non-survivors, in addition to a higher average glucose level among those who died.

TABLE 4 - Demographic and clinical characteristics of patients hospitalized with severe or critical illness analyzed according to outcome within 180 days after the onset of COVID-19 symptoms.

Demografic and Clinical Conditions	$Death_{180}$ Psev		Stathistic (df1) OR [95%CI]
	No N=209 (57.3%)	**Yes** N=156 (34.3%)	
Age Mean (years)	60.5(±15.5)	71.6 (±14.3)	ΔM=11.1 [8.2-14.0] p=0.001 OR=1.052 [1.036 -1.067]
Gender			
Female	99 (47.4)	65 (41.0)	χ2=0.46 p=0.497
Male	110 (52.6)	92 (59.0)	
Ethinicity			
Caucasian	165 (78.9)	130 (83.3)	χ2=1.11 p=0.497
Non-caucasian	44 (21.1)	26 (16.7)	
Conditions	243 (87.7)	148 (94.3)	χ2=2.17 p=0.141
Neurocognitive	16 (7.7)	39 (25.0)	χ2=23.69 p<0.001 OR=4.02 [2.15 – 7.52]
Pulmonary	18 (8.6)	19 (12.2)	χ2=1.25 p=0.264
DM	71 (34.0)	78 (50.0)	χ2=9.50 p=0.002 OR=1.94 [1.27– 2.97]
Heart Disease	29 (13.9)	34 (21.8)	χ2=3.92 p=0.048 OR=1.73 [1.00 – 2.99]
HAS	120 (57.4)	100 (64.1)	χ2=1.67 p=0.197
Obesity	64 (30.6)	37 (23.7)	χ2=2.13 p=0.145
Orotracheal Intubation	27 (21.6)	98 (78.4)	χ2=98.78 p<0.001 OR=11.39 [6.78-19.13]

Df: degrees of freedom; Categorical variables were obtained by simple percentage frequency (n%). and age by mean and standard deviation (±SD). For analysis of the difference between categories, Chi-square test (χ^2) with standardized adjusted residues (z test) and significance corrected by the Bonferroni method (p<0.0125) were used, when appropriate, with significance at p<0.05. Risk estimation Odds Ratio (OR) and Confidence intervall [95CI] is available for significant difference between groups by logistic binary regression. Age stathistic performed through t-Student test with bootstrap 1000 times interpreted by ΔM (mean difference) and Bias Corrected and Acelerated (Bca) Confidence Interval 95% (95CI on non-normality data distribuition.

TABLE 5 – Differences in laboratory parameter means among COVID-19 patients hospitalized with severe or critical clinical conditions, based on outcome risk within 180 days.

Laboratory Analysis	$Death_{180}$ Psev		Stathistic (df1) ΔM [95CI] and OR [95CI]
	No (N=209)	**Yes** (N=156)	
	Median (±SD)		
Leucocyte (x10^3/μL)	7.47 (3.49)	9.39 (4.98)	ΔM=1.92 [0.98 – 2.80] p=0.001 OR=1.000 [1.000 -1.000]
Neutrophil (x10^3/μL)	5.85 (3.21)	7.52 (4.20)	ΔM=1.68 [0.85– 2.49] p<0.001 OR=1.013 [1.007 -1.019]
NLRatio	9.77 (10.48)	13.62(10.20)	ΔM=3.85 [1.61– 5.89] p=0.001 OR=1.036 [1.015 -1.058]
CRP (mg/dL)	119.8 (76.7)	156.7 (97.7)	ΔM=36.90 [17.8 - 54.9] p=0.001 OR=1.005 [1.002 -1.007]
LDH (U/L)	330.4 (133.2)	412.2 (200.9)	ΔM=81.72 [45.85-118.60] p<0.001 OR=1.003 [1.002-1.005]
AST (U/L)	48.9 (32.3)	58.2 (62.8)	ΔM=9.3 [-0.6 – 20.8] p=0.120
IL-6 (ng/mL)º	157.2 (153.9)	402.7 (620.6)	ΔM=245.5 [118.6-404.7] p=0.013 OR=1.003 [1.002-1.005] p=0.004
Ferritin (ng/mL)	1355.4 (2811.3)	1612.4 (2260)	ΔM=267.9 [-264.9-795.5] p=0.338
D-dimer (ng/mL)∞	1649.9 (9388)	1968,9 (6120)	ΔM=319,0[-1604 – 2022] p=0,760
CPK(U/L)	218,4 (305)	248,4 (305)	ΔM=30,0[-57,8 – 101,5] p=0,480
Glucose (mg/dL)	161,6 (79,8)	188,7 (99,1)	ΔM=27,1 [9,3 -46,6] p=0,006 OR=1,003 [1,001 -1,006] p=0,005
Lactate (mg/dL)	1,81 (0,76)	2,21 (2,08)	ΔM=0,39 [0,09 - 0,75] p=0,046

Df1: 1 degree of freedom; NLR: Neutrophil-Lymphocyte Ratio; CRP: C Reactive Protein; LDH: Lactate Dehydrogenase; AST: Aspartate Aminotransferase; IL-6: 6-Interleukin; CPK: The results were expressed as mean and Standard Deviattion (±SD). Stathistic performed through t-Student test with bootstrap 1000 times interpreted by ΔM and Bias Corrected and Acelerated (Bca) Confidence Interval 95% (CI95); ºN=134 (59/75) patients; ∞N=268 (157/11) patients difer from general N.

5.4 DISCUSSION

5.4.1 Sex and Age of COVID-19 Patients

According to data from the Brazilian Institute of Geography and Statistics (13), 48.9% of the Brazilian population would be male in 2020. In this study, the Pmild, which at the time was mainly convened among health professionals, could imply a bias in the selection of the female sex, but among the other groups, the predominance of men clearly exceeds the limits of Brazilian demography. On the other hand, sex seems to be a factor that influences the worsening of the clinical condition, but does not imply a higher risk of death for the patient already hospitalized.

Data generated during the pandemic in other populations showed the same phenomenon (14,15), possibly influenced by socio-cultural factors but, essentially, based on the genetic-immunological-endocrine context of the male sex for the worsening of COVID-19 (16,17). Females typically develop more effectively innate, humoral and cellular immune responses to viral infections, as well as negatively experience more autoimmune diseases (16).

Thus, as in other inflammatory models, in the disease induced by SARS-CoV-2, males and females differ significantly in the behavior of innate immunity and show different profiles of regulation and expression of viral receptors related to other vital functions that influence the severity of COVID-19 in males, such as angiotensin-converting enzyme 2 (ACE2) (15,18,19).

These components of the immune system and body physiology are affected by the age of the patient who contracts COVID-19, resulting in poorer outcomes for elderly patients (20). According to data compiled by the World Health Organization (21), a majority of COVID-19 cases were identified in adults aged 25 to 64 throughout the pandemic. However, this population accounted for less than one-third of deaths.

Among the participants of the Pmild study, the frequency of cases examined in individuals over the age of 60 was relatively balanced (17.36%), similar to estimates of the Brazilian population in the Southern region (16.2%) (13). The preponderance of this age group in Pmod cases and particularly in Psev highlights senility as a crucial risk factor for severity and mortality in SARS-CoV-2 infection. This may coincide with other susceptibility factors like sex and comorbidities.

Physiologically, ACE2 expression is increased in older adults (18,19) potentially leading to higher viral replication rates (22). Conversely, the efficacy of interferons in protecting cells from new infections declines significantly with age (23).

The anticipated and coordinated effects for an effective antiviral cellular response involve three elements: 1) Th1 and NK responses, macrophage activation, and cytotoxic effects on infected cells; 2) a regulatory response to limit inflammation; and 3) a Th2 response focused on antibody production and immunological memory. These effects are decreased with age, a process known as immunosenescence (24). With aging, the immune response is directed towards phenotypes more focused on cell migration (Th17) and less regulatory profiles (25,26), determining the exacerbated cellular response characteristic of that observed in COVID-19 pulmonary infiltrates (27) and cytokine release syndrome profiles (28).

This study found that hospitalization with COVID-19 after the age of 60 increases the risk of death by at least 3.6% each year old, which is more severe than the risk observed in a large sample from USA during the same time period (29).

Variations in COVID-19 mortality rates can be attributed to several factors, including disparities in accessing health care technology across different regions, as well as significant socioeconomic and cultural circumstances (30–32). These factors can be challenging to clearly quantify. Physiologically, as an individual ages, they experience a gradual decline in their functional abilities and develop comorbidities that increase the risk of negative outcomes in infectious diseases (33,34). Thus, certain pre-existing conditions and habits have been frequently linked to worse progression of COVID-19, specifically diabetes, cardiovascular and respiratory diseases, neurocognitive disorders, obesity, immunosuppressive conditions, kidney failure, and tobacco use, among others (35,36).

5.4.2 Comorbidities

The impact of at least one comorbidity on the severity or mortality rate of COVID-19 was estimated to be 1.1 to 1.5 times greater than that of patients with no comorbidity diagnoses (37). We observed higher estimates among hospitalized patients when death occurred later after symptom onset (OR=2.3 on D180) in our

study, potentially because comorbidities complicate clinical conditions and make long-term patient recovery even more difficult (38–40).

At least 50% of Pmod and Psev patients had arterial hypertension (AH) among the investigated comorbidities, aligning with findings from larger sample studies (37,41). This clinical condition appears to be the most frequent comorbidity in hospitalizations due to COVID-19, including among younger adult patients (37,42,43) and its physiology is intertwined - homeostatic regulator ACE2 pathway and viral receptor - with SARS-CoV-2 infection (44,45).

In addition, this study found that most comorbidities assessed had a significant association with moderate or severe cases of COVID-19 in contrast to patients with mild conditions, with the exception of hypothyroidism. Surprisingly, at the first analyses obesity also exhibited no association (46,47).

Nevertheless, we noted that 26.0% of hospitalized patients were obese, and this condition affected 50% and 37.5% of patients in the age ranges under 60 years (**Figure 2)** as previously reported (48,49). These findings surpass the 2019 estimated national average for BMI over 30kg/m^2 in adult individuals, which was 19.8% (13).

Obesity correlates with COVID-19 severity and manifests as a chronic inflammatory status and suboptimal immune response to the vírus (50). Furthermore, being overweight increases the risk of diabetes and cardiovascular disease, exacerbating the condition even in patients who don't have aging as their primary risk factor (51).

Although sometimes controversial, the cause-and-effect relationships between obesity and the worse outcome of obese patients in other infections (47,52,53) involve the secretion of adipokines such as leptin (increase), adiponectin (decrease), angiotensin II (increase) by adipose tissue, and the expression of their genetically determined receptors (50,53), and by the availability of coronaviruses ligands in adipose tissue, such as dipeptidyl peptidase 4 (DPP4) (54). All of these interact decisively for metabolic and immunological deregulation (51).

Thus, discoveries about the relationships between adipose tissue and the immune system indicate that obesity impairs chemotaxis and alters macrophage differentiation, NK and dendritic cell functionality, in addition to enhancing the release

of cytokines related to the immunological syndrome, such as monocyte chemoattractant protein (MCP-1), TNF-α, IL-17A and IL-6 (50–52,55).

In anyway, molecular mechanisms and epidemiology show a convergence of epidemics of hypertension, obesity and COVID-19 (51,56), findings already observed in the H1N1 influenza pandemic (53,57) as well as MERS-CoV (58).

Furthermore, we observed that hospitalized patients with heart disease and diabetes are at increased risk for coronaviruses disease progression, as demonstrated by comparable studies (43). In COVID-19, myocardial injury was significantly associated with mortality and cardiovascular events, independent of previous cardiovascular disease, and likely due to the severity of the acute illness rather than exacerbation of pre-existing disease (59,60). The impact of the virus and thromboembolism on the inflammatory response, as well as endothelial injury were the driving mechanisms of this association (59,61).

In the same bidirectional pattern, type 1 and type 2 diabetes (DM) can determine more severe conditions in COVID-19 (62–64) and also prevails as one of the most exacerbated or diagnosed conditions after infection by SARS-CoV-2 (62,65,66). Interestingly, hospital admission hyperglycemia is a predictor of COVID-19 severity and mortality regardless of diabetes status (67), probably linked to low levels of adiponectin as well as insulin resistance, both induced by adipose cell dysfunction and beta cell faillure (68,69) in consequence of inflamatory status.

Thus, stress hyperglycemia and uncontrolled DM in terms of glucotoxicity promote endothelial damage and oxidative stress cell injury, as well as mitochondrial impairment, all of them identified outside (70) or in the context of acute SARS-CoV-2 infection (68).

The overall results obtained indicate the association of neurocognitive disorders with death (OR=4.02), without the interaction with other factors associated with the severity of COVID-19 (heart disease and DM) being able to jointly predict a greater likelihood of death in patients already severely ill or even in older people. Then, it requires interpretation with caution, mainly because of the patientes clinical diversity and the small sample.

Common complications of COVID-19 include observed psychiatric changes (71,72), sensory alterations and memory impairment (73,74), as well as clinical worsening of neurodegenerative diseases (75) and stroke (76). Nevertheless, the studies observing the primary impact of these multiple conditions on the severity and mortality of infection are heterogeneous (77), as is the variability of the grouped pathologies, making it difficult to interpret the results even in well-conducted studies (78).

On the other hand, both younger and older patients with neurological disorders show greater vulnerability to SARS-CoV infection (77,79,80), and complications pos-COVID-19 are associated with worsening schizophrenia, Parkinson's disease, and Alzheimer's disease (75,77,81). This is likely due to their self-care fragility, indicating the necessity of prevention practices and targeted care for these disorders.

In summary, hypertension, heart disease, obesity, diabetes and neurocognitive disorders are pathophysiologically intertwined, driven by the inflammatory process, and contribute to the severity and worst outcomes of the disease initially caused by coronaviruses, but the infection may also become the missing trigger for the manifestation of functional diseases.

5.4.3 Chest Tomography (CT) Score

Typical findings on computed tomography (CT) scans included bilateral ground-glass pulmonary parenchyma and consolidative pulmonary opacities, occasionally exhibiting a rounded morphology and showing a lung distribution at the periphery (82). The pathophysiology of ARDS includes damage to the alveolar epithelium that causing hyaline membrane formation, interstitial edema, and alveolar edema (83).

The commonly held belief that there is a correlation between the severity of respiratory disease and the extent of radiological involvement (84) no longer holds true, given that almost all COVID-19 patients seeking specialized treatment present with chest radiological changes, and only a small subset of these require hospitalization (83). These findings are similar with our data. Except >75% changes (score5), there

is no observed proportionality between pulmonary involvement seen on CT scans and mortality rates in hospitalized patients (**Figure 5**).

The chest CT kinetics for patients without ARDS show that abnormalities are evident in the first few days of symptoms with the most variability appearing between days 5 to 8. The greatest scale impairment is observed between days 10 and 14 when recovery starts (85).

The mean time to imaging for the patients examined was 6 days, which could have influenced the variation in scores for individuals who presented severe clinical conditions. This may have also led to no apparent variation in the need for ventilatory support between the scores.

Changes in chest CT characteristics may provide valuable insights for early identification of severe cases, including the risk of mortality (86). Therefore, it is crucial to consider the timing of the analysis when interpreting the images. In any case, the most important discoveries are ground glass opacities corresponding to a score of 5, which can predict the need for invasive oxygen therapy, and death in conditions that are already clinically more severe (87).

5.4.4 Laboratory Analysis Parameters

Although neutrophilia can be a finding in patients with COVID-19, dysfunctional or apoptotic cells in the peripheral blood are observed in severe clinical conditions (88). Neutrophils play a crucial role, directly or indirectly, in the pathophysiology of tissue infiltration and vasculopathy during the hyperinflammatory state (89–93). On the opposite side, lymphopenia can be considered as an indicator of the severity and poor prognosis of COVID-19 (93–97).

Among the patients studied, it is difficult to observe the trophic effect on neutrophils ($p=0.389$) and the apoptosis of circulating monocyte cells (97) because neutrophils and lymphocytes ($p=0.301$) did not differ between the groups of severity.

The correlation between the absolute number of polymorphonuclear neutrophils and lymphomononuclear cells (NLR) in peripheral blood has been used as a surrogate marker to predict the severity of COVID-19 and hospital admission in

patients (98–103). Thus, it aids in interpreting the pathological effects on severity ($p< 0.001$) and death (OR=1.036 [1.015 – 1.058] p=0.001).

In the examined cases, a correlation between mortality and NLR, CRP, LDH, IL-6, and blood glucose biomarkers has been identified. Moreover, some of these biomarkers exhibit increased levels in other infectious diseases, including CRP (104–107), which is the most extensively studied acute phase protein biomarker in cases of sepsis (108). Although clinical manifestations of COVID-19 are less severe, they exhibit similarities to septic conditions based on serum levels of others inflammatory markers such as ferritin, IL-6, TNF-α, LDH, and certain liver enzymes such as AST and ALT (5,6,106,107,109,110).

In a pro-inflammatory context of COVID-19, IL-6 is a pleiotropic cytokine that is produced in numerous tissues and exerts a wide range of effects on both innate and adaptive immunity (111). And, as reported, high levels of IL-6 are observed in several viral infections, but the upregulation of these interleukin can worsen clinical manifestations and promote viral survival (112).

Thus, IL-6 levels appear to be the only cytokine among IL-1β, IL-6 and TNF-α that can safely and independently predict an increased risk of death based on disease severity, despite there being positive correlations between these cytokines (113). In COVID-19 patients, serum IL-6 levels can increase by 10 to 1000 times (IR:5pg/mL), which is dependent on the severity of tissue injury. In fact, mechanical ventilation and changes in lung imaging are proportionally associated with IL-6 (6,114), making this cytokine one of the best markers of poor prognosis for the disease, as well as for patients with sepsis (115).

In addition to immune response markers, the LDH enzyme is also considered an indicator of pyroptosis, the death of infected cells (116). This phenomenon likely results from apoptotic lymphopenia, as well as from the interplay between neutrophils, pulmonary infiltrate, and other cells during the infectious process (117). Thus, LDH may serve as a valuable and easily accessible biomarker to forecast the severity and mortality rates of COVID-19 and other pulmonary viral infections (118,119) and may also reflect hemolysis as a pathological process of SARS-CoV-2 (120).

Overall, the inflammatory response also impacts both glycolysis and mitochondrial dysfunction (121). Conversely, hyperglycemia exacerbates oxidative

stress, provokes cytokine release, boosts ACE2 expression in lungs, and causes microangiopathy. This generates a self-perpetuating cycle that can lead to adverse outcomes for COVID-19 patients (64).

Thus, multiple interaction of risk factors and inflamatory and metablolic paramethers are expected. Indeed, this study found positive correlations into cellular responses and other inflammatory markers. However, certain results exhibited small and difficult-to-explain positive or negative effects that require interpretation within a highly variable context. Despite these challenges, the interactions among parameters related to iron metabolism are noteworthy.

The iron not needed for its various organic functions is stored in cells bound to ferritin, particularly in hepatocytes and macrophages (122). When necessary, these reserves are mobilized via ferroportin under the modulating effect of hepcidin and are transported in association with transferrin. Excessive iron is extensively detrimental, hence, its intake provides important protection against oxidative damage to tissues (123).

Conversely, cellular iron deficiency disrupts proper bodily functions by impairing the activity of iron-dependent proteins. The manifestation of anemia in both laboratory and clinical settings is a clear indication of this phenomenon (124). Surprisingly, regardless of sex, only 18.14% of Psev cases exhibited anemia with hemoglobin (HB) levels below 12g/dL, while hypoferrinemia (<50µg/dL) occurred in 86.96% and 90.38% of Pmod and Psev cases, respectively. Additionally, there was no observed correlation between low iron levels and altered HB levels in the collected data.

Furthermore, iron serum levels decreased as COVID-19 severity increased (**Table 3** and **Figure 3)**, while ferriitin levels remained consistently high in both Pmod and Psev patients.

Restricting free iron is one of the first lines of defense against invading pathogens (125), including viruses (126) because lower levels of serum iron can produce free transferrin, which binds non-transferrin-bound iron released during tissue injury and hemolysis. This effective process could prevent the enhancement of pathogen proliferation (125). However, iron depletion may affect the activation and

proliferation of T and B lymphocytes, making it a crucial immunomodulator effects (127).

On the other hand, viruses could induce changes in iron transport and iron homeostasis, both of which are associated with altered COVID-19 disease states (126).

Iron-related parameters in hospitalized COVID-19 patients demonstrate significant hypoferremia and predict disease severity, and the change in iron dynamics is also manifested in the results obtained, including decreased transferrin levels (TIBC), and transferrin saturation as reported in other studies (123,124), mainly in Psev. In the same way, hypoferremia is also partly correlated with inflammatory markers, such as CRP and IL-6 (128). Thus, our data shows a negative correlation between Pmod iron levels and NLR and PCR. However, in Psev, this negative correlation exists only with isolated IL-6 levels.

During the inflammatory process, IL-6 has a positive effect on ferritin as well as hepatic hepicidin synthesis, a peptide involved in the regulation of iron available to cells by blockade of ferroportin (129,130). As a result, iron storage tends toward retention in macrophage cells rather than the more conventional ubiquitous storage tissues associated with ferritin (125). Thus, iron uptake in the reticuloendothelial system, inflammation, and anemia are linked through the action of hepcidin (129) and, indirectly, of IL-6 (130).

Of note, hepcidin negatively correlates with the PaO2/FiO2 ratio and predicts mortality in ICU patients independent of age, lung function, and other inflammatory biomarkers (131), suggesting that this molecule may be involved in unidentified mechanisms linking hypoferrinemia and the inflammatory process in COVID-19 (128,131).

Notably, evidence suggests that hepcidin may be required for chemokine (C-X-C motif) ligand 1 (CXCL1) production and neutrophil recruitment in another infection model (132), just as the correlation between lower lymphocyte proliferation and low iron levels (127) may explain the negative correlation between serum iron and NLR in Pmod patients.

We also looked for a possible correlation between O_2 saturation on admission and iron-related parameters in Psev, to observe whether oxygen requirement (indirect) would be influenced by iron deficiency (128). Spearman's correlation was null between serum iron and PO_2Sat (r=0.133), and linear regression failed to show an influence of the former on the latter parameter (p=0.1557).

Data collected did not show an association between ferritin levels and Psev outcome, but it was correlated with other acute phase indicator molecules such as CRP, LDH and AST in Pmod and Psev. While previous studies have indicated a link between ferritin levels and COVID-19 severity (104,105,133–135), we observed no significant differences in ferritin levels that would allow us to determine the effect of severity and outcome on this parameter, mainly due to the high and uniform ferritin levels in a majority of hospitalized patients.

Contrary to previous studies (133,134), hospitalized diabetic and non-diabetic patients have similar mean ferritin levels (1389.8ng/mL and 1391.0ng/mL), and there is no correlation between age and ferritinemia (p=0.314). Despite age being the primary risk factor for the severity of COVID-19, other studies have shown a positive correlation (133,135) which was not observed in this study.

Hyperferritinemia is present in various conditions that involve inflammatory processes and tissue damage, including autoimmune diseases, cancer, stroke, sepsis and other infections (136). This was also a common finding in almost all hospitalized patients in our study, who also had low serum iron levels and elevated other inflammatory parameters.

In these disorders, the ferritin molecule functions as a chelator due to its extensive iron-binding capacity. Additionally, ferritin is a regulator of the oxidative processes that determine iron-mediated cellular injury and apoptosis ferro (122,136), since the metal is turned into a cytotoxic agent (ferroptosis) as a result of its idutive release from H-, L- and mitochondrial ferritins (ferritinophagy) (137).

In addition to autophagic turnover, there is a consensus that liver cells and macrophages have the ability to secrete ferritin (138), which also contributes to the elevation of serum levels of this protein.

Therefore, iron metabolism is believed to play a crucial role in the pathogenesis of COVID-19, particularly by establishing a harmful cycle between hyperferritinemia and tissue damage caused by oxidative stress (120,122,137,139). From this perspective, ferritin would transition from a simple reflection of iron storage and an indicator of inflammation to a key modulator of the pathogenesis of the disease to be controlled.

One limitation of this study is its retrospective approach and the restricted sample size of Pmild and Pmod for laboratory measurements. Accordingly, in-depth studies are still necessary to make clearer the relationship between these biomarkers and COVID-19 pathogenesis, particularly its applicability to other periods of this respiratory virus infection after vaccination.

5. 5 CONCLUSION

The COVID-19 pandemic had a significant impact on many scientific fields and generated an unprecedented number of studies on the epidemiology and pathophysiology of a single infection in such a short period of time.

The findings from these studies, as well as those presented herein, indicate that age, gender, and coexisting medical conditions, such as obesity, diabetes and heart disease, significantly contribute to the deterioration of COVID-19 cases and are positively correlated with higher mortality rates among hospitalized patients.

During the COVID-19 pandemic, laboratory and imaging parameters proved to be valuable tools in predicting disease prognosis. This is particularly true in light of the existing knowledge base surrounding other infectious or inflammatory disorders, which is consistently supported by the literature.

Hence, we posit that the accumulation of COVID-19 data gathered through both prospective and retrospective means in epidemiological research, along with investigations into the pathophysiology of the disease, will consistently aid in developing uniform protocols for enhancing the diagnostic and therapeutic capabilities of coronavirus and other emerging viral infections that may affect susceptible populations.

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RISK AND PROTECTION FROM COVID-19 MORTALITY ASSOCIATED WITH MINOR ALLELES OF p2rx7 VARIANTS rs37511143 AND rs23933799. PELISSON, Marsileni; DANELI, Tiago; LOZOVOY, Marcell Alysson Batisti; CHIARAMONTE, Julia Pelisson; VESPERO, Eliana Carolina.

ABSTRACT

Background:

The recognized functional features of the P2X7 purinergic receptor are closely related to the pathogenesis and progression of COVID-19. Consequently, polymorphisms within the *p2rx7* gene, which alter the functionality of this receptor, have already been associated with less favorable responses to infections and may have significant implications for the disease course. This cross-sectional study aimed to investigate the potential correlation between single nucleotide polymorphisms (SNPs), specifically (rs3751143A>C and rs2393799C>T), and the severity of COVID-19, including the proportional mortality rate in cases classified as severe-critical.

Materials and Methods:

Clinical, epidemiological, and genetic aspects were analyzed in 601 adult patients diagnosed with COVID-19. Genotyping of *p2rx7* rs3751143A > C (*rsA*) and rs2393799C > T (*rsB*) was conducted using the qPCR Taqman® VIC™/FAN™ kit, while serum cytokine concentrations were quantified via chemiluminescence. SNPs were assessed for allelic, genotypic, and haplotypic frequencies – ^{Ht}I: ^{rsA}[AA] and ^{rsB}[CC]; II: ^{rsA} [AC;CC] and ^{rsB}[CC]; III: ^{rsA}[AA] and ^{rsB}[CT;TT]; IV: ^{rsA}[C;CC] and ^{rsB}[CT;TT] – in order to investigate their association with COVID-19 severity and mortality risk. Statistical analyses included the Chi-squared test for associations, one-way ANOVA (*F**) (bootstrap 1,000 times) for mean comparisons, and Cox regression for mortality risk estimations at 30 (D30), 60 (D60), and 180 days (D180).

Results:

The study determined no significant association between the analyzed polymorphisms and the exacerbation of COVID-19 cases. However, at day 180 (D180), a proportional increase in risk mortality was observed in the group with the ^{Ht}II haplotype compared to the other haplotypes – ^{Ht}II x ^{Ht}I (*P*<0.042, HR=1.586 [1.017 – 2.476]); ^{Ht}IIx ^{Ht}III (*P*<0.001, HR=2.869 [1.875 – 4.392]); ^{Ht}II x ^{Ht}IV (*P* 0.013, HR=1.976 [1.155 – 3.382], while the ^{rsB}[T] allele provided protection to patients with severe disease.

Conclusion:

In summary, the study concluded that the haplotype containing the allelic variant rs3751143 [C] within the gene *p2rx7* is associated with an increased risk of mortality when compared to individuals carrying the haplotype containing the [T] allele at rs2393799. This association indicates distinct risk and protective profiles associated with the respective SNPs concerning COVID-19 mortality.

Key-words : P2RX7; genetic polymorphism; COVID-19 severity; mortality.

6. 1 INTRODUCTION

The approximately 7 million COVID-19-related deaths (WHO 2023 data), along with subsequent post-infection complications, represent unparalleled legacies within the contemporary medical scenario, underscoring the notion that the pathogenesis and therapeutics of this disease continue to provide fertile ground for clinical and experimental research.

In this context, the pronounced clinical manifestations mediated by the cytokine release syndrome (1) are intricately linked to the heightened sensitivity of pulmonary macrophages to the maturation of IL-1β via the P2X7-inflammasome (2,3). Inflammatory processes exacerbate tissue damage, induce hypoxia, and culminate in a substantial escalation of extracellular adenosine triphosphate (eATP) concentrations within the lungs (4).

As a result, eATP serves as the danger signal detected by transmembrane sensors, such as P2X7 receptors on macrophages and T lymphocytes, translating into the activation of signaling pathways (5,6), These pathways lead to the release of inflammatory mediators (TNF-α, IL-6, IL-18, and IL-1β), which, in COVID-19 and other infections (7), induce cellular migration, apoptosis of infected cells (pyroptosis), and fibrosis (8).

The characteristic biphasic nature of COVID-19 (9), and the diversity of its complications (10) may reflect the variability in the response of the purinergic receptor P2X7 to various stimuli (11), whether tropic or related to cell death (12,13). Moreover, this receptor is also involved in limiting infection and permitting viral replication (14).

Furthermore, the *p2rx7 gene* is highly polymorphic, with *splicing* and SNPs contributing to this genetic diversity (12), and susceptibility to diseases linked to the immunological context of the receptor (15). Notably, the rs3751143 (1513A>C) (*rsA*), located within exon 13, serves as both a *p2rx7* transcriptional enhancer region and a binding site for transcriptional regulators (16). The resulting $Glu^{496}Ala$ alteration in the cytoplasmic portion of the protein leads to partial ^{rsA}[AC] or complete ^{rsA}[CC] loss of P2X7 receptor function (17). This loss can hinder the clearance intracellular pathogens by macrophages, as evidenced in tuberculosis (TB) (18) and toxoplasmosis (19).

Less extensively studied, the rs2393799 (*rsB*) is an intronic variant within the promoter region (-762C>T) whose effects on receptor expression and functionality

are not well-defined, nor is its association with diseases (20). Interestingly, the wild-type *rsB*[CC], rather than its mutated allele, has been associated with an increased risk of lymphatic TB in children (21) Conversely, *rsB*[TT] has been linked to a reduced likelihood of the eosinophilic phenotype in asthmatic children (22).

These known functions of the P2X7 receptor intersect with the inflammatory profile observed in COVID-19 (23–26). Moreover, elevated serum levels of P2X7 reflect the disease severity, indicating increased receptor expression or cellular shedding during the disease (27,28).

Given this complex interplay of factors and the lack of comprehensive studies on *p2rx7* polymorphisms in the context of COVID-19, our work aimed to investigate the association between rs3751143 (*rsA*) and rs2393799 (*rsB*) with comorbidities predicting disease severity, as well as the disease severity itself and the short- and long-term clinical outcomes of patients post-hospitalization. Thus, the primary goal of this research is to contribute to understanding the role of P2X7 in the pathogenesis of COVID-19.

6. 2 MATERIAL AND METHODS

6.2.1 Ethic

This research protocol was approved by the Institutional Research Ethics Committee of the State University of Londrina, Paraná, Brazil (CAAE:31656420.0.0000.5231) and all participants and their guardians were informed in detail about the research and signed the free and informed consent.

6.2.2 Study Design

To achieve this, we conducted a cross-sectional descriptive study involving 434 patients (≥18 years) admitted to a reference hospital with a diagnosis of SARS-CoV-2 infection. These patients were categorized as follows: 69 with moderate severity (P_{mod}), 365 with severe/critical disease (P_{sev}). And an additional 167 symptomatic adults with mild symptoms (P_{mid}) who had recovered from COVID-19 were also included in the study.

Cancer, chronic infections, autoimmune disorders, and the continuous use of

anti-inflammatory and immunomodulatory medications served as exclusion criteria. Patients were queried via a structured questionnaire, and their electronic medical records were evaluated. The laboratory researcher, both in wet and genetic labs, were blinded to the clinical assessments.

Blood samples were collected upon the hospital admission of Pmod and Psev and during the Pmild interview. Plasma and buffy coat were stored at a temperature of −80 °C until the analysis.

Patient deaths were monitored for a period of 30, 60, and up to 180 days after experiencing symptoms of COVID-19.

6.2.3 PCR- Genotyping and Single Nucleotide Polymorphisms (SNP)

Genomic DNA was extracted and purified from the buffy coat by column resin, following the manufacturer's protocol (Biopur, Mobius Life Science - Pinhais, Paraná, Brazil) and quantified in Nanodrop 2000c™ Spectrophotometer (ThermoFisher Scientific - Watman, Massachusetts, USA) at 260nm. Real-time polymerase chain reaction (qPCR) with TaqMan® VIC™/FAN™ (ThermoFisher Scientific - Waltham, Massachusetts, USA) probes at final concentration of 200mM was applied to identify *p2rx7* rs3751143A > C and rs2393799C > T genotypes, [AA, CC, AC] and [CC, TT, CT], respectively. QuantStudio™ 6 Pro Real-Time PCR System and Analysis Software version 1.6 (ThermoFisher Scientific - Waltham, Massachusetts, USA) were used for the reaction and genotypes discrimination.

SNPs were studied in terms of allele (*Al*) frequencies (wild type vs. mutant); genotype (*Gt*) frequencies for ^{rsA}[AA;AC;CC] and ^{rsB}[CC;CT;TT], and the grouping (*Gr*) of mutant alleles: ^{rsA}[AA], ^{rsA}[AC;CC], ^{rsB}[CC], ^{rsB}[CT;TT], as well as haplotype (*Ht*) frequencies – I: ^{rsA}[AA] and ^{rsB}[CC]; II: ^{rsA}[AC;CC] and ^{rsB}[CC]; III: ^{rsA}[AA] and ^{rsB}[CT;TT]; IV: ^{rsA}[C;CC] and ^{rsB}[CT;TT].

6.2.4 Interleukins Dosage

After that, portion of plasma and serum, preserved at a temperature of -80°C, were analyzed in a panel of 134 sequentially-sampled Psev patients for the serum levels of interleukins (IL) such as IL-1β, IL-6, IL-17, and Tumor Necrosis Factor (TNF-α). Each haplotype's sample (N) was proportionally distributed and evaluated. The

analysis was conducted using the Human ProcataPlexTM Multiplex Immunoassay manufactured by ThermoFisher Scientific (Waltham, Massachusetts, USA).

6.2.5 Statistics

Our analyses aimed to investigate the association of genetic variants with demographic data, comorbidities, disease severity, and the mortality outcomes at 30 (D30), 60 (D60), or 180 (D180) days following the onset of COVID-19 symptoms. Non-probabilistic sampling was used, and data collection occurred between March and September 2020. Normality and variance homogeneity were assessed using the Komogorov-Smirnov or Shapiro-Wilk tests and Levene's test, respectively.

Categorical data were expressed as simple (n) and relative frequency (%). Chi-squared (X^2) or Fisher's exact (EF) tests were applied for bivariate analysis of genotype and haplotype associations with predictor variables. Bonferroni correction and *post-hoc* pairwise comparisons using adjusted residuals (z-test) were employed when appropriate. The estimated risk for association was expressed as Odds Ratio (OR) with a 95% confidence interval [95CI].

For continuous variables, median and interquartile ranges (IQ25 - IQ75) as well as mean and standard deviation (±SD) were reported for data with and without normal distribution (Komogorov-Smirnov or Shapiro-Wilk tests). One-way ANOVA with bootstrap analysis (1,000 replications) was used for non-normally distributed data, Brown-Forsythe ANOVA (F*) for data without homoscedascity (Levene; $P<0.05$), and Games-Howell *post-hoc* analysis for ANOVA with $P<0.05$. Mean difference (ΔM) and a bias-corrected and accelerated 95% confidence interval (Bca95CI) were used for pairwise comparisons. The proportion of variance attributed to independent variables was reported as *η2.* Moreover, the Kruskal-Wallis (H) test was applied to non-parametric data when appropriate.

The coefficient of variation (CV%) was used to assess the homogeneity of continuous variables concerning intragroup means.

Survival curves were constructed using Kaplan-Meier analysis with Log-Rank test for simple bivariate analysis, and Cox regression models were used for multivariate analysis to obtain Hazard Ratios (HR) estimating the proportional risk of death overtime associated with covariables.

IBM SPSS Statistics *version 20.0* (IBM Corporation, Armonk, NY, USA) and

GraphPad Prism *version 8.0.0* (GraphPad Software, San Diego, California, USA), both for *Windows*, were used for statistical analyses and graph generation, respectively. All analyses were conducted at an initial significance level of $P<0.05$.

6.3 RESULTS

The allelic frequencies for the SNPs were determined as follows: *rsA* [A=0.8278; C=0.1722] and *rsB*[C=0.6722; T=0.3288] among the 601 patients. Within the 156 P_{sev}-$D_{180,}$ we observed *rsA*[A=0.8213; C=0.1987] and *rsB*[C=0.6923; T=0.3077]. The allelic frequencies were consistent with Hardy-Weinberg equilibrium in the studied population compared to the expected frequencies, i.e., *rsA* χ^2=0.0499/χ^2c=3.841(1) (α<0.05) and *rsB*[χ^2 =0.2277/χ^2c=3.841(1) (α<0.05).

Non-Caucasian patients exhibited a higher prevalence of ^{Ht}III e ^{Ht}IV, which contained the minor allelic variants of *rsB* (**Table 1**). Thus, the analysis comparing *rsB*[CC] to *rsB*[CT;TT] determined a significant association of genotypes containing [T] with non-Caucasian ethnicity in COVID-19 (χ^2=15.66 (1); *P*<0.001; OR= 2.36 [95CI:1.53- 2.40]. However, no allelic differences were observed between surviving and non-surviving patients when grouped by ethnicity, nor in survival between Caucasians –130/295 (44.1%) – and non-Caucasians – 26/70 (37.1%); (Log Rank χ2=0.652 (1) *P* 0.419).

Similarly, the results did not reveal differences in *AI*, *Gt,* or *Gr* within the SNPs or the *Ht* for almost all pre-existing clinical conditions. Nonetheless, 21 individuals (11.1%) with *rsA*[AC;CC] were diagnosed with hypothyroidism (HT), whereas only 5.6% of those with *rsA*[AA] (χ2=5.837 (1) *P* 0.016), (α 0.0125) had this condition. Conversely, in the case of *rsB,* the highest frequency was observed in individuals without the mutant allele [T]: 26 (9.5%) *rsB*[CC] with HT compared to 18 (5.5%) with *rsB*[CT;TT] (*P* 0.062).

AI, *Gt*, *Gr*, and *Ht* were compared in terms of their association with the severity of COVID-19 and mortality in Psev at D30, D60, and D180. Since the results did not differ significantly, we reported the effects observed at D180 (**Table 2**).

The results indicated that genotypic variables are not correlated with disease severity. In regression models incorporating demographic variables and comorbidities, searching for a significant proportional risk of death was not productive. However, there are indications of a higher proportional risk of mortality at D180 in

Psev carrying the mutated allele *rsA*[C] as compared to *rsA*[A], *Al C-A*OR=1.61 [1.06 – 2.42]. Additionally, a comparative analysis revealed that *Ht*II carries a higher mortality risk at D180, with an OR of 1.82 [1.02 – 3.28] (**Table 2**).

The following results were observed for survival curves at D180 for Psev grouped according to the combinations *rsA*[AA] x *rsA*[AC;CC] (Log Rank χ2=2.918 (1) *P* =0.183); *rsA*[AA] x [AC] x [CC] (Log Rank χ2=3.146 (2); *P* =0.207) and *rsB*[CC] x *rsB*[CT;TT] (Log Rank χ2 =1.188 (1) *P*=0.170); *rsB*[CC] x [CT] x [TT] (Log Rank χ2=2.255 (2) *P* 0.196). Regarding the genotypes (*Gt)*, there was no difference between mortality and survival at D180 within the haplotypes (*Ht)* (Log Rank χ2=5.122 (3); *P* 0.163).

On the other hand, through Cox regression analysis, models were examined to discriminate estimates of proportional risk (HR, 95CI) for *Gt* and *Ht* grouped pairwise over the time intervals D30, D60, and D180 (**Figure 1).** In the analysis, *Ht*II displayed approximately threefold higher likelihood of death than *Ht*III. Furthermore, a protective association of *Ht*III over patients carrying *Ht*I was observed, along with a less significant risk at D180 between *Ht*II and *Ht*I (HR=1.611 [1.076 - 2.412] *P* 0.021).

Figure 2 displays the survival functions of P_{sev} at D180 for genotypes and haplotypes. The regression model for *rsA*[AC] x *rsA*[AA] proved suitable for estimating the risk among *Gt* within the 66 patients *Ht*II (HR=1.611 [1.076 – 2.412] *P*=0.021; χ2=5.254 (1) *P*=0.022); however, owing to the sample limitation, it was not possible to estimate the difference between *rsA*[CC] x *rsA*[AA] in the respective model (*P* 0.461). Similarly, adequacy was not obtained for *rsB*[CC] x *rsB* [TT] (HR=1.678 [0.980 – 2.876], *P* 0.059; χ2=3.770, *P*=0.052). Nevertheless, it was possible to determine that the *rsB*[CC] genotype conferred a risk of death approximately two times greater than *rsB*[CT]: HR=2.228 [1.510 - 3.289] *P* <0.001; χ2=15.251 (1) *P* <0.001, among the 152 *Ht*III patients.

The CV(%) values demonstrated a tendency for more significant variability in serum interleukin levels among P_{sev} individuals compared to the means for *Ht*I and *Ht*IV. On average, IL-17A levels were lower in *Ht*III than *Ht*I, or in mutated allele carriers *rsB*[CT;TT] compared to non-mutated *rsB*[CC], although the mutation had a small effect on the measurements (η^2=0,05). Similarly, for TNFα, there was a significant difference in the distribution levels among haplotypes (χ2= 12.43 (176, 3) *P*=0.006). The means of *Ht*I and *Ht*III differed significantly, with a moderate effect of *rsB*[CT;TT] on

the level of TNF-α (η^2=0.06) in P_{sev} **(Table 3).** Due to limitations in the sample, the multivariate analysis to determine the impact of *Ht* on the cytokine set measured was unable to performed.

TABLE 1 - Demographic Characteristics and Comorbidities of Patients with Mild, Moderate, and Severe COVID-19 and Time Elapsed from Symptoms to Hospitalization of Patients with Moderate or Severe Disease.

	p2rx7 SNP Haplotypes (^{Ht}) rs3751143 (^{rsA}) and rs2393799 (^{rsB})				
	I	**II**	**III**	**IV**	**Stathistics**
	[AA] [CC] N=158 (26.3%)	**[AC;CC] [CC] N=116 (19.3%)**	**[AA] [CT;TT] N=254 (42.3%)**	**[AC;CC] [CT;TT] N=73 (12.1%)**	
Age Median (IQ25;IQ75)	61 (46;73)	59 (45;68)	59 (43;74)	63 (47;76)	χ2=2.25 *P*=0,781
Sex n (%)					χ2=2.25 *P*=0,506
Female	77 (48.7)	54 (46.6)	128 (50.4)	31 (42.5)	
Male	81 (51.3)	62 (53.4)	126 (49.6)	42 (57.5)	
Ethnicity n (%)					χ2=18.86 *P*<0.001
Caucasian [a]	142 (89.9)	97 (83.6)	185 (72.8)	58 (79.5)	
Non Caucasian [b]	16 (10.1)	19 (16.4)	69 (27.2)	15 (20.5)	
Conditions n (%)					
Neurocognitive Disorder[1]	19 (12.03)	11 (9.48)	28 (11.02)	7 (9.59)	χ2=0.57 *P*=0.901
Pulmonary Disease [3]	7 (4.4)	6 (5.2)	21 (8.3)	8 (11.0)	χ2=4.58 *P*=0.204
Kidney Disease [2]	14 (8.9)	10 (8.6)	26 (10.2)	7 (9.6)	χ2=0.34 *P*=0.952
Diabetes (1 and 2)[3]	42 (26.6)	39 (33.6)	81 (31.9)	22 (30.1)	χ2=1.90 *P*=0.592
Hypothyroidism[3]	13 (8.2)	13 (11.2)	1 (3.9)	8 (11.0)	χ2=8.48 *P*=0.037
Heart Disease [3]	19 (12.0)	17 (14.7)	31 (12.2)	6 (8.2)	χ2=1.74 *P*=0.628
Arterial Hypertension[3]	72 (45.6)	59 (50.9)	119 (46.9)	35 (47.9)	χ2=0.80 *P*=0.847
Dyslipidaemia[3]	17 (10.8)	10 (8.6)	19 (7.5)	4 (5.5)	χ2=2.25 *P*=0.521
Obesity (BMI>30Kg/m^2)	42 (26.6)	30 (25.9)	54 (21.3)	18 (24.7)	χ2=1.86 *P*=0.601
Mean days of symptoms	6.8 (±3.9)	6.1(±4.0)	6.7 (±3.8)	6.7 (±4.4)	*F*=0.68 *P*=0.563

Fisher exact test or Pearson chi-squared test (χ2) with standardized adjusted residuals (z-test) were applied to categorical variables. Kruskal-Wallis test or one-way ANOVA (F) were applied to age and time to hospitalization, respectively. All tests were performed with a significance of P<0.05. Kruskal-Wallis test or one way ANOVA (F) were applied age and time elapsed until hospitalization. respectively. All tests performed with significance of *P*<0.05. [1] Neurological disease under treatment, dementia, or due to cerebrovascular accident; [2] Dialysis or not; [3] Conditions with medication treatment or not. BMI: Body mass index.

TABLE 2 - Alleles (Al), Genotypes (Gt), Groups (Gr) and Haplotypes (Ht) Analysis of *p2rx7* rs3751143 (rsA) and rs2393799 (rsB) According to COVID-19 Severity and Death in 180 Days After Manifestation of Symptoms (Death180).

	Genotypes					
	rs3751143A>C			**rs2393799C>T**		
N (%)	**AA**	**AC**	**CC**	**CC**	**CT**	**TT**
Total	412 (68.6)	171 (28.4)	18 (3.0)	274 (45.6)	260 (43.3)	67 (11.1)
Covid Severity						
Mild (P_{mild})	115 (27.9)	47 (27.5)	5 (27.8)	76 (27.7)	72 (27.7)	19 (28.3)
Moderate (P_{mod})	47 (11.4)	20 (11.7)	2 (11.1)	34 (12.4)	30 (11.5)	5 (7.5)
Severe/Critical (P_{sev})	250 (60.7)	104 (60.8)	11 (61.1)	164 (59.9)	158 (60.8)	43 (64.2)
Stathistics	$^{Gt}\chi 2$=0.01 (2) *P* 0.993			$^{Gt}\chi 2$=0.44 (2) *P* 0.802		
Psev Death$_{180}$	100 (40.0)	50 (48.1)	6 (54.5)	78 (47.6)	60 (38.0)	18 (41.9)
Stathistics	$^{Al\ C\text{-}A}\chi 2$=5.0 (1) *P* 0.025; OR=1.61 [1.06 - 2.42]			$^{Al\ T\text{-}\ C}\chi 2$=0.04 (1) *P* 0.829		
	$^{Gt}\chi 2$=1.26 (2) *P* 0.532			$^{Gt}\chi 2$=3.03 (2) *P* 0.219		
	$^{Gr}\chi 2$=2.43 (1) *P* 0.119			$^{Gr}\chi 2$=2.83 (1) *P* 0.093		
	Haplotypes					
	I	**II**		**III**		**IV**
Total	158 (26.3)	116 (19.3)		254 (42.3)		73 (12.1)
Covid Severity						
Mild (P_{mild})	41 (25.94)	35 (30.17)		74 (29.13)		17 (23.29)
Moderate (P_{mod})	19 (12.02)	15 (12.93)		28 (11.02)		7 (9.59)
Severe/Critical (P_{sev})	98 (62.04)	66 (56.90)		152 (59.85)		49 (67.12)
Stathistics	$\chi 2$=2.50 (3) *P* 0.868					
Psev Death$_{180}$	44 (44.9)	34 (51.5)		56 (36.8)		22 (44.9)
Stathistics	$^{Ht}\chi 2$=4.52 (3) *P* 0.211 $^{HtII\text{-}\ III}\chi 2$=4.09 (1) *P* 0.043; OR=1.82 [1.02 – 3.28]					

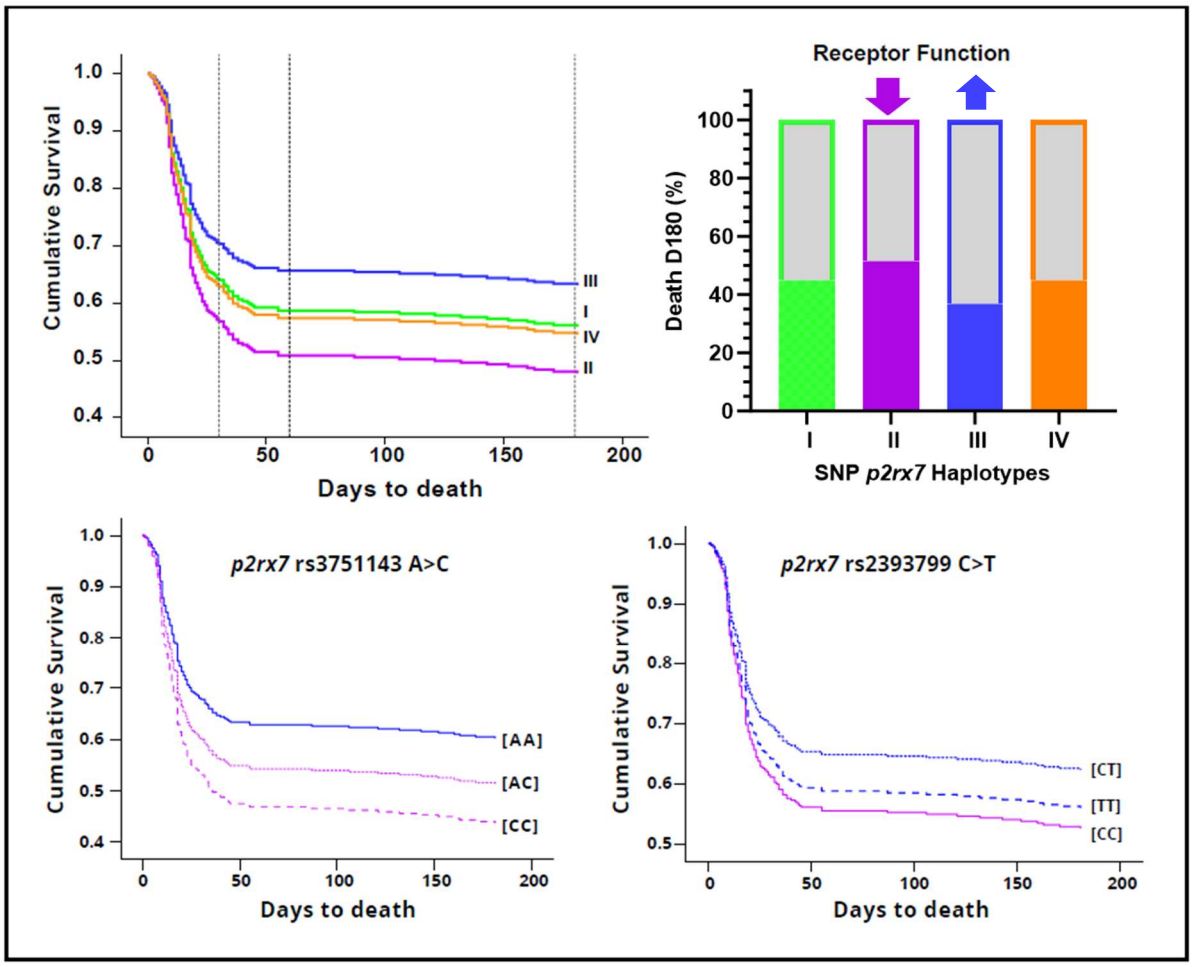
Results for categorical variables were presented as simple frequencies and percentages. Fisher exact test or chi-squared test ($\chi 2$) with standardized adjusted residues (z-test) were applied when appropriate. OR: odds ratio and [95CI]. *p2rx7* ^{rsA} and ^{rsB} haplotypes (^{Ht}), respectively: I - [AA] and [CC]; II – [AC;CC] and [CC]; III – [AA] and [CT;TT]; IV- [AC;CC] and [TC;TT]. p2rx7 ^{rsA} and ^{rsB} group (^{Gr}), respectively: [AA], [AC;CC] and [CC], [CT;TT].

FIGURE 1 – *p2rx7* SNP Haplotypes Pairwise and Proportional Risks of Death at 30 Days (D30), 60 Days (D60), and 180 Days (D180) From the Onset of COVID-19 Symptoms in Patients with Severe or Critical Disease.

HAPLOTYPE → PAIRWISE		RISK OF DEATH AFTER COVID-19 SYMPTOMS ONSET		
		D30	D60	D180
III	I	*P* 0.003 **HR=0.511** [0.330 - 0.789]	*P* <0.001 **HR=0.435** [0.289 - 0.655]	*P* 0.003 **HR=0.437** [0.295 - 0.648]
I	III	*P* 0.003 **HR=1.959** [1.267 - 3.029]	*P* <0.001 **HR=2.300** [1.527 - 3.464]	*P* 0.003 **HR=2.287** [1.542 – 3.390]
III	II	*P*<0.001 **HR=0.378** [0.234 - 0.585]	*P* <0.001 **HR=0.333** [0.215 - 0.316]	*P* <0.001 **HR=0.348** [0.228 - 0.533]
II	III	*P*<0.001. **HR=2.705** [1.710 - 4.277]	*P* <0.001. **HR=3.002** [1.939 - 4.647]	*P* <0.001. **HR=2.869** [1.875 - 4.392]
II	IV	*P* 0.015. **HR=2.098** [1.152 - 3.820]	*P* 0.014. **HR=1.992** [1.152 - 3.446]	*P* 0.013. **HR=1.976** [1.155 - 3.382]

Analyses were performed by Cox regression to obtain the hazard ratio (HR) and its 95% confidence interval [95CI]; HR>1 indicates that the first haplotype has a higher instantaneous risk of death than the second, and HR<1 indicates relative protection to death from the first to the second haplotype. *p2rx7* rs3751143 and rs2393799 haplotypes, respectively: I - [AA] and [CC]; II - [AC;CC] and [CC]; III - [AA] and [CT;TT]; IV - [AC;CC] and [TC;TT]. Arrow: in pairs from left to right.

FIGURE 2 – Frequency of Death within 180 Days and Cox Survival Function for Patients with Severe COVID-19 According to *p2rx7* SNP rs3751143A>C and rs2393799C>T Grouped into Haplotypes I, II, II and IV and their Genotype Variants.



p2rx7 rs3751143 and rs2393799 haplotypes respectively: I - [AA] and [CC]; II - [AC;CC] and [CC]; III - [AA] and [CT;TT]; IV - [AC;CC] and [TC;TT]. Cumulative survival from Cox regression with outcome death at D30. D60 and D180 after COVID-19 first symptoms. Arrows at the top right indicate the likely effect of mutations on the p2rx7 gene receptor (down: possible loss; up: possible gain of function).

TABLE 3. **Interleukin Levels in Hospitalized Patients with Severe or Critical COVID-19, Grouped According to the Haplotypes of the *p2rx7* SNP (Ht).**

Interleukin	SNP *p2rx7* Haplotype				Stathistic
	I	**II**	**III**	**IV**	
	N=34	N=21	N=51	N=28	
IL-6 (pg/mL)	234.8 (±356.9)	168.1 (±201.1)	215.7 (±281.7)	497.7 (±894.3)	*F**=2.295 (3) *P* 0.091
CV (%)	152.0	119.6	130.6	179.7	
IL-17A (pg/mL)	22.3[a] (±39.74)	9.1 (±31.04)	8.8[b] (±9.59)	7.6 (±13.91)	*F**=3.232 (3) ***P=0.029** [ab]ΔM=7.35 [1.56 – 13.52] η^2=0.05
CV (%)	174.5	139.9	109.4	192.9	
TNF-α (pg/mL)	20.6[a] (±18.6)	15.2 (±6.5)	12.7[b] (±5.94)	18.9 (±15.4)	F*=2.999 (3) ***P=0.036** [ab]ΔM=6.72 [1.88-13.03] η^2=0.06
CV (%)	90.2	42.8	46.9	81.6	
IL-1β (pg/mL)	8.8 (±10.79)	7.5 (±3.89)	7.0 (±3.04)	6.5 (±3.63)	*F**=0.804 (3) *P*=0.4973
CV (%)	123.2	51.9	43.6	55.4	

p2rx7 rs3751143 and rs2393799 haplotypes, respectively: I - [AA] and [CC]; II – [AC;CC] and [CC]; III – [AA] and [CT;TT]; IV - [AC;CC] and [TC;TT].The results are expressed by mean and standard deviation (±SD). Statistics obtained by one-way ANOVA of Brown-Forsythe (F*) after 1000-fold bootstrap, applied to data without normality distribution; CV: Coefficient of variation (%); ΔM: mean difference; [ab]: significant mean difference between groups; *η2*: eta squared ordinal represents the effect size of the predictor variable. All tests are performed with a significance of *P*<0.05. Bold indicates significant data.

6.4 DISCUSSION

The gene encoding the P2X7 receptor harbors several polymorphisms (12,15), which may result in unpredictable functional consequences or modulate the course of the infection-related inflammatory processes. In this regard, genetic association studies can provide valuable information about gene sequences that might help elucidate uncertainties concerning the interplay between the disease and this receptor.

In this context, the study did not succeed in investigating the association of *rsA* and *rsB* with the severity of SARS-CoV-2-induced disease. Still, it revealed the relative contribution of these SNPs to mortality, possibly because the receptor is decisive for the syndromic response to virus-induced pulmonary changes characterized by high eATP (7,29), as well as its significant involvement in subsequent pulmonary fibrosis (8). Conversely, the consistency in the results of the predictor variables across different *Ht* groups facilitated the interpretation of specific findings of this study.

The allelic frequencies of *rsA* are in concordance with global studies, in which *rsA*[C] frequencies range from 0.1596 to 0.1906, as reported by the NCBI Allele Frequency Aggregator (ALFA) dataset (30). In contrast, the frequency of the *rsB*[T] is divergent, particularly between global (0.3856) and African (0.6354) studies. Hence, we suggest that the diverse range of Brazilian ethnic backgrounds provides a foundation for associating *rsB*[T] with non-Caucasian ethnicity in COVID-19 (**Table 1**).

Although we acknowledge the limitations of the sample size and the self-reported nature of this study, we propose that the distribution of *rsB*[T] and the protective effects of *Ht*III may provide a foundation for removing the genetic basis for the greater lethality of COVID-19 in people of African descent than in Caucasians in Brazil (31). Of note, much has been studied about the low incidence and morbidity of COVID-19 in Africa, ranging from its association with co-infections such as malária (32), to demographic, social, immunologic, and genetic parameters (33). The study's findings are aligned with these investigations and suggest a comprehensive evaluation of P2X7 *rsB*[T]'s protective capacity against COVID-19 in African descendants, while considering the multifaceted aspects of ethnicity's impact.

Notably, haplotype *Ht*III exhibits a lower frequency of HT (3.9%) and confers a protective effect at D180 **(Figure 1).** Although there is no direct evidence linking this condition to mortality, it has been established that HT influences platelet aggregation (34), an aspect that should be considered along with outer pro-thrombotic determinants in COVID-19 (35). Moreover, the additional stimulation of ligand-gated purinergic receptors in HT like P2X7 may exert exceptional effects on tissues susceptible to COVID-19 complications (36). Consequently, more comprehensive data, including subclinical HT and inadequate therapeutic interventions, may help elucidate the relationship of P2X7 – HT – COVID-19.

IL-6 is a prognostic biomarker for COVID-19 severity and mortality (37). Accordingly, the expectation of elevated IL-6 levels (>35pg/mL) in haplotypes was confirmed, along with a decrease in IL-1β and IL-6 levels attributed to the hypofunctional state of P2X7 (38) em *Ht*II. Conversely, increased cytokine levels in *Ht*I, except for IL-6, signify expressed and functional P2X7, capable of directing the immune response through the release of inflammatory mediators, primarily driven by IL-1β.

Simultaneously, in *Ht*III, the lowest cytokine levels and their CV, which differs from the polymorphic haplotype (*Ht*I) in terms of TNF-α and IL-17A, indicate that the

pro-inflammatory profile of P2X7 may be attenuated in the presence of [T] in the intronic SNP. These findings are substantiated by the protective effect of ^{Ht}III compared to other haplotypes regarding linear D180 mortality.

Conversely, the small difference between *Ht* groups in IL-1β concentrations, as well as the challenges in interpreting TNF-α levels, may originate from the source of the stimulus, namely, the pulmonary microenvironment, without a proportional reflection in plasma during acute SARS-CoV-2 infection or this prompt clearence (39).

Similar to cytokines, the effects of the ^{rsA}[C] allele among genotypes and haplotypes (^{Ht}II x ^{Ht}I) exhibit a proportionally detrimental effect at D180. This effect resembles what is observed in susceptibility to tuberculosis and the survival of its etiological agent in infected macrophages (40). Thus, it is plausible that the receptor, influenced by ^{rsA}[C], reduces apoptosis and the ability to detect and modulate the virus via the cytoplasmic sensor (14) despite attenuating the pro-inflammatory cytokine-mediated profile (41). The increased risk of death attributed to ^{Ht}II compared to other haplotypes highlights apoptosis as a critical component of the immune response against viral infections (42) in COVID-19.

Notably, the survival of ^{IV}Ht seems to characterize a compensatory mechanism among the studied SNPs, prompting consideration of an enhanced antiviral response as a potential alteration in P2X7 functionality due to rs2393799.

6.5 Conclusion

Our findings show no discernible association between the allelic variants *p2rx7* rs3751143 and rs2393799 with COVID-19 severity. Nevertheless, this study underscores that in cases of severe disease, individuals carrying the [C] alleles in both SNPs face an increased mortality risk due to severe illness. Furthermore, haplotypes harboring the allelic variant rs3751143 [C] within the *p2rx7* gene exhibited a mortality risk at least 2.7 times higher than those carrying haplotypes containing the allele [T] at rs2393799. These results suggest distinct risk and protection profiles associated with these SNPs concerning COVID-19 mortality. Therefore, we recommend that studies correlating purinergic signaling and therapeutic approaches for SARS-CoV-2 infections incorporate the *p2rx7* allelic profile of the target population as a relevant variable for meticulous consideration.

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7 CONCLUSÃO

Os resultados deste estudo demonstram que a idade maior que 60 anos, o sexo masculino e as comorbidades como o diabetes, as doenças cardíacas e desordens neurocognitivas contribuíram significativamente para a deterioração dos casos de COVID-19 e foram positivamente associados ao aumento das taxas de mortalidade entre pacientes hospitalizados. Além disso, os parâmetros laboratoriais e de imagem utilizados para prognóstico da COVID-19 foram úteis em conjunto para a associação com a severidade e o risco de óbito na doença moderada e severa-crítica. IL-6, PCR e a relação Neutrófilo/Linfócito estão associadas à maior gravidade dos casos de COVID-19. Além disso, os resultados das associações dos parâmetros demográficos, clínicos e laboratoriais observados com a severidade e o óbito na COVID-19 são objetivamente apoiados por uma ampla e significativa literatura médica.

Por outro lado, os resultados experimentais não demonstraram associação discernível entre as variantes alélicas *p2rx7* rs3751143 e rs2393799 e a severidade da COVID-19. No entanto, este estudo ressalta que, em casos de doença grave, os indivíduos portadores dos alelos [C] em ambos os SNPs enfrentam um risco aumentado de mortalidade devido a doença grave. Além disso, os haplótipos que abrigam a variante alélica rs3751143 [C] dentro do gene *p2rx7* exibiram um risco de mortalidade pelo menos 2,7 vezes maior do que aqueles que carregam os haplótipos contendo o alelo [T] em rs2393799. Estes resultados sugerem perfis distintos de risco e proteção associados a estes SNPs em relação à mortalidade por COVID-19.

Assim, recomendamos que estudos que correlacionem sinalização purinérgica e abordagens terapêuticas para infecções por SARS-CoV-2 incorporem o perfil alélico *p2rx7* da população-alvo como uma variável relevante a ser considerada.

8 CONSIDERAÇÕES FINAIS

Embora incontáveis infecções assintomáticas e casos leves compunham o maior conjunto na pandemia da COVID-19, os quase 7 milhões de óbitos notificados e a condição pós-COVID-19 são os legados respectivos de fracasso e, por outro lado, consequências a longo prazo da pandemia para a assistência à saúde.

Esperamos que os resultados e conclusões deste estudo venham a contabilizar informações epidemiológicas pertinentes sobre a pandemia no contexto regionalizado de assistência terciária à COVID-19.

Ademais, e de forma profícua, acreditamos que este trabalho cria a perspectiva de novos estudos que respondam “como?”, “quando?”, e “em quais indivíduos?” o receptor purinérgico P2X7 se torna “anjo ou demônio” em doenças infecciosas, de maneira a impulsionar a busca por terapêuticas que atuem como ferramentas efetivas para minimizar o impacto de doenças como a COVID-19 na saúde das populações.

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ANEXO A

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Investigação de fatores genéticos e imunológicos na infecção por SARS-CoV-2: associação com o prognóstico, morbidade e mortalidade

Pesquisador: Andréa Name Colado Simão

Área Temática:

Versão: 2

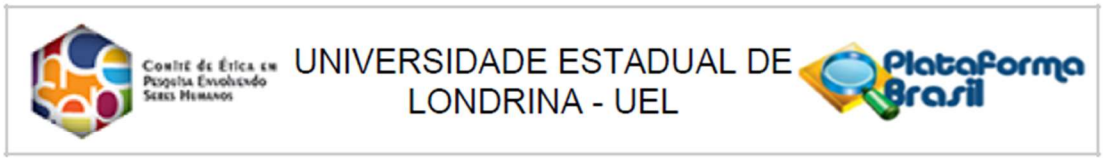
CAAE: 31656420.0.0000.5231

Instituição Proponente: CCS - Departamento de Patologia, Análises Clínicas e Toxicologias

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 4.053.033



Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

LONDRINA, 27 de Maio de 2020

Assinado por:
Adriana Lourenço Soares Russo
(Coordenador(a))

ANEXO B

Termo de Consentimento Livre e Esclarecido (TCLE)

"INVESTIGAÇÃO DE FATORES GENÉTICOS E IMUNOLÓGICOS NA INFECÇÃO POR SARS-CoV-2: ASSOCIAÇÃO COM O PROGNÓSTICO, MORBIDADE E MORTALIDADE"

Prezado(a) Senhor(a):

Gostaríamos de convidá-lo (a) para participar da pesquisa **"INVESTIGAÇÃO DE FATORES GENÉTICOS E IMUNOLÓGICOS NA INFECÇÃO POR SARS-CoV-2: ASSOCIAÇÃO COM O PROGNÓSTICO, MORBIDADE E MORTALIDADE"**, a ser realizada no Hospital Universitário de Londrina (HU). O objetivo deste estudo é avaliar biomarcadores genéticos e imunológicos associados ao prognóstico, morbidade e mortalidade da doença causada por SARS-CoV-2.

Este estudo está selecionando pacientes atendidos no HU com diagnóstico de Síndrome Gripal, que pode ter como causa o SARS-CoV2 ou outros vírus respiratórios. Ao aceitar participar, após o diagnóstico laboratorial, você poderá ser alocado em um dos dois grupos a seguir: Grupo Outros Vírus Respiratórios (OVR) e Grupo COVID-19. Os pacientes alocados em qualquer dos dois grupos necessitarão fornecer informações clínicas e realizar uma coleta de sangue, no momento da internação (tempo zero do estudo). Serão realizados exames laboratoriais que para determinar quantitativamente as subpopulações de linfócitos, os níveis circulantes de citocinas e os genes relacionados [*IFNG* (rs2069718, rs1861493, rs1861494), *TGFBR2* (rs308465, rs9831477, rs6790424), *TGFBRI* (rs1800468, rs1800469, rs1800470, rs1800471, rs1800472), *IL10* (rs1800896, rs1800871, rs1800872), *FOXP3* (rs2232365, rs3761548), *APOBEC* (APOBEC3A/B deletion), *TNFA, IL6* (rs1800795, rs1800796), *IL17R* (rs2241043, rs2241049 e rs6518661), *IL17* (rs3819024, rs2275913 e rs3819025), *IL22R* (rs3795300, rs4233051 e rs4292900), *IL22* (rs2227484, rs2227485 e rs2227513), *IL23R* (rs12401432 e rs6656929), *IL23* (rs2066808 e rs2371494)]. No entanto, caso você seja diagnosticado com COVID-19 você será acompanhado pela equipe de pesquisadores por 28 dias, e os dados laboratoriais e clínicos associados a morbidade e mortalidade da doença serão obtidos de seu prontuário no 7°, 14° e 28° dia após o início do estudo.

Esclarecemos, que suas informações serão utilizadas somente para os fins desta pesquisa e serão tratadas com o mais absoluto sigilo e confidencialidade, de modo a preservar a sua identidade. Todos os dados coletados, clínicos e laboratoriais, serão descartados após a publicação do estudo. Esclarecemos que sua participação é totalmente voluntária, podendo você: recusar-se a participar, ou mesmo desistir a qualquer momento, sem que isto acarrete qualquer ônus ou prejuízo à sua pessoa. Esclarecemos que você não pagará e nem será remunerado(a) por sua participação. Garantimos, porém, que todas as despesas decorrentes da pesquisa serão ressarcidas, quando devidas e decorrentes especificamente de sua participação

A sua participação neste estudo não resultará em benefícios diretos à você. No entanto, ressaltamos que sua participação contribuíra para o melhor entendimento dos mecanismos fisiopatológicos envolvidos no desenvolvimento e progressão da doença, resultando em benefícios para a sociedade como um todo.

Quanto aos riscos, informamos que sua participação não acarretará qualquer risco à sua saúde nem alteração de qualquer um dos seus tratamentos. A coleta de sangue pode ocasionar sinais decorrentes da punção venosa e consiste: dor no local da punção venosa ou pequeno hematoma e, muito raramente, vermelhidão ou infecção local. Mesmo sendo mínimos, caso ocorra algum tipo de desconforto o participante será prontamente atendido e amparado pelos farmacêuticos responsáveis pela coleta de sangue e um dos pesquisadores deste estudo.

Caso você tenha dúvidas ou necessite de maiores esclarecimentos poderá nos contatar **(Walton Luiz Del Tedesco Júnior, Avenida Robert Koch 60, telefone: 99650-0552, drwaltontedesco@gmail.com ou Andréa Name Colado Simão, Avenida Robert Koch 60, telefone**

*Termo de Consentimento Livre Esclarecido apresentado conforme normas da Resolução 466/2012 de 12 de dezembro de 2012.

99627-8181), ou procurar o Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Londrina, situado junto ao prédio do LABESC – Laboratório Escola, no Campus Universitário, telefone 3371-5455, e-mail: cep268@uel.br.
Este termo deverá ser preenchido em duas vias de igual teor, sendo uma delas devidamente preenchida, assinada e entregue a você.

Este termo deverá ser preenchido em duas vias de igual teor, sendo uma delas devidamente preenchida, assinada e entregue a você.

Londrina, ___ de _____de 20__.

Pesquisador Responsável
Walton Luiz Del Tedesco Júnior
RG: 7.511.648-9
Tel: 99650-0552

Andréa Name Colado Simão
RG: 6.226.736-4
Tel: 99627-8181

Eu, (**colocar nome por extenso do participante da pesquisa**), tendo sido devidamente esclarecido sobre os procedimentos da pesquisa, concordo em participar voluntariamente da pesquisa descrita acima.

Assinatura (ou impressão dactiloscópica): _____
Data:_____

*Termo de Consentimento Livre Esclarecido apresentado conforme normas da Resolução 466/2012 de 12 de dezembro de 2012.

APÊNDICE A

Paciente: _____Prontuário: _____ Tel _____
DN: __/__/____ Peso: _____: Altura:_____ IMC: _____ B ( ) P ( ) N ( ) A ( ) Outro ( )

Comorbidades (S/N)		Sintomas				Medicações (especificar)	
HAS		Diarreia		Febre medida		Corticoides (?)	
IC		Náusea e vômitos		Dores no corpo		Antibióticos (?)	
IR (D) (ND)		Conjuntivite		Fadiga		Tamiflu	
IP (?)		Odinofagia		Mal estar geral		Ivermectina	
DM (1) (2)		Coriza		Tosse		Outros:	
ID		Perda de olfato		Dif. Respiratória		Uso contínuo:	
Neurológicas (?)		Perda de paladar		Outros: (?)			
Dislipidemia		Cefaleia				Tabaco (usa) usou ( )	

APÊNDICE B

Comprovante de submissão à revista Journal of Research in Medical Sciences

Journal of Research in Medical Sciences <editor@jmsjournal.net> 12:50 (há 1 hora)

para mim

inglês › português Traduzir mensagem Desativar para: inglês

Dear Prof Prof. MARSILENI PELISSON,

Journal of Research in Medical Sciences has received your manuscript entitled "RISK AND PROTECTION FROM COVID-19 MORTALITY ASSOCIATED WITH MINOR ALLELES OF P2RX7 VARIANTS RS37511143 AND RS23933799" for consideration for publication. The reference number for this manuscript is "jrms_677_23". Kindly quote this in future correspondences related to this manuscript.

The manuscript is being reviewed for possible publication with the understanding that it is being submitted to ONE journal at a time and has NOT been published, simultaneously submitted, or already accepted for publication elsewhere either as a whole or in a part.

Online submission of this article implies that the corresponding author has written consent from all the contributors to act as the corresponding author.

The co-authors are requested to send their agreement response on the **Digital Copyright** sent via a link to their associated emails, within 1 week of submission. The status can be viewed in the 'Manuscript Information page' from the submitting author's area. The decision about the manuscript will be conveyed only on receipt of the agreement on copyright form received from all contributors.

High-resolution images are required at the time of acceptance, you should be notified separately for the same if images uploaded by you are not of printable quality.

The Editors will review the submitted manuscript initially. If found suitable, it will follow a double-blinded peer review. We aim to finish this review process within a short time frame, at the end of which a decision on the suitability or otherwise of the manuscript will be conveyed to you via this system.

During this process, you are free to check the progress of the manuscript through various phases from our online manuscript processing site https://review.jow.medknow.com/jrms.

We thank you for submitting your valuable work to the Journal of Research in Medical Sciences.

Yours sincerely,

Editorial Team

Journal of Research in Medical Sciences