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AVALIAÇÃO DE MARCADORES DE TRANSLOCAÇÃO MICROBIANA NA COVID-19: IMPACTO NA RESPOSTA INFLAMATÓRIA E PERFIL DE MONÓCITOS.

Porto Alegre
2022

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Dissertação submetida ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Federal de Ciências da Saúde de Porto Alegre como requisito para a obtenção do grau de Mestre.

Orientador: Dr. Pedro Roosevelt Torres Romão
Coorientador: Dr. Gilson Pires Dorneles

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Essa dissertação é um convite para que todos, pais, alunos, sociedade, repensemos nossos papéis e nossas atitudes, pois com ela demonstramos o compromisso com a saúde e com a ciência em tempos tão difíceis. Aos professores e pesquisadores, fica o convite para que não descuidem de sua missão de educar e de descobrir, nem desanimem diante dos desafios. Pois se a educação sozinha não transforma a sociedade, sem ela, tampouco, a sociedade muda. (Paulo Freire)

“Existem muitas hipóteses na ciência que estão erradas. Isso é perfeitamente aceitável, elas são abertura para achar as que estão certas”.

Carl Sagan

RESUMO

Esta dissertação teve como objetivo avaliar o impacto das alterações da barreira da mucosa gastrointestinal na resposta inflamatória sistêmica e subpopulações de monócitos na patogênese da COVID-19. Para isto, foram desenvolvidos dois estudos originais com foco na patogenia da infecção aguda pelo SARS-CoV-2, agente causador da COVID-19. No estudo 1, amostras de sangue periférico de pacientes com a forma grave da COVID-19 com desfecho de óbito apresentaram níveis aumentados de marcadores de translocação microbiana e de citocinas e quimicionas pró-inflamatórias (IL-6, TNF-α, CCL2/MCP-1 e CCL5/RANTES) ao longo da hospitalização. A incubação *in vitro* de monócitos com plasma de pacientes COVID-19 não sobreviventes no T2 (fim da hospitalização) levou a uma maior expressão de TLR4, CCR2, CCR5, CCR7 e CD69. Conclui-se que há uma coexistência entre aumento da translocação microbiana, maior ativação de monócitos e hiperinflamação de pacientes com COVID-19 grave. O estudo 2 sugere que maiores níveis de endotoxinas na circulação podem indicar um estado comprometido da resposta imune do hospedeiro contra coinfecções em pacientes com COVID-19 grave. A estimulação *ex vivo* de sangue total de pacientes graves com COVID-19, com diferentes concentrações de LPS (1 e 10 ng/mL), induziu menor produção de IL-6, IL-10 e TNF-α na condição de 1 ng/mL e menor produção de IL-6, L-10, CCL2 e TNF-α após estimulação de 10 ng/mL em comparação com os controles. Ambas concentrações de endotoxina reduziram a frequência de monócitos CD14+HLA-DRlow em pacientes graves com COVID-19, enquanto que os aumentos na expressão de TLR-4 e ativação de NF-κB foram mais pronunciados em CD14+ HLA-DR^{low} e CD14+HLA-DR^{high} monócitos de controles saudáveis e pacientes leves com COVID-19 em comparação com o grupo grave de COVID-19, sugerindo que a infecção aguda por SARS CoV-2 está associado a diminuição da resposta à endotoxina em monócitos. Diante disso, a infecção aguda por SARS CoV-2 e o desenvolvimento dos piores desfechos clínicos na COVID-19 podem estar associadas ao aumento da translocação microbiana, que coexiste com hiperinflamação e imunossupressão.

Palavras-chave: COVID-19. Inflamação. Citocinas. Translocação microbiana. Lipopolissacarídeo. Monócitos.

ABSTRACT

This dissertation aimed to assess the impact of changes in the gastrointestinal mucosal barrier on the systemic inflammatory response and monocyte subpopulations on the pathogenesis of COVID-19. For this, two original studies were developed focusing on acute infection by SARS-CoV-2, the causative agent of COVID-19. In study 1, peripheral blood samples from patients with the severe form of COVID-19 with an outcome of death had increased levels of microbial translocation markers, and of pro-inflammatory cytokines and chemokines (IL-6, TNF-α, CCL2/ MCP-1 and CCL5/RANTES) throughout hospitalization. *In vitro* incubation of monocytes with plasma from non-surviving COVID-19 patients at T2 (end of hospitalization) led to increased expression of TLR4, CCR2, CCR5, CCR7 and CD69. It is concluded that there is a coexistence between increased microbial translocation, increased monocyte activation and hyperinflammation of patients with severe COVID 19. Study 2 suggests that higher levels of circulating endotoxins may indicate a compromised state of the host's immune response against co-infections in patients with severe COVID-19. *Ex vivo* stimulation of whole blood from critically ill patients with COVID-19 with different concentrations of LPS (1 and 10 ng/mL) induced lower production of IL-6, IL-10 and TNF-α in the condition of 1 ng/mL, and lower production of IL-6, L-10, CCL2 and TNF-α after stimulation of 10 ng/mL compared to controls. Both endotoxin concentrations reduced the frequency of CD14+ HLA-DR^{low} monocytes in critically ill patients with COVID-19, while increases in TLR-4 expression and NF-κB activation were more pronounced in CD14+ HLA-DR^{low} and HLA-DR^{high} monocytes from healthy controls and mild COVID-19 patients compared with the severe COVID 19 group, suggesting that acute SARS CoV-2 infection is associated with diminished endotoxin response in monocytes. Therefore, acute SARS CoV-2 infection and the development of worse clinical outcomes in COVID-19 may be associated with increased microbial translocation, which coexists with hyperinflammation and immunosuppression.

Keywords: SARS-CoV-2. Inflammation Cytokines. Microbial translocation. Lipopolysaccharide. Monocyte.

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LISTA DE ABREVIAÇÕES

CCR – Receptor de quimiocina C-C

CD – Cluster de diferenciação

CTLA-4 - Proteína Associada aos Linfócitos T Citotóxicos-4

CXCL2 – Quimiocina do tipo C-X-C - 2

CXCL3 - Quimiocina do tipo C-X-C-3

CXCL10- Quimiocina do tipO C-X-C-10

CXCR – Receptores de quimiocina tipo CXC

DAMPs – Padrões moleculares associados ao dano

PAMPs – Padrões moleculares associados ao patógeno

PRRs- Receptor de reconhecimento de padrão

ELISA - Ensaio de imunoabsorção enzimática

HLA-DR – Antígeno de leucócito humano-DR

IgA – Imunoglobulina A

IL – Interleucina

LPS – lipopolissacarídeo

MD-2 - Fator de diferenciação mieloide

MyD88 - Fator de diferenciação mieloide 88

MCP-1 – Proteína de quimioatração de monócitos – 1

NF-κB – Fator de transcrição nuclear – kappaB

NK – Células Natural Killers

PD-1 – Proteína de morte celular programada – 1

RANTES – Ligante de quimiocina do tipo C-C-5

RNA – Ácido ribonucleico

RNAm – RNAmensageiro

Th – Células T auxiliar

THP-1 - Linhagem monócito humana

TLR – Receptor do tipo Toll

TLR-4 - Receptor Toll-like 4

TNF-α – Fator de necrose tumoral-alfa

[ ] - Concentração

↑ - Aumento

↓ - Diminui

SUMÁRIO

1 APRESENTAÇÃO

Essa dissertação de mestrado foi motivada devido ao surto pandêmico causado pelo vírus SARS-CoV-2, o qual intrigou a comunidade científica, principalmente pesquisadores da área de imunologia, que foram induzidos a investigar e interpretar os aspectos imunopatogênicos da doença causada pelo coronavírus (COVID-19). A ideia central desta dissertação surgiu a partir dos questionamentos sobre o impacto da infecção aguda pelo SARS-CoV-2 sobre a resposta imune inata, procurando identificar novos biomarcadores, bem como aspectos imunopatogênicos em relação à gravidade e desfechos clínicos da COVID-19.

Desta forma, a presente dissertação está estruturada do seguinte modo:

1) Capítulo 1:

   Introdução e justificativas para a pesquisa.

2) Capítulo 2:

   Artigo original intitulado “Increased LPS levels coexist with systemic inflammation and result in monocyte activation in severe COVID-19 patients” publicado no periódico *International Immunopharmacology,* de fator de impacto 5.71 e Qualis B1 no extrato Medicina I da CAPES.

3) Capítulo 3:

   Artigo intitulado “Endotoxin tolerance in acute SARS-CoV-2 infection occurs through the low activation of TLR-4/NF-κB axis in CD14+HLA-DRlow/+ monocytes” submetido no periódico Journal of Molecular Medicine, de fator de impacto 5.60 e Qualis A1 no extrato de Medicina 1 da CAPES.

4) Capítulo 4:

   Este capítulo é composto por discussão geral dos dados da dissertação, com comentários dos resultados, apresentados separadamente nos manuscritos originais e perspectivas para o desenvolvimento de estudos futuros.

As demais produções desenvolvidas ao longo do período de mestrado estão apresentadas na forma de documentos anexos a esta dissertação.

2 CAPÍTULO 1

2.1 INTRODUÇÃO

A doença coronavírus 2019 (COVID-19), causada pelo coronavírus da síndrome respiratória aguda grave 2 (SARS-CoV-2), emergiu em Wuhan, na China, em dezembro de 2019 (LUO; MCHENRY; LETTERIO, 2020). Os primeiros relatos da COVID-19 surgiram a partir das observações de uma nova doença respiratória que frequentemente provocava quadros inflamatórios e pneumonias (ZUO *et al*., 2020). Hoje já se sabe que COVID-19 apresenta diferentes manifestações clínicas na população, entretanto a grande maioria dos pacientes infectados com SARS-CoV-2 manifesta a fase aguda da doença de forma assintomática ou leve, apresentando sintomas de infecções das vias aéreas superiores, incluindo tosse, dispneia e anosmia (FRAGA-SILVA *et al*., 2020). Nos casos graves, a infecção pelo SARS-CoV-2 pode envolver vários órgãos e evoluir para desfechos clínicos mais lesivos, como síndrome do desconforto respiratório agudo (SDRA), sepse, falência de múltiplos órgãos e morte (CHEN *et al*.,2020). Segundo registros de RITCHIE (2022), o SARS-CoV2 já infectou cerca de 581 milhões de pessoas e causou a morte de mais de 6,41 milhões de pacientes, desde o início do surto pandêmico da COVID-19. No Brasil, 33,9 milhões indivíduos foram infectados e 679 mil brasileiros foram a óbito pela COVID-19. No presente momento, cerca de 33 mil novos casos de infecção pelo SARS-CoV-2 são diagnosticados diariamente no Brasil e aproximadamente 1.147 mil novos casos são registrados no mundo.

Ademais, estudos recentes identificaram a persistência dos sintomas em pacientes infectados por SARS-CoV-2, mesmo semanas ou meses após desenvolver a fase aguda da COVID-19, esta condição clínica foi denominada como COVID-19 longa, Síndrome pós-COVID (SCP) ou *Post Acute-sequelae of SARS-CoV-2* (PASC) (AIYEGBUSI *et al*., 2021; ANAYA *et al*., 2021). Os sintomas recorrentes identificados na COVID-19 longa variam principalmente entre sequelas no músculo esquelético, e em diversos tecidos e órgãos do corpo humano, incluindo pulmonar, digestivo e neurológico (ANAYA *et al*., 2021). Atualmente muitos estudos estão sendo publicados

com o intuito de compreender e especificar por que pacientes evoluem para quadros clínicos mais graves da COVID-19, desenvolvendo sepse, falência de múltiplos órgãos e/ou apresentam a persistência de sintomas entre outras características clínicas que comprometem o funcionamento e a saúde do corpo humano.

A imunopatogenia da COVID-19 está relacionada a uma hiperativação imunológica e uma liberação excessiva de citocinas pró-inflamatórias de resposta precoce, evento nomeado como hiperinflamação (JOSE; MANUEL, 2020). A hiperinflamação gera uma desregulação do sistema imunológico inato, a exemplo de uma forte resposta de células imunes inatas e ativação de linfócitos de perfil exausto (VABRET *et al*., 2020). As citocinas desempenham um papel importante na imunopatogenia durante a infecção viral. Uma resposta imune inata rápida e bem coordenada é a primeira linha de defesa contra a infecção viral (VABRET *et al*., 2020). No entanto, respostas imunológicas desreguladas e excessivas podem causar danos imunológicos ao corpo humano, além de facilitar a infecção e o agravamento de infecções oportunistas por outros patógenos (YE *et al*., 2020). Dessa forma, desfechos clínicos como COVID-19 grave e a morte decorrente de falência de órgãos ou por falência respiratória podem estar associados às altas concentrações de citocinas sistêmicas (HU *et al*., 2020). Segundo Yang e colaboradores, 2020, descreveram que o SARS-CoV-2 interrompe as respostas imunes (coordenadas) normais, compromete o sistema imunológico e gera respostas inflamatórias descontroladas em pacientes graves e críticos com COVID-19. Pacientes graves registram ativação e disfunção de linfócitos, quadros de linfopenia, redução na frequência de Linfócitos T CD4+/CD8+, redução de Células NK e Células B (ANKA *et al,* 2020); registram altos níveis de citocinas, imunoglobulina G (IgG) e anticorpos totais, apresentam anormalidades de monócitos e granulócitos, desenvolvem eosinopenia e os neutrófilos estão aumentados no sangue periféricos desses pacientes, os quais podem estar associados às condições de linfopenia (YANG *et al*., 2020).

Foi comprovado que o trato respiratório é o principal local de infecção pelo SARS-CoV-2, mas outros estudos demonstraram que o vírus também possui tropismo para diversos órgãos e sistemas como: coração, fígado, rins, intestino e vasos sanguíneos (OLIVA *et al*., 2021). Nesse sentido, a enzima conversora de angiotensina-2 (ACE-2), principal receptor do novo coronavírus, expressa-se não apenas no trato respiratório superior e inferior, mas também nas células endoteliais, intestinais e tireoidianas, contribuindo para caracterizar a natureza sistêmica da

doença (OLIVA *et al.*, 2021). A infecção do SARS-CoV-2, no sistema gastrointestinal ganha a atenção, devido aos relatos de sintomatologia como diarreia e distensão abdominal durante o curso da doença (ZUO *et al.*, 2020). Além disso, estudos demonstraram altas taxas de replicação viral na mucosa do intestino delgado, e foi encontrado o material genético do vírus, RNA do SARS-CoV-2, em amostras de fezes de pacientes com COVID-19 (DHAR; MOHANTY, 2020). Pacientes infectados pelo SARS-CoV-2 apresentaram modificações no perfil da microbiota intestinal, caracterizando um aumento do número de patógenos oportunistas e uma depleção de bactérias comensais durante a hospitalização (ZUO *et al*., 2020). Por exemplo, foi identificado que bactérias como: Ruminococcus gnavus, Ruminococcus torques, Bacteroides dorei e Bacteroides vulgatus na COVID-19 são responsáveis por uma consistente interferência na desregulação imune mediada por microorganismos, além de estarem implicados em várias doenças inflamatórias intestinais, como doença do intestino irritável e colite ulcerativa (YEOH et al., 2021; MATSUOKA et al., 2015; HALL et al., 2017 e AHMED et al., 2020). Diante disso, surge a possibilidade de que a translocação bacteriana e produtos microbianos do trato GI para o sangue periférico possam contribuir para o estado hiperinflamatório e a gravidade da COVID-19 (SHARMA; RIVA, 2020). Estudos observacionais também indicaram a existência de uma persistência viral, ou de resíduos do SARS-CoV-2, no microbioma intestinal de pacientes pós-COVID-19, sugerindo alterações na composição da microbiota e possível persistência na resposta inflamatória tecidual (LIU *et al*., 2022).

A função do microbioma intestinal nas infecções virais respiratórias em geral, e especificamente em pacientes com COVID-19, está apenas começando a ser compreendido. Há evidências que indicam que a infecção grave por SARS-CoV-2, desenvolvida por meio da inflamação sistêmica pulmonar pode induzir a ruptura da barreira intestinal, contribuindo para a disseminação sistêmica de bactérias e produtos microbianos que afetam a resposta do hospedeiro à infecção (KIPKORIR *et al*., 2020; ZUO *et al*., 2020). Estudos com modelos animais de infecção pelo vírus influenza descobriram mecanismos pelos quais o microbioma influencia a imunidade antiviral e, por sua vez, a infecção viral demonstrou romper a barreira intestinal de camundongos, danificando a microbiota intestinal (ICHINOHE *et al*., 2011; STEED *et al.*, 2017) e gerando uma hiperinflação sistêmica. Curiosamente, o vírus SARS-CoV-2 pode estar gerando semelhante translocação microbiana e induzindo uma hiperativação das

células imunes inatas, principalmente de monócitos nos pacientes com COVID-19 (KIPKORIR *et al*., 2020).

O sistema imune inato reconhece padrões moleculares associados a patógenos (PAMPs) e diversos patógenos e agentes não-próprios, a exemplo das endotoxinas como o Lipopolissacarídeo (LPS), presente nas membranas externas das bactérias gram negativas. Os PAMPs podem atuar como um forte imunoativador quando suas estruturas moleculares são reconhecidas através de receptores de reconhecimento de padrões (PRRs) de células imunes inatas, sendo a interação entre o LPS e o receptor toll-like (TLR-4) um dos mecanismos de ativação imune mais bem estabelecidos (LU; YEH; OHASHI, 2008). Assim, a ativação das células do sistema imune inato, após o reconhecimento de PAMPs, gera uma forte reação inflamatória, caracterizada pela síntese elevada de mediadores pró-inflamatórios, a exemplo de quimiocinas (CCL2, MCP-1, CCL5, RANTES) e citocinas, incluindo IL-6 e fator de necrose tumoral alfa (TNF-α) (FREDE *et al.*,2006).

A ativação imunológica alicerçada à translocação microbiana é recorrente em muitas infecções patogênicas, principalmente de origem viral, e em quadros de sepse em pacientes críticos. A exemplo do vírus HIV, no qual a patogênese, morbidade e mortalidade dos indivíduos infectados estão vinculados à inflamação persistente e disfunção imunológica, condições essas, associadas à ruptura e/ou disfunção da mucosa intestinal, aumento da permeabilidade da barreira epitelial, translocação de produtos microbianos para a circulação e disbiosa do microbioma (BURGENER; MCGOWAN; KLATT, 2015; CASTRO *et al.*, 2019; SOMSOUK *et al.,* 2015). Pacientes críticos, com quadro de sepsemia, em Unidades de Terapia Intensiva (UTI), apresentam grande prevalência de falência múltipla de órgãos decorrente da resposta imune desregulada ao hospedeiro (HAAK; WIERSINGA, 2017; HUERTA; RICE, 2018).

Diante disso, a presente dissertação tem por objetivo investigar a associação entre marcadores de translocação microbiana com inflamação sistêmica induzindo ativação de monócitos, e assim relacionar com a gravidade e desfechos clínicos da COVID-19.

2.2 SARS-COV-2 E AS INFECÇÕES HUMANAS

Evidências indicam que os coronavírus (CoVs) são uma grande família viral, conhecidos desde os anos de 1960, responsáveis por três surtos infecciosos em grande escala nas últimas duas décadas, sendo eles: Síndrome Respiratória Aguda Grave (SARS) em 2002, Síndrome Respiratória do Oriente Médio (MERS) em 2012 e atualmente a COVID-19 (HARRISON; LIN; WANG, 2020). O primeiro caso de infecção por SARS-CoV-2 foi relatado em Wuhan, China, em dezembro de 2019, os pacientes apresentavam sintomas de pneumonia atípica. Este caso foi confirmado como sendo causado pelo novo coronavírus, SARS-CoV-2, desde então ocorreu uma rápida transmissão entre humanos e uma disseminação intercontinental se seguiu, sendo declarada uma pandemia pela Organização Mundial da Saúde (*World Healt Organization - WHO*) em março de 2020 (HARRISON; LIN; WANG, 2020). No presente momento, OMS contabiliza o número aproximado de 511 milhões de pessoas que foram infectadas com SARS-CoV-2, com mais de 6 milhões de mortes no mundo (OMS, 2022).

Segundo Rabaan e colaboradores (2020), os coronavírus apresentam em sua taxonomia uma subfamília composta de quatro gêneros: coronavírus alfa, beta, gama e delta. Os coronavírus que infectam humanos (HCoVs) pertencem a dois desses gêneros (alfa coronavírus e beta coronavírus). Os alfas coronavírus que infectam humanos são HCoV-229E e HCoVNL63, e os beta coronavírus que infectam humanos são HCoV-HKU1, HCoV-OC43, Síndrome respiratória do Oriente Médio (MERSCoV), a Síndrome respiratória aguda grave (SARS-CoV) e SARS-CoV-2 (HARRISON; LIN; WANG, 2020).

O novo betacoronavírus SARS-CoV-2, responsável por causar a COVID19, apresenta em sua estrutura um RNA fita simples de sentido positivo, é encapsulado e codifica quatro proteínas estruturais: glicoproteína espicular (S), proteína do envelope (E), glicoproteína da membrana (M) e proteína do nucleocapsídeo (N) (FELSENSTEIN *et al.*, 2020). Estes vírus utilizam a proteína Spike (S) para se ligar ao receptor e mediar a fusão à membrana e à entrada na célula. A proteína Spike (S) apresenta afinidade pelo receptor da enzima conversora da angiotensina 2 (ACE2), que são expressos, principalmente, nas superfícies das células epiteliais respiratórias, intestinais, coração, rins, fígado, íleo terminal, cólon e vasos sanguíneos (WAN *et al*., 2020). Dessa forma, o SARS-CoV-2 consegue entrar em diversas células do

hospedeiro e infectar diferentes sistemas, desenvolvendo a COVID-19 com várias manifestações clínicas.

Segundo a Organização Mundial de Saúde (*OMS*), pacientes infectados pelo novo coronavírus podem desenvolver a doença em diversos aspectos clínicos, podendo variar entre casos assintomáticos e manifestações clínicas leves até quadros moderados, graves e críticos, conforme demonstrado na tabela 1.

Tabela 1 - Classificação dos casos e principais manifestações clínicas da COVID-19.

CLASSIFICAÇÃO DOS PACIENTES COM COVID-19		
CASO	**MANIFESTAÇÕES CLÍNICAS**	**EXEMPLOS**
Assintomático	Ausência de sintomas	Teste laboratorial positivo
Leve	Sintomas não específicos	Tosse, dor garganta, coriza seguido ou não de anosmia, ageusia, diarreia, dor abdominal, febre, mialgia, calafrios, fadiga e/ou cafeleia
Moderado	Sintomas pouco específicos	Tosse (seca ou produtiva) e febre persistente, adinamia, prostração, mal-estar, hiporexia e pneumonia leve
Grave	Sintomas específicos	Síndrome respiratória aguda, síndrome gripal com dispneia/desconforto respiratório e pressão persistente do tórax, saturação de O2 < 95% em ar ambiente, coloração azulada de lábios e rosto
Crítico	Sintomas específicos	Síndrome do desconforto respiratório agudo (SDRA), insuficiência respiratória grave, pneumonia grave sepse, choque séptico, disfunção de múltiplos órgãos, auxílio de suporte respiratórios e internação em UTI

Fonte: Adaptado de OMS, 2022 e MS, 2021.

2.3 COVID-19 GRAVE E SISTEMA INFLAMATÓRIO: O EIXO ENTRE PULMÃO E SISTEMA GASTROINTESTINAL

O desenvolvimento de uma doença infecciosa em um indivíduo suscetível envolve interações complexas entre os microrganismos e o seu hospedeiro. Os primeiros eventos durante a infecção incluem a entrada do patógeno, invasão e colonização dos tecidos do hospedeiro, ativação da resposta imune do hospedeiro, lesão tecidual ou dano funcional. Dessa forma, microrganismos podem desencadear

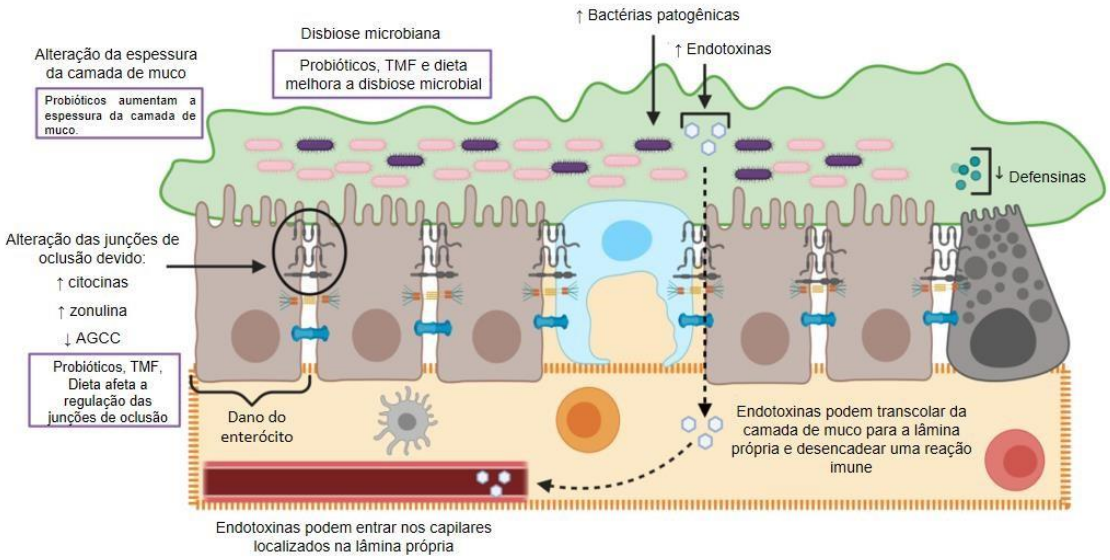
eventos de morte de células infectadas do hospedeiro e liberação toxinas que podem desencadear danos funcionais às células vizinhas de tecidos distintos. Além disso, a infecção viral estimula uma desregulação na resposta imunológica que prejudica tanto o tecido infectado quanto os tecidos normais (FINLAY; MCFADDEN, 2006).

A infecção pelo SARS-CoV-2 no trato respiratório e a destruição das células pulmonares desencadeiam uma resposta imune local, recrutando macrófagos que respondem à infecção e liberam citocinas, e geram resposta imune de células da imunidade adaptativa, linfócitos T e B. Na maioria dos casos, esse processo é capaz de solucionar a infecção sem a exacerbação de sintomas ou prejuízos sistêmicos ao hospedeiro (TAY *et al*., 2020). No entanto, o desenvolvimento da COVID-19 grave está relacionado a uma resposta inflamatória excessiva ao SARS-CoV-2, e não unicamente à carga viral do vírus. Notavelmente os perfis sistêmicos de citocinas analisadas em pacientes com COVID-19 grave apresentaram semelhanças com as analisadas em síndromes de liberação de citocinas, como a exemplo de ativação macrofágica, que apresenta aumento de produção de citocinas como IL-6, IL-7 e fator de necrose tumoral alfa (TNF-α), como também há um aumento nas concentrações de quimiocinas inflamatórias incluindo CC-ligante de quimiciona 2 (CCL2), CCL3 e CXC-ligante de quimiocina 10 (CXCL10), os quais representam uma ativação desregulada do compartimento do fagócito mononuclear que contribui para a hiperinflamação relacionada a COVID-19 (MERAD; MARTIN, 2020). Além disso, a maioria dos casos graves de COVID-19 foram associados a altos níveis sistêmicos de TNF-α, IL-1β e IL-6 (GUBERNATOROVA *et al*., 2020), onde concentrações de IL-6 foram determinados como os preditores clínicos mais significativos da mortalidade associada à COVID-19. A elevação contínua de IL-6 é conhecida por promover a replicação viral e provocar inflamação e lesão dos tecidos pulmonares (VELAZQUEZ-SALINAS *et al*., 2019), e predispor a uma alta mortalidade associada à SARS-CoV-2 (BONAFÈ *et al*., 2020).

Ademais, novos estudos demonstram que tanto o sistema pulmonar quanto o gastrointestinal podem estar associados ao desenvolvimento da COVID-19 grave, à medida que marcadores de translocação microbiana podem estar induzindo a ativação do sistema imune e contribuem para o estado hiperinflamatório da infecção. Hipóteses da ruptura da barreira gastrointestinal em pacientes portadores da COVID-19 grave, estão alicerçados em dois eixos principais, isto é, o eixo indireto da quebra da barreira gastrointestinal, o qual está vinculado à infecção ou lesão pulmonar que causa a

inflamação sistêmica e gera perda da integridade da barreira intestinal e aumento da permeabilidade a micróbios intestinais e produtos microbianos (GIRON *et al*., 2021). O segundo eixo, denominado como direto, é quando o vírus SARS-CoV-2 infecta diretamente o epitélio das células gastrointestinais, as quais também expressam a angiotensina 2 (ACE-2), que facilitam a entrada, o alojamento e a replicação do novo coronavírus no sistema gastrointestinal. Assim, o SARS-CoV-2 consegue induzir a ruptura da barreira intestinal e ocasionar o extravasamento e a migração de produtos bacterianos para o sistema circulatório, conforme ilustrada na figura 1, que podem sensibilizar e ativar o sistema imune inato e contribuir para a tempestade de citocinas, característico da COVID-19 grave (GIRON *et al*., 2021).

Figura 1 **-** Representação esquemática da translocação microbiana induzida pela infecção do SARS-CoV-2.



Fonte: Adaptada de Sharma; Riva (2020).

É característico que doenças infecciosas apresentem a capacidade de interferir na estabilidade da barreira gastrointestinal. Segundo Shi e colaboradores 2017, a microbiota intestinal se coordena para moldar a imunidade do hospedeiro e contribuir para manter a homeostase intestinal e inibir a inflamação. Uma interação prejudicada entre a microbiota intestinal e o sistema imunológico da mucosa está associada à patogênese de doenças inflamatórias, infecciosas e quadros de sepse.

2.4 TRANSLOCAÇÃO MICROBIANA

O microbioma humano é composto por uma variedade de microrganismos, como bactérias, fungos, protozoários e vírus, que residem na superfície da barreira epitelial do nosso corpo (LI *et al.*, 2019). A microbiota desempenha muitas funções importantes em pessoas saudáveis, como por exemplo proteção contra organismos patogênicos, facilitar a digestão de carboidratos e vegetais, regulação do metabolismo de gorduras, e produção de pequenas moléculas que interagem com o ambiente hospedeiro (CLEMENTE; MANASSON; SCHER, 2018). Desse modo, a relação simbiótica, estipulada entre o microbiota intestinal e o hospedeiro é fundamental para manter a homeostase e a saúde do indivíduo (FERREIRA; VIANA; REIS, 2020).

O epitélio intestinal é composto primariamente por enterócitos conectados por junções firmes (zônulas de oclusão), formando uma barreira com permeabilidade seletiva entre a bainha epitelial e o conteúdo luminal (QUEIROZ *et al*., 2016). Em condições saudáveis, a barreira intestinal apresenta em seu lado luminal uma variedade de microrganismos intestinais, enquanto o lado basolateral permanece estéril (NAGPAL; YADAV, 2017). A camada epitelial intestinal tem função bimodal de maximizar a absorção de nutrientes digeridos, enquanto impede a passagem dos componentes luminais, tais como bactérias e componentes intactos de alimentos (QUEIROZ *et al.*, 2016).

No entanto, se o sistema imunológico da mucosa intestinal estiver desregulado e os mecanismos de defesa falharem, pode ocorrer a passagem não fisiológica de bactérias e/ou produtos bacterianos do lado luminal do trato gastrointestinal para sítios estéreis, este processo é denominado como translocação microbiana (BERG, 1995). Desse modo, três mecanismos principais estimulam a translocação bacteriana: i) supercrescimento bacteriano intestinal, ii) deficiências nas defesas imunológicas do hospedeiro e iii) aumento da permeabilidade ou dano à barreira da mucosa intestinal (BERG, 1995).

Na infecção por SARS-CoV-2, hipóteses indicam que o motivo da translocação bacteriana está associado ao dano intestinal relacionado à infecção do tecido, disfunção imunológica induzida por inflamação sistêmica e dano vascular difuso mediado por IL-6, à medida que a replicação e a infecção do SARS-CoV-2 resultam na ativação da imunidade inata e adaptativa e do complemento, que geram respostas imunes locais e sistêmicas (CARDINALE *et al*.,2020). A exemplo da tempestade de

citocinas, uma liberação excessiva de citocinas pró-inflamatórias, que induz a migração de células imunes como macrófagos, células dendríticas, natural killer (NK), neutrófilos, células T da circulação para os sítios de infecção, que resultam na desestabilização das células endoteliais e epiteliais, as quais geram danos na barreira vascular e também da mucosa intestinal (RAGAB *et al.*, 2020).

Além disso, a resposta de citocinas inflamatórias na COVID-19 polariza a resposta adaptativa de células T CD4+ para um padrão Th1/Th17, caracterizado por alta produção de interleucina (IL-17) e outras citocinas pró-inflamatórias, as quais são células determinantes para a quebra da integridade da barreira gastrointestinal (CARDINALE *et al.,* 2020). Entretanto, a liberação de derivados de linfócitos e de monócitos / macrófagos, bem como o acúmulo de neutrófilos e macrófagos nos tecidos, também contribuem para dano adicional da barreira gastrointestinal e perpetuam inflamação sistêmica (SCHETT; STICHERLING; NEURATH, 2020).

Deste modo, a desestabilização na barreira intestino-sangue em indivíduos infectados pelo SARS-CoV-2 permite a translocação de produtos microbianos para a circulação, causando um evento denominado como endotoxemia sistêmica (SANDLER; DOUEK, 2012). Isto é, a presença no sangue periférico de substâncias como o LPS, componente bacteriano que tem sido relacionado com a inflamação sistêmica e ativação do sistema imune (ANCUTA *et al*., 2008).

As linhas de defesas para inibir a translocação microbiana são mediadas por macromoléculas da camada de muco no interior do lúmen do trato gastrointestinal: proteínas, fosfolipídios, eletrólitos e água, como também, as imunoglobulinas A (IgA) luminal podem se ligar e eliminar as bactérias, assim restringir a capacidade de translocação (BRENCHLEY; DOUEK, 2012). Além disso, a barreira epitelial do trato gastrointestinal, por si só, representa um impedimento físico contra a translocação microbiana. Se os produtos microbianos ultrapassarem o muco e a barreira epitelial, eles serão identificados por um grande número de macrófagos residentes especializados que impedem o acesso de substâncias estranhas na circulação sistêmica (SMITH; OCHSENBAUER-JAMBOR; SMYTHIES, 2005). No entanto, caso os macrófagos do trato gastrointestinal não contenham os produtos microbianos, eles são drenados pela veia porta para fígado; assim o fígado realiza a depuração das substâncias estranhas oriundas do trato gastrointestinal. Diante disso, para entrar na circulação sistêmica, os antígenos microbianos que se originam no trato gastrointestinal devem passar por meio do muco luminal e IgA, atravessar a barreira

epitelial fechada, escapar da captação pelos macrófagos da lâmina própria do trato gastrointestinal e evitar a depuração hepática (BRENCHLEY; DOUEK, 2012).

As alterações causadas pela translocação microbiana resultam em inúmeras e complexas interações patógeno-hospedeiro, resultando no aumento dos níveis de produtos microbianos, como o lipopolissacarídeos (LPS) e substâncias inflamatórias como CD14 solúvel (sCD14), as quais são comumente encontrados, em altas concentrações, no plasma de pacientes com infecções virais, a exemplo do HIV e/ou em quadros de sepse (KRASTINOVA *et al*.,2015; CASTRO FOF et al., 2019).

Uma vez na circulação, a presença de LPS pode sensibilizar uma resposta mediada pelo hospedeiro, regulada por receptores de superfície celular que detectam e circulam fatores que se ligam e eliminam produtos microbianos (OLIVA et al., 2021). As células do sistema imune como monócitos, macrófagos, células dendríticas e neutrófilos ativados expressam a proteína de superfície CD14, que apresenta a funcionalidade de ser receptor celular dos LPS, (BRENCHLEY; DOUEK, 2012; WRIGHT *et al.*,1990), além de ativar a produção de fatores solúveis como CD14 solúvel (sCD14) e de proteínas de ligação ao LPS (LBP). A presença de LPS no plasma induz a síntese hepática da proteína de ligação a LPS (LBP), responsável pelo aumento da ligação do LPS ao receptor CD14 adjacente ao receptor toll-like 4 (TLR4) (PŁÓCIENNIKOWSKA *et al.*, 2015), ou seja, após a estimulação com LPS, monócitos/macrófagos CD14+ secretam sCD14 e liberam CD14 de superfície, que se liga ao LPS. Portanto, a quantificação de LPS e/ou LBP e sCD14 podem identificar indiretamente os efeitos da translocação microbiana (LICHTFUSS *et al.*, 2011).

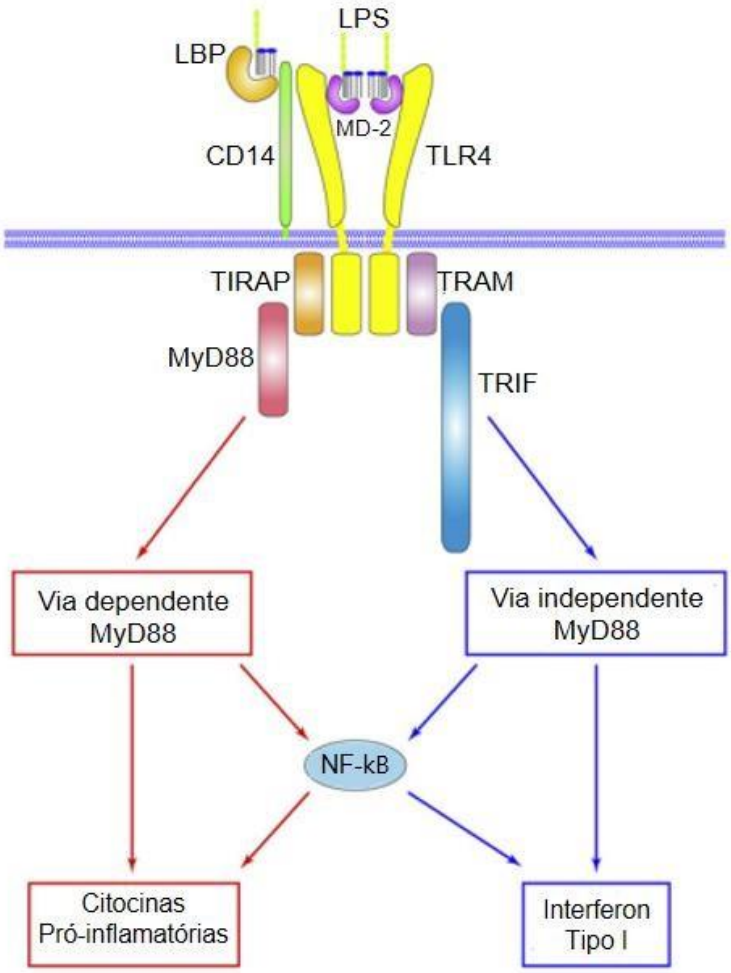
2.5 ENDOTOXINAS E ATIVAÇÃO DO SISTEMA IMUNE

Os aspectos históricos do papel das endotoxinas na patogênese bacteriana (Beutler e Rietschel, 2003) e a caracterização química e estrutural dos lipopolissacarídeos (LPS) (RAETZ *et al*., 2007; RAETZ; WHITFIELD, 2002) têm sido objeto de estudo de algumas revisões. O LPS e/ou endotoxina é uma molécula altamente imunogênica que compõe a membrana externa de bactérias gram-negativas (CIESIELSKA; MATYJEK; KWIATKOWSKA, 2021). Essas estruturas desempenham importante função na interação hospedeiropatógeno ao ser responsável pela ativação do sistema imunológico (FREUDENBERG *et al.*, 2001). O LPS apresenta grande importância clínica, à medida que altas concentrações dessas

endotoxinas, no plasma ou sangue periférico de pacientes, podem induzir febre, aumentar a frequência cardíaca, gerar quadros de sepse, choque séptico e levar à morte (MAYR; YENDE; ANGUS, 2014).

O LPS é uma molécula de carga negativa que pode ser dividida em três estruturas: lipídio A, açúcares centrais e repetições do antígeno O (RAETZ; WHITFIELD, 2002). O lipídio A é o centro imunorreativo do LPS, de modo que é sensibilizado e reconhece estruturas celulares e humorais da imunidade inata do hospedeiro (MEDZHITOV, 2001), sendo responsável pelos efeitos tóxicos das infecções por bactérias gram-negativas (GALANOS *et al*., 1985). O receptor TLR-4 liga-se ao LPS com auxílio da proteína de ligação ao LPS (LBP), CD14 e da proteína MD-2 (fator de diferenciação mieloide) (CIESIELSKA; MATYJEK; KWIATKOWSKA, 2021), essas estruturas formam um complexo imunorreativo, conforme ilustrado na figura 2, onde cada componente apresenta uma funcionalidade específica. Diante disso, TLR-4 sozinho não reconhece o LPS, em primeiro momento o receptor TRL-4 precisa formar um heterodímero ao ligar-se ao MD-2 para iniciar uma sinalização específica para o complexo, após a ligação com LPS (GNAUCK; LENTLE; KRUGER, 2016). No entanto, as células imunes de origem mieloide e não mieloide não conseguem mediar uma resposta direta ao LPS, e também o LPS não tem a capacidade de se ligar diretamente ao complexo receptor TLR4/MD-2, para isso, é necessário a presença do CD14. O CD14, tanto na sua forma solúvel (sCD14), quanto na forma ligado à membrana (mCD14), tem a capacidade de se ligar à molécula de LPS e apresentar o mesmo para MD-2 (KITCHENS; THOMPSON, 2005). Entretanto, ambas as formas de CD14 (sCD14 e mCD14) possuem baixa afinidade ao LPS. Essa afinidade é aumentada na presença da proteína de ligação ao LPS (LBP) (HAILMAN *et al*.,1994).

Figura 2 - Representação esquemática da sinalização LPS/TLR4. O reconhecimento LPS é viabilizado por LBP e CD14, e mediado pelo complexo receptor TLR4/MD-2.



Fonte: Adaptada de LU; YEH; OHASHI (2008).

A interação do complexo TLR4-MD-2, após a ligação do LPS, leva à ativação de vários componentes de sinalização, incluindo vias dependentes de MyD88 (Fator de diferenciação mieloide 88) e independentes de MyD88 (dependentes de TRIF), NF-kB, IRF3, e a subsequente produção de citocinas pró-inflamatórias (GNAUCK; LENTLE; KRUGER, 2016; CIESIELSKA; MATYJEK; KWIATKOWSKA, 2021; LU; YEH; OHASHI, 2008). A via dependente de MyD88 é responsável pela expressão de citocinas pró-inflamatórias, enquanto a via independente de MyD88 medeia a ativação de interferons Tipo I e genes induzíveis por interferon (LU; YEH; OHASHI, 2008).

2.6 MONÓCITOS E COVID-19, PRINCIPAIS MODIFICAÇÕES/FENÓTIPOS E ATIVIDADE FUNCIONAL

Os monócitos são membros do sistema fagocitário mononuclear, uma família de células de origem mieloide que compreende monócitos ou diferenciação destes em células dendríticas (DCs) e/ou macrófagos (JAKUBZICK; RANDOLPH; HENSON, 2017, SHI; PAMER, 2011). Os monócitos são definidos como células sanguíneas circulantes que constituem aproximadamente 10% dos leucócitos periféricos em humanos (YONA; JUNG, 2010). Após o nascimento, os monócitos derivam principalmente de precursores hematológicos na medula óssea e entram na circulação sanguínea, onde são recrutados para os tecidos do corpo (JAKUBZICK; RANDOLPH; HENSON, 2017). A saída dos monócitos da circulação e a migração destas células para os tecidos inicia o processo de diferenciação celular, onde células dendríticas e macrófagos são definidas como “células derivadas de monócitos” (RANDOLPH; JAKUBZICK; QU, 2008).

Os monócitos são conhecidos como um grupo celular heterogêneo que se subdivide em subpopulações/subgrupos com características e funções distintas (GINHOUX; GUILLIAMS, 2016; VLACIL *et al.*, 2019). Essas subpopulações foram diferenciadas por suas propriedades fenotípicas e funções (VLACIL *et al.*, 2019). Durante a homeostase e em condições patológicas, cada subpopulação pode desempenhar um papel diferente. A heterogeneidade dos monócitos contribui para a compreensão da patogênese da inflamação e ser biomarcador de doenças (WONG et al.,2012). Desse modo, as subpopulações de monócitos estão subdivididas em três subgrupos, com base na expressão diferencial de receptores CD14 e CD16 (ZIEGLER-HEITBROCK *et al.* 2010), conforme demonstrado na figura 3. Esses subgrupos são denominados como monócitos Clássicos ($CD14^{++}CD16^{-}$), Intermediários ($CD14^{++}CD16^{+}$) e Não clássicos ($CD14^{+}CD16^{++}$) (ZIEGLER-HEITBROCK *et al.* 2010). As diferenças na expressão de citocinas, moléculas de adesão e receptores de quimiocinas indicam que os subtipos de monócitos são funcionalmente distintos (ILDZKOWSKA et al., 2015), sugerindo que os subtipos podem representar diferentes estágios na sequência de diferenciação celular (YONA et al., 2013).

Figura 3 - Subpopulação de monócitos humanos e expressão diferencial dos marcadores de superfície CD14 e CD16.

Fonte: Modificada de Ziegler-Heitbrock (2010).

As células de linhagem monocítica são fundamentais para imunidade humoral e celular, à medida que são importantes fagócitos que atuam na apresentação de antígeno às células T e produtoras de citocinas reguladoras como TNF-α, IL-1, IL-6, INF α/β que são importantes para a hematopoese (BELGE *et al.*, 2002; DALE; BOXER; LILES, 2008). As funções de fagocitose, apresentadores de antígenos e imunomodulação tornam as células mononucleares fundamentais para coordenação e regulação da resposta imune (DALE; BOXER; LILES, 2008). Além disso, a produção de citocinas pelos monócitos e macrófagos estão vinculadas à ativação do receptor Toll-like, esse receptor tem a função de promover a cascata de sinalização que provoca a síntese de citocinas e regula a resposta imune por meio da ativação celular (BELGE *et al.*, 2002).

Ademais, monócitos são células altamente plásticas que podem se diferenciar de acordo com a microbiota (NAHRENDORF *et al*., 2007; YANG *et al*., 2014), assim tem papel fundamental na inflamação e pode apresentar atividades prejudiciais e benéficas em diferentes condições. Diante disso, os subgrupos de monócitos humanos estão basicamente alicerçados nas condições inflamatórias induzidas pela expressão dos receptores superficiais das células monocíticas (FRANÇA *et al*., 2017).

A função microbicida elevada é comumente atribuída aos monócitos clássicos, enquanto que as funções de monócitos não clássicos são menos definidas até o momento (CROS et al., 2010). Monócitos Clássicos ($CD14^{++}CD16^{-}$) são considerados células inflamatórias, que expressam altos níveis de CCR2 (receptor de quimiocina da

proteína quimioatrativa de monócitos1, MCP -1). Essas células são recrutadas da medula óssea e dos reservatórios esplênicos para locais de inflamação ativa e respondem a diferentes estímulos gerados por tecidos danificados e/ou infectados, produzindo citocinas inflamatórias, como interleucina (IL-1, IL-12 e necrose tumoral fator-α (TNF-α) (MEISNER; SONG; PRICE, 2012; SWIRSKI, 2011). Já monócitos não clássicos ($CD14^{+}CD16^{++}$) apresentam baixa expressão de CCR2 (AUFFRAY *et al.*, 2007; CARLIN *et al.*, 2013) e são os produtores primários de TNF-α e IL-1β, além de serem células com maior atividade de patrulhamento de tecidos, migração e estarem envolvidos na sobrevivência e reparo tecidual (CROS et al., 2010; AUFFRAY et al., 2007). Esse subconjunto tem uma presença intravascular e tecidual estendida (AUFFRAY *et al.*, 2007)

Monócitos intermediários ($CD14^{++}CD16^{+}$) compartilham características fenotípicas e funcionais de Monócitos Clássicos e Não clássicos e estão relacionados na produção de Espécie Reativa de Oxigênio (ROS) (FRANÇA *et al*., 2017), mediadores pró-inflamatórios como TNF-α e IL-1β, como também produzem citocinas anti-inflamatórias IL-10 (MERINO *et al.*, 2011), e expressam, em células endoteliais, marcadores de angiogênese (SCHAUER *et al*., 2014). Neste sentido, monócitos intermediários estão principalmente envolvidos no processamento e apresentação de antígenos, possuindo alta expressão de MHC classe II além de uma alta capacidade de ativar a imunidade adaptativa e indução da capacidade proliferativa de linfócitos (ZAWADA et al., 2011). Desse modo, a proliferação e ativação de monócitos/macrófagos é o eixo mais significativo no início da imunopatogênese de uma variedade de infecções e processos inflamatórios (RITTIG *et al*., 2018).

Estudos recentes correlacionaram a extensão e gravidade da patogênese da COVID-19 à ativação desregulada do compartimento dos fagócitos mononucleares (MERAD *et al.*, 2022) e a incapacidade de montar uma resposta imune eficaz contra o novo coronavírus (MERAD *et al*., 2022). Estudos sobre infecções virais, a exemplo dos vírus Influenza, Chikungunya, herpes humano e Zika identificaram, que após a infecção viral, os monócitos respondem com expressão elevada de moléculas sinalizadoras pró-inflamatórias e respostas antivirais (NIKITINA *et al*., 2018). Curiosamente, novos achados, em amostras de líquidos broncoalveolar (LBA) de pacientes COVID-19 graves, detectaram alta expressão de CCL2 e CCL7, quimiocinas recrutadoras de monócitos CCR2+, o que significa diferenciação de monócitos de

fenótipo inflamatórias (ZHOU *et al*., 2020), somado à depleção de macrófagos alveolares residentes em tecido e abundância de macrófagos derivados de monócitos inflamatórios nesses pacientes (LIAO et *al*., 2020). Monócitos clássicos são uma fonte significativa de IL-6 e outras citocinas inflamatórias (CHEVRIER *et al*., 2020; ZHOU *et al*., 2020) que justificam a quadro de hiperinflação, característico da infecção pelo SARS-CoV-2, e contribuindo para patogênese da pneumonia, tempestade de citocinas e o desenvolvimento de SDRA na COVID-19.

Pacientes COVID-19 graves apresentaram disfunção e esgotamento de células NK e células T efetoras no sangue periféricos, macrófagos alveolares, também estão em estado severo de esgotamento nos pulmões e células dendríticas estão reduzidas no sangue e pulmões desses pacientes (MERAD *et al*., 2022). Curiosamente, manifestações extrapulmonares como disbiose intestinal pode estar associado a quadros graves da COVID-19 (ZHAO *et al.*, 2022), de forma que a integridade da barreira intestinal pode ser comprometida, pois células imunes congênitas e adaptativas podem apresentar perfil inflamatório, como monócitos clássicos, e ativar e secretar citocinas pró-inflamatórias para o sistema circulatório, resultando em inflamação sistêmica (ZHANG *et al.*, 2020), assim, justifica achados sobre a indução do processo de translocação microbiana na presença de perfis inflamatórios em pacientes com COVID-19.

2.7 COVID-19 GRAVE E IMUNOSSUPRESSÃO OU ESTADO INFLAMATÓRIO

A COVID-19 pode resultar em doença grave caracterizada por uma imunopatologia significativa, que é estimulada por uma resposta imune inata excessiva e desregulada, somado a uma resposta adaptativa pobre (LOWERY; SARIOL; PERLMAN, 2021). Respostas imunes limitadas e retardadas de interferon I (IFN-I) e IFN-III resultam na rápida replicação do vírus SARS-CoV-2, no recrutamento de monócitos/macrófagos inflamatórios para os locais de infecção (GALANI *et al*., 2021; LOWERY; SARIOL; PERLMAN, 2021), na produção exacerbada de citocinas pró-inflamatórias e extensos infiltrados celulares no trato respiratório, dessa forma, caracterizando a infecção pelo novo coronavírus, em uma patologia inicialmente pulmonar (LOWERY; SARIOL; PERLMAN, 2021).

Curiosamente a interleucina (IL) -10, tipicamente classificada como citocina anti-inflamatória e imunossupressora, os efeitos da IL-10 são dependentes do contexto e existem vários cenários em que a IL-10 aumenta a ativação e proliferação de células imunes causando a liberação de citocinas pró-inflamatórias. (ISLAM *et al*., 2021). Estudos recentes indicam que, no contexto, a infecção pelo SARS-CoV-2 a IL-10 apresenta um comportamento ambíguo, ou seja, níveis aumentados de IL-10 podem ser interpretados como uma tentativa de moderar a hiperinflamação e prevenir danos nos tecidos ou até mesmo indicar falha na supressão da hiperinflamação e predispor a desfechos clínicos mais graves na COVID-19 (ISLAM *et al*., 2021; ANTONIV; IVASHKIV, 2006).

Níveis elevados de IL-10 e citocinas pró-inflamatórias, incluindo interleucina (IL) -6, IL-1 e fator de necrose tumoral (TNF) -α foram associados aos casos graves da infecção por SARS-CoV-2 (CHEN *et al*., 2020; HUANG *et al*., 2020; TAY *et al*., 2020). Estudos recentes identificaram que as principais causas de morte por COVID-19 incluem insuficiência respiratória e desenvolvimento de sepse, condição que tem sido observada em muitos pacientes falecidos (GIAMARELLOS-BOURBOULIS *et al*., 2020). Diante disso, estudos vinculam resposta imune exacerbada mediada pela elevada produção de citocinas pró-inflamatória a lesão pulmonar, lesão de múltiplos órgãos e um estado pró-coagulante em pacientes COVID-19 (TAY *et al*., 2020). Em contrapartida, complicações geradas pela infecção por SARS-CoV-2 podem estar associadas à condição de imunossupressão (REMY *et al*., 2020), uma superprodução de citocinas inflamatórias pode gerar um estado de colapso imunológico e inflamação estéril que predispõe o hospedeiro a uma infecção secundária (COLANTUONI *et al*., 2020). Esse colapso da imunidade inata do hospedeiro se manifesta como uma falha no controle da replicação e disseminação viral desenfreada com citotoxicidade direta no hospedeiro (REMY *et al*., 2020). O suporte para esta teoria contrastante é baseado na linfopenia progressiva e profunda observada, muitas vezes, em pacientes infectados por HIV (SERETI *et al*., 2009). Evidências recentes mostram que a linfopenia é incessante em pacientes criticamente doentes com COVID-19 e se correlaciona com o aumento de infecções secundárias, quadros de sepse, choque séptico e morte (HUANG et al., 2020; WANG *et al*., 2020). Estudos recentes de revisão sistemáticas que avaliaram a carga de coinfecções em pacientes infectados pelo SARS-COV-2 identificaram que, em geral, 7% dos pacientes hospitalizados graves

tiveram coinfecção secundária aumentada para 14.3% de infecções bacterianas, virais e fúngicas, entre os agentes oportunistas estão: as bactérias M.pneumoniae, P.aeruginosa, H.influenzae e K.pneumoniae, os vírus Sincicial Respiratório e Influenza A e os fungos Candida albicans, Aspergillus fumigatus e flavus, os quais geram piora no prognóstico desses pacientes (LANBURY et al., 2020 ; SARKAR et al., 2021; MOSER et al., 2021). Ademais, estudos com ensaios *ex vivo* de superinfecções fúngicas, candidíase associada ao COVID-19, identificaram que a liberação de citocinas e expressão de fenótipos de leucócitos dos pacientes COVID-19 grave estão reduzidas na presença de coinfecções secundárias (MOSER et al., 2021).

A sepse é caracterizada como uma disfunção orgânica com risco de vida causada por uma resposta desregulada do hospedeiro à infecção (VENET; MONNERET, 2018). As manifestações clínicas na sepse são bem definidas e estão associadas às condições de febre, tempestade citocinas, disfunção de múltiplos órgãos, trombocitopenia, microtrombose vascular, coagulopatia, falência respiratória, leucopenia, hipotensão e aumento da predisposição a infecções oportunistas (OLWAL *et al*., 2021). Curiosamente, essas condições clínicas também são observadas em pacientes graves infectados pelo SARS-CoV-2. No entanto, a sepse é caracterizada por um desequilíbrio entre as respostas pró e anti-inflamatórias (CZAIKOSKI *et al.*, 2016), em que suas principais respostas pró-inflamatórias incluem a ativação do sistema complemento, do sistema de coagulação, do endotélio vascular, neutrófilos e plaquetas, enquanto a imunossupressão é causada principalmente pela reprogramação das células apresentadoras de antígenos e pela apoptose e exaustão de linfócitos (POLL *et al.*, 2017).

A persistência de patógenos circulantes, após a complexas interações de resposta imune, pode induzir o quadro de sepse. A intensa produção de mediadores pró-inflamatórios pode resultar em tempestade de citocinas, que desregula a resposta imune e ativa distúrbios inflamatórios patológicos (CHOUSTERMAN; SWIRSKI; WEBER, 2017). Ademais, o estado anti-inflamatório e/ou imunossupressivo induzido pela sepse envolve tanto sistema imune adaptativo quanto o inato, é caracterizado pelo processo de apoptose celular que marca a depleção de células imunes, incluindo células T CD4+ e CD8+, células B e células dendríticas (HOTCHKISS; MONNERET; PAYEN, 2013), resultando na linfopenia. Além disso, é observada a redução da expressão de moléculas do Antígeno Leucocitário Humano (HLA) - DR nos monócitos do sangue, regulação positiva das citocinas anti-inflamatórias (IL-10) e dificuldade de

produzir citocinas pró-inflamatórias, que causa imunoparalisia (POLL *et al.*, 2017) e predispõe à invasão de patógenos oportunistas (CHANG, 2019). Segundo Hotchkiss e colaboradores, 2013, em seus estudos post-mortem hipotenizaram que a sepse prolongada é predominantemente caracterizada por imunossupressão sistêmica, que leva a uma falha na erradicação de infecções primárias e à aquisição de infecções secundárias letais. É possível que a imunossupressão, e não a hiperinflamação, seja a força motriz predominante para a morbidade e mortalidade na sepse. Desse modo, os quadros de sepse observados em pacientes graves com COVID-19 podem ser vinculados à sobreposição do estado de imunossupressão, ao contrário da hiperinflamação, e se relacionar como causa de gravidade e morte desses pacientes.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar os marcadores de translocação microbiana, resposta inflamatória e fenótipo de monócitos em pacientes com diferentes graus de COVID-19.

3.2 OBJETIVOS ESPECÍFICOS

3.2.1 Objetivos específicos do artigo 1

- Quantificar concentrações de Lipopolissacarídeos (LPS) e CD14 solúvel (sCD14) em amostras de plasmas de pacientes saudáveis e pacientes COVID-19 grave, sobreviventes e não sobreviventes.
- Avaliar o nível sistêmico de citocinas e quimiocinas: IL-6, IL-10, TNF-α, TGF-β, IFN-γ, CCL2/MCP-1, CCL4/MIP-1β e CCL5/RANTES em amostras de plasma de pacientes COVID-19 grave, sobreviventes e não sobreviventes.
- Avaliar o efeito do plasma de pacientes COVID-19 grave, sobreviventes e não sobreviventes sobre o fenótipo de células THP-1 in vitro.

Para responder a estes objetivos, a coleta do sangue periférico de indivíduos saudáveis foi realizada uma única vez e dos pacientes COVID-19 grave sobreviventes e não sobreviventes, foi realizada em dois períodos. A primeira coleta dos pacientes COVID-19 grave foi efetuada após a internação (T1) e a segunda antes da alta hospitalar (T2: 0 a 72 horas antes da alta hospitalar ou óbito). As amostras de plasma

foram obtidas por centrifugação (2.000g, 10 minutos). A quantificação das concentrações de LPS foi obtida por espectrometria de massa acoplada a HPLC (LC-MS). A produção de citocinas e quimiocinas, IL-6, IL-10, TNF-α, TGF-β, IFN-γ, CCL2/MCP-1, CCL4/MIP-1β e CCL5/RANTES, bem como, os níveis de sCD14 foram avaliados pelo método de ELISA. Para avaliar o efeito de fatores solúveis plasmáticos de pacientes com COVID-19 grave na modulação de monócitos, células da linhagem THP1 foram cultivadas em meio de cultura com RPMI mais soro fetal bovino a 10% ou o plasma obtido dos pacientes e o fenotípico das células THP1 foi analisado por citometria de fluxo utilizando os anticorpos monoclonais antiTLR-4, anti-CD16, anti-CD69, anti-CCR2, anti-CCR5, anti-CCR7, anti-CD14 e anti-HLA-DR.

3.2.2 Objetivos específicos do artigo 2

- Avaliar a expressão do receptor Toll-like 4 (TLR-4) e ativação do Fator de Transcrição (NF-kB) em monócitos $CD14^{+}HLA\text{-}DR^{low}$ em amostras sangue periférico de pacientes COVID-19 leve e grave não estimulados e estimulados por LPS em duas concentrações diferentes (1ng/mL e 10ng/mL).
- Avaliar a produção de citocinas e quimiocinas: IL-1β, IL-6, IL-10, PGE-2, CCL-2 e TNF-α em amostras de plasma de pacientes COVID-19 leve e grave não estimulados e estimulados por LPS em duas concentrações diferentes (1ng/mL e 10ng/mL).
- Quantificar concentrações de Lipopolissacarídeos (LPS) e CD14 solúvel (sCD14) em amostras de plasmas de pacientes COVID-19 leve e grave não estimulados.

Para responder a estes objetivos, a coleta de sangue dos indivíduos saudáveis, pacientes COVID-19 leve e grave foram realizadas uma única vez. Amostras de pacientes graves foram coletas até 6 horas a admissão hospitalar. As amostras de plasma foram obtidas por centrifugação (2.000g, 10 minutos). A quantificação de citocinas e quimiocinas IL-6, IL-10, CCL-2 TNF-α foram realizadas por ensaios multiplex usando a tecnologia Luminex e seguindo as recomendações do fabricante (MILLIPLEX MAP Human Cytokine/Chemokine, Millipore, EUA). Já CD14 solúvel, IL-1β e PGE-2 foram analisados por Kit de ELISA seguindo as recomendações dos fabricantes. A quantificação das concentrações de LPS foram obtidas por

espectrometria de massa acoplada a HPLC (LC-MS). A expressão do receptor Toll-like 4 (TLR-4), ativação do Fator de Transcrição (NF-kB) e fenótipo de monócitos $CD14^{+}HLA\text{-}DR^{low}$ foram analisados por citometria de fluxo utilizando os anticorpos monoclonais anti-CD14, HLA-DR, NF-κBp65 e TLR-4.

4 CAPÍTULO II

4.1 ARTIGO ORIGINAL - INCREASED LPS LEVELS COEXIST WITH SYSTEMIC INFLAMMATION AND RESULT IN MONOCYTE ACTIVATION IN SEVERE COVID-19 PATIENTS

Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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Increased LPS levels coexist with systemic inflammation and result in monocyte activation in severe COVID-19 patients

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ABSTRACT

Mucosal barrier alterations may play a role in the pathogenesis of several diseases, including COVID-19. In this study we evaluate the association between bacterial translocation markers and systemic inflammation at the earliest time-point after hospitalization and at the last 72 h of hospitalization in survivors and non-survivors COVID-19 patients. Sixty-six SARS-CoV-2 RT-PCR positive patients and nine non-COVID-19 pneumonia controls were admitted in this study. Blood samples were collected at hospital admission (T1) (Controls and COVID-19 patients) and 0–72 h before hospital discharge (T2, alive or dead) to analyze systemic cytokines and chemokines, lipopolysaccharide (LPS) concentrations and soluble CD14 (sCD14) levels. THP-1 human monocytic cell line was incubated with plasma from survivors and non-survivors COVID-19 patients and their phenotype, activation status, TLR4, and chemokine receptors were analyzed by flow cytometry. COVID-19 patients presented higher IL-6, IFN-γ, TNF-α, TGF-β1, CCL2/MCP-1, CCL4/MIP-1β, and CCL5/RANTES levels than controls. Moreover, LPS and sCD14 were higher at hospital admission in SARS-CoV-2-infected patients. Non-survivors COVID-19 patients had increased LPS levels concomitant with higher IL-6, TNF-α, CCL2/MCP-1, and CCL5/RANTES levels at T2. Increased expression of CD16 and CCR5 were identified in THP-1 cells incubated with the plasma of survivor patients obtained at T2. The incubation of THP-1 with T2 plasma of non-survivors COVID-19 leads to higher TLR4, CCR2, CCR5, CCR7, and CD69 expression. In conclusion, the coexistence of increased microbial translocation and hyperinflammation in patients with severe COVID-19 may lead to higher monocyte activation, which may be associated with worsening outcomes, such as death.

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection causes Coronavirus disease 2019 (COVID-19), a disease with diverse clinical manifestations [1]. While most COVID-19 cases are asymptomatic or mild, the severe form of COVID-19 can occur with detrimental manifestations such as acute respiratory distress syndrome (ARDS), multi-organ failure, and death [2]. The COVID-19 immunopathology is mainly characterized by a hyperinflammatory state, strong innate cells response, and lymphocyte activation with an exhausted profile [3,4].

The mechanism underlying the inflammatory state of COVID-19 is

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not fully understood at this time. Although the respiratory tract is the main site of infection for COVID-19, the pathogenesis of the disease can also involve different organs and systems, such as the heart, kidneys, intestines, vasculature, and liver [3]. In the gastrointestinal (GI) tract, symptoms such as diarrhea and abdominal distention have been reported in several patients [5] and enterocytes in the ileum and colon express the angiotensin-converting enzyme 2 (ACE-2) receptor and may act as a site for SARS-CoV-2 entrance and predispose to enteric infection [6]. Furthermore, the transmembrane serine protease 2 (TMPRSS2), also highly expressed in enterocytes, is able to prime the spike protein of SARS-CoV-2 and mediate the virus entry into cells [7]. Additionally, RNA viral particles were detected in stool samples, arising the possibility that bacterial translocation and microbial products from the GI tract to the peripheral blood might contribute to the hyperinflammatory state and COVID-19 severity [8]. The gut and lungs are anatomically distinct but the proximal communication and complex pathways involving their respective microbiota indicate the existence of a gut-lung axis. In this sense, airways infections by viruses and bacteria are accompanied by alterations in the composition of intestinal microbiota, increased GI symptoms, gut dysfunction and barrier disruption [8]. Ahlawat and Sharma [9] and others [10–12] proposed a gut-lung axis during the SARS-CoV-2 infection through the hyperactive host immune system releasing higher levels of inflammatory cytokines with consequent lung hyper-permeability, gut infection, and disruption of the intestinal permeability that leads to the leakage of gut microbes and associated metabolites into circulation. Interestingly, disruption in gut barriers and microbial translocation is more likely to occur in older individuals and those with chronic diseases, which contribute to the exacerbation of systemic inflammation by activating innate immune cells, mainly monocytes and neutrophils [10].

Lipopolysaccharide (LPS) is a product derived from the membrane of gram-negative bacteria that acts as a potent immune-activating stimulus when recognized by innate immune cells expressing toll-like receptor 4 (TLR4) [13]. Then, innate immune cells become activated and produce high amounts of proinflammatory mediators including cytokines, such as interleukin (IL)-6 and tumor necrosis factor-alpha (TNF-α) and chemokines (i.e., CCL2/MCP-1 and CCL5/RANTES) [14]. In this sense, peripheral blood LPS is a well-recognized marker of translocation of bacterial products after events that compromise the integrity of the intestinal mucosa [15,16]. Moreover, soluble CD14 (sCD14) is released after cleavage from the membrane form (mCD14) on the surface of monocytes through exposure to the LPS stimulation and is considered a marker of microbial translocation [16,17].

There is emerging evidence indicates that severe SARS-CoV-2 infection induces disruption in the gut barrier contributing to the systemic spread of bacteria and microbial products which affect the host's response to the infection [11,18,19]. Mechanistically, it was proposed that SARS-CoV-2 infects enterocytes and causes microbial translocation through transcellular permeability and paracellular pathway using the tight junctions. Additionally, viral infection and replication culminate in the activation of innate and adaptive immunity that induces intestinal damage and increase the intestinal permeability precipitating intestinal barrier failure and bacterial translocation, which in turn auto-fuel vicious cycles of systemic inflammation and tissue damage [18,19]. However, the time course of the presence of microbial product in the peripheral blood of COVID-19 patients during hospitalization remains unknown. Here, we investigated the association between microbial translocation markers and systemic inflammation in the hospital admission and at the discharge time from the hospital in survivors and non-survivors COVID-19 patients. We hypothesized that microbial translocation markers are associated with systemic inflammation and could contribute to the death of hospitalized COVID-19 patients.

2. Methods

2.1. Study population

Hospitalized COVID-19 patients with confirmed SARS-CoV-2 RT-PCR positive test on nasopharyngeal specimens (n = 66) were consecutively recruited after admission in the COVID-19 Unit of Hospital São Camilo (Esteio/RS, Brazil) between June/2020 and December/2020 to a clinical cohort study. The study was approved by the UFCSPA Ethics Committee (CAAE: 38886220.0.0000). Informed consent was obtained from those legally responsible for the patients after the research objectives and procedures were explained. The authors signed an agreement to preserve patient and staff anonymity related to the use of this data. All patients were diagnosed with COVID-19 infection according to the World Health Organization interim guidance and Diagnosis and Treatment Guideline for Novel Coronavirus Pneumonia and were positive for at least two nucleic acid tests for SARS-CoV-2 [20].

Clinical and socio-demographic data were collected from the patient's electronic medical records upon admission to the unit. Blood samples from 9 age- and sex-matched SARS-CoV-2 RT-PCR negative controls admitted to hospital with pneumonia were also obtained.

2.2. Blood collection and laboratory analysis

Blood samples were collected from the controls (n = 9) and all COVID-19 patients (n = 66) from the antecubital vein into 4 mL tubes with EDTA as anti-coagulant at the earliest time-point after hospital admission (T1). Additional blood samples were collected from 14 survivors and 12 non-survivors COVID-19 patients at the discharge time from the hospital (T2: 0 to 72 h before leaving hospital or death). Plasma samples were obtained by centrifugation (2000g, 10 min), aliquoted and immediately kept at −80 °C until analysis.

Plasma levels of IL-6, IL-10, TNF-α, TGF-β1 (all from Invitrogen Life Sciences, USA), IFN-γ, CCL2/MCP-1, CCL4/MIP-1β, and CCL5/RANTES (all from Peprotech, USA) were analyzed by *Enzyme Linked Immunosorbent Assay* (ELISA) following the manufacturer's procedure using a microplate reader (EzBiochrom, USA). The intra-assay coefficient of variability was <7.5%. The detection limits of each cytokine were: IL-6, 2–200 pg/mL; IL-10, 4–200 pg/mL; IFN-γ, 10–300 pg/mL; TNF-α, 2–200 pg/mL; TGF-β1, 2–500 pg/mL; CCL5/RANTES, 20–1000 pg/mL; CCL4/MIP-1β, 50–1500 pg/mL; CCL2/MCP-1, 50–1000 pg/mL. The soluble form of CD14 was analyzed using Human CD14 ELISA Kit from Invitrogen (USA), with detection limits ranging 8.23–8000 ng/mL.

2.3. Liquid chromatography-tandem mass spectrometry determination of LPS

LPS concentration was determined by quantitation of 3-hydroxytetradecanoic acid as described by Pais de Barros and colleagues [21]. Briefly, 150 μL of plasma was hydrolyzed with 75 μL of NaCl 150 mM, 300 μL of HCl 8 mol for 4 h at 90 °C and extracted with 4 mL of a hexane. Samples were dried under a nitrogen stream and resuspended in 50 μL of methanol immediately prior to the injection in the analytical system. A Nexera UFLC system coupled to a LCMS-8040 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan) was used for the analysis. The electrospray parameters were set in the negative ion mode ($[M-H]^-$) as follows: capillary voltage, 3000 V; desolvation line temperature, 250 °C; heating block temperature, 500 °C; drying gas, 18/L min; and nebulizing gas, 2 L/min. Collision-induced dissociation was obtained with 230 kPa argon pressure. Analyses were carried out with multiple reaction monitoring (MRM) by using the following fragmentation: m/z 243.1 → m/z 59.0. The chromatographic separation was conducted with an Acquity UPLC® C18 column (2.1 × 50 mm, 1.7 μm particle size) (Waters Corporation, Ireland). The analyses were performed in gradient elution mode with a flow rate of 0.4 mL/min and the gradient mobile phase system consisted of water (solvent A) and acetonitrile (solvent B) both

fortified with 0.2% acetic acid as follow: 0–1.5 min, 75–100% of B; 1.5–2.0 min, 100% of B; 2.0–2.1 min, 100–75% of B; 2.1–5.5 min, 75% B. The column oven was kept at 50 °C. The data were processed using LabSolutions software (Shimadzu, Kyoto, Japan). Calibration curves were constructed at intervals of 0.2–1000 ng/mL.

2.4. Cell culture and flow cytometry

THP-1 human monocyte cells (ATCC TIB-202) (2×10^5 cells/well) were cultured in RPMI media (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin and streptomycin (both from Sigma-Aldrich) in 96 well plates for 24 h. Afterwards, the media plus FBS were removed; cells were washed with phosphate-saline buffer and incubated with RPMI supplemented with 10% serum of survivors and non-survivors COVID-19 patients for 15 h. Then, cells were labelled with monoclonal antibodies (all anti-human) conjugated with specific fluorochromes: CD14 Fitc, CD16 Pe, TLR4 Pe, CD69 Percp-Cy5.5, CD192 (CCR2) Pe, CD195 (CCR5), CD197 (CCR7) Fitc, HLA-DR PercP-Cy5.5 (all from EbioScience, ThermoFisher, USA). 10.000 events were acquired using CELLQuest Pro Software (BD Bioscience, USA) on a FACSCalibur flow cytometer (BD Bioscience, USA) to determine cell phenotype. THP-1 were identified and gated according to each forward scatter (FSC) and side scatter (SSC) profile, and the mean fluorescence intensity (MFI) was evaluated.

2.5. Statistical analysis

Normality of data was checked by Kolmogorov-Smirnov, with values presented as mean ± 95 %CI for demographic and clinical characteristics and mean ± standard deviation (SD) for inflammatory mediators. Participant's characteristics and inflammatory analysis were between-groups compared through a one-way ANOVA followed by Bonferroni's post-hoc for multiple comparisons. The time-course of inflammatory response and immune variables were analyzed by a generalized linear mixed model. Correlation analyses between microbial translocation markers and inflammatory mediators were performed using Pearson's Coefficient of Correlation. P values ≤ 0.05 were considered statistically significant. The SPSS 22.0 (IBM Inc, EUA) software was used in all analyses.

Table 1
Clinical and demographic subject's characteristics.

		COVID-19		
	Controls (n = 9)	All (n = 66)	Survivors (n = 43)	Non-survivors (n = 23)
Age (years)	73.7 (65.2–82.2)	64.8 (60.3–69.3)	65.8 (59.5–72.1)	63.6 (56.6–70.6)
Gender (female/male, %)	33.3/66.6	47/53	46.5/53.5	47.8/52.2
Body mass (kg)	61.7 (38.2–85.2)	86.7 (79.5–94.0)[A]	79.7 (72.5–87.0)[A]	95.8 (82.4 –109.2)[A,B]
BMI (kg/m^2)	23.6 (12.4–34.8)	30.4 (27.8–33.1)[A]	28.7 (25.8–31.5)[A]	32.7 (27.7–37.8)[A,B]
Nutritional Status (%)				
Lean	50	17.9	22.7	11.8
Overweight	25	28.2	36.4	17.6
Obesity	25	53.8	40.9	70.6
Length of in-hospital stay (days)	18.8 (8.4–29.3)	21.1 (15.6–26.6)	21.6 (14.0–29.3)	20.2 (12.8–27.6)
SpO2 (%)	–	92.3 (89–8–94.7)	94.2 (92.6–95.9)	88.6 (82.3–94.9)
Oxygen use during hospitalization (%)	–	53.4	42.6	84.2
Intensive care unit admission (%)	77.8	84.8	81.4	91.3
Death (%)	–	34.8	–	100
Symptoms (%)				
Fever		74.2	80	72.7
Dyspnea		62.8	–	80
Cough		58.5	46.6	61.8
Body aches		22.8	60	16.3
Sore throat		22.8	26.6	21.8
Flu-like syndrome		15.7	40	18.8
Fatigue		11.4	13.3	16.3
Myalgia		8.5	20	9.0
Nausea or vomiting		8.5	20	5.4
Headache		8.5	20	5.4
Coryza		8.5	13.3	7.2
Diarrhea		7.1	13.3	7.2
Underlying medical condition (%)				
Hypertension		21.3	19	22.7
Diabetes Mellitus		18.7	16.7	18.2
Neurological diseases		14.7	19	4.5
Cardiovascular diseases		12	4.8	22.7[B]
Rheumatic diseases		1.3	2.4	–
Cancer		1.3	2.4	–
Chronic kidney disease		1.3	2.4	–
Asthma		1.3	2.4	–
Pharmacological treatment (%)				
Azithromycin		24.3	26.7	21.5
Corticosteroids		23.7	23.3	24.1
Ceftriaxone		7.7	5.8	10.1
Levofloxacine		5.9	5.8	6.3
Cefepime		5.9	7	5.1
Vancomycin		4.7	2.3	5.1

Data are presented as mean ± standard deviation.
A Denotes statistical difference compared to Controls ($p < 0.05$).
B Denotes statistical difference compared to COVID-19 Survivors ($p < 0.05$).

3. Results

3.1. Characteristics of the study population

Firstly, we analyzed the baseline characteristics and inflammatory mediators in controls and COVID-19 hospitalized patients. To this, 66 patients admitted at COVID-19 care unit due to COVID-19 complications were enrolled in this study. Additionally, 9 COVID-19 negative controls were analyzed. COVID-19 patients presented higher body mass and body mass index than controls ($p < 0.01$), and 53.8% of patients were classified as obese. The mean length of stay in the hospital was 21.1 (15.6–26.6) days for COVID-19 patients, 84.8% of them were admitted to the intensive care unit (ICU) due to COVID-19 complications, and 34.8% patients died due to complications of SARS-CoV-2 infection. Forty-three [43) patients survived to SARS-CoV-2 infection and were classified as COVID-19 survivors. Patients and control data are shown in Table 1.

Interestingly, COVID-19 non-survivors ($n = 23$, 30.7% of COVID-19 patients) were heavier than controls (body mass, $p = 0.01$; BMI, $p = 0.01$) and COVID-19 survivors ($n = 43$, 57.3% COVID-19 patients) (body mass, $p = 0.03$; BMI, $p = 0.02$), but no difference between groups was found in the length of stay in the hospital ($p > 0.05$). COVID-19 non-survivors required more oxygen use during hospitalization than COVID-19 survivors ($p = 0.002$), but similar rates of ICU admission were observed ($p > 0.05$). The most reported symptoms by COVID-19 patients were fever (74.2%), dyspnea (62.8%), and cough (58.5%). Furthermore, 21.7% of COVID-19 patients were diagnosed with hypertension, 18.7% with diabetes mellitus 2, 14.7% with some neurologic disease, and 12% with some cardiovascular disease. Regarding pharmacological treatment, azithromycin (24.3%) and corticosteroids therapy (23.7%) were the predominant medicine adopted by medical staff during hospitalization. There was no difference between groups regarding symptoms, comorbidities, or pharmacological therapy during hospitalization ($p < 0.05$), unless the prevalence of cardiovascular diseases (COVID-19 survivors, 4.8%; COVID-19 non-survivors, 22.7%; $p = 0.04$).

Table 2
Systemic inflammatory mediators in survivors and non-survivors COVID-19 hospitalized patients.

		COVID-19		
	Controls (n = 9)	All (n = 66)	Survivors (n = 43)	Non-survivors (n = 23)
Monocytes (cells/mm^3)	640.7 ± 308.3	592.7 ± 380.7	685.9 ± 396.3	418.3 ± 282.2[B]
IL-6, pg/mL	18.7 ± 6.7	36.5 ± 18.7 [A]	36.3 ± 22.0 [A]	36.9 ± 10.7 [A]
IL-10, pg/mL	42.8 ± 22.4	37.1 ± 16.5	38.2 ± 17.8	35.0 ± 13.7
IFN-γ, pg/mL	29.5 ± 13.2	38.3 ± 12.5 [A]	36.7 ± 10.5	41.3 ± 15.2 [A]
TNF-α, pg/mL	11.2 ± 5.1	16.3 ± 7.3 [A]	14.7 ± 6.4	19.5 ± 8.0 [A, B]
TGF-β1, pg/mL	137.7 ± 94.7	80.8 ± 48.0 [A]	78.6 ± 53.1 [A]	84.8 ± 37.2 [A]
CCL2, pg/mL	438.7 ± 175.7	538.4 ± 118.3 [A]	548.6 ± 116.5 [A]	519.9 ± 121.8 [A]
CCL4, pg/mL	359.7 ± 160.4	556.0 ± 197.0 [A]	597.7 ± 215.1 [A]	477.8 ± 128.8 [A,B]
CCL5, pg/mL	231.9 ± 68.0	378.2 ± 290.2 [A]	448.5 ± 336.7 [A]	246.6 ± 67.9

Data are presented as mean ± standard deviation. Statistical significance was determined using the one-way ANOVA test followed by Bonferroni's post-hoc.
A Denotes statistical difference compared to controls ($p < 0.05$).
B Denotes statistical difference compared to COVID-19 survivors ($p < 0.05$).

3.2. Systemic inflammatory mediators in survivors and non-survivors COVID-19 patients

COVID-19 patients presented a hyperinflammatory profile at hospital admission compared to controls as presented in Table 2. COVID-19 patients had increased systemic levels of IL-6 ($p < 0.01$), IFN-γ ($p < 0.01$), TNF-α ($p < 0.01$), CCL5/RANTES ($p < 0.01$), CCL4/MIP-1β ($p < 0.01$), and CCL2/MCP-1 ($p < 0.01$) but diminished circulating levels of TGF-β1 ($p < 0.05$). When COVID-19 patients were stratified in survivors and non-survivors, both groups presented higher IL-6 ($p < 0.01$), CCL2/MCP-1 ($p < 0.01$), and CCL4/MIP-1β ($p < 0.01$) levels at hospital admission compared to controls.

However, only non-survivors had increased IFN-γ ($p < 0.01$) and TNF-α ($p < 0.05$) compared to controls, and higher TNF-α levels ($p < 0.05$) than survivors. Conversely, survivors had higher levels of CCL5/RANTES ($p < 0.01$) and CCL4/MIP-1β ($p < 0.05$) compared to control and non-survivors groups, respectively. Furthermore, reduced TGF-β1 levels were found in both survivors and non-survivors COVID-19 patients compared to controls ($p < 0.01$). Finally, a strong monocytopenia was observed in COVID-19 non-survivors compared to COVID-19 survivors ($p = 0.01$). These data indicate that COVID-19 patients have higher levels of inflammatory cytokines/chemokines at the hospital admission and higher TNF-α plasma levels that may be associated with death.

3.3. Increased markers of microbial translocation in plasma of COVID patients are associated with systemic inflammation

Markers of microbial translocation were found in the peripheral blood of COVID-19 hospitalized patients. In fact, COVID-19 subjects, independently of the outcome group, presented higher sCD14s ($p < 0.001$ for all comparisons) and LPS (controls vs. COVID-19 patients, $p < 0.001$; controls vs. COVID-19 survivors, $p < 0.001$; controls vs. COVID-19 non-survivors, $p < 0.05$) levels at time of hospital admission (Fig. 1). We examined the Pearson's Coefficient Correlation between inflammatory mediators, sCD14 and LPS for each participant. We highlight the significant correlations of sCD14 with TGF-β ($r = -0.46$; $p < 0.001$), CCL4/MIP-1β ($r = 0.46$; $p < 0.001$) and CCL2/MCP-1 ($r = 0.29$; $p = 0.03$). LPS was correlated with TNF-α ($r = -0.32$; $p < 0.004$), CCL5/RANTES ($r = 0.49$; $p < 0.001$), and CCL4/MIP-1β ($r = 0.27$; $p = 0.01$) (Fig. 2).

3.4. Systemic inflammatory cytokines and chemokines and microbial translocation markers in COVID-19 survivors and non-survivors at T1 and T2

The time course of systemic inflammatory markers and variables related to microbial translocation in 14 survivors and 12 non-survivors COVID-19 patients were evaluated in two times. Peripheral blood of patients was collected at hospital admission (T1) and at the discharge time from the hospital (T2: 0 to 72 h before leaving hospital or death). The time course of systemic inflammatory mediators was presented in Fig. 3. Increases in IFN-γ ($p < 0.01$) and TNF-α ($p < 0.05$) concentrations were identified in survivors COVID-19 patients at T2. Non-survivors COVID-19 patients presented increased levels of IL-6 ($p < 0.01$), TNF-α ($p < 0.05$), CCL5/RANTES ($p < 0.05$), and CCL4/MCP-1 ($p < 0.01$) at T2.

Non-survivor patients presented higher LPS levels in T2 compared to T1 ($p < 0.05$), but sCD14 only tended to increase in T2 ($p = 0.055$). On the other hand, no changes were identified in sCD14 and LPS at the end of hospitalization in COVID-19 survivors. Furthermore, the delta value (Δ, T2 - T1) of LPS levels positively correlated with Δ CCL2/MCP-1 levels ($r = 0.54$; $p = 0.008$) (Fig. 4). Together, these data indicate that the increase in LPS levels may be associated with death.

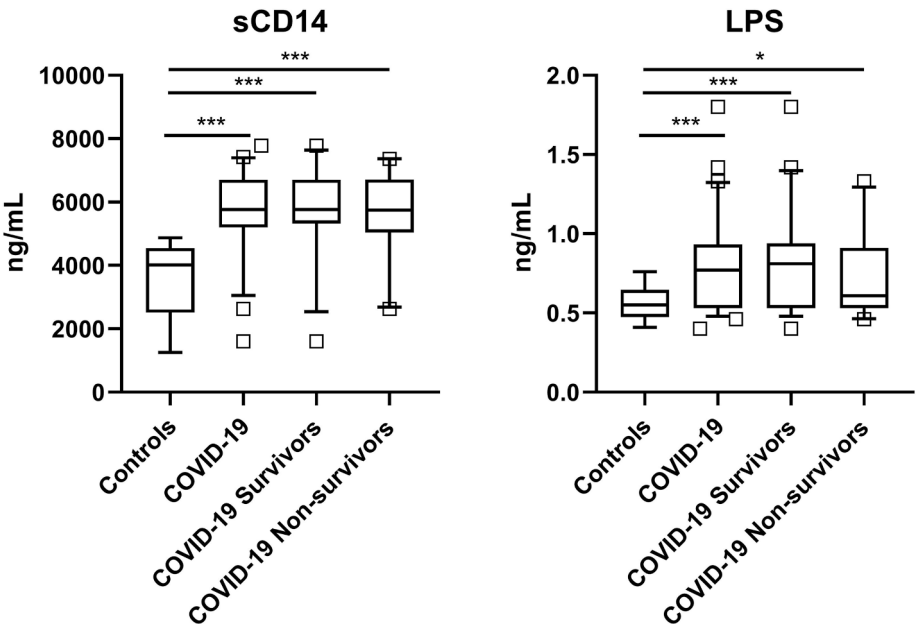

Fig. 1. Microbial translocation markers in plasma of COVID-19 patients at time of hospital admission. The systemic levels of soluble CD14 (A) and LPS (B) in controls (n = 9) and COVID-19 survivors (n = 43) and non-survivors (n = 23) patients were compared using one-way ANOVA followed by Bonferroni's post-hoc for multiple comparisons ($p < 0.05$). Box with 5–95 percentile data were used to show each data.* Indicates $p < 0.05$.*** Indicates $p < 0.001$.

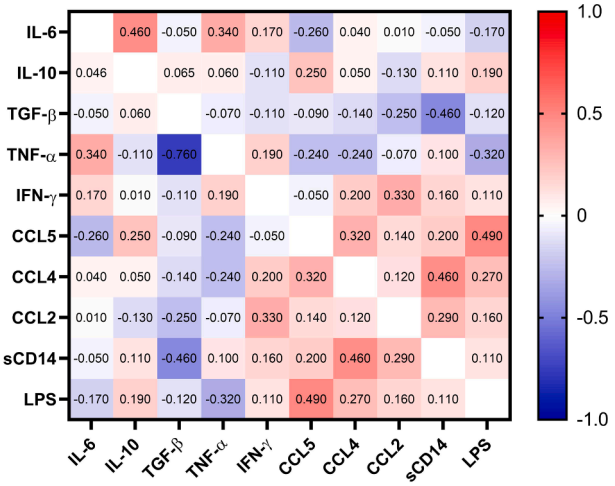

Fig. 2. Correlation heat map including IL-6, IL-10, TGF-β, TNF-α, IFN-γ, CCL2/MCP-1, CCL4/MIP-1β, CCL5/RANTES, sCD14 and LPS, at admission to the hospital. Pearson's correlation coefficients are plotted. Cells were colored according to the strength and trend of correlations (shades of red = positive, shades of blue = negative correlations). Statistical analysis performed through Pearson's coefficient correlation.

3.5. Effect of plasma from COVID survivors and non-survivors on THP-1 cell phenotype

As hospitalized non-survivors COVID-19 patients presented higher levels of microbial translocation markers with concomitant increase in some plasmatic inflammatory mediators, we hypothesized if the plasma from COVID-19 patients alters the phenotype of monocytes including subpopulation, and activation markers and TLR4 and chemokine receptors expression. To evaluate this hypothesis, we incubated THP-1 human monocyte lineage with plasma of survivors (n = 8) or non-survivors (n = 8) COVID-19 patients collected at hospital admission (T1) and at the end of hospitalization (T2) to evaluate the expression of TLR4, CD14, CD16, CD69, HLA-DR, CCR2, CCR5, and CCR7 (Fig. 5). Interestingly, plasma from COVID-19 non-survivor patients obtained at T2 increased the expression of TLR4 ($p = 0.03$), CCR2 ($p = 0.05$), CCR5 ($p = 0.02$) and CCR7 ($p = 0.02$) and the activation marker CD69 ($p = 0.02$) on the cell surface of THP-1 cells compared to T1 plasma from the same group. The expression of CD16 ($p = 0.04$) and CCR5 ($p = 0.03$) were higher in THP-1 cells incubated with plasma from COVID-19 survivor patients collected at T2. No statistical differences were found in CD14 and HLA-DR expression ($p < 0.05$). These data indicate that non-survival COVID-19 patients presented higher systemic levels of microbial translocation products and inflammatory mediators in the plasma that can increase activation/migration status of monocytes.

4. Discussion

Hospitalized COVID-19 patients display a broad spectrum of inflammatory responses that contributes to immune activation, disease severity, and the outcome of the disease [22]. In the present study, we hypothesized that microbial translocation markers could impact on inflammatory response in COVID-19 patients. Our main findings can be summarized as follows: a) higher levels of microbial translocation markers were found in COVID-19 patients on hospital admission; b) COVID-19 patients exhibited a proinflammatory status as shown by higher levels of IL-6, IFN-γ, TNF-α, CCL5/RANTES, CCL4/MIP-1β and CCL2/MCP-1; c) non-survivors COVID-19 patients showed significant increase in the amount of LPS and sCD14 during hospitalization associated with increased systemic levels of IL-6, TNF-α, CCL5/RANTES and CCL2/MCP-1.; d) survivors COVID-19 patients maintained LPS and sCD14 levels at stable values concomitant with increases in IFN-γ levels; e) the incubation of THP-1 cells with plasma of COVID-19 patients obtained at T2 increased TLR4, CCR2, CCR5, and CD69 expression on the cell surface; while THP-1 cells incubated with plasma of survivors at T2 presented higher CCR5 and CD16 expression. Together, our data indicate that increased microbial translocation coexists with the hyperinflammatory condition induced by SARS-CoV-2 infection and may lead to increased monocyte activation that may be associated with worse outcomes in severe COVID-19, such as death (Fig. 6).

Angiotensin-Converting Enzyme 2 (ACE-2), which is the most recognized receptor that binds to the Spike protein of SARS-CoV-2 and allows the host infection, is widely expressed in epithelial cells along

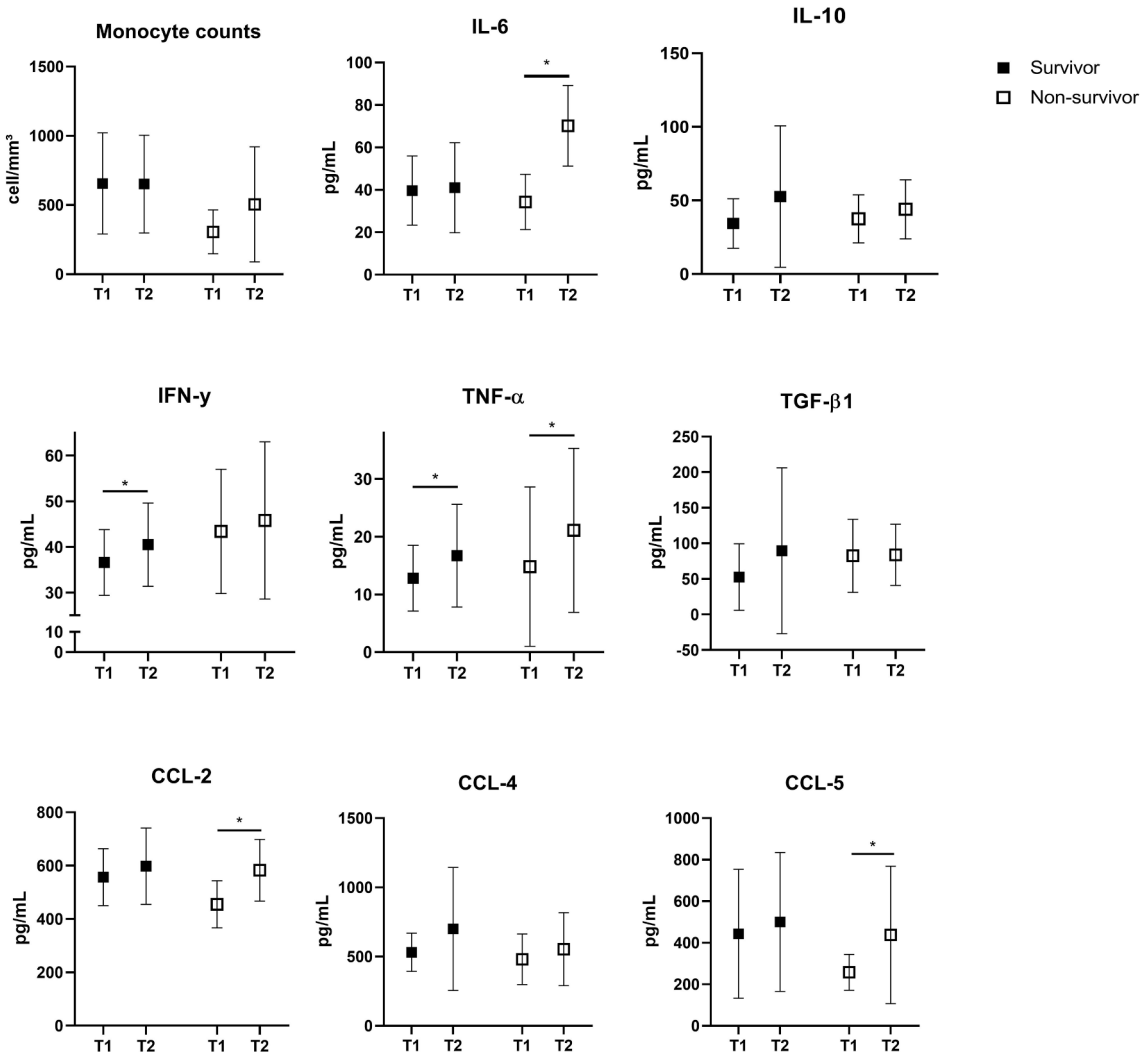

Fig. 3. The systemic levels of IL-6, IL-10, IFN-γ, TNF-α, TGF-β1, CCL5/RANTES, CCL4/MIP-1β, and CCL2/MCP-1 in survivors and non-survivors COVID-19 patients at the hospital admission (T1) and at the end of hospitalization (T2). Data presented as mean ± SD. Two-way ANOVA followed by Bonferroni's post hoc were applied to verify time and group effects ($p < 0.05$). * Indicates $p < 0.05$.

with the gastrointestinal (GI) tract [10,23]. Thus, SARS-CoV-2 can invade the intestinal tract causing GI dysfunction that disrupt intestinal barriers, increasing the translocation of microbial products [11,23]. Increased endotoxemia has been previously described in several pathological conditions associated with hospitalization, becoming a recognized marker of critical ill suffering from sepsis [24–26]. Furthermore, recent studies described disrupted gut barrier integrity and increased circulating bacteriome in moderate and severe COVID-19 patients [11,18,19].

In fact, probiotics, fecal microbiota transplantation and dietary therapies have been proposed to ameliorate barrier dysfunctions in patients with COVID-19 [7]. Here, we found increased LPS and sCD14 levels in the plasma of hospitalized COVID-19 patients at admission and during ICU hospitalization. Arunachalam and colleagues [22] found higher levels of bacterial DNA products, as measured by PCR quantification of 16S ribosomal RNA gene product, and LPS levels in severe ICU patients. The increased bacterial products in the peripheral circulation may contribute to the enhanced macrophage activation in infected tissue (i.e. lungs and gastrointestinal tissues) and to the failure in supressing the hyperinflammation during SARS-CoV-2 infection [9]. In fact, elevated levels of gut leakage markers were previously associated with inflammasome activation which leads to cardiac injury during the course of hospitalization in COVID-19 patients [19]. Corroborating these data, other studies show an important role for endotoxin and sCD14 in heart failure [27,28].

Microbial translocation to the peripheral blood also contributes to an early activation of monocytic cells. Indeed, we observed higher sCD14 in survivors and non-survivors COVID-19 patients. In line with this, Bowman and coworkers [29] found increased sCD14 levels in COVID-19 patients independent of the severity of disease. Furthermore, the authors revealed that COVID-19 critical ill patients who recovered or deceased had the highest sCD14 and lipopolysaccharide-binding protein (LBP, another marker of endotoxemia) values compared to the other groups [29]. These data highlighted the presence of strong activation of the monocytic lineage on hospital admission due to the SARS-CoV-2 infection in addition to the LPS translocation and inflammatory exacerbation in ICU patients [30]. In accordance with this study, systemic markers of monocyte activation, mainly sCD14 and sCD163, correlated with inflammatory cytokines in a non-intensive care unit COVID-19 cohort patients [31]. Giron and coworkers [32] found strong correlations between markers of tight junction permeability and microbial translocation with systemic inflammatory molecules, which corroborates our data.

It was previously postulated that perturbations in gut microbiota and microbial translocation may exacerbate the severity of hyperinflammation in COVID-19 [33]. Moreover, sCD14 tended to be higher

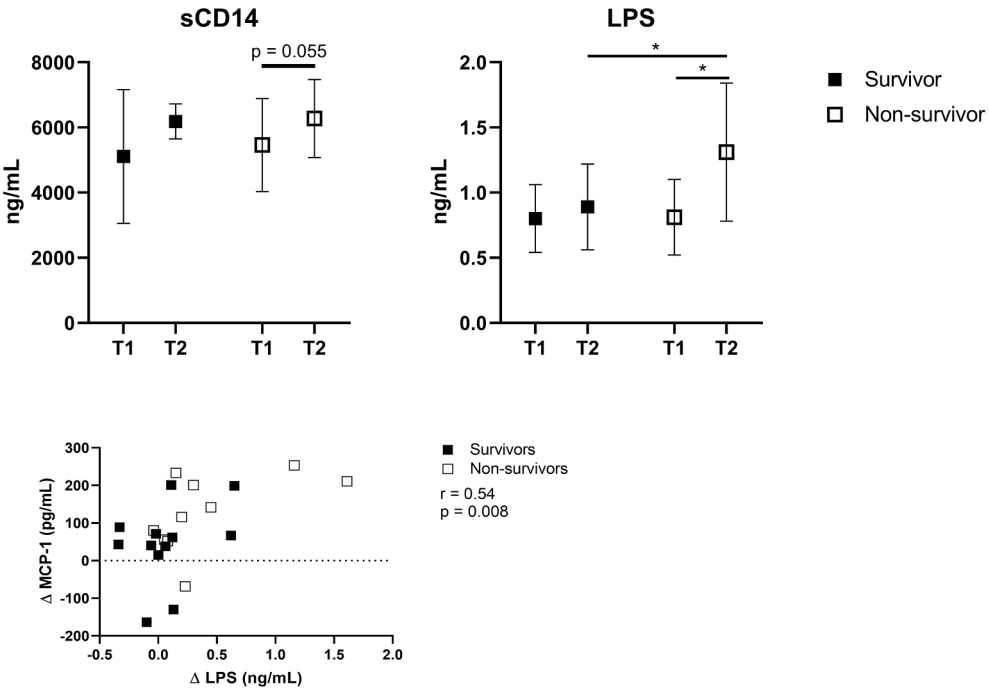

Fig. 4. The systemic levels of soluble CD14 (A) and LPS (B) in survivors and non-survivors COVID-19 patients at the hospital admission (T1) and at the end of hospitalization (T2). Correlation between Δ MCP-1 and Δ LPS levels of COVID survivors and non-survivors are represented (C). Two-way ANOVA followed by Bonferroni's post hoc were applied to verify time and group effects. ($p < 0.05$). * Indicates $p < 0.05$. Pearson's Coefficient test were applied to verify the correlation between inflammatory markers and LPS levels ($p < 0.05$).

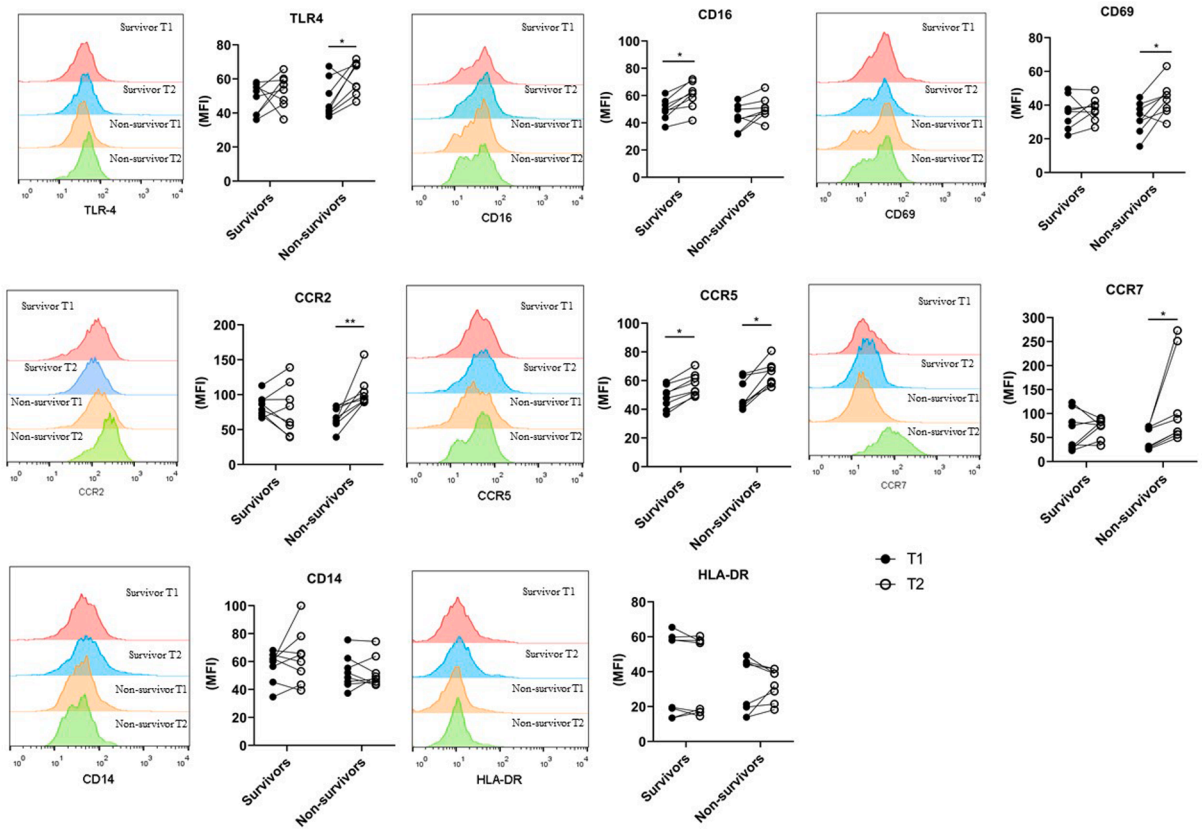

Fig. 5. Effect of plasma from COVID survivors and non-survivors on THP-1 cell phenotype. THP-1 cells were incubated with plasma from COVID survivors and non-survivors at T1 and T2 for 15 h, then the expression of monocyte phenotype, activation markers and chemokine receptors were evaluated by flow cytometry. Media Fluorescence Intensity of T1 and T2 are represented. Two-way ANOVA followed by Bonferroni's post hoc were applied to verify time and group effects. ($p < 0.05$).

at T2 in non-survivors COVID-19 patients. Interestingly, heat map correlations revealed that sCD14 correlated with CCL4/MIP-1β and CCL2/MCP-1, while LPS correlated with CCL5/RANTES and CCL4/MIP-1β. These results suggest that microbial translocation may contribute to the increases in chemokines, which recruits innate immune cells to the infectious site. In fact, chemokines release is directly involved with the

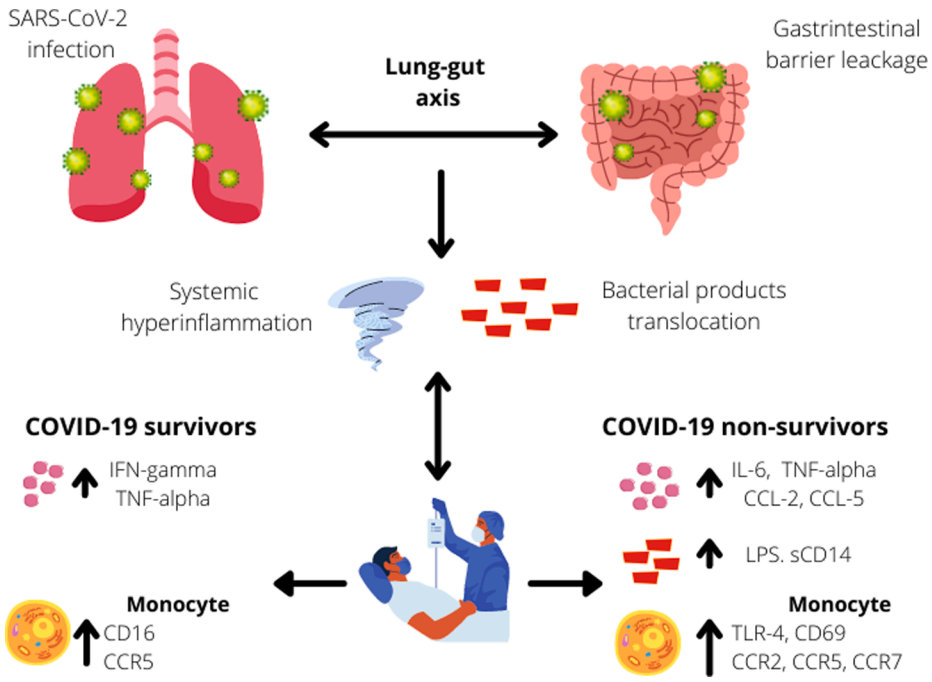

Fig. 6. Representation our hypothesis that SARS-CoV-2 infection promotes lung-gut axis alterations, gut barrier dysfunction and translocation of bacterial products into the systemic circulation resulting in hyperinflammation, monocyte activation and worse outcomes such as death of severe COVID-19 patients. The increased LPS and sCD14 markers during the hospitalization were associated with higher systemic levels of IL-6, TNF-α, CCL5/RANTES and CCL2/MCP-1 in non-survivors COVID-19 patients. On the other hand, survivors patients maintained LPS and sCD14 levels at stable values concomitant with increases in IFN-γ and TNF-α levels. Finally, the incubation of THP-1 cells with plasma samples from non-survivors, obtained at the end of hospitalization (T2), increased the expression of TLR4, CCR2, CCR5, and CD69 on the cell surface of monocytic lineage cells, while THP-1 cells incubated with plasma of survivor patients at T2 presented higher CCR5 and CD16 expression.

acute respiratory disease syndrome, a major complication leading to death in severe COVID-19 infection [34,35]. All the chemokines evaluated here, CCL4/MIP-1β, CCL2/MCP-1 and CCL5/RANTES, are upregulated early post SARS-CoV-2 infection and contribute to the migration of inflammatory monocytes, natural killer cells and T cells to the infection site [36]. Thus, chemokines seem to be crucial players during COVID-19 pathogenesis and the hyperinflammatory condition of the severe disease. Our data also indicate that there was no difference between COVID-19 groups at hospital admission regarding chemokine or cytokine concentrations.

However, we found increases in the systemic LPS levels concomitant with higher IL-6, TNF-α, CCL/5/RANTES, and CCL2/MCP-1 concentrations in the plasma of non-survivors group collected 24–72 h before the death of critical COVID-19 patients. Lucas and coworkers [4] described a deregulated increase in inflammatory cytokines and chemokines in severe COVID-19 illness patients who deceased during the hospitalization, suggesting a maladapted immune response, exacerbated inflammatory induction associated with severe COVID-19 and poor clinical outcome. Interestingly, COVID-19 patients admitted to intensive care units have strong cytokine response compared to other types of pneumonia, elevations of IL-6 levels during hospitalization predicts ICU stay and mortality [37]. Thus, the increases of inflammatory mediators occur with concomitant increased microbial translocations, as suggested by increased LPS and sCD14 levels, in the blood of COVID-19 deceased patients.

The enhanced hyperinflammatory condition associated with higher LPS levels at T2 in non-survivors COVID-19 patients may contribute to systemic organ dysfunction. Gut permeability may increase during the hospitalization course and contribute to the worsening of clinical outcomes due to the respiratory distress syndrome caused by SARS-CoV-2 infection. In agreement, Ferreira and coworkers [38] hypothesized that gut microbiota dysbiosis and a leakage in the gut barrier integrity may be associated with poor clinical outcomes, such as ICU admission and death. Bacterial translocation during the COVID-19 hospitalization strongly correlates with immune activation, increased proinflammatory production by innate immune cells, and a higher mortality rate [39]. However, the interplay between systemic inflammation and LPS release in the blood remains to be elucidated in future studies involving COVID-19 cohorts.

Here, we hypothesized that plasma factors, such as LPS and inflammatory cytokines and chemokines, may directly impact the phenotype of monocytes. To address this question, we incubated monocytic THP-1 lineage with the plasma samples of hospitalized survivors or non-survivors COVID-19 patients obtained at hospital admission (T1) and in a time compromising 0–72 h before hospital discharge (T2, alive or dead). Interestingly, TLR4, the LPS ligand, increased only in non-survivors COVID-19 patients, which is corroborated by the higher systemic LPS levels in this group. In fact, critically ill COVID-19 patients presented upregulation in the TLR4 expression and higher pro-inflammatory cytokine and chemokine production after monocyte TLR4 stimulation similarly to the observed in bacterial sepsis [40]. Furthermore, the spike protein binds to LPS and lead to TLR4/NF-kB axis activation, modulating the phenotype of monocytes. In this sense, we found increased CD69 expression on THP-1 cells incubated plasma of non-survivors COVID-19 patients collected at T2, indicating an early monocyte activation. However, no difference was observed in the HLA-DR expression on THP-1 cells after incubation with plasma of both groups. Plasma obtained from COVID-19 patients may have no impact on the antigen presentation and long-term activation of monocytes. On the other hand, plasma from COVID-19 survivors collected at T2 moment increased CD16 expression in THP-1 cells. Data from literature described higher percentages of $CD14^{+}CD16^{high}$ monocytes and very low expression of HLA-DR in all $CD14^{+}$ monocyte subsets in severe COVID-19 patients [41–43]. The mobilization of newly bone marrow monocytes associated with continuous higher systemic and organ inflammation along the course of the acute infection, rather than short-term stimulation, may impact on the changes of CD16 and HLA-DR expression in circulating monocytes.

Severe COVID-19 patients presented an inflammatory phenotype of monocytes associated with early activation and downregulation of HLA-DR [42,44]. Monocytes upregulate the CD69 expression, which promotes tissue infiltration and retention [45,46]. Increased activation and CD69 expression in monocytes of severe COVID-19 subjects were associated with diapedesis and tissue infiltration, contributing to the monocytopenia observed in the peripheral circulation of diseased patients [47]. The increased expression of CCR2, CCR5, and CCR7 in THP-1 incubated with plasma at T2 of non-survivors COVID-19 patients may indicate the pro-inflammatory action of soluble factors produced during

the SARS-CoV-2 infection. Moreover, THP-1 cells also presented higher CCR5 expression after the incubation with plasma of COVID-19 survivors obtained at T2. Infiltrating monocytes constitute the majority of leukocytes migrating into the infected lungs, contributing to the severe lung inflammation in COVID-19 [44]. In fact, increased transcription of CCR2 and CCR5 was found in macrophages present in the bronchial alveolar lavage fluid of severe COVID-19 patients [48]. Furthermore, increased $CCR2^{+}$ monocytes elicit calcium influx, inducing higher levels of reactive oxygen radicals and upregulating the integrin expression which recruit neutrophils and mast cells, exacerbating the inflammatory local response and induces tissue damage [49–51]. The activation of $CCR5^{+}$ in macrophages is linked to the signal transduction downstream of Gαi/PI3K/AKT and Gαi/MEK/ERK pathways that induce anti-apoptotic activity, maintaining inflammatory exhausted macrophages in the inflamed lung [52]. In addition, markers of microbial translocation and systemic inflammation were strongly associated with a shift in several biologically active molecules, which likely contributes to the altered immunometabolism inflamed/exhausted phenotype of innate immune cells [32]. Interestingly, COVID-19 patients treated with leronlimab, an monoclonal antibody used to block CCR5, induced a rapid reduction in plasmatic IL-6, restoration of the CD4/CD8 ratio, and a significant decrease in the SARS-CoV-2 plasma viremia [53]. Antigen uptake by human monocyte derived dendritic cells, during viral infections, increases the expression of CCR7 that mediates the migration of antigen-bearing cells to lymphatic tissue [54]. Thus, the upregulation of CCR7 is crucial for the migration of antigen presenting cells to secondary lymphoid organs during viral infections [55]. Due to their pivotal role in the migration of inflammatory monocytes to the infected tissues and lymphoid organs, future experimental studies should be conducted to evaluate the impact of monoclonal antibody therapies that blockade the overexpression of chemokine receptors in severe COVID-19 hospitalization.

In conclusion, severe COVID-19 is related to increased microbial translocation during hospitalization coexisting with the inflammatory condition of SARS-CoV-2 infection and could lead to higher monocyte activation and worsening clinical outcomes, such as death.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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5 CAPÍTULO III

5.1 ARTIGO ORIGINAL - ENDOTOXIN TOLERANCE IN ACUTE SARS-COV-2 INFECTION OCCURS THROUGH THE LOW ACTIVATION OF TLR-4/NF-KB AXIS IN CD14+HLA-DRLOW/+ MONOCYTES

Endotoxin tolerance and low activation of TLR-4/NF-κB axis in monocytes of COVID-19 patients

Running Ahead Title: Endotoxin tolerance in COVID-19

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Endotoxin tolerance through the low activation of TLR-4/NF-κB axis in monocytes of COVID-19 patients

Abstract

Higher endotoxin in the circulation may indicate a compromised state of host immune response against coinfections in severe COVID-19 patients. We evaluated the inflammatory response of monocytes from COVID-19 patients after lipopolysaccharide (LPS) challenge. Whole blood samples of healthy controls, patients with mild COVID-19 and severe COVID-19 where incubated with LPS for 2 hours. Severe COVID-19 patients presented higher LPS and sCD14 levels in the plasma than healthy controls and mild COVID-19 patients. In non-stimulated *in vitro* condition, severe COVID-19 patients presented higher inflammatory cytokines and PGE-2 levels and CD14+HLA-DR^{low} monocytes frequency than controls. Moreover, severe COVID-19 patients presented higher NF-κB activation in CD14+HLA-DR^{low}, as well as higher expression of TLR-4 and NF-κB activation in CD14+HLA-DR^{high} compared to controls. The stimulation of LPS in whole blood of severe COVID-19 patients leads to lower cytokine production but higher PGE-2 levels compared to controls. Endotoxin challenge

with both concentrations reduced the frequency of CD14+HLA-DR^{low} in severe COVID-19 patients, but the increases in TLR-4 expression and NF-κB activation were more pronounced in both CD14+ monocytes of healthy controls and mild COVID-19 patients compared to severe COVID-19 group. We conclude that acute SARS-CoV-2 infection is associated with diminished endotoxin response in monocytes.

Key-word: COVID-19, inflammation, monocytes, lipopolysaccharide.

INTRODUCTION

The Coronavirus Disease-2019 (COVID-19) is the disease caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that induces a multiorgan damage and impairs several functional physiological units, such as the cardiorespiratory and neurological systems (1). The clinical characteristics of COVID-19 is widely diverse, although most subjects are asymptomatic or exhibit only mild symptoms, a significant number of patients require hospitalization, including intensive care unit (ICU) admission (2). Despite several progresses were recently made to understand the immunopathology of COVID-19, the decisive pathological processes that are responsible for patient morbidity and mortality remain unknown.

The main causes of death by COVID-19 include respiratory failure and the development of sepsis, a condition that has been observed in several deceased patients(3). In this sense, the predominant theory is that an overexuberant immune response mediated by strong proinflammatory cytokinemia drives lung injury, multiorgan lesion, and a procoagulant state (4,5). On the other hand, an overproduction of inflammatory cytokines may lead to a state of immunologic collapse and sterile inflammation that predisposes the host to a secondary

infection (3,6). The higher levels of pro- and anti-inflammatory cytokines, including but not only interleukin(IL)-6, IL-10, tumor necrosis factor-alpha (TNF-α) and interferons, contribute to the augment of the tissue damage in parallel with functional paralysis of both innate and adaptive immune cells (7,8). In such scenario, new bacterial or viral infections increases the risk of mortality and morbidity (9–11).

In fact, we and others recently reported higher levels of bacterial translocation markers in the peripheral blood of ICU COVID-19, which was associated with death (7,12,13). Severe COVID-19 shares several immunopathological features with septic conditions, including T cell exhaustion, lymphocyte apoptosis, leukopenia, and increased immunosuppressive myeloid-derived suppressor cells (CD14+HLA-DR^{low}) phenotype (13,14) Moreover, endotoxin tolerance is one of the main mechanisms responsible for the appearance of secondary infections due to impairment of proinflammatory cytokine production after a secondary immunogenic stimulus, low antigen presentation and high phagocytic ability (15,16). Here, we aimed to investigate the impact of lipopolysaccharide (LPS) whole blood assay on the cytokine production and the expression of toll-like receptor-4 (TLR-4) and the activation of the master inflammatory transcription NF-κB in CD14+HLA-DR$^{low/+}$ monocytes of COVID-19 patients.

METHODS

Ethics procedure and participants

This study was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Moinhos de Vento Ethics

Committee N 4932566 (Porto Alegre/Brazil). All the participants provided an informed consent. Patients who fulfilled the clinical criteria for mild and severe COVID-19 and have the diagnosis confirmed by SARS-CoV-2 nucleic acid testing of nasal and pharyngeal throat swab specimens using real-time RT-PCR assay were included in this study (7). The clinical data of the patients included in the study are summarized in Supplementary Table I. Blood samples (5 mL) were obtained from controls and severe COVID-19 patients into K2EDTA tubes (Becton & Dickinson, USA) within 6 h from hospital admission. All healthy controls have no history of any significant systemic diseases or malignancy and were negative for SARS-CoV-2 acute infection (based on a routine diagnostic RT-PCR test).

Disease severity was classified according to the World Health Organization classification after completing the follow-up questionnaire. Clinical and socio-demographic data were collected from the patient's electronic medical records upon admission to the unit.

Table 1. Demographic and clinical participant's characteristics

	Healthy controls (n=5)	Mild COVID-19 (n=8)	Severe COVID-19 (n=7)	p
Age (y)	52.3 ± 18.5	47.6 ± 12.3	56.6 ± 17.2	0.08
Sex (male)	23.1	42.9	57.1	0.30
Racial or ethnic group				
• Caucasian	75	69.6	77.3	0.34
• Non-Caucasian	25	30.4	22.7	
Body mass (kg)	-	79.1 ± 16.7	84.5 ± 11.7	0.42
BMI (kg/m²)	-	28.7 ± 3.9	29.6 ± 1.9	0.33
Days from symptom onset	-	3.7 ± 1.4	9.3 ± 2.4	**0.001**
Underlying medical condition (%)				
• Hypertension		14.3	28.6	**0.002**
• Diabetes Mellitus		-	28.6	**0.001**
• COPD		14.3	-	**0.001**
• Asthma		-	9.1	**0.001**
• HIV		-	14.3	**0.001**
• Dyslipidemia		-	14.3	**0.001**
Oxygen use during hospitalization	-	-	42.9	**0.001**

Quantitative data presented as mean ± standard deviation. Between groups comparison through one way ANOVA ($p<0.05$).

BMI, body mass index; COPD, chronic obstructive pulmonary disease; HIV huma immunodeficiency virus.

Qualitative data presented as relative frequency. Between groups comparison through Qui-square test ($p<0.05$).

Liquid chromatography-tandem mass spectrometry determination of LPS

LPS concentration was determined by quantitation of 3-hydroxytetradecanoic acid as described previously (7). Briefly, 150 µL of plasma was hydrolyzed with 75 µL of NaCl 150 mM, 300 µL of HCl 8 mol for 4 h at 90 °C and extracted with 4 mL of a hexane. Samples were dried under a nitrogen stream and resuspended in 50 µL of methanol immediately prior to the injection in the analytical system. A Nexera UFLC system coupled to a LCMS-8040 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan) was used for the analysis. The electrospray parameters were set in the negative ion mode ($[M-H]^-$) as follows: capillary voltage, 3000 V; desolvation line temperature, 250 ºC; heating block temperature, 500 ºC; drying gas, 18/L min; and nebulizing gas, 2 L/min. Collision-induced dissociation was obtained with 230 kPa argon pressure.

Analyses were carried out with multiple reaction monitoring (MRM) by using the following fragmentation: *m/z* 243.1 → *m/z* 59.0. The chromatographic separation was conducted with an Acquity UPLC® C18 column (2.1 x 50 mm, 1.7 µm particle size) (Waters Corporation, Ireland). The analyses were performed in gradient elution mode with a flow rate of 0.4 mL/min and the gradient mobile phase system consisted of water (solvent A) and acetonitrile (solvent B) both fortified with 0.2 % acetic acid as follow: 0 – 1.5 min, 75 – 100% of B; 1.5 – 2.0 min, 100% of B; 2.0 – 2.1 min, 100 – 75 % of B; 2.1 – 5.5 min, 75 % B. The column oven was kept at 50 °C. The data were processed using LabSolutions software (Shimadzu, Kyoto, Japan).

Whole blood ex vivo assay

Blood samples collected into k3EDTA tubes were aliquoted in tubes to a volume of 1 mL and incubated (5%CO_2, 37ºC) with two different LPS concentrations (1 and 10 ng/mL, Sigma-Aldrich, USA) or without immunogenic stimulus for 2 hours (17). At the end of the assay, the tubes were centrifuged, the plasma was stored in microtubes and frozen at -80ºC until the day of cytokine analysis. The remaining blood was used to analyze the expression of TLR-4 and the activation of NF-κB in CD14+HLA-$DR^{-/+}$ monocytes.

sCD14, PGE2, and cytokine analyzes

The supernatant concentrations of interleukin (IL)-6, IL-10, C-C motif ligand 2 (CCL2) and tumor necrosis factor-alpha (TNF-α) were analyzed by bead-based multiplex assays using Luminex Technology following manufacturer's recommendations (MILLIPLEX MAP Human Cytokine/Chemokine, Millipore,

USA). The soluble form of CD14 was analyzed using Human CD14 ELISA Kit from Invitrogen (USA), with detection limits ranging 8.23–8000 ng/mL. Prostaglandin E2 (PGE2, Invitrogen, ThermoFisher, USA, detection limits of 39.1 – 10.000 pg/mL) and IL-1β (Intitrogen, USA, detection limits of 2.0 – 500 pg/mL) concentrations were analyzed in plasma and supernatant by enzyme-linked immunosorbent assay (ELISA) in microplate reader (EzReader, EUA) following manufacturer's recommendations.

Flow cytometry

Whole blood samples (100 µL) were incubated with monoclonal surface antibodies (all anti-human) at 4ºC for 20 min in accordance with the following combination: a) FITC-conjugated anti-CD14, Pe-conjugated anti-CD284; Percp-Cy5.5 anti-HLA-DR; b) FITC-conjugated anti-CD14, Pe-conjugated anti-NF-κBp65 (Ser529); Percp-Cy5.5 anti-HLA-DR. Then, samples were incubated for 10 min with lysing buffer (BD Biosciences) and then centrifuged at 500g for 5 min. The supernatant was discarded, and samples were resuspended in *phosphate buffered saline* (PBS - 1mL) and then centrifuged at 500g for 5 min. Finally, the samples were resuspended in 0.5 mL PBS and analyzed in flow cytometry. For intracellular analysis, tubes with whole blood stained with anti-CD4 or anti-CD14 antibodies were incubated with fixation and permeabilization buffers following manufacturer's recommendation (Ebioscience, EUA). After the period, samples were incubated with Phospo-NF-κBp65 (Ser529) Pe (Ebioscience, EUA) monoclonal during 30 min. Then, cells were washed and resuspended in Flow Cytometry Staining Buffer (Ebioscience, EUA). Cell phenotype was acquired

using CELLQuest Pro Software (BD Bioscience) on a FACSCalibur flow cytometer (BD Bioscience).

Statistical Analysis

Data were analyzed using SPSS 20.0 (SPSS Inc, IBM, Chicago, IL). After the normality test by Shapiro-Wilk, all values were presented as mean ± standard deviation (SD). Categorical variables were presented as relative frequency and analyzed by chi-square test. To identify associations between LPS and symptoms we used Mann-Whitney U-test to compare SARS-CoV-2 infected patients with and without specific disease symptoms. Pearson's Coefficient Test was performed to check correlations between LPS and the clinical variables of severe patients. Two-way repeated measurement analysis of variance, taking time and group as factors, were performed followed by Bonferroni's post hoc for multiple comparisons. A $p \leq 0.05$ was adopted in all analysis.

RESULTS

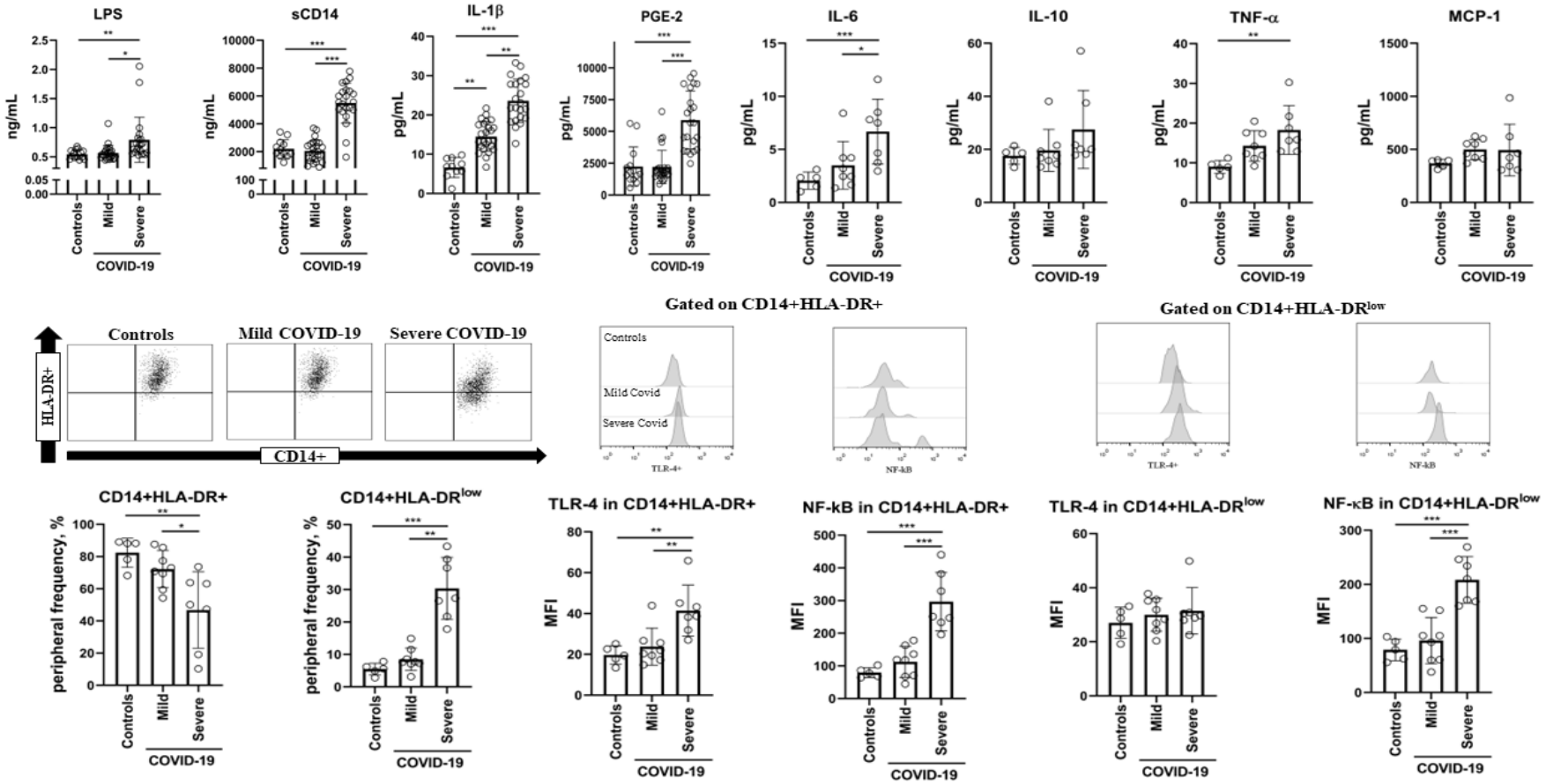
3.1 Increased systemic lipopolysaccharide and cytokines levels and altered monocyte phenotype in the peripheral blood of severe COVID-19 patients

Moreover, significant increase in TLR-4 expression (p=0.01 vs. healthy controls; p<0.05 vs. mild COVID-19) and NF-κB activation (p=0.001 vs. healthy controls; p=0.01 vs. mild COVID-19) were found in CD14+HLA-DR+ monocytes of severe COVID-19 patients. In addition, higher frequency of CD14+HLA-DR^{low} monocytes (p=0.001 vs. healthy controls; p=0.01 vs. mild COVID-19) presenting higher NF-κB activation (p=0.001 vs. both healthy controls and mild COVID-19 patients) as well as lower CD14+HLA-DR+ monocytes (p=0.01 vs. healthy

controls; $p<0.05$ vs. mild COVID-19 patients) expressing higher TLR-4 ($p=0.01$ vs. both control and mild COVID-19 groups) and NF-κB activation ($p<0.001$ vs. both groups comparison) were identified in the peripheral blood of severe COVID-19 subjects.

Figure 1 shows the concentrations of LPS, sCD14, IL-1β and PGE-2 (healthy controls, n=14; mild COVID-19, n=25; severe COVID-19, n=22) as well as the levels of cytokines (healthy controls, n=5; mild COVID-19, n=8; severe COVID-19, n=7) in the blood of healthy controls and COVID-19 patients. Higher LPS levels ($p=0.05$ vs. healthy controls and mild COVID-19 groups), sCD14 ($p=0.001$ vs. healthy controls and mild COVID-19), IL-1β ($p=0.001$ vs. healthy controls, $p=0.01$ vs. mild COVID-19), PGE-2 ($p<0.001$ vs. healthy control and mild COVID-19), IL-6 ($p=0.001$ vs. healthy controls; $p<0.05$ vs. mild COVID-19) and TNF-α ($p=0.01$ vs. healthy controls) were identified in the blood of severe COVID-19 subjects. Mild COVID-19 patients presented higher IL-1β plasma concentrations than healthy controls ($p<0.01$).

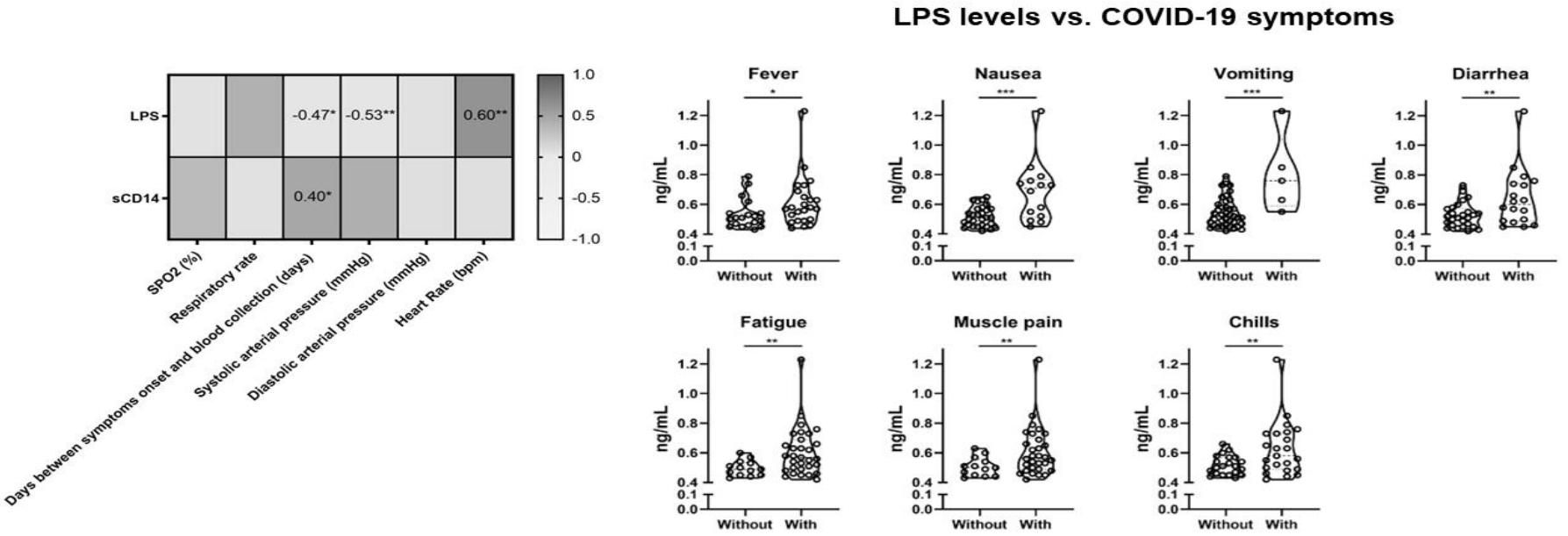
Figure 1. Analysis of systemic LPS, cytokines and the peripheral frequency of CD14+HLA-DR$^{low/+}$ monocytes expressing TLR-4 and NF-κB in healthy controls and in mild and severe COVID-19 patients. Between groups comparison performed through one-way ANOVA followed by Bonferroni's post hoc. ($p<0.05$).



3.2 Systemic LPS concentrations associates with COVID-19 symptoms and clinical cardiovascular parameters

Figure 2 presents the systemic LPS concentrations in COVID-19 patients with or without clinical disease symptoms. Interestingly, LPS levels were higher in patients reporting fever ($p=0.05$), nausea ($p<0.001$), vomiting ($p<0.001$), diarrhea ($p<0.01$), fatigue ($p<0.01$), muscle pain ($p<0.01$) and chills ($p<0.01$). Then, we correlated clinical cardiorespiratory parameters of severe COVID-19 patients with LPS and sCD14 levels. Systemic LPS inversely correlated with systolic blood pressure ($r= -0.53$; $p<0.01$) but positively correlated with heart rate ($r=0.60$; $p<0.01$). Days between symptoms onset and blood collection correlated with LPS ($r= -0.47$, $p<0.05$) and sCD14 ($r=0.40$; $p<0.05$).

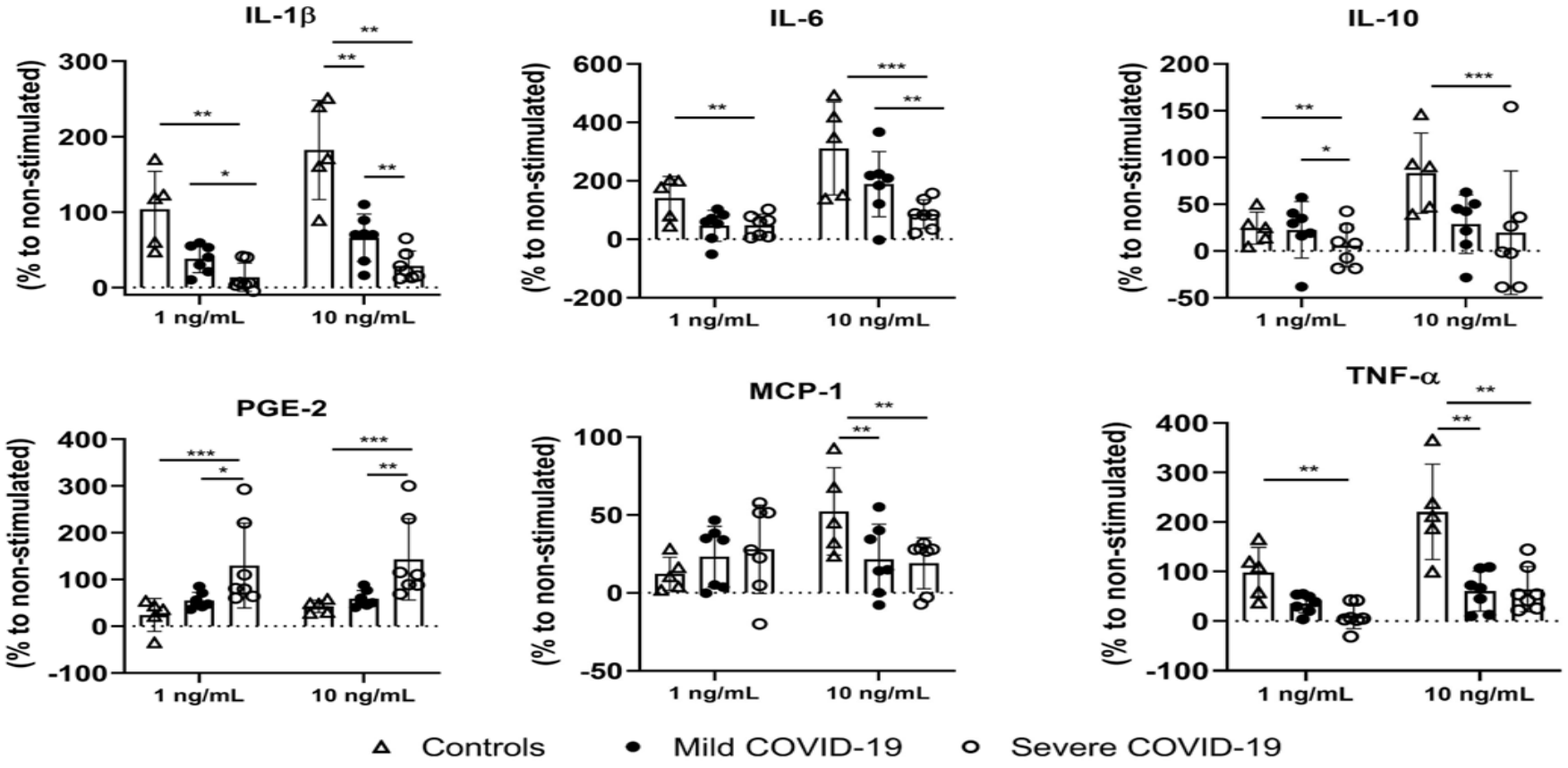
Figure 2. The association of systemic LPS concentrations with clinical symptoms of acute SARS-CoV-2 infection. LPS and sCD14 levels were correlated with clinical cardiorespiratory signs of SARS-CoV-2 infection by Pearson's Coefficient Correlation test ($p<0.05$). The levels of LPS were compared between patients with and without clinical symptoms through Mann-Whitney U-test and the significant results were presented ($p<0.05$). ***** $p<0.05$; ** $p<0.01$; *** $p<0.001$.



3.3 Whole blood LPS stimulation induces lower inflammatory response in severe COVID-19

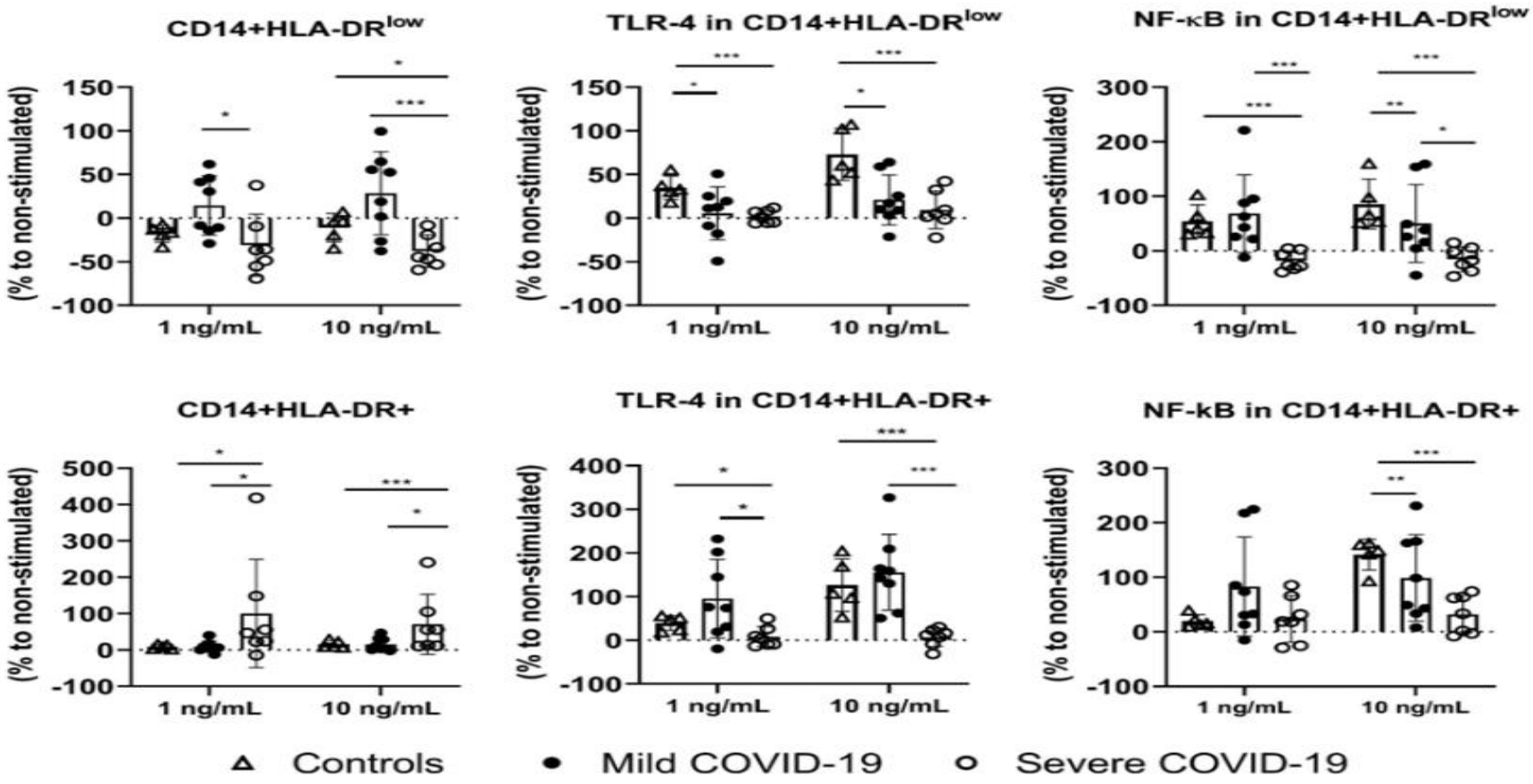
Next, we performed a whole blood *ex vivo* stimulation with LPS at two different concentrations (1 ng/mL or 10 ng/mL) for 2 hours (5%CO2, 37ºC). After the incubation period the plasma was collected and kept at -80ºC to analyze cytokines production, while the remaining blood was used to verify the expression of TLR-4 and NF-κB in CD14+HLA-DR$^{low/+}$ monocytes by flow cytometry. After stimulation with LPS at 1 ng/mL, increased IL-1β (p=0.01), IL-6 (p=0.01), IL-10 (p=0.01) and TNF-α (p=0.01) production were found in healthy controls compared to severe COVID-19 patients, and higher IL-1β and IL-10 production by whole blood of mild compared to severe COVID-19 (p<0.05) (Fig 3). *LPS (10 ng/mL)-stimulated whole blood* cultures of healthy controls elicited a great increase in the production of IL-1β (p=0.01 vs. mild COVID-19 and severe COVID-19), IL-6 (p=0.001 vs. severe COVID-19), IL-10 (p=0.001 vs. severe COVID-19), CCL2 (p=0.01 vs. mild COVID-19; p=0.01 vs. severe COVID-19), and TNF-α (p=0.01 vs. mild COVID-19; p=0.01 vs. severe COVID-19). Increased IL-1β and IL-6 production was also found after *ex vivo* LPS (10 ng/mL) stimulation of blood cells of mild COVID-19 patients compared to severe COVID-19 group (p=0.01). Furthermore, LPS-stimulated whole blood of severe COVID-19 patients produced higher PGE-2 after 1 ng/mL (p<0.001 vs. healthy controls; p<0.05 vs. mild COVID-19) and 10 ng/mL (p<0.001 vs. healthy controls; p<0.01 vs. mild COVID-19) stimulation.

Figure 3. The production of IL-1β, IL-6, IL-10, CCL2, PGE-2, TNF-α by *ex-vivo* LPS-stimulated whole blood of healthy controls and mild and severe COVID-19 patients. The production of cytokines was evaluated by the % of difference compared to the non-stimulated condition. ** (p<0.01), *** (p<0.001) denote statistical difference (p<0.01).



Severe COVID-19 patients presented lower proportions of CD14+HLA-DR^{low} (1 ng/mL, $p<0.05$ vs. mild COVID-19; 10 ng/mL, $p<0.05$ vs. healthy controls; 10 ng/mL, $p=0.001$ vs. mild COVID-19) (Fig 4) and increased frequencies of CD14+HLA-DR+ (1 ng/mL, $p<0.05$ vs. healthy controls; 1 ng/mL, $p<0.05$ vs. mild COVID-19; 10 ng/mL, $p<0.001$ vs. healthy controls; 10 ng/mL, $p<0.05$ vs. mild COVID-19) after ex-vivo LPS challenge. Furthermore, healthy controls presented increased TLR-4 in CD14+HLA-DR^{low} after both 1 ng/mL ($p<0.05$ vs. mild COVID-19; $p=0.001$ vs. severe COVID-19) and 10 ng/mL LPS challenge ($p=0.05$ vs. mild COVID-19; $p=0.001$ vs. severe COVID-19). The NF-κB activation was lower in CD14+HLA-DR^{low} monocytes of severe COVID-19 patients after treatment with 1 ng/mL ($p=0.001$ vs. healthy controls; $p=0.001$ vs. mild COVID-19) and 10 ng/mL of LPS ($p=0.001$ vs. healthy controls; $p<0.05$ vs. mild COVID-19), although significant difference was also found in healthy controls vs. mild COVID-19 comparison ($p=0.01$) after 10 ng/mL LPS stimulation. In CD14+HLA-DR+ monocytes of severe COVID-19, LPS stimulation induced lower TLR-4 expression (1 ng/mL, $p<0.05$ vs. healthy controls; 1 ng/mL, $p<0.05$ vs. mild COVID-19; 10 ng/mL, $p=0.001$ vs. healthy controls; 10 ng/mL, $p<0.005$ vs. mild COVID-19). Finally, healthy controls presented higher NF-κB activation in CD14+HLA-DR+ monocytes compared to mild COVID-19 ($p=0.01$) and severe COVID-19 ($p=0.001$) patients.

Figure 4. The frequencies of CD14+HLA-DR^{low} and CD14+HLA-DR+ monocytes expressing TLR-4 and NF-κB in ex-vivo LPS-stimulated whole blood of healthy controls and mild and severe COVID-19 patients. The frequencies of each cell subtype were evaluated by the % of difference compared to the non-stimulated condition. * (p<0.05), ** (p<0.01), *** (p<0.001) denote statistical difference (p<0.01).



DISCUSSION

Here, we aimed to evaluate the acute inflammatory response and the modulation of monocyte phenotype after an *ex vivo* whole blood LPS challenge in patients with different degrees of COVID-19 severity. We hypothesized that acute SARS-CoV-2 infection impairs the ability of immune system to respond against a secondary pathogen, suggesting a temporary immunosuppression. We main found that severe COVID-19 patients presented: a) Increased LPS and sCD14 concentrations in their peripheral blood associated with clinical COVID-19 symptoms and cardiovascular data; b) Increased frequencies of CD14+HLA-DR^{low} and lower CD14+HLA-DR+ monocytes in the peripheral blood; c) Increased TLR-4 expression in CD14+HLA-DR+ and higher NF-κB expression in both CD14+HLA-DR^{low} and CD14+HLA-DR+ monocytes. Furthermore, after the stimulation of whole blood with different concentrations of the classic TLR-4 ligand LPS, we main found a) lower production of inflammatory cytokines associated with increased PGE-2 production by stimulated whole blood of severe COVID-19 patients; b) Severe COVID-19 group presented decreased CD14+HLA-DR^{low} and higher CD14+HLA-DR+ monocytes after 1 and 10 ng/mL LPS stimulation; c) Impaired TLR-4 expression and NF-κB expression were found in both CD14+HLA-DR^{low} and CD14+HLA-DR+ monocytes of mild and severe COVID-19 patients after 1 and 10 ng/mL LPS stimulation; d) Severe COVID-19 patients presented the most pronounced impairment in monocytes modulation after LPS stimulation. Taken together, our results denote that acute SARS-CoV-2 infection is associated with diminished endotoxin response in monocytes of patients with severe disease.

The present results are in line with previous data published by our group (7) and others (18) demonstrating an association between increased markers of bacterial translocation and proinflammatory cytokines in severe COVID-19 subjects at hospital admission. Strong endotoxemia were also linked to tissue damage and multiorgan disease after SARS-CoV-2 once that increased LPS-binding protein (LBP) remained higher during hospitalization in patients with severe COVID-19 (12). Here, we also observed increased PGE-2 levels in severe COVID-19 patients. Higher pro-resolving and inflammatory lipid mediators, such as eicosanoids and docosanoids, were found in the blood and bronchoalveolar lavage of severe COVID-19 patients which suggests a role of pro- and anti-inflammatory lipids to contribute to the progression of disease severity (19). The production of PGE-2 during active SARS-CoV-2 infection occurs through the upregulation of cyclo-oxygenase-2 and reduced 15-hydroxyprostaglandin-dehydrogenase enzyme activity in infected lung epithelial cells (20). Furthermore, higher levels of PGE-2 was found in the blood of severe COVID-19 patients with high viral load (21) and reduces anti-viral immune response by targeting B-cell proliferation and differentiation(20). Finally, systemic immune activation and chronic inflammation are linked to microbial translocation and endotoxemia after viral infection in patients with HIV infection (22,23). Collectively, these data indicate that viral infections, such as SARS-CoV-2, can induce an enhanced endotoxemia that may contribute to the systemic inflammation and hamper the immune response.

Gut leakage and microbial products translocation were previously associated with cardiovascular involvement and inflammasome activation in hospitalized COVID-19 patients (12). In fact, we identified a positive correlation

between systemic LPS levels and heart rate in severe COVID-19 group. Thus, tachycardia observed during severe SARS-CoV-2 infection (24) may be related by the disruption of sympathetic nervous system and endothelial damage induced by hyperinflammation and endotoxemia. Furthermore, LPS levels inversely correlated with systolic arterial pressure which suggest that alterations in blood perfusion and conductance are impacted by endotoxins. In fact, endotoxemic shock patients present falls in blood pressure, and LPS infusion in rats reduces mean arterial blood pressure (MAP) by deregulation in molecular pathways involved in oxidative stress response and coagulation (25). Finally, we identified higher levels of LPS in SARS-CoV-2 infected patients presenting gastrointestinal (i.e., nausea, vomiting and diarrhea) or systemic inflammatory symptoms (i.e., fever, fatigue, muscle pain and chills). In this sense, gastrointestinal symptoms may indicate intestinal damage and systemic microbial translocation during SARS-CoV-2 infection. Furthermore, since LPS levels were higher in severe COVID-19, the data presented here reinforces the implications of disruption in gut functions as a potential force to COVID-19 severity (7).

We also observed elevated frequencies of CD14+HLA-DR^{low} monocytes, an immunosuppressive phenotype of myeloid cells, in the peripheral blood of severe COVID-19 patients. This result is in line with previous studies who also found increased proportions of HLA-DR^{low} immature monocytes in hospitalized and ICU COVID-19 patients(26,27). CD14+HLA-DR^{low} monocytes are part of an immature cells that can mediate suppression of the immune response (28). Interestingly, immunosuppressive monocytic phenotype remained unchanged over the hospitalization and is linked to a dysfunctional immune response. When transitioning to an immunosuppressive state, monocytes are highly sensitive and

become inactive, leading to immunoparalysis (29). Furthermore, immunosuppressive myeloid phenotype in severe COVID-19 patients was associated with the development of secondary infections, and 28- and 60- day mortality (26). Thus, taken together, increased endotoxemia, inflammatory cytokines and the accumulation of CD14+ HLA-DR^{low} monocytes in the peripheral blood of severe COVID-19 patients may be related to the immunosuppressive condition and higher secondary infection rates observed in these patients.

Low grade endotoxemia is a common event in SARS-CoV-2 infected patients (30), and usually related to a continuous stimulation of TLR-4 in innate immune cells. Furthermore, the spike protein of SARS-CoV-2 synergically interact with LPS, leading to a boosting of proinflammatory actions mediated by the phosphorylation of NF-κB in monocytic THP-1 cell line (31). Similarly, it was found that the interaction between spike protein and LPS enhances *in vivo* pro-inflammatory cytokine production, suggesting that an early innate immune response occurs by the activation of transcription inflammatory genes after the co-presence of LPS and proteins related to SARS-CoV-2(32–34). Here, we found an increased expression of TLR-4 on CD14+HLA-DR+ monocytes in conjunct with higher NF-κB activation in both CD14+HLA-DR+ and CD14+HLA-DR^{low} monocytes of severe COVID-19 patients. In fact, is was previously reported an overactivation of TLR-4 by the concomitant presence of systemic endotoxemia and SARS-CoV-2 related proteins observed in *in vitro* experiments conducted in previous reports (34–36). Furthermore, previous *in silico* studies predicted cell surface TLR-4 is most likely to be involved in recognizing molecular patterns of SARS-CoV-2 to induce inflammatory response at early stages of infection and the inhibition of NF-κB was able to suppress IL-1β production induced by spike

protein (37), confirming the crucial role of TLR-4/NF-κB axis in the hyperinflammatory response of COVID-19 pathogenesis.

Clinically, TLR-4 and signaling molecules are upregulated in leukocytes from COVID-19 patients in a condition that mimics bacterial sepsis. Furthermore, Shambat and colleagues (38) showed that a higher degree of SARS-CoV-2 viremia mediates hypercytokinemia and is positively associated with bacterial superinfections. Interestingly, the authors showed that TNF-α and other cytokines synergistically mediate impaired antibacterial effector functions and downregulates the HLA-DR expression of monocytes from COVID-19 patients (38).

Here, we employed an *ex vivo* LPS-whole blood stimulation to verify the ability of systemic immune cells of SARS-CoV-2-infected patients to respond to an endotoxin challenge. Interestingly, severe COVID-19 patients presented lower production of IL-1β, IL-6, IL-10, CCL2 and TNF-α after LPS stimulation at 1 and 10 ng/mL. Thus, our results suggest that SARS-CoV-2 infection hampers the cytokine production after a secondary stimulus, which may predispose the host to secondary infections. The impaired cytokine production after endotoxin challenge found here may be related to the higher PGE-2 production identified in the supernatant of whole blood stimulation. Previous *in vitro* studies identified low cytokine production and impaired cell activation by LPS-stimulated macrophages after PGE-2 treatment, reinforcing the immunomodulatory role of lipid mediators during inflammation (39).

PGE-2 is produced by macrophage during high endotoxemia and contributes to vasodilation, pain, fever, and multiple organ failure during conditions such as septic shock syndrome (40). Furthermore, PGE-2 has a negative feedback loop

through the enhancement in the expression of phosphatase 1 that impairs the activity of MAPK p38 and suppress cytokine release during disturbances of inflammatory response (41). In this sense, it seems that major immunologic abnormalities in COVID-19 is a profound defect in host immune response due to high inflammation condition induced by primary SARS-CoV-2 infection. There was no evidence of exaggerated TNF-α production in response to *ex vivo* LPS stimulation of mononuclear cells from COVID-19 patients compared to septic or critically illness non septic patients, despite increased plasma inflammatory cytokines (42). Furthermore, mononuclear cells from healthy volunteers presented impaired TNF-α and IL-6 production after exposure to spike and nucleocapsid recombinant proteins from SARS-CoV-2 and then challenged with LPS, indicating endotoxin tolerance induced by SARS-CoV-2 proteins. Collectively, the data indicate that COVID-19 induces an immunocollapse that predisposes the patients to a secondary infection.

Since LPS triggers NF-κB activation and cytokine production through the engagement with TLR-4 on the cell surface of CD14+ monocytes, we evaluated the role of both innate inflammatory mediators after an *ex vivo* endotoxin challenge in whole blood from COVID-19 patients. We identified increased proportions of CD14+HLA-DR+ and lower CD14+HLA-DR^{low} in the whole blood of severe COVID-19 patients compared to both mild COVID-19 and healthy control groups after *ex vivo* endotoxin stimulation. These results are in contrast with previous data that reported lower HLA-DR expression in monocytes after antigenic challenge (16,38,43). The divergent data may be linked to the different conditions used for stimulation, since the reported impaired immune response to *Candida albicans* (43)*, Staphylococcus aureus* and *Streptococcus pneumoniae*

(38) by monocytes from COVID-19 patients may indicate that others pattern recognition receptors (PRRs) are impaired during acute SARS-CoV-2. Future studies should be conducted to clarify the impact of COVID-19 on innate PRRs response.

We observed for the first time that compared to healthy controls, both mild and severe COVID-19 patients presented impaired TLR-4 expression in CD14+HLA-DR^{low} monocytes after *ex-vivo* LPS stimulation and lower TLR-4 expression in CD14+HLA-DR+ monocytes from severe COVID-19 patients following LPS challenge. Collectively, these data may indicate that SARS-CoV-2 infection induces a lower endotoxin recognition by the decreased expression of TLR-4 despite the increases in activation status as demonstrated by higher HLA-DR expression in monocytes. Furthermore, in response to LPS, the NF-κB activation was lower in CD14+HLA-DR$^{low/+}$ monocytes of severe COVID-19 patients. LPS tolerance is a critical event observed in septic condition (44) and others critical ill situations induced by several negative regulators of TLR-4 signal transduction, such as IRAK-M, aryl hydrocarbon receptor, tryptophan catabolism and other molecules that negatively regulate NF-κB activation, mainly BCL-3 and NF-κBp50 (45). In this sense, SARS-CoV-2 antigens may contribute to the LPS tolerance during acute infection, since the continuous stimulation of TLR-4 and NF-κB by viral peptides may exhaust the innate pathways (31,37). The activation and translocation of NF-κB occur rapidly following exposure of monocytes to spike protein of coronavirus, suggesting that the recognition pattern by TLR is continuously activated during acute SARS-CoV infection (46). Furthermore, SARS-CoV-2 nucleocapsid protein triggers the phosphorylation of NF-κB p65 *in vitro* and promotes M1 macrophage polarization which increases lung injury in

mice model (47). On the other hand, PGE-2 reduces the activation of NF-κB and PI3k-Akt signaling during endotoxin stimulation with strong implications in cytokine production and monocyte activation (48). Thus, the continuously stimulation of TLR-4/NF-κB axis during acute SARS-CoV-2 infection may saturate the inflammatory pathways in a model to reduce the ability of innate response against a secondary endotoxin/LPS stimuli, which may indicate reduced immunosurveillance against a secondary infection.

CONCLUSION

In summary, our data provide evidence indicating that SARS-CoV-2 infection induces lower cytokine production and TLR-4/NF-κB activation in monocytes from severe COVID-19 after LPS stimulation. In addition, COVID-19 patients had systemic alterations in the peripheral blood, such as increased LPS and cytokine levels, higher frequency of immunosuppressive CD14+HLA-DR^{low} and higher expression of TLR-4 and NF-κB activation. Taken together, these results may indicate that the pro-inflammatory condition and microbial translocation markers observed in acute SARS-CoV-2 infection may induce endotoxin tolerance associated with the potential development of secondary infections.

Availability of data and materials

After request, first author can provide data.

Ethics Approval and Consent to Participate

Experimental protocols were approved by the Moinhos de Vento Ethics Committee N 4932566 (Porto Alegre/Brazil) and performed according to the

declaration of Helsinki. All participants were informed before entering the study and informed consent was obtained from the subjects.

Consent for Publication

All authors express their consent to the publication of this manuscript in this journal.

Competing interests

The authors declare no competing interests.

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Authors' contribution

GP Dorneles, PC Teixeira, A Peres and S Eller, SG Fonseca and PRT Romão conceived and planned the experiments. GP Dorneles, PC Teixeira, S Eller and TF Oliveira carried out the experiments. A Peres, LCR Júnior, SG da Fonseca, MC Monteiro and EM Wedland contributed to the interpretation of the results. SG da Fonseca and PRT Romão took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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6 CAPÍTULO IV

6.1 VISÃO GERAL

O SARS-CoV-2 é um vírus complexo e dinâmico que aflige uma série de alterações em diversos tecidos do hospedeiro. Esta dissertação identificou que a imunopatogenia durante a COVID-19 possibilita um aumento da translocação microbiana em conjunto ao ambiente hiperinflamatório. Muitos mecanismos da infecção e ativação do sistema imune já foram elucidados, no entanto, existem vias de sinalização e infecção que ainda são desconhecidas ou há divergências entre pesquisadores. Nosso estudo contribuiu para o entendimento da fase aguda da doença da COVID-19 e possíveis fatores que causam gravidade e hospitalização dos pacientes infectados pelo SARS-CoV-2. Vinculamos discussões sobre: translocação microbiana, hiperinflamação, tolerância a endotoxina e imunossupressão como eixos principais para o desenvolvimento de desfechos clínicos graves na COVID-19.

6.2 ESTUDO I

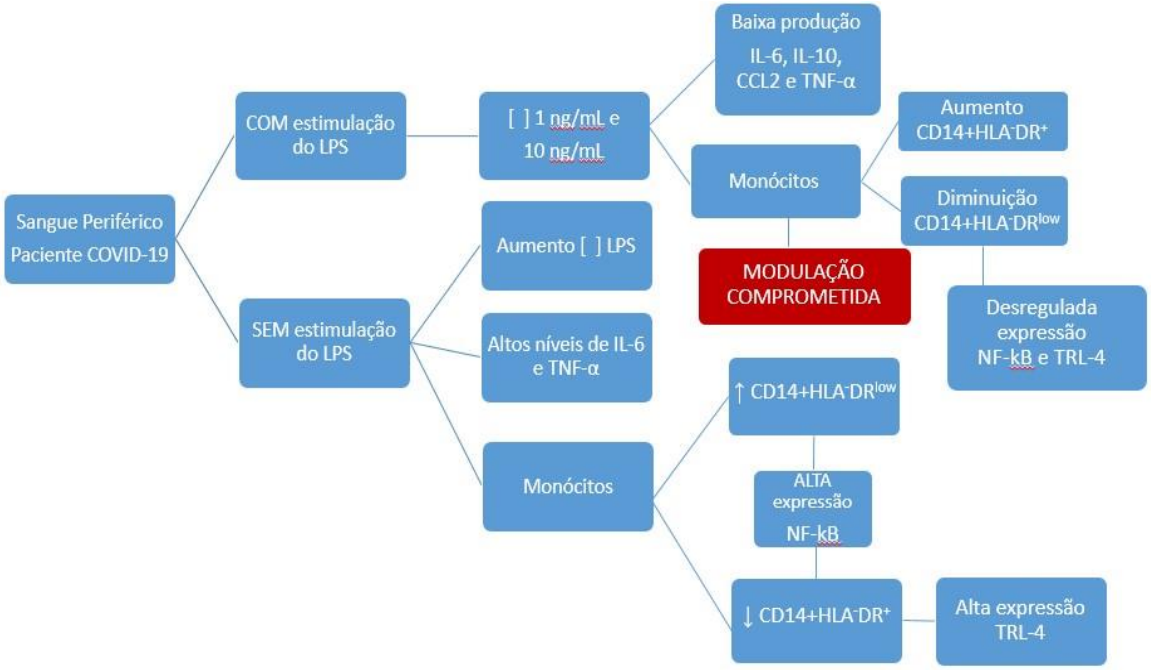
O estudo I nesta dissertação, inserida no capítulo II, teve objetivo de investigar a associação entre marcadores de translocação microbiana e inflamação sistêmica no momento da admissão hospitalar e na alta hospitalar em pacientes sobreviventes e não sobreviventes com COVID-19. Nossa hipótese foi que marcadores de translocação microbiana estão associados à inflamação sistêmica e podem influenciar na piora dos quadros clínicos e acarretar no óbito dos pacientes hospitalizados com COVID-19. Os principais achados deste estudo indicaram que níveis aumentados de LPS e sCD14 no sangue periférico de pacientes COVID-19 foram mais elevados na admissão hospitalar, os pacientes com COVID-19 apresentaram níveis elevados de IL-6, IFNγ, TNF-α, CCL5 / RANTES, CCL4 / MIP-1β e CCL2 / MCP-1, o que é indicativo de estado pró-inflamatórios; enquanto pacientes com COVID-19 não sobreviventes apresentaram aumento de LPS e sCD14 durante a internação e apresentaram aumento dos níveis sistêmicos de IL-6, TNF-α, CCL5 / RANTES e CCL2 / MCP-1. Por outro lado, os pacientes COVID-19 sobreviventes mantiveram os níveis de

LPS e sCD14 em valores estáveis concomitantes com aumentos nos níveis de IFN-γ. Dessa forma, nossos dados indicam que o aumento da translocação microbiana durante a hospitalização coexiste com a condição inflamatória da infecção por SARS-CoV-2 e pode induzir a maior ativação de monócitos, os quais podem estar alicerçados à piora dos desfechos de COVID-19.

6.3 ESTUDO II

O estudo II nesta dissertação, inserida no capítulo III, observamos que altas concentrações de endotoxinas na circulação podem indicar um estado comprometido da resposta imune do hospedeiro e gerar uma e maior suscetibilidade para aquisição de infecções oportunistas em pacientes infectados pelo SARS-CoV-2, a qual poderiam estar vinculadas à imunossupressão do sistema imune ao contrário da hiperinflamação. Desse modo, avaliamos a resposta inflamatória aguda e a modulação do fenótipo de monócitos em experimentos *ex vivo* com a estimulação de LPS em sangue total em pacientes com diferentes aspectos de gravidade da COVID-19. Acreditamos que a infecção aguda pelo SARS-CoV-2 pode comprometer a capacidade do sistema imunológico de responder contra patógenos oportunistas e, assim, gerar uma imunossupressão momentânea. Nossas análises foram realizadas em 3 condições diferentes das amostras de sangue periférico de pacientes COVID-19, sendo elas: amostras sem estimulação prévia de LPS e amostras com estimulação de LPS (Lipopolissacarídeos de Escherichia coli O26:B6 – Sigma-Aldrich) em diferentes concentrações (1ng/mL e 10 ng/mL). Nossos principais resultados estão demonstrados na figura 4.

Figura 4 - Fluxograma dos principais resultados do estudo II.



Esses resultados indicam que a infecção aguda por SARS-CoV-2 está vinculada à diminuição da resposta de endotoxina em monócitos de pacientes com COVID-19 grave.

7 CONCLUSÃO

Os dados da presente dissertação de mestrado indicam que pacientes falecidos apresentam uma condição caracterizada por translocação microbiana que coexiste com a condição inflamatória durante a COVID-19 e pode contribuir para a ativação de monócitos e induzir estado hiperinflamatório. Ademais, os dados do segundo estudo demonstram que a infecção por SARS-CoV-2 induz menor produção de citocinas e ativação de TLR-4/NF-κB em monócitos de COVID-19 grave após estimulação com LPS. Em conjunto, esses resultados podem indicar que a condição pró-inflamatória e os marcadores de translocação microbiana observados na infecção aguda por SARS CoV-2 podem induzir a tolerância à endotoxina e gerar suscetibilidade ao desenvolvimento de infecções secundárias e contribuir para desfechos clínicos graves da COVID-19.

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ANEXOS

ANEXO I – Parecer de aprovação no Comitê de Ética em Pesquisa – UFCSPA e Hospital Moinhos de Vento

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Análise epidemiológica e clínica de portadores de sintomas respiratórios e COVID-19 no Município de Esteio/RS

Pesquisador: CLAUDIA ELIZABETH THOMPSON

Área Temática:

Versão: 2

CAAE: 38886220.0.0000.5345

Instituição Proponente: Universidade Federal de Ciências da Saúde de Porto Alegre

Patrocinador Principal: MUNICIPIO DE ESTEIO

DADOS DO PARECER

Número do Parecer: 4.357.120

Apresentação do Projeto:

Trata-se de um estudo observacional longitudinal prospectivo de acompanhamento da evolução clínica, do momento do diagnóstico, da hospitalização até a alta hospitalar ou óbito, nos casos graves e do período de recuperação de todos os pacientes diagnosticados com COVID-19 no município de Esteio, com sequenciamento viral das amostras positivas.

Objetivo da Pesquisa:

Objetivo Primário: Analisar dados epidemiológicos e acompanhar a evolução clínica de portadores de sintomas respiratórios que buscaram assistência na rede de saúde do Município de Esteio, Rio Grande do Sul, durante a pandemia de Coronavírus (COVID-19).

Objetivo Secundário: I. Descrever os sintomáticos respiratórios no período da pandemia de COVID-19 em Esteio. II. Analisar a população de sintomáticos e suas características sociodemográficas, formas de exposição/infecção e contatos. III. Descrever a sintomatologia apresentada pela população em estudo com testes positivos para o coronavírus. IV. Acompanhar a evolução clínica dos pacientes graves, a partir de marcadores laboratoriais, tempo de permanência no hospital, tipo de hospitalização (UTI ou enfermaria), tempo de hospitalização, ventilação mecânica ou não, procedimentos realizados, dados clínicos laboratoriais e medicações utilizadas. V. Monitorar a evolução clínica dos casos

leves ou moderados mantidos na comunidade após o diagnóstico de COVID-19. VI. Acompanhar, nos casos incidentes, a presença do material genético (RNA) do vírus a partir de RT-qPCR realizadas periódicamente a partir do primeiro exame positivo e teste rápido (TR) para detecção de anticorpos, também periodicamente a partir do 10o dia de positividade na RT-qPCR, bem como
realizar o acompanhamento de biomarcadores laboratoriais, até o final do estudo. VII. Sequenciar o genoma viral de todas as amostras de pacientes positivos para COVID-19. VIII. Criar um banco de dados que contenha todas as informações clínicas, sociodemográficas e de resultados laboratoriais dos sintomáticos respiratórios no período da pandemia de COVID-19 em Esteio. IX. Avaliar níveis sistêmicos de citocinas (GM-CSF, IFN-a, IL-1, IL-6, TNF-a) no plasma de pacientes com COVID-19. X. Avaliar a expressão de NF-kB em monócitos e a frequência periférica de monócitos CD14+CD16- (clássicos), CD14+CD16+ (intermediários), CD14-CD16+(não-clássicos), CD14+CD16-/+HLA-DR+ /CD69+ (estado de ativação celular) de pacientes com COVID-19 e indivíduos
controles não infectados. XI. Avaliar apoptose, potencial de membrana mitocondrial e geração de espécies reativas de oxigênio em monócitos de pacientes com COVID e controles saudáveis. XII. Avaliar a frequência periférica de linfócitos T CD4+CD25+CD39+, CD4+CD25-CD39+, CD4+CD25+CD39+CD73+, CD4+CD25highCD127low, CD8+CD27+CD28+, CD8+CD27+CD28-, CD8+CD27-CD28-, CD4-/+CD8-/+PD-1+, CD4-/+CD8-/KLRG-1+
de pacientes com COVID-19 e indivíduos controles não infectados.

Avaliação dos Riscos e Benefícios:

Riscos: Pode ser considerado um estudo de riscos mínimos, por poder trazer constrangimentos a partir da aplicação de questionário. A punção digital e
venosa e a obtenção de amostras por swab orofaríngeo podem ser incluídas na classificação de riscos mínimos. Em caso de alguma complicação relacionada à coleta das amostras, o indivíduo será atendido pela própria rede de assistência à saúde do município que apoia a realização do projeto. Nenhum procedimento que tenha outras consequências será realizado.

Benefícios: Os beneficios para os participantes estão relacionados, especialmente, ao acompanhamento da evolução da doença de uma patologia ainda pouco estudada e conhecida, na qual a maioria das pessoas apresenta apenas sintomas leves, mas que pode resultar em quadro severo que responde melhor as medidas terapeuticas quando estas sao empregadas precocemente.

Comentários e Considerações sobre a Pesquisa:

Todas as pendências e inadequações foram atendidas.

Considerações sobre os Termos de apresentação obrigatória:

Todos os termos de apresentação obrigatória foram apresentados

Conclusões ou Pendências e Lista de Inadequações:

Todas as pendências e inadequações foram atendidas.

Considerações Finais a critério do CEP:

De acordo com o parecer do Relator.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1638466.pdf	14/10/2020 18:20:10		Aceito
Outros	TermoCompromissoRelatorioFinal.pdf	14/10/2020 18:19:33	CLAUDIA ELIZABETH THOMPSON	Aceito
Outros	CARTA_RESPOSTA_CEP_UFCSPA.pdf	14/10/2020 18:15:46	CLAUDIA ELIZABETH THOMPSON	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_Projeto_OUT2020.docx	14/10/2020 18:04:49	CLAUDIA ELIZABETH THOMPSON	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_Projeto_OUT2020_Responsaveis.docx	14/10/2020 18:04:21	CLAUDIA ELIZABETH THOMPSON	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_Projeto_OUT2020_Responsaveis.pdf	14/10/2020 18:04:12	CLAUDIA ELIZABETH THOMPSON	Aceito
Declaração de Instituição e Infraestrutura	Termo_Anuencia_LabImunologiaUFCSPA.pdf	14/10/2020 17:54:15	CLAUDIA ELIZABETH THOMPSON	Aceito
Declaração de Instituição e Infraestrutura	Termo_Anuencia_Lab_AnalisesClinicasUFCSPA.pdf	14/10/2020 17:54:07	CLAUDIA ELIZABETH THOMPSON	Aceito
Projeto Detalhado / Brochura Investigador	Projeto_COVID19_ESTEIO_14out.pdf	14/10/2020 17:52:56	CLAUDIA ELIZABETH THOMPSON	Aceito

Projeto Detalhado / Brochura Investigador	Projeto_COVID19_ESTEIO_14out.docx	14/10/2020 17:52:40	CLAUDIA ELIZABETH THOMPSON	Aceito
Folha de Rosto	FolhaRosto_CONEP_CThompson.pdf	03/10/2020 21:16:23	CLAUDIA ELIZABETH THOMPSON	Aceito
Declaração de Manuseio Material Biológico / Biorepositório / Biobanco	TermoCompromisso_NovaPesquisa.pdf	29/09/2020 13:19:57	CLAUDIA ELIZABETH THOMPSON	Aceito
Declaração do Patrocinador	DeclaracaoPatrocinador.pdf	29/09/2020 13:02:35	CLAUDIA ELIZABETH THOMPSON	Aceito
Declaração de Instituição e Infraestrutura	TermoAnuencia_HospitalSaoCamilo.pdf	28/09/2020 14:56:54	CLAUDIA ELIZABETH THOMPSON	Aceito
Declaração de Pesquisadores	Declaracao_Pesquisadores.pdf	28/09/2020 14:50:16	CLAUDIA ELIZABETH THOMPSON	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

PORTO ALEGRE, 23 de Outubro de 2020

Assinado por:
Fernanda Bordignon Nunes
(Coordenador(a))

PARECER CONSUBSTANCIADO DO CEP

DADOS DA EMENDA

Título da Pesquisa: Avaliação de características clínicas, fatores de risco, método diagnóstico e resposta imune de COVID-19: um estudo de vida real em dois hospitais no sul do Brasil

Pesquisador: Renato Tetelbom Stein
Área Temática:
Versão: 11
CAAE: 30749720.4.1001.5330
Instituição Proponente: Hospital Moinhos de Vento - HMV
Patrocinador Principal: Hospital Moinhos de Vento - HMV

DADOS DO PARECER

Número do Parecer: 4.932.566

Apresentação do Projeto:
Trata-se de um estudo que visa realizar a avaliação de características clínicas, fatores de risco, método diagnóstico e resposta imune de COVID-19 em dois hospitais no sul do Brasil.
A presente emenda solicita a inclusão da Pontifícia Universidade Católica do Rio Grande do Sul como centro participante do estudo visando a realização dos testes de oscilometria, tendo em vista a expertise desta instituição, a existência de um laboratório de função pulmonar já estruturado e dos profissionais parceiros desse local na realização deste exame. A fim de cumprir o cronograma planejado e atingir os objetivos propostos do subestudo de oscilometria e ergoespirometria,os investigadores solicitam a aprovação da inclusão de dois novos grupos de participantes para realização e comparação de impedância pulmonar por oscilometria, sendo eles:
a) Participantes internados por SARS-COV-2, com até três meses do quadro clínico (em subamostra de participantes internados na PUCRS);
b) Participantes voluntários saudáveis, que não tenham história clínica ou laboratorial de SARS-COV-2.

Objetivo da Pesquisa:
Objetivo Primário:

Avaliar a prevalência da infecção por SARS-CoV-2 em todas as faixas etárias, descrevendo e analisando suas características clínicas, fatores de risco, prevalência e papel das coinfecções por outros vírus respiratórios e/ou tuberculose, acurácia de métodos diagnósticos e características da resposta imune em pacientes que buscam atendimento em sala de emergência ou que são hospitalizados com suspeita de SARSC-V2 em dois hospitais do sul do Brasil.

Objetivo Secundário:
1. Avaliar a prevalência de infecções por SARS-CoV-2 entre os participantes ambulatoriais ou hospitalizados com quadro clínico suspeito de COVID-19 em atendimento nos dois hospitais do estudo, durante o período da pandemia de 2020;
2. Identificar a frequência de coinfecções (vírus influenza A e B e VSR) com o SARS-CoV-2 nos participantes hospitalizados e ambulatoriais, avaliando seu impacto como fator de risco para gravidade;
3. Identificar a frequência de infecção por Mycobacterium tuberculosis em participantes ambulatoriais do estudo com suspeita clínica de SARS-CoV-2 em sub-amostra de participantes no HRES) através de coleta de escarro, avaliando seu impacto como fator de risco para gravidade;
4. Descrever a evolução clínica dos participantes com diagnóstico confirmado de acordo com a faixa etária e comorbidades, após 4 semanas da inclusão no estudo (participantes ambulatoriais) ou da alta hospitalar (participantes internados);
5. Descrever a evolução clínica dos participantes encaminhados para isolamento domiciliar ou após alta hospitalar através de ligação telefônica;
6. Avaliar a acurácia do teste rápido sorológico comparado à técnica padrão-ouro de biologia molecular RTPCR;
7.Comparar a técnica padrão-ouro de biologia molecular RT-PCR para identificação do SARS-CoV-2 em diferentes materiais: nasal/orofaríngeo (padrão, realizado em toda a coorte), escarro espontâneo, saliva, e aspirado traqueal (estas três últimas em
subamostras);
8. Entender as relações entre uso de bloqueadores do sistema RAS (iECA ou ARA II) com gravidade e prognóstico da COVID-19, ajustando para comorbidades dosagem e uso, recente ou prévio;
9.Descrever etiologia, características e gravidade das doenças cardíacas pré-existentes (hipertensiva, isquêmica, insuficiência cardíaca, valvulopatias) com gravidade e prognóstico da COVID-19, ajustando para outras comorbidades;
10.Descrever a evolução temporal da troponina (ultra-sensível) e BNP (ou NT-pro-BNP)

nosparticipantes hospitalizados com COVID-19 (doença leve a moderada ou grave) e sua relação com gravidade e prognóstico da COVID-19;
11.Comparar o perfil da resposta imunológica (análise de células imunes no sangue por citometria de fluxo e de anticorpos neutralizantes) e evolução clínica entre crianças e adultos com teste positivo para SARS-CoV2 e controles saudáveis, avaliando fatores protetores e de gravidade;
12.Caracterizar o uso de receptores antigênicos específicos – células T e B – para compreender o espectro da resposta protetora gerada;
13.Quantificação da carga viral SARS-CoV-2 por RT-PCR quantitativa em diferentes tipos de amostras biológicas (saliva, nasofaringe e oral), avaliando fatores de gravidade;
14.Analisar a resposta inata e adaptativa em diferentes graus de doença, buscando marcadores preditivos;
15. Nos participantes incluídos após a internação ocorrida nos primeiros 2 meses de pandemia no HMV, serão avaliados os objetivos 4, 8, 9 e 10;
16. Descrever a prevalência de colonização nasofaríngea por Streptococcus pneumoniae e seus sorotipos de acordo com status;
17. Avaliar a duração dos anticorpos anti-SARS-CoV-2 gerados após a infecção nos indivíduos incluídos nesta análise em amostras sequenciais;
18. Avaliar a duração dos anticorpos neutralizantes anti-SARS-CoV-2 gerados após a infecção nos indivíduos incluídos nesta análise em amostras sequenciais;
19. Descrever a resposta T específica correlata com proteção comparado com a geração de anticorpos neutralizantes específicos para o SARS-CoV-2 em amostras sequenciais; 20. Descrever a imunidade gerada em crianças, e sua correlação com a ausência ou presença de sintomas.

Avaliação dos Riscos e Benefícios:

Riscos:

Os riscos atribuídos à participação no estudo incluem aqueles inerentes às coletas de material biológico, devido a punção venosa, aspiração de secreção e desconforto ao responder alguma questão da entrevista. Nesse caso o entrevistado pode optar por não responder. A coleta de sangue tem riscos inerentes à punção da veia como desconforto, dor, sangramento local, ou hematoma no local da picada. A coleta de teste rápido tem o risco de dor,sangramento e hematoma no local da punção com lanceta em extremidade. A coleta de secreção respiratória tem riscos inerentes ao uso do swab para a coleta como desconforto, dor no local, sangramento nasal discreto, vômito, tosse ou espirro devido à coleta. O

procedimento será feito por profissional devidamente capacitado e com capacidade de avaliar o estado do paciente e suspender a coleta, caso necessário. Todas as coletas serão realizadas com material descartável e de uso individual, portanto,sem riscos de contaminação.Exames coletados por indicação médica da equipe assistente durante a internação serão registrados como dados de prontuário. Os principais tipos de riscos potenciais que envolvem a oscilometria são: riscos com energia elétrica, o que é comum para um equipamento eletrônico,risco de reações de bioincompatibilidade do equipamento se os materiais utilizados não forem
adequadamente selecionados e a possibilidade de imprecisão na medição do resultado, bem como problemas de software ou hardware. Contudo, a equipe do estudo tomará as devidas medidas para que esses riscos não ocorram. Os possíveis riscos da realização de ergoespirometria são o desenvolvimento de síncope, arritmia, infarto do miocárdio, parada cardíaca e morte. Estes riscos são extremamente baixos, e estudos envolvendo centenas de pacientes demonstraram que este exame é seguro.Além disso, o participante da pesquisa estará sendo acompanhado durante todo o exame por profissional de saúde habilitado e experiente.

Benefícios:
Estarão sendo ofertados ao paciente testes diagnósticos de alta sensibilidade e especificidade para quadro respiratório (SARS-COV-2, Influenza, VSR e, no HRES também para tuberculose), com resultados disponíveis no mesmo dia (tuberculose, Influenza e VSR) ou em até aproximadamente 48 horas (SARS-CoV -2), sendo que estes não são disponibilizados desta forma rotineiramente no SUS e na saúde suplementar.
Os benefícios diretos obtidos com a ergoespirometria realizados neste estudo podem dar ao participante de pesquisa a confiança necessária para realizar suas atividades usuais após alta hospitalar, além de fornecer informações para a prescrição de reabilitação cardiopulmonar. As informações obtidas podem servir para aprimorar o atendimento futuro de pacientes que se recuperaram da COVID-19, podendo influenciar na orientação e prescrição de reabilitação cardiopulmonar, melhorando a qualidade de vida dos pacientes.

Comentários e Considerações sobre a Pesquisa:
Na presente emenda, os documentos encaminhados para apreciação são:
1. Emenda8_COVIDa_versao_marcada_julho2021.pdf
2. Emenda8_COVIDa_versao_limpa_julho2021.pdf
3. TCLE_oscilometria_ergoespirometria_versao_marcada_julho2021.pdf

4. TCLE_oscilometria_ergoespirometria_versao_limpa_julho2021.pdf

As seguintes alterações são propostas nesta emenda:
1. Inclusão da Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS)como centro participante;
2. Ajuste do Objetivo Secundário “4.2.21”, alterando os comparando três diferentes subgrupos para: “1) Participantes recuperados da infecção por SARS-COV-2, que tenham necessitado internação hospitalar, há mais de seis meses do quadro clínico (em subamostra de participantes internados no HMV). 2) Participantes internados por SARS-COV-2, com até três meses do quadro clínico (em subamostra de participantes na PUCRS) e 3) Participantes voluntários saudáveis, que não tenham história clínica ou laboratorial de SARS-COV-2”;
3. Alteração do item “5.10” do tópico PRINCIPAIS HIPÓTESES: de “pacientes internados por pneumonia de outra etiologia” para “participantes voluntários sem história clínica ou laboratorial de SARS-COV-2”;
4. Inclusão do parágrafo “4” no item “6.2 Descrição da pesquisa, local e período de realização”: “4. Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS): análise de oscilometria nos participantes incluídos no Hospital Moinhos de Vento recuperados da infecção SARS-COV-2 e que tenham necessitado internação hospitalar, após ao menos seis meses do quadro clínico; análise de oscilometria em participantes internados no Hospital São Lucas da PUCRS por SARS-COV-2 até três meses do quadro clínico; e participantes voluntários saudáveis sem quadro clínico ou laboratorial de SARS-COV-2”;
5. Alteração no item “6.5 Critérios de inclusão”: exclusão da palavra “apenas” e inclusão de “participantes hospitalizados na PUCRS e participantes voluntários sem história clínica ou laboratorial de SARS-COV-2”;
6. Alteração do parágrafo: de “oscilometria pulmonar** após a alta hospitalar e reavaliados entre 120 - 240 dias após a alta.” para “Outros três grupos serão convidados a realizar oscilometria pulmonar: a) participantes incluídos no HMV recuperados da infecção por SARS-COV-2 e que tenham necessitado internação hospitalar, após seis meses do quadro clínico; b) participantes internados no Hospital São Lucas da PUCRS por SARS-COV-2 até três meses do quadro clínico e; c) participantes voluntários saudáveis sem quadro clínico ou laboratorial de SARS-COV-2”;

7. Alteração no item 6.14.1.4: Inclusão de “pós seis meses do quadro clínico”,
exclusão da palavra “pneumonia” e inclusão de “até três meses do quadro clínico”;
8. Inclusão no “cronograma proposto” das atividades referentes ao segundo semestre
de 2021,sendo elas:“Revisão bibliográfica, Realização dos diagnósticos laboratoriais, Realização deanálises imunológicas e moleculares, Realização da coleta de dados, Avaliação dos Resultados, Elaboração de artigos para publicação e Elaboração de material didático para o Ministério da Saúde”;
9. Inclusão no Termo de Consentimento Livre e Esclarecido–Participante de Pesquisa com Idade 18 Anos – OSCILOMETRIA e ERGOESPIROMETRIA da frase “e no Hospital São
Lucas da Pontifícia Universidade Católica do Rio Grande do Sul.” e inclusão da opção “Não se aplica”, a pergunta: “2) Você concorda em realizar o teste cardiopulmonar de exercício (ergoespirometria)?”;
10. Ajuste no Termo de Consentimento Livre e Esclarecido.

Considerações sobre os Termos de apresentação obrigatória:

Termos de apresentação obrigatórios devidamente preenchidos, assinados e adaptados conforme atualizações propostas nesta emenda.

Conclusões ou Pendências e Lista de Inadequações:

Emenda administrativa que comunica inclusão de centro e de dois novos grupos de participantes. Vide "comentários e considerações sobre a pesquisa" para mais informações. Não há impedimento ético para a aprovação desta emenda e continuidade do estudo.

Considerações Finais a critério do CEP:

Diante do exposto, o Comitê de Ética em Pesquisa em Seres Humanos do Hospital Moinhos de Vento, de acordo com as atribuições definidas na Resolução 466/2012 do CNS e complementares, e pela Norma Operacional Nº 001/2013 do CNS, manifesta-se pela aprovação da Emenda.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_1780936_E8.pdf	25/08/2021 16:53:13		Aceito
Outros	Carta_resposta_ao_CEP_agosto_2021.pdf	25/08/2021 16:36:14	Ingrid Rodrigues Fernandes	Aceito

Continuação do Parecer: 4.932.566

Projeto Detalhado / Brochura Investigador	Emenda8_COVIDa_versao_marcada_julho2021.pdf	01/07/2021 12:58:42	Gabriela Oliveira Zavaglia	Aceito
Projeto Detalhado / Brochura Investigador	Emenda8_COVIDa_versao_limpa_julho2021.pdf	01/07/2021 12:58:17	Gabriela Oliveira Zavaglia	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_oscilometria_ergoespirometria_versao_marcada_julho2021.pdf	01/07/2021 12:55:02	Gabriela Oliveira Zavaglia	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_oscilometria_ergoespirometria_versao_limpa_julho2021.pdf	01/07/2021 12:54:27	Gabriela Oliveira Zavaglia	Aceito
Outros	Carta_ao_CEP_julho_2021.pdf	01/07/2021 12:53:36	Gabriela Oliveira Zavaglia	Aceito
Outros	termo_responsabilidade_30dezembro2020.pdf	30/12/2020 22:14:56	Ingrid Rodrigues Fernandes	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	01_TCLE_adulto_imuno_05out2020.pdf	06/10/2020 16:18:30	Ingrid Rodrigues Fernandes	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	02_TCLE_crianca_imuno_05out2020.pdf	06/10/2020 16:15:18	Ingrid Rodrigues Fernandes	Aceito
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Situação do Parecer:
Aprovado

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PORTO ALEGRE, 26 de Agosto de 2021

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ANEXO II - Normas de formatação para as revistas científicas

Seguindo as recomendações da coordenação do PPG-Ciências da Saúde, optou-se por inserir apenas o link para as normas online de cada revista.

Artigo I
Revista International Immunopharmacology
https://www.elsevier.com/journals/international-immunopharmacology/1567-5769/guide-for-authors

Artigo II **Revista Journal of Molecular Medicine**
https://www.springer.com/journal/109/submission-guidelines

ANEXO III - Artigos produzidos ao longo do período de mestrado

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RESEARCH ARTICLE

Journal of Cellular Physiology WILEY

Alterations in CD39/CD73 axis of T cells associated with COVID-19 severity

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Abstract

Purinergic signaling modulates immune function and is involved in the immunopathogenesis of several viral infections. This study aimed to investigate alterations in purinergic pathways in coronavirus disease 2019 (COVID-19) patients. Mild and severe COVID-19 patients had lower extracellular adenosine triphosphate and adenosine levels, and higher cytokines than healthy controls. Mild COVID-19 patients presented lower frequencies of $CD4^+CD25^+CD39^+$ (activated/memory regulatory T cell [mTreg]) and increased frequencies of high-differentiated ($CD27^-CD28^-$) $CD8^+$ T cells compared with healthy controls. Severe COVID-19 patients also showed higher frequencies of $CD4^+CD39^+$, $CD4^+CD25^-CD39^+$ (memory T effector cell), and high-differentiated $CD8^+$ T cells ($CD27^-CD28^-$), and diminished frequencies of $CD4^+CD73^+$, $CD4^+CD25^+CD39^+$ mTreg cell, $CD8^+CD73^+$, and low-differentiated $CD8^+$ T cells ($CD27^+CD28^+$) in the blood in relation to mild COVID-19 patients and controls. Moreover, severe COVID-19 patients presented higher expression of PD-1 on low-differentiated $CD8^+$ T cells. Both severe and mild COVID-19 patients presented higher frequencies of $CD4^+$Annexin-V^+ and $CD8^+$Annexin-V^+ T cells, indicating increased T-cell apoptosis. Plasma samples collected from severe COVID-19 patients were able to decrease the expression of CD73 on $CD4^+$ and $CD8^+$ T cells of a healthy donor. Interestingly, the in vitro incubation of peripheral blood mononuclear cell from severe COVID-19 patients with adenosine

reduced the nuclear factor-κB activation in T cells and monocytes. Together, these data add new knowledge to the COVID-19 immunopathology through purinergic regulation.

KEYWORDS
adenosine, ATP, inflammation, purines, SARS-CoV-2, T lymphocytes

1 | INTRODUCTION

The coronavirus disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2; coronavirus disease 2019 [COVID-19]) is a hyperinflammatory condition that can be asymptomatic or manifested as a broad spectrum of disease ranging from few symptoms (mild COVID-19 cases) to severe pneumonia that may evolve to SARS and death (severe COVID-19 cases; Chen et al., 2020). Although several SARS-CoV-2 infections generate asymptomatic or mild COVID-19, a considerable proportion of the remaining cases show severe and critical pneumonia requiring oxygen support and mechanical ventilation (Pascarella et al., 2020).

Host factors and the physiological environment determine the type and the strength of the immune response during the viral infection, as well as the disease outcomes. Thus, the biology of several immune cells, including lymphocytes, can be influenced by blood extracellular adenine nucleotides—adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP), and nucleoside (adenosine; Cekic & Linden, 2016). Purinergic signaling regulates a number of immune cell functions, such as cell-to-cell interactions, cell death, cytokine and chemokine secretion, surface antigen shedding, and cell proliferation (Antonioli et al., 2013). During infections, once released by damaged cells, ATP acts as a damage-associated molecular pattern (DAMP) through P2 receptors (P2X and P2Y receptors) activating immune cells and inducing strong proinflammatory effects (Idzko et al., 2014). Interestingly, severe COVID-19 patients had higher ATP content in the bronchoalveolar lavage supernatant associated with P2RX7-inflammasome activation in macrophage compared with non-COVID-19 patients (Wauters et al., 2021). Furthermore, ATP and ADP levels had positive correlations with markers of blood coagulation and leukocytes showed an upregulation of the several purinergic receptors and ectonucleotidases activities (Schultz et al., 2022). Thus, alterations in adenine-based purine molecules may contribute to the hyperinflammation and immune dysfunction observed in COVID-19 pathophysiology.

The ectoenzyme CD39 (ecto-nucleoside triphosphate diphosphohydrolase 1) converts extracellular ATP into AMP and then CD73 (ecto-5′-nucleotidase) dephosphorylates AMP into adenosine (Antonioli et al., 2013; Deaglio et al., 2007). Adenosine signaling is mediated by G-protein-coupled adenosine receptors, which act as a mechanism for regulating intracellular cyclic AMP levels to induce immunosuppressive events (Mandapathil et al., 2010).

The expression and activity of both CD39 and CD73 change under the pathological context of acute and chronic viral infections (Aeffner et al., 2015; He et al., 2015; Leal et al., 2005). In addition, CD39 expression in $CD8^+$ T cells has also been described as a marker of exhaustion in virus infection (Gupta et al., 2015). There are interconnected changes in the immune system and in the purinergic signaling. Those changes are mediated by the dynamics of adenine-based purine molecules and CD39/CD73 axis that directly impact the host immune response to viral infection (Hasan et al., 2022). During SARS-CoV-2 infection, the active replication and release of the virus can cause the host cells to undergo pyroptosis, promoting the outpour of ATP, which acts as a DAMP in the extracellular environment activating several purinergic components. On the other hand, the dephosphorylation of ATP to adenosine activates immunosuppressive actions mediated mainly by regulatory T cells (Treg cells). In this sense, the modulation of purinergic signaling of T cells expressing CD39/CD73 ectonucleotidases and the extracellular levels of adenine-based purine molecules may contribute to the impaired control of acute hyperinflammatory response through a failure of immunosuppressive axis in COVID-19 (Zarei et al., 2021). The understanding of how purinergic signaling acts during acute SARS-CoV-2 infection could contribute to the development of potential pharmacological targets in the treatment of the disease and may attenuate the progression of severe COVID-19 cases and bad outcomes.

Ahmadi et al. (2020) found lower proportions of $CD8^+CD73^-$ T cells in the peripheral blood of COVID-19 patients and demonstrated that these cells possess a significantly higher cytotoxic effector phenotype. To better understand the role of the CD39/CD73 pathway in the immunopathology of SARS-CoV-2 infection, this study evaluated the systemic levels of ATP, ADP, AMP, and adenosine and the frequencies of $CD4^+$ and $CD8^+$ T cells expressing CD39 and CD73 ectonucleotidases, the proportions of $CD8^+CD27^{-/+}CD28^{-/+}$ expressing PD-1, as well as the rates of lymphocyte apoptosis in the peripheral blood from mild and severe COVID-19 patients.

2 | METHODS

2.1 | Study patients and ethics

We prospectively evaluated a convenience sample of mild and severe COVID-19-positive patients admitted at the Hospital Moinhos de

Vento between July 2020 and November 2020, and volunteering uninfected healthy individuals. Infection with SARS-CoV-2 was confirmed by reverse-transcription polymerase chain reaction (RT-PCR) with nasopharyngeal and oropharyngeal swab samples. This study was approved by the Moinhos de Vento Ethics Committee N 3977144 (Porto Alegre/Brazil) and informed consent was obtained from all participants. Blood samples (5 ml) were obtained from patients with mild or severe COVID-19 into K2EDTA tubes (Becton & Dickinson) within 6 h from hospital admission and were kept refrigerated (2–4°C) until the moment of processing (2 h after the collection). Flow cytometry experiments (described below) were conducted with fresh blood. The remaining blood was centrifuged (1500*g*, 10 min), plasma was aliquoted, and was kept at −80°C until plasma analysis.

Disease severity was classified according to the World Health Organization classification after completing the follow-up questionnaire (WHO, 2021). Clinical and sociodemographic data were collected from the patient's electronic medical records upon admission to the unit. Body mass index was calculated from weight and height data.

2.2 | Analysis of adenine-based purine concentrations and lipopolysaccharide (LPS) levels by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

An aliquot of 150 µl of acetonitrile was added to the plasma samples (50 µl) and the mixture was shaken for 60 s. After centrifugation for 6 min at 9000*g*, the supernatant was collected and a 25 µl aliquot was directly injected into the LC-MS/MS system. The analytical system consisted of a Nexera UFLC system coupled to a LCMS-8040 triple quadrupole mass spectrometer (Shimadzu). The electrospray ionization-MS/MS parameters were set in positive and negative ion modes (polarity switch, 25 ms) as follows: capillary voltage, positive 4500 V, and negative 3000 V; desolvation line temperature, 200°C; heating block temperature, 500°C; drying gas, 18 L/min; and nebulizing gas, 2 L/min. Analyses were carried out with multiple reaction monitoring (MRM) by using the following fragmentations: *m/z* 268.0 → *m/z* 136.0 for detection of adenosine ($[M + H]^+$); *m/z* 346.0 → *m/z* 210.1 for detection of AMP ($[M - H]^-$); *m/z* 426.0 → *m/z* 327.4 for detection of ADP ($[M - H]^-$); and *m/z* 506.0 → *m/z* 207.5 for detection of ATP ($[M - H]^-$). The chromatographic separation was conducted with a Shim-pack GISS column (2.1 × 100 mm, 1.9 µm particle size; Shimadzu) eluted with flow rate of 0.3 ml/min. The gradient mobile phase system consisted of water (Solvent A) and acetonitrile (Solvent B) both fortified with 0.2% acetic acid and 0.1% tributylamine as follow: 0–4.5 min, 10–90% of B; 4.5–5.5 min, 90% of B; 5.5–5.6 min, 90–10% of B; 5.6–10 min, 10% B. The column oven was kept at 30°C. The data were processed using LabSolutions software (Shimadzu). In addition, LPS concentration was determined by quantification of 3-hydroxytetradecanoic acid as described by Teixeira et al. (2021).

2.3 | Cytokine levels

The plasma concentrations of interleukin-1A (IL-1A; from PeproTech) and IL-6, IL-10, and transforming growth factor-β (TGF-β; all from eBioscience, ThermoFisher) were quantified by enzyme-linked immunosorbent assay (ELISA) in a microplate reader (EzReader). The detection limits of each cytokine were IL-6, 2–200 pg/ml; IL-10, 2–300 pg/ml; IL-1A, 2–1000 pg/ml; TGF-β, 20–500 pg/ml.

2.4 | Immunophenotyping

Whole blood samples (100 µl) were incubated with monoclonal surface antibodies (all antihuman) at 4°C for 20 min in accordance with the following combination: (a) Fluorescein isothiocyanate (FITC)-conjugated anti-CD4, Pe-conjugated anti-CD25, PerCP-Cy5.5-conjugated anti-CD39, allophycocyanin (-conjugated anti-CD73; (b) FITC-conjugated anti-CD8, Pe-conjugated anti-CD39, APC-conjugated anti-CD73; and (c) FITC-conjugated anti-CD8, Pe-conjugated anti-CD28, PerCP-Cy5.5-conjugated anti-CD27, APC-conjugated anti-PD-1. Then, samples were incubated for 10 min with lysing buffer (BD Biosciences) and then centrifuged at 500*g* for 5 min. The supernatant was discarded and samples were resuspended in phosphate buffered saline (PBS; 1ml, pH 7.2) and then centrifuged at 500*g* for 5 min. Finally, the samples were resuspended in 0.5 ml PBS and analyzed in flow cytometry. Cell phenotype was acquired using CELLQuest Pro Software (BD Bioscience) on a FACSCalibur flow cytometer (BD Bioscience). A minimal of 50,000 events/tubes were acquired, and granulocytes, lymphocytes, and monocytes were identified and gated according to each forward scatter and side scatter profiles. In the $CD8^+$ T cells gate, cells were further characterized by CD27/CD28 expression: $CD27^+CD28^+$ were defined as low-differentiated cells and $CD27^-CD28^-$ were defined as high-differentiated cells (van Aalderen et al., 2015). PD-1 expression was evaluated in both $CD8^+CD27^-CD28^-$ and $CD8^+CD27^+CD28^+$ T cells subsets and presented as mean fluorescence intensity.

2.5 | Mitochondrial membrane polarization and apoptosis analysis

The mitochondrial membrane potential (ΔΨm) was quantified according to a method previously described (Ferlini & Scambia, 2007), using the fluorescent dye rhodamine 123 (Rh 123, Sigma-Aldrich). The detection of apoptosis was performed by using additional labeling with Annexin V–FITC following manufacturer's guidelines (Invitrogen, ThermoFisher). Analyses were performed by using CELLQuest Pro Software (BD Bioscience) on a FACSCalibur flow cytometer (BD Bioscience).

2.6 | Cell culture and in vitro experiments

Peripheral blood mononuclear cells (PBMCs) of a healthy noninfected donor were isolated from peripheral blood using histopaque gradient

solution, Histopaque 1077 (Sigma-Aldrich) as previously described (Dorneles et al., 2020). Then, the cells were washed and suspended in Roswell Park Memorial Institute-1640 medium (Sigma-Aldrich) supplemented with 2 g/L sodium bicarbonate, 2% glutamine, 100 U/ml penicillin–0.1 mg/ml streptomycin (Sigma-Aldrich), and 10% of plasma acquired from mild COVID-19 ($n = 9$), severe COVID-19 ($n = 9$), or healthy controls ($n = 9$) for 15 h (37°C, 5% CO_2). PBMCs were collected, washed with PBS 1×, and stained with anti-CD4 Pe, anti-CD8 FITC, anti-CD39 Percp-Cy5.5, and anti-CD73 APC (all antihuman antibodies from BD Bioscience) for Flow Cytometry analysis in BD FacsCalibur (BD).

To test the effect of in vitro adenosine treatment on COVID-19-related hyperinflammation, PBMC (1×10^6 cells/well) of severe ($n = 3$) COVID-19 patients were seeded in 12-well plates and treated with adenosine (100 µM) or PBS as control for 24 h (37°C, 5% CO_2). The supernatants were frozen at −80°C until cytokine production analysis. Then, cells were stained with 5 µl monoclonal antibodies (all antihuman) conjugated with specific fluorochromes: CD3 FITC (EbioScience) or CD14 FITC (BIOGEMS). Thereafter, cells were washed with 1.5 ml PBS. For intracellular analysis, cells stained with anti-CD3 or anti-CD14 antibodies were incubated with fixation and permeabilization buffers following the manufacturer's recommendation (Ebioscience). After the period, samples were incubated with Phospo-NF-κBp65 (Ser529) Pe (Ebioscience) monoclonal antibody during 30 min. Then, cells were washed and resuspended in Flow Cytometry Staining Buffer (Ebioscience) and analyzed by flow cytometry as described above. The levels of tumor necrosis factor-α (TNF-α), IL-1β, IL-17a, and IL-10 were evaluated in the culture supernatant by ELISA using commercial kits (all from Ebioscience, ThermoFisher). The detection limits of each cytokine were IL-1β, 2–400 pg/ml; IL-10, 2–300 pg/ml; IL-17A, 2–1000 pg/ml; TNF-α, 2–500 pg/ml.

2.7 | Statistical analysis

Normality of data was checked by Kolmogorov–Smirnov test and the values were presented as mean ± SD. Categorical variables were presented as relative frequency and analyzed by χ^2 test. To identify associations between adenine-based purine levels and symptoms, we used Mann–Whitney *U*-test to compare SARS-CoV-2-infected patients with and without specific disease symptoms. A one-way analysis of variance followed by Bonferroni's post hoc test for multiple comparisons was used to verify between-groups differences. Pearson's coefficient test was performed to check correlations between the variables. $p \leq 0.05$ was considered statistically significant. The SPSS 20.0 (IBM Inc.) software was used in all analysis.

3 | RESULTS

3.1 | Participant's characteristics

The characteristics of severe ($n = 22$), mild ($n = 24$) COVID-19 patients, and healthy control ($n = 13$) participants are presented in Table S1. All patients had COVID-19 confirmed by RT-PCR. Severe COVID-19 patients were older ($p < 0.001$), presented more days from symptom onset ($p < 0.001$), and required oxygen use during hospitalization ($p < 0.001$) compared with mild COVID-19 patients. Furthermore, several symptoms were reported by severe COVID-19 patients, including cough ($p = 0.007$), dysgeusia ($p = 0.04$), anosmia ($p = 0.001$), vomiting ($p = 0.007$), diarrhea ($p = 0.004$), skin rash ($p = 0.01$), fatigue ($p = 0.01$), and stuffy nose ($p = 0.005$). Regarding associated medical condition, severe COVID-19 patients reported hypertension ($p = 0.002$), diabetes mellitus ($p = 0.001$), cardiovascular diseases ($p = 0.001$), heart failure ($p = 0.001$), chronic obstructive pulmonary disease ($p = 0.001$), asthma ($p = 0.001$), and dyslipidemia ($p = 0.001$).

3.2 | COVID-19 impacts on systemic purinergic molecules and cytokine levels

The plasma concentrations of adenine-based purines ATP, ADP, AMP, adenosine, and cytokines are shown in Figure 1. ATP levels were decreased in mild ($p = 0.03$) and severe ($p = 0.03$) COVID-19 patients compared with that in healthy controls. Systemic adenosine levels were higher in healthy controls compared with mild ($p = 0.03$) and severe ($p = 0.02$) COVID-19 patients. Severe COVID-19 presented higher systemic IL-6 and IL-10 levels compared with that in healthy controls and mild COVID-19 patients ($p < 0.01$ for all comparisons). In addition, lower IL-17A levels were identified in the peripheral blood of healthy controls compared with that in mild ($p = 0.03$) and severe COVID-19 ($p = 0.02$) patients.

Then, we compared the systemic levels of adenine-based purines in patients with or without clinical symptoms of COVID-19. COVID-19 patients with nausea symptom ($n = 13$) presented lower adenosine levels ($p = 0.04$) and a tendency to decreased AMP levels ($p = 0.07$). Furthermore, higher ADP levels were found in patients reporting stuffy nose symptom compared to patients without stuffy nose ($p = 0.02$, $n = 19$). Skin rash was reported by two patients and presented lower ATP ($p = 0.01$), ADP ($p = 0.01$), and AMP ($p = 0.01$) levels (Figure 2). Interestingly, LPS levels, a microbial translocation marker who is elevated in COVID-19 patients, were higher in patients with nausea symptom ($p = 0.02$) and inversely correlated with the systemic adenosine levels ($r = -0.54$; $p = 0.03$).

3.3 | Altered CD39 and CD73 expression in T lymphocytes of COVID-19 patients

The T-cell subsets was evaluated in healthy controls ($n = 8$), mild COVID-19 ($n = 12$), and severe COVID-19 patients ($n = 13$; Figure S1). Lower frequencies of $CD4^+$ and $CD8^+$ T cells ($p < 0.001$ vs. healthy controls; $p = 0.03$ vs. mild COVID-19) were found in mild and severe COVID-19 patients. Reduced peripheral frequency of $CD4^+CD25^-$ T cells were also found in mild COVID-19 ($p = 0.01$) and severe COVID-19 ($p = 0.001$) groups compared with healthy controls.

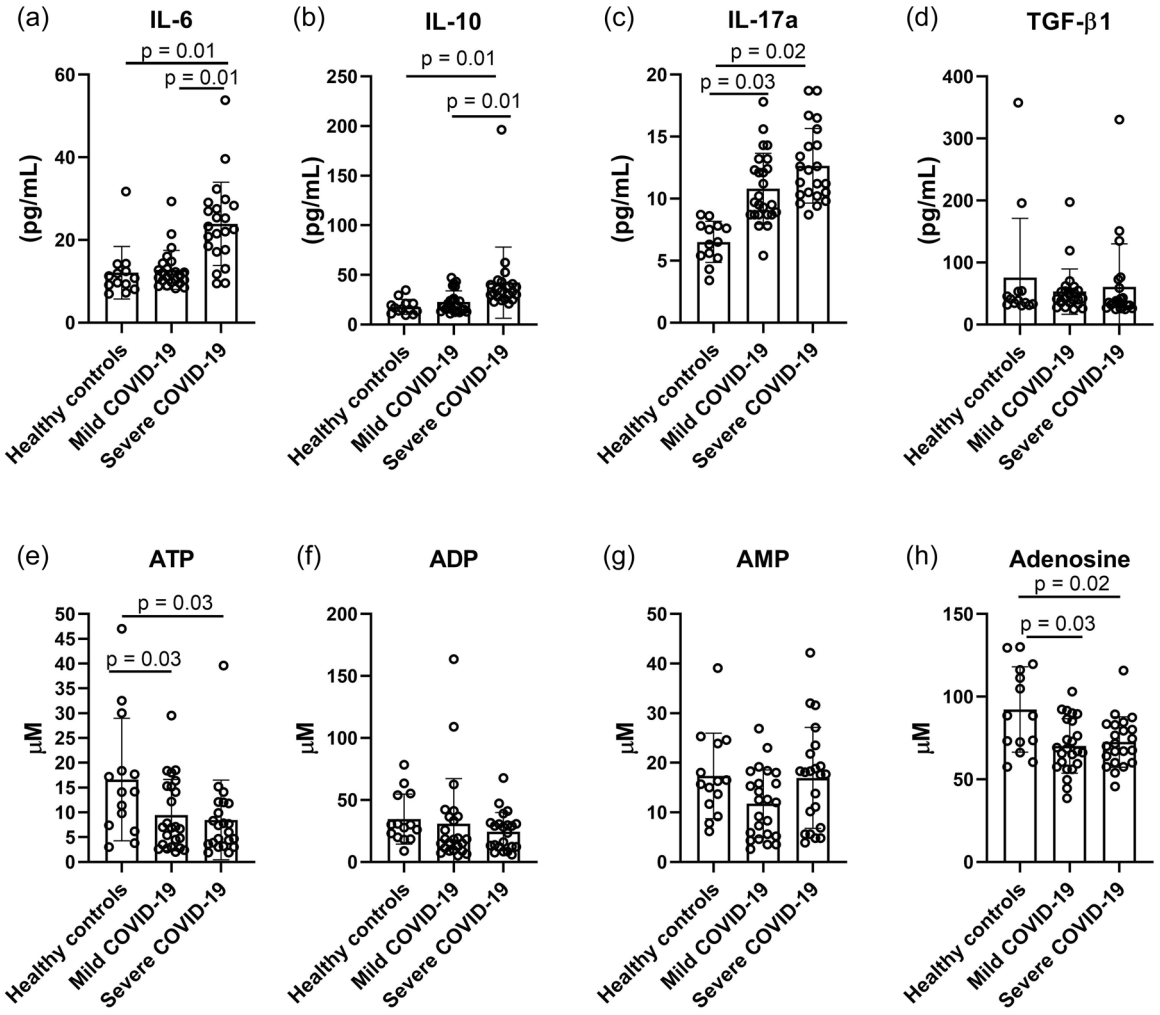

FIGURE 1 The systemic levels of systemic cytokines (a, interleukin-6 [IL-6]; b, IL-10; c, IL-17A; d, transforming growth factor-β1 [TGF-β1]) and adenine-based purines (e, adenosine triphosphate [ATP]; f, adenosine diphosphate [ADP]; g, adenosine monophosphate [AMP]; h, Adenosine) of healthy controls, mild and severe coronavirus disease 2019 (COVID-19) patients. Data are presented as mean ± SD. Group comparisons were performed by one-way analysis of variance with Bonferroni's post hoc test ($p \leq 0.05$).

Figure 3 shows the frequency of CD4, $CD8^+$, $CD4^+CD25^-$, $CD4^+CD25^+$ T cells expressing $CD39^+$ and $CD73^+$, and the proportions of $CD8^+CD27^{-/+}CD28^{-/+}$ T cells expressing PD-1. The frequency of $CD4^+$ ($p = 0.01$ vs. controls; $p = 0.03$ vs. mild COVID-19) and $CD4^+CD25^-$ ($p = 0.002$ vs. controls; $p = 0.004$ vs. mild COVID-19) T cells expressing $CD39^+$ were higher in severe COVID-19. On the other hand, the proportions of $CD4^+CD73^+$ ($p = 0.04$ vs. controls), $CD4^+CD25^+CD39^+$ ($p = 0.001$ vs. controls; $p = 0.002$ vs. mild COVID-19) and $CD8^+CD73^+$ ($p = 0.001$ vs. controls; $p = 0.002$ vs. mild COVID-19) T cells were lower in severe COVID-19 patients. Higher frequency of high-differentiated ($CD27^-CD28^-$) $CD8^+$ T cells was identified in the peripheral blood of mild COVID-19 patients ($p = 0.001$ vs. healthy control) and severe COVID-19 subjects ($p = 0.001$ vs. healthy control), and lower proportions of low-differentiated ($CD27^+CD28^+$) $CD8^+$ T cells in the severe COVID-19 group ($p = 0.05$ vs. healthy controls). The expression of PD-1 was higher in low-differentiated $CD8^+$ T cells of severe COVID-19 patients ($p = 0.008$ vs. healthy controls).

3.4 | Higher apoptosis and mitochondrial dysfunction in lymphocytes of COVID-19 patients

Next, we evaluated the apoptosis and mitochondrial membrane polarization in healthy controls ($n = 6$), mild COVID-19 ($n = 6$) and severe COVID-19 ($n = 6$) individuals (Figure 4). COVID-19 patients presented decreased mitochondrial membrane polarization (severe COVID-19 vs. healthy control, $p < 0.001$; mild vs. healthy control, $p = 0.03$; Figure 4a), indicating mitochondrial membrane depolarization. COVID-19 patients presented higher $CD4^+$Annexin V^+ (severe COVID-19 vs. healthy controls, $p = 0.001$; mild COVID-19 vs. healthy controls, $p = 0.01$) and $CD8^+$Annexin V^+ (severe COVID-19 vs. healthy controls, $p = 0.001$; mild COVID-19 vs. healthy controls, $p = 0.01$). Severe COVID-19 patients also presented higher $CD8^+$Annexin V^+ T cells compared with mild COVID-19 subjects ($p = 0.03$; Figure 4b).

Figure 4c shows the heat map of Pearson's coefficient correlation test. ATP levels inversely correlated with $CD8^+CD27^-CD28^-$

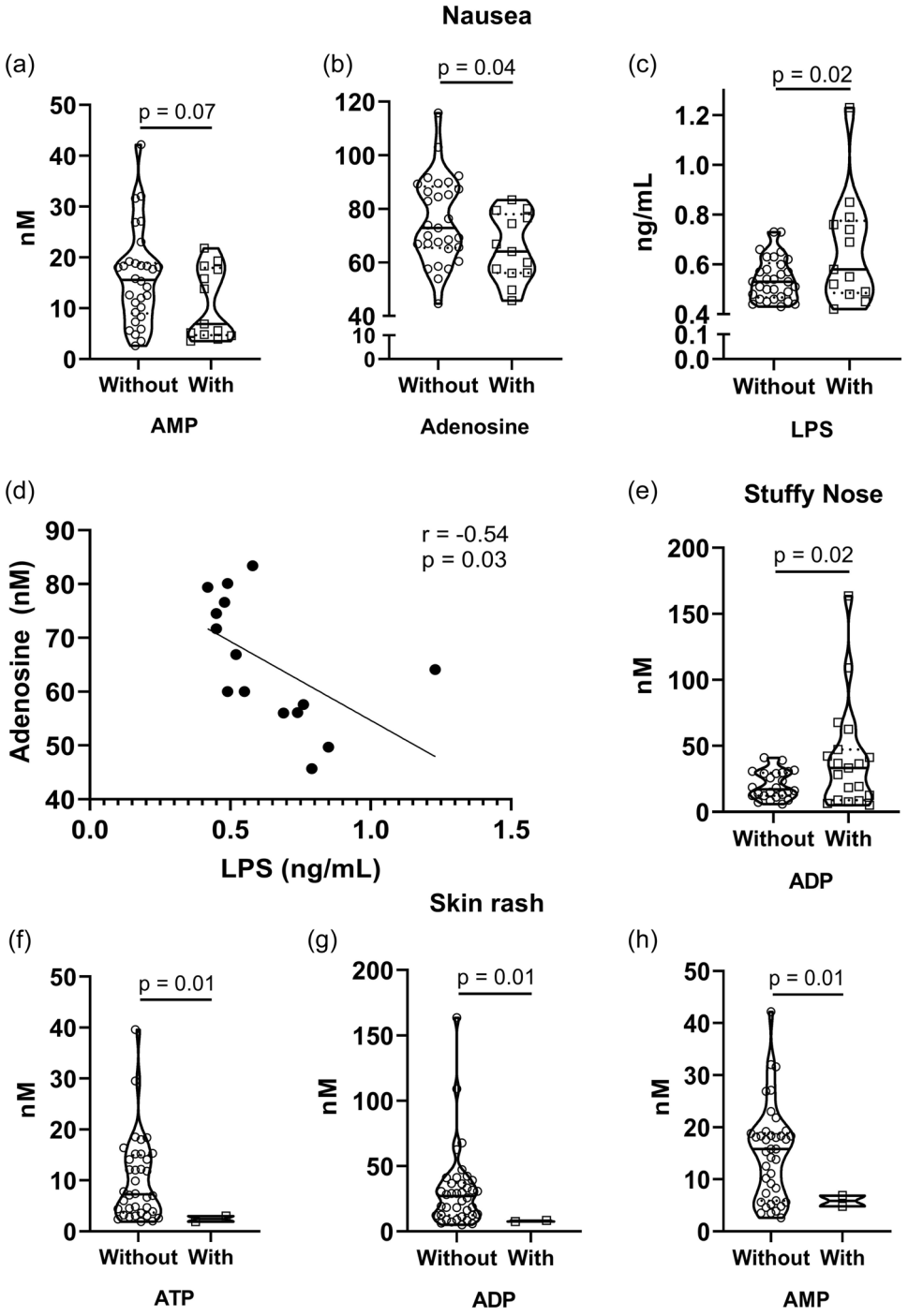

FIGURE 2 The association of adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), and adenosine levels, and lipopolysaccharide (LPS) concentrations with clinical symptoms of acute severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. Significant differences of AMP (a), Adenosine (b), and LPS (c) were found in individuals reporting nausea. Adenosine inversely correlated with LPS (d) in coronavirus disease 2019 (COVID-19) patients. Higher ADP levels were found in patients reporting stuffy nose symptom compared to patients without stuffy nose $p = 0.02$, $n = 19$ (e). COVID-19 individuals reporting stuffy nose presented higher ADP levels (f). Lower ATP (g), ADP (h), and AMP (i) were found in COVID-19 patients reporting skin rash. The levels of adenine-based purine molecules and endotoxin were compared between patients with and without clinical symptoms through Mann–Whitney *U* test and the significant results were presented ($p < 0.05$). The correlation between adenosine levels and LPS concentrations in patients reporting nausea symptom were performed by Pearson's coefficient correlation test ($p < 0.05$).

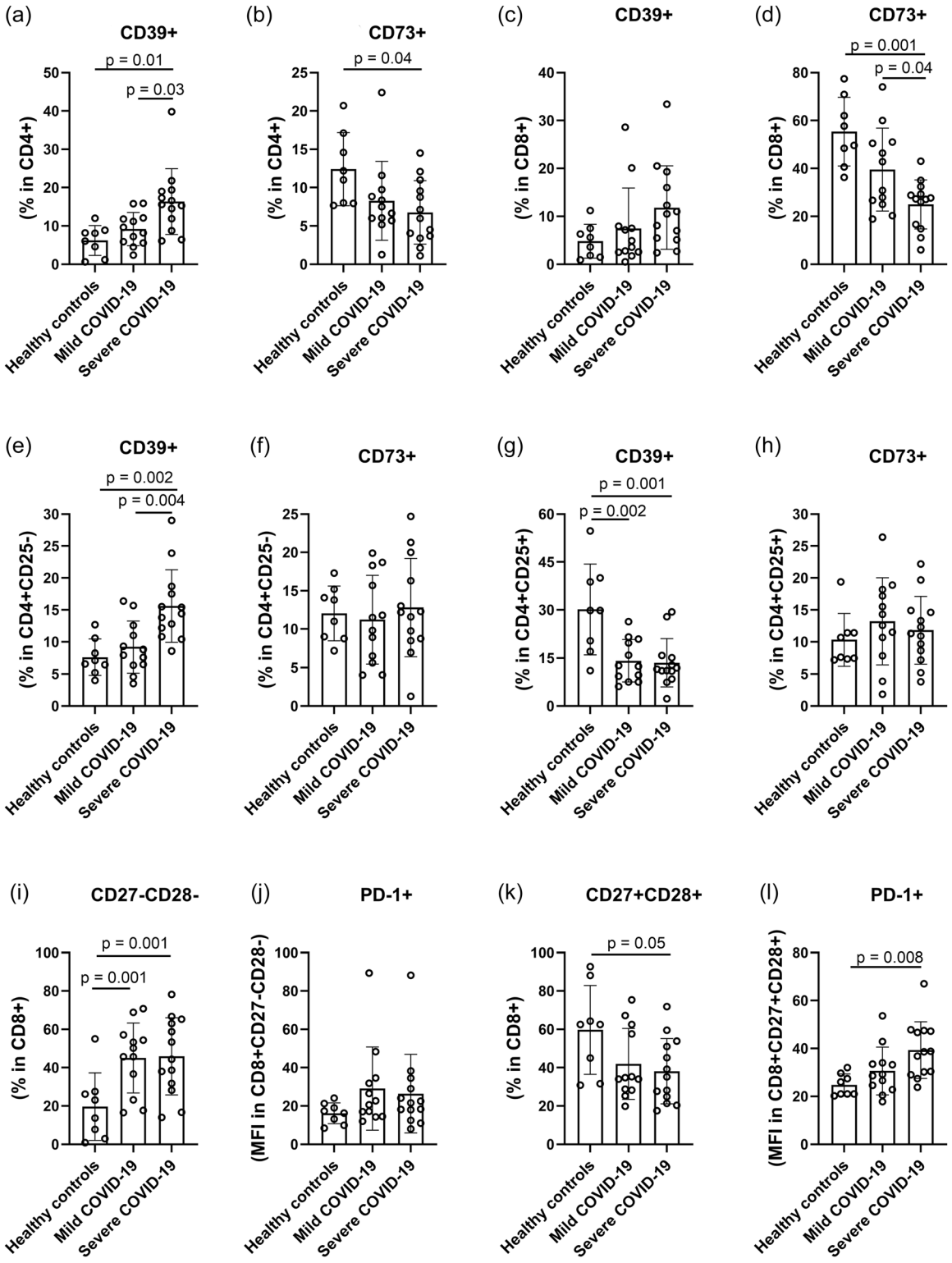

FIGURE 3 The frequency of CD4, $CD8^+$, $CD4^+CD25^-$, $CD4^+CD25^+$ T cells expressing $CD39^+$ and $CD73^+$, and the proportions of $CD8^+CD27^{-/+}CD28^{-/+}$ T cells expressing PD-1 in the peripheral blood of healthy controls, mild, and severe COVID-19 patients. Data are presented as mean ± SD. Group comparisons were performed by one-way analysis of variance with Bonferroni's post hoc test ($p \leq 0.05$).

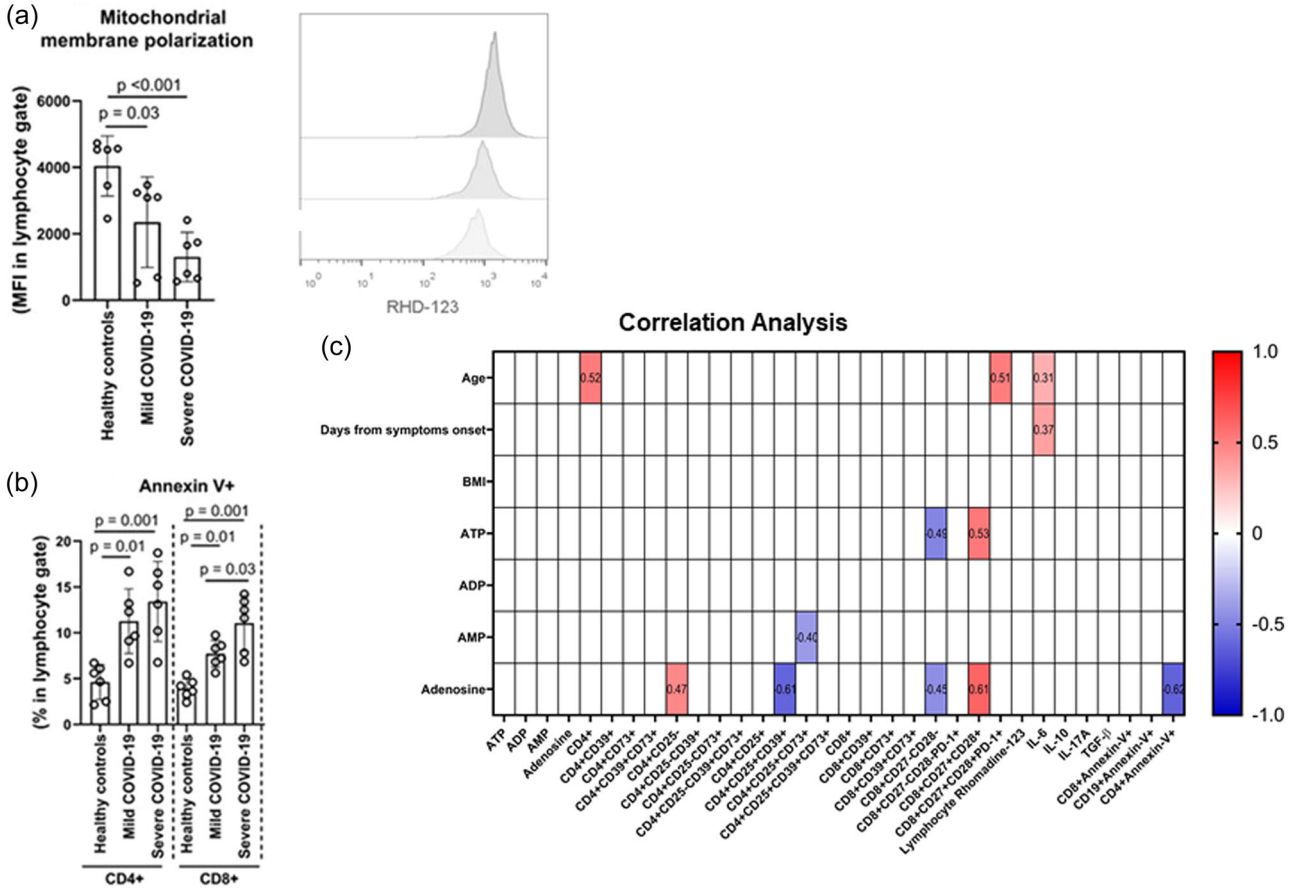

FIGURE 4 Mitochondrial membrane polarization (a) and apoptosis (b) of lymphocytes in coronavirus disease 2019 (COVID-19) patients and healthy controls. Data are presented as mean ± SD. Group comparisons were performed by one-way analysis of variance with Bonferroni's post hoc test ($p \leq 0.05$). The Correlation heat map including adenine-based purines and immune variables at admission to the Hospital (c). Pearson's correlation coefficients are plotted. Cells were colored according to the strength and trend of correlations (shades of red = positive, shades of blue = negative correlations). Statistical analysis performed through Pearson's coefficient correlation.

($r = -0.49$; $p = 0.01$) and positively correlated with $CD8^+CD27^+CD28^+$ T-cell proportions ($r = 0.53$; $p = 0.03$). AMP levels inversely correlated with $CD4^+CD25^+CD73^+$ ($r = -0.40$; $p = 0.04$). Adenosine levels positively correlated with the peripheral frequency of $CD4^+CD25^-$ ($r = 0.47$; $p = 0.01$) and $CD8^+CD27^+CD28^+$ ($r = 0.61$; $p = 0.001$), but negatively correlated with $CD4^+CD25^-CD39^+$ ($r = -0.61$; $p = 0.001$), $CD4^+CDCD27^+CD28^+$ ($r = -0.45$; $p = 0.02$) and $CD4^+$Annexin-V^+ ($r = -0.62$; $p = 0.03$) proportions. The age of the COVID-19 patients correlated with the frequency of $CD4^+$ T cells ($r = 0.52$; $p = 0.03$), the expression of PD-1^+ on $CD8^+CD27^+CD28^+$ T cells ($r = 0.51$; $p = 0.02$) and the IL-6 levels ($r = 0.31$; $p = 0.04$). Days from symptoms onset correlated only with the plasma IL-6 levels ($r = 0.37$; $p = 0.02$).

3.5 | In vitro adenosine treatment reduces nuclear factor-κB (NF-κB) expression in PBMC

The incubation of PBMC from a healthy noninfected control donor with the plasma of severe COVID-19 patients decreased the expression of CD73 on the cell surface of $CD4^+$ (Figure 5a; $p = 0.01$ vs. healthy controls; $p = 0.02$ vs. mild COVID-19) and $CD8^+$ (Figure 5b; $p = 0.01$ vs. healthy controls; $p = 0.02$ vs. mild COVID-19) T cells, without changes in CD39 expression. Interestingly, PBMC from severe COVID-19 patients in vitro treated with adenosine (100 μM) reduced the NF-κB activation in both $CD3^+$ T cells (Figure 5c, $p = 0.04$) and $CD14^+$ monocytes (Figure 5d, $p = 0.04$). Moreover, lower levels of IL-1β and IL-17a ($p < 0.05$ for both) were found in the culture supernatant of PBMC treated with adenosine, despite no changes in IL-10 and TNF-α production.

4 | DISCUSSION

This study identified a relationship between COVID-19 severity and lower systemic adenine-based purines and the frequency of circulating lymphocytes expressing CD39 and CD73 ectonucleotidases. We found decreased plasma levels of ATP and adenosine in COVID-19 patients, concomitant with markedly altered frequencies of $CD4^+$ and $CD8^+$ T cells expressing CD39 and CD73 enzymes on the cell surface. In addition, plasma samples collected from severe COVID-19 patients

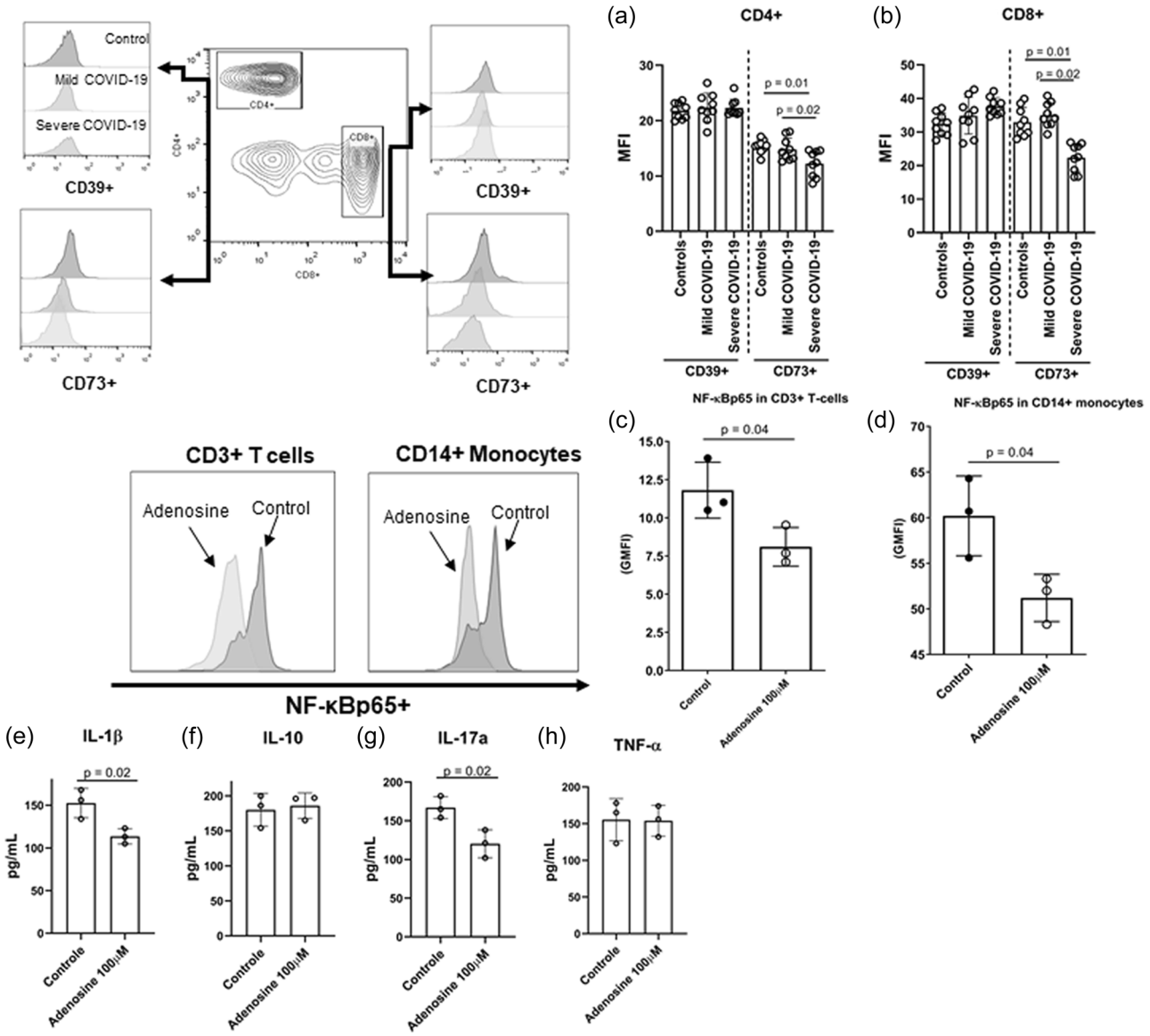

FIGURE 5 CD39 and CD73 expression in $CD4^+$ (a) and $CD8^+$ (b) T cells of a healthy noninfected donor after the incubation with plasma obtained from controls, mild coronavirus disease 2019 (COVID-19) or severe COVID-19 patients. In addition, peripheral blood mononuclear cell (PBMC) of severe COVID-19 ($n = 3$) were treated in vitro with adenosine and the activation of NF-κBp65 were evaluated in $CD3^+$ T cells (c) and $CD14^+$ monocytes (d), as well as the production of interleukin (IL)-1β (e), IL-10 (f), IL-17a (g), and tumor necrosis factor-α (TNF-α) (h) were evaluated. Data are presented as mean ± SD. Group comparisons were performed by one-way analysis of variance with Bonferroni's post hoc test ($p \leq 0.05$).

were able to decrease the expression of CD73 on $CD4^+$ and $CD8^+$ T cells of a healthy donor and the in vitro incubation of PBMC from severe COVID-19 patients with adenosine reduced the NF-κB activation in T cells and monocytes, and lower levels of IL-1β and IL-17a in the supernatant of cell culture. Furthermore, COVID-19 severity was associated with an accumulation of highly differentiated $CD27^-CD28^-$ $CD8^+$ T cells proportions and decreased low-differentiated $CD27^+CD28^+CD8^+$ T-cell frequency in the peripheral blood concomitant with higher expression of the exhaustion marker PD-1 in this population. COVID-19 patients also presented higher rates of lymphocyte mitochondrial membrane depolarization and increased apoptosis rates in $CD4^+$ and $CD8^+$ T cells.

Lower ATP plasma levels are a surprising new result in SARS-CoV-2 infection. Extracellular ATP was found at high concentrations in bronchoalveolar lavage fluid from patients with acute respiratory distress syndrome and in the blood of septic patients (Cicko et al., 2018). On the other hand, extracellular ATP facilitates interferon (IFN) type I secretion through p38/JNK/ATF-2 signaling pathway in vesicular stomatitis virus-infected mice (Zhang et al., 2017). Thus, limiting the extracellular levels of ATP in SARS-CoV-2 infection may contribute to the delay in IFN type I, turning off the initial alarm of the purinergic system. SARS-CoV-2 infection impairs the rapid IFN-I pathway activation in mild and severe patients, leading to lower antiviral response and worse outcomes in critically ill COVID-19

patients (Arunachalam et al., 2020). Furthermore, we identified lower levels of AMP and adenosine in COVID-19 patients reporting nausea, higher ADP levels in COVID-19 patients reporting stuffy noses, and diminished levels of ATP, ADP, and AMP in individuals reporting skin rash. Although the biological and clinical significance is not clear, these results suggest that the modulation of extracellular adenine-based purine molecules may be involved in the clinical symptoms of COVID-19. In this sense, the deregulation in purinergic signaling is associated with the hyperinflammatory and coagulation status of COVID-19, as well as causing exacerbations in clinical symptoms. Interestingly, a pilot clinical trial demonstrated that elderly COVID-19 patients taking between 1.2 and 1.6 g of oral ATP daily had improved COVID-19 survival and fewer clinical symptoms compared to the control infected group (Abraham et al., 2021). Thus, the association between adenine-based purine molecules and clinical symptoms may indicate that purinergic signaling could be a target for pharmacologic therapies as previously suggested by hypotheses studies (Berger et al., 2022; Hasan et al., 2022; Simões et al., 2021). Also, the correlation analysis revealed a positive correlation between extracellular ATP levels and the peripheral frequency of low differentiated $CD8^+$ T cells. This result may suggest that decreased extracellular ATP during COVID-19 disease may impact the regulation of total $CD8^+$ T-cell differentiation.

Here we showed that COVID-19 patients present lower adenosine levels in the blood, which may contribute to the uncontrolled inflammation, and severity of the disease. Recent data highlighted the increased inosine levels and upregulated adenosine deaminase activity in the blood of COVID-19 patients, suggesting increased adenosine metabolization during active SARS-CoV-2 infection (Schultz et al., 2022). On the other hand, the hydrolysis of ATP seems to be reduced by PBMC, while platelets showed the highest nucleotide hydrolysis in severe and moderate COVID-19 patients (da Silva et al., 2022). Adenosine binds the P1 receptors, mainly the AR2A subtype, of immune cells (i.e., lymphocytes, monocytes, and macrophages) to induce several events to suppress the inflammatory response, such as the downregulation of the master inflammatory transcription factor NF-κB (Linden & Cekic, 2012; Mandapathil et al., 2010; Sitkovsky et al., 2004). Furthermore, our data revealed higher levels of IL-6, IL-10, and IL-17A plasma concentrations in the severe COVID-19 group, indicating a Th1/Th17 inflammatory polarization. Reinforcing the regulatory effect of adenosine in the inflammation and cytokine storm, the use of inhaled adenosine in COVID-19 patients has successfully decreased the length of hospitalization and improved the prognosis of patients (M et al., 2021; Spiess et al., 2021). We found that in vitro adenosine treatment lowers the NF-κB activation in both T cells and monocytes, suggesting a role for the therapeutic adenosine treatment in the reduction of hyperinflammation in COVID-19. Collectively, our data indicate alterations in adenine-based purinergic molecules, mainly ATP and adenosine levels, during the initial phase of SARS-CoV-2 infection.

Here we also described a negative correlation between plasma adenosine levels and the $CD4^+$ T-cell apoptosis rate. Previous studies have suggested that adenosine and its analog are related to antiapoptotic events through A2A receptor activation in $CD4^+$ T cells (Himer et al., 2010). On the other hand, mitochondrial dysfunction is involved in the induction of apoptosis, thus increasing the depolarization of transmembrane potential (Wang & Youle, 2009). Here, lymphocytes of COVID-19 patients presented a state of mitochondrial membrane depolarization and higher rates of apoptosis. Interesting, mitochondria have a central role in T-cell activation by producing ATP and higher mitochondrial dysfunction may lead to failure in the purinergic regulation and cell homeostasis (Ledderose et al., 2014).

The increased expression of CD39 and CD73 in lymphocytes rapidly converts extracellular ATP to adenosine to induce anti-inflammatory effects (Antonioli et al., 2013). Here we described increased proportions of $CD4^+CD39^+$ T cells and lower frequencies of $CD4^+CD73^+$ and $CD8^+CD73^+$ in the peripheral blood of severe COVID-19 patients. Our results contrast, at least in part, with previous data from da Silva et al. (2022) who found increased expression of both CD39 and CD73 ectonucleotidases in $CD45^+$ leukocytes. In this sense, these results indicate that purinergic signaling may be compromised in several blood immune cells, including monocytes and neutrophils. The higher expression of CD39 together with lower CD73 on T cells may explain the diminished extracellular levels of both ATP and adenosine concomitant to an exacerbated inflammatory state in COVID-19 patients.

Schultz et al. (2022) found increased CD39 expression in leukocytes of COVID-19 patients by using a bioinformatics approach. In COVID-19 patients, higher $CD39^+$ T-cell frequency was previously associated with the expression of PD-1, confirming the hypotheses that SARS-CoV-2 infection induces a hyperactivated exhausted lymphocyte profile (Mathew, Giles, Baxter, Greenplate, et al., 2020), contributing to the T-cell dysfunction in COVID-19. Interestingly, Wang, Veurich, Kalbasi, Graham, et al. (2021) identified increased CD39 expression in the lung, liver, spleen, and PBMCs from severe COVID-19 patients, which correlated with days in the hospital, days in intensive care unit (ICU), and markers of coagulation, indicating associations between ectonucleotidases and disease progression. Similarly, CD39 messenger RNA (mRNA) as upregulated in peripheral blood leukocytes in association with higher soluble CD39 levels in severe COVID-19 patients, which were related to the length of hospital day and ICU admission (Díaz-García et al., 2022). Furthermore, upregulation in CD39 expression in late-stage COVID-19 was associated with heightened levels of STAT-3 and HIF-1α, but decreased levels of CD39-antisense RNA, possibly a purinergic imbalance that contributes to metabolic changes and T-cell dysfunction (Wang, Veurich, Kalbasi, Shaefi, et al., 2021). $CD4^+CD39^+$ T cells identify an effector lymphocyte that is prone to apoptosis in older adults (Fang et al., 2016), corroborating with our data regarding higher apoptosis rate in the $CD4^+$ T cells of COVID-19 patients. On the other hand, lower frequencies of $CD4^+CD73^+$ and $CD8^+CD73^+$ T cells were identified in severe COVID-19 patients. These results are in line with some recent data that demonstrated an association between skewing of T cell receptor repertoire with early $CD4^+$ and

$CD8^+$ activation, and altered expression of several immune checkpoints, such as Tim-3, PD-1, and CD73 (Schultheiß et al., 2020).

Similar to our study, Shahbaz et al. (2021) showed that patients with COVID-19 infection presented higher expression of CD39 but lower CD73 expression in $CD4^+$ T cells, which may contribute to the inflammatory exacerbation. The lack of CD73 expression in T cells may impact lymphocyte homing to draining lymph nodes, as adenosine generated by CD73 regulates lymphocyte migration through $A_{2B}A2$ signaling (Takedachi et al., 2008). The downregulation of CD73 impairs not only the extracellular adenosine generation and anti-inflammatory response but also the modulation of innate immune activation during the viral immune response (Aeffner et al., 2015). In severe COVID-19 patients, $CD8^+$ T cells lacking CD73 expression possess a significantly cytotoxic effector functionality compared with $CD8^+CD73^+$ T cells and correlate with plasma ferritin level, a surrounding clinical marker of uncontrolled systemic inflammation (Ahmadi et al., 2020). Here, the diminished proportions of $CD8^+CD73^+$ T cells were accompanied by low frequencies of low differentiated T cells and higher proportions of $CD8^+CD27^-CD28^-$ T cells in the peripheral blood of COVID-19 patients. Highly differentiated $CD27^-CD28^-$ cytotoxic T cells presents low telomerase activity and decreased Akt phosphorylation, which limits the ability of these cells to be maintained in continuous proliferation after antigen recognition (Plunkett et al., 2007). Although we were unable to determine the memory phenotype of T cells due to methodological reasons, our results indicate that COVID-19 patients display a complex alteration in differentiation status and memory acquisition in adaptive immunity-related to disturbances in purinergic signaling. Moreover, we found increased PD-1 expression in low differentiated $CD8^+CD27^+CD28^+$ T cells of the severe COVID-19 group, indicating an exhaustion profile. Similarly, COVID-19 patients presented $CD8^+$ T cells enriched for expression of CD38, HLA-DR, KI67, CD39, and PD-1, highlighting the co-expression of activation markers with features of inhibitory/exhausted phenotype (Mathew, Giles, Baxter, Oldridge, et al., 2020).

The combining analysis of CD25 and CD39 expression in $CD4^+$ T cells reveals distinct phenotypes: $CD4^+CD25^+CD39^+$ (activated/memory Treg, mTreg) and $CD4^+CD25^-CD39^+$ (memory T effector cell; mTeff; Dorneles et al., 2019; Dwyer et al., 2010). These populations dynamically change during the acute inflammatory response in response to extracellular purine molecules accumulation (Mandapathil et al., 2010). We found an accumulation of mTeff cells in severe COVID-19 patients. $CD39^+$ mTeff cells are nonsuppressive and have a memory phenotype that expresses higher levels of mRNA Th-lineage-specific cytokine profile, mainly Th1 and Th17 subsets (Zhou et al., 2009). In this sense, the accumulation of $CD4^+CD25^-CD39^+$ T cells in the peripheral blood of severe COVID-19 patients may contribute to the skewing toward the Th17 profile. In humans, $CD4^+CD25^-CD39^+$ T cells are $CD45RO^+$, $Foxp3^-$, secrete higher amounts of IFN-γ and IL-17, and are increased following autoantigen recognition (Moncrieffe et al., 2010).

$CD4^+CD25^+CD39^+$ mTreg cells were decreased in mild and severe COVID-19 patients. Furthermore, the proportions of $CD4^+CD25^+$ T cells co-expressing CD39 and CD73 were also reduced in COVID-19 patients. These data indicate a failure in the immunosuppressive axis of adenosine generation by Treg cells during SARS-CoV-2 infection. Contrary to us, an upregulation in ENTPD1 (CD39) gene expression within the Treg pathway in peripheral tissues of severe COVID-19 patients was previously reported, suggesting higher Treg suppression mediated by the CD39 axis may occur in the infected tissues during acute SARS-CoV-2 infection that may contribute to secondary infections by others pathogens (Wang, Veurich, Kalbasi, Shaefi, et al., 2021). On the other hand, Meckiff et al. (2020) described an imbalance of Tregs cells and cytotoxic reactive $CD4^+$ T cells in severe COVID-19. Considering that severe COVID-19 patients presented lower extracellular adenosine levels in the peripheral blood, we postulated that there a distinct Treg-expressing CD39/CD73 ectonucleotidases in the peripheral blood compared with infected tissues during COVID-19. As Treg cells are known to play an important role in limiting the host antiviral response and the consequent tissue immunopathology, the present data shows decreased CD39/CD73 axis in Treg that may be related to the decreased Treg response to SARS-CoV-2 and have a relevant impact on fueling systemic inflammation in COVID-19 patients (Belkaid & Tarbell, 2009).

Considering the COVID-19 pandemic status, efforts to understand the immunopathological role of SARS-CoV-2 infection have been made by several research groups. Here we found significant findings that indicate the alterations in adenine-based purine molecules and T-cell phenotype in COVID-19 and both seem to be correlated. The range of disease severity directly impacts in the alterations of $CD4^+$ and $CD8^+$ T cells expressing CD39/CD73 ectonucleotidases, which suggests that disturbances in purinergic signaling may result in poor clinical outcomes.

5 | CONCLUSION

In summary, we describe for the first time an imbalance in the levels of extracellular adenine-based purine molecules and alterations in CD39/CD73 ectonucleotidases axis in $CD4^+$ and $CD8^+$ T cells of COVID-19 patients with different degrees of disease severity. The reduced adenosine extracellular levels and alterations in T cells phenotype may impact on COVID-19 severity. Collectively, these data add new knowledge regarding the immunopathology of COVID-19 through purinergic regulation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

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RESEARCH ARTICLE

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Systemic redox imbalance in severe COVID-19 patients

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Abstract

The aim of this study was to evaluate the systemic redox state and inflammatory markers in intensive care unit (ICU) or non-ICU severe COVID-19 patients during the hospitalization period. Blood samples were collected at hospital admission (T1) (Controls and COVID-19 patients), 5–7 days after admission (T2: 5–7 days after hospital admission), and at the discharge time from the hospital (T3: 0–72 h before leaving hospital or death) to analyze systemic oxidative stress markers and inflammatory variables. The reactive oxygen species (ROS) production and mitochondrial membrane potential (MMP) were analyzed in peripheral granulocytes and monocytes. THP-1 human monocytic cell line was incubated with plasma from non-ICU and ICU COVID-19 patients and cell viability and apoptosis rate were analyzed. Higher total antioxidant capacity, protein oxidation, lipid peroxidation, and IL-6 at hospital admission were identified in both non-ICU and ICU COVID-19 patients. ICU COVID-19 patients presented increased C-reactive protein, ROS levels, and protein oxidation over hospitalization period compared to non-ICU patients, despite increased antioxidant status. Granulocytes and monocytes of non-ICU and ICU COVID-19 patients presented lower MMP and higher ROS production compared to the healthy controls, with the highest values found in ICU COVID-19 group. Finally, the incubation of THP-1 cells with plasma acquired from ICU COVID-19 patients at T3 hospitalization period decreased cell viability and apoptosis rate. In conclusion, disturbance in redox state is a hallmark of severe COVID-19 and is associated with cell damage and death.

KEYWORDS
apoptosis, inflammation, oxidative stress, reactive oxygen species, SARS-CoV-2

1 | INTRODUCTION

In the beginning of 2020, the severe acute respiratory syndrome (SARS) coronavirus 2 (SARS-CoV-2) rapidly spread worldwide and became a pandemic affecting the population.[1] Individuals infected might manifest the disease in different degrees, including asymptomatic cases or report mild to severe symptoms, and around of 20% are considered severe cases that require hospitalization or intensive care.[2,3] The most common symptoms are like other viral infections that commit the respiratory system, as flu-like syndrome, fever,

Isabelle A. L. Postiga and Paula C. Teixeira are considered the first authors of this study.

cough, fatigue, coryza, sore throat. However, most severe cases are associated with complications in pulmonary, cardiovascular, and neurological systems that lead to the necessity to intensive care and increased mortality. In this line, a disturb in both cellular and systemic host metabolism could contribute to viral replication and expression of pro-inflammatory cytokines.[4,5]

The host response to the infection driven the severity of the diseases, being observed that more severe cases are associated with increased immune response of the host that cause activation of multisystemic inflammatory responses and respiratory dysfunction.[6,7] The SARS-CoV-2 pathogenesis had as pivot mechanism the redox system and the pro-inflammatory cytokines release and is related to hypoxia and oxidative stress.[8] The immune innate response begins when the virus enters into the airways and it activates macrophages and dendritic cells that release inflammatory cytokines and produce reactive oxygen species (ROS). The viral replication and the multisystemic pro-inflammatory environment contributes to the constant production of ROS, due to respiratory burst activity of immune cells (macrophages and neutrophils mainly), alterations in the activity of enzymes as NADPH oxidase, xanthine oxidase, and respiratory chain in the mitochondria.[9,10] In this scenario, the virus acts diminishing the antioxidant defense through the transcriptor factor nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2) inhibition, which is responsible for the increase of antioxidant enzymes activity. Consequently, the increased production of ROS and the inhibition of antioxidant defense culminates in impaired redox balance, affecting membrane lipids and cytoplasmic proteins.[11] Moreover, the effects of ROS mechanism on pulmonary cells might have an important role in hypoxic respiratory failure diagnosed in severe cases of SARS-CoV-2 infection.[6]

Therefore, oxidative stress is directly related to the severity of the infection caused by SARS-CoV-2 and the follow-up of alterations in redox status biomarkers (with clinical relevance) could be used to identify high-risk patients and to contribute to the identification of possible targets for therapeutic approaches. The aim of this study was to evaluate the systemic markers of oxidative stress and IL-6 in patients infected with SARS-CoV-2 with different severity degrees during the hospitalization period.

Significance statement

COVID-19 induces a strong inflammatory response that disturbs immunological response resulting in multiorgan impairment and systemic damage on several tissues. This study provided information regarding the redox state in SARS-CoV-2 severely infected patients over hospitalization. In summary, the progression of the hospitalization increased protein oxidation and reactive oxygen species (ROS) generation in intensive care unit (ICU) COVID-19 patients. Furthermore, we showed that peripheral leukocytes produce a higher amount of ROS during severe SARS-CoV-2. Finally, the alterations in plasma obtained from ICU COVID-19 at the end of the hospitalization resulted in lower cell viability and higher apoptosis rate. These findings suggest an interesting dynamic in the biomarkers with a potential use in clinical management of the disease.

2 | METHODS

2.1 | Study population

This is a clinical cohort study that enrolled 30 hospitalized patients with positive diagnosis for SARS-CoV-2 reverse transcription–polymerase chain reaction (RT-PCR) test. Hospitalized patients were recruited after admission to the COVID-19 Unit of Hospital São Camilo (Esteio/RS, Brazil) between June and December of 2020. The present study was approved by the Ethics Committee of UFCSPA (CAAE: 38886220.0.0000). We use the diagnoses criteria for COVID-19 from World Health Organization interim guidance and Diagnosis and Treatment Guideline for Novel Coronavirus Pneumonia, being considered positive to SARS-CoV-2 infection patients with positive result for at least two nucleic acid tests for SARS-CoV-2. The patient's electronic medical records were used to collect clinical and sociodemographic data upon admission to the unit. Additionally, we included as control a group of 12 individuals with age and sex-matched admitted to hospital with pneumonia that tested negative to SARS-CoV-2 RT-PCR. All patients or those legally responsible for the patients received the explanation of the research objectives and procedures and informed consent to participate in the study. All authors sign an agreement to preserve patients and staff anonymity regarding the use of the present data.

2.2 | Blood collection

Blood samples of controls (n = 12) and COVID-19 patients (n = 29; 18 non-intensive care unit [ICU] and 11 ICU COVID-19) were collected right after hospital admission (T1) from the antecubital vein into 4 ml tubes with EDTA as anticoagulant. Further samples of blood were collected from non-ICU COVID-19 and ICU COVID-19 patients during the hospitalization, 5–7 days after admission (T2: 5–7 days after hospital admission), and at the discharge time from the hospital (T3: 0–72 h before leaving hospital or death). Blood was centrifuged (2000g, 10 min) to obtained plasma samples, which was aliquoted and immediately kept at −80°C until analysis.

2.3 | Thiol concentration

The thiol concentration measure was based in the interaction between thiol group and 5-5′-dithiobis 2-nitrobenzoic acid (DTNB). The analytical method was previously described by Ellman.[12] Briefly, we used 50 µl of sample, 25 µl of phosphate saline solution

(10 mmol/L, pH 7.4), and 25 µl of DTNB in a plate of 96 wells. The assay was incubated for 30 min, protected from light, and then read on a microplate reader (Spectra Max 250; Molecular Devices) at 412 nm. The results were expressed in µmol/ml.

2.4 | Nonenzymatic antioxidant status

The nonenzymatic antioxidant status in plasma was assessed through the reduction of the 3-[4,5-dimethylthiazol-2-yl]−2,5-diphenylte trazolium bromide (MTT) by molecules with antioxidant action, according Medina et al.[13] In a test tube, 100 µl of sample and 20 µl of MTT were added with 380 µl of phosphate-saline solution (10 mmol/L, pH 7.4). This solution was incubated in a water bath for 60 min at 37°C, protected from light. Then, 1 ml of a solution of isopropyl alcohol with hydrochloric acid (0.4 N) was added and centrifuged at 3500 rpm for 10 min at room temperature. The reaction supernatant was transferred to a 96-well plate and analyzed in the microplate reader (Spectra Max 250; Molecular Devices) at 570nm. The evaluation of the antioxidant status was expressed according to the absorbance obtained.

2.5 | Total antioxidant capacity (TAC)

The TAC was evaluated based on the oxidation of 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) according to the method reported by Miller et al.[14] In this assay, a solution of ABTS (7 mM) with potassium persulfate (2.4 mM) was prepared, kept protected from light, and later diluted in methanol. This final solution was reacted with 10 µl of sample of plasma in a 96-well plate. The calibration curve was made using Trolox reagent. The antioxidant capacity was determined by reading on a microplate reader (Spectra Max 250; Molecular Devices) at 740 nm. Data were expressed in µmol/L.

2.6 | Nitrite levels

The nitrite levels in plasma was determined by the Griess method.[15] Briefly, a Griess solution was prepared with 0.2% sulfanilic acid, 0.2% *N*-(1-naphthyl) ethylenediamine in 5% phosphoric acid. First, we performed the deproteinization of the sample by adding 200 µl of sample with 20 µl of zinc chloride (1 M), followed by centrifugation at 3500 rpm for 10 min. Then, 50 µl of sample was pipetted, which was reacted with the Griess solution, followed by the addition of 50 µl of vanadium (150 µM). Finally, this solution was incubated for 60 min at 37°C. The standard curve was performed using sodium nitrate ($NaNO_2$). The analysis was conducted in a 96-well plate using a microplate reader (Spectra Max 250; Molecular Devices) at 540 nm. Data were expressed in µmol/L.

2.7 | ROS

The ROS content was evaluated in the plasma through the fluorescence intensity of the redox-sensitive dye 2′,7′-dichlorodihydrofluorescein diacetate (DCFH, 100 µM; Sigma-Aldrich). DCFH is permeable through the cell membrane and it undergoes the process of deacetylation by intracellular esterases enzymes, forming the intermediate compound 2′,7′-dichlorodihydrofluorescein in cytoplasm, in which reacts with ROS forming oxidized 2′,7′ dichlorofluorescein (DCF), a fluorescent molecule. The analysis was performed with excitation and emission wavelengths of 480 and 535 nm, respectively, using SpectraMax M2e (Molecular Devices).

2.8 | Protein oxidation

The evaluation of protein and amino acid oxidation was performed using the method proposed by Witko-Sarsat et al.[16] This method determines the levels of advanced oxidation protein products (AOPP) in plasma. Briefly, 50 µl of plasma was used, which was diluted in 450 µl of phosphate-saline solution (10 mmol/L, pH 7.4). In the final solution was added 50 µl of potassium iodide (1.16 ml/L) followed by 100 µl of citric acid (0.2 mol/L). The control was done with a Bovine serum albumin solution. The absorbance was measured in a microplate reader (Spectra Max 250; Molecular Devices) with an absorbance of 340 nm. The result was calculated by a standard curve with chloramine equivalents and was expressed in µmol/L.

2.9 | Lipid peroxidation

Lipid peroxidation was evaluated by the thiobarbituric acid reactive substances (TBARS) method.[17] This test measures the concentrations of malondialdehyde (MDA), products formed from the oxidation of lipids, through their reactivity with thiobarbituric acid. For this assay, 200 µl of plasma reacted with 250 µl of acetic acid (2.5 M, pH 3.4), 50 µl of sodium dodecyl sulfate (8%), and 250 µl of thiobarbituric acid (0.8%). This mixture was vortexed and then incubated in a water bath for 60 min at 100°C. Afterwards, the samples were centrifuged at 3500 rpm for 10 min at 4°C. The supernatant was transferred to a 96-well plate and analyzed in the microplate reader (Spectra Max 250; Molecular Devices) at 532nm. The result was expressed in nmol/ml.

2.10 | C-reactive protein and IL-6 analyzes

C-reactive protein (CRP) levels were determined using automated equipment that uses the principle of chemiluminescence to determine plasma CRP levels. The IL-6 concentration (Invitrongen Life Sciences, EUA) was analyzed by enzyme linked immunosorbent assay according to manufacturer's instructions for procedure using a

microplate reader (EzBiochrom). The variability coefficient of variability was was <7.5%. The detection limit was 2–200 pg/ml.

2.11 | ROS measurement in peripheral leukocytes

ROS production and mitochondrial membrane potential (MMP) were analyzed in peripheral granulocytes and monocytes from healthy controls ($n = 8$), non-ICU COVID-19 ($n = 8$), and ICU COVID-19 ($n = 8$) patients at hospital admission. The MMP using the fluorescent dye rhodamine 123 (Rh 123; Sigma-Aldrich®) as previously described. Briefly, whole blood (100 µl) was incubated with 500 µl of Rh 123 (1 µg/ml) for 10 min at 37°C. After being washed, the cells were resuspended in 0.5 ml of phosphate-buffered saline (PBS). The generation of ROS was quantified by the cell-permeant dye 2′,7′-DCF diacetate (DCF-DA; Sigma-Aldrich), which becomes fluorescent after oxidation to DCF. Whole blood was incubated with 0.5 ml of 10 µM DCF-DA for 30 min at room temperature. After being washed with PBS, the cells were resuspended in 0.5 ml PBS. The analysis of MMP and ROS production was conducted using BD FACSCalibur (Becton-Dickinson) flow cytometer and CellQuest Pro software (Joseph Trotter; Scripps Research Institute) using the blue argon-ion 488 nm laser with the FL1 filter channel. A minimal of 30.000 events/tubes was acquired, and granulocytes and monocytes were identified and gated according to each forward scatter (FSC) and side scatter (SSC) profiles.

2.12 | Cell viability and apoptosis in THP-1 monocytic cell line

THP-1 human monocyte cells (ATCC TIB-202) (2×10^5 cells/well) were cultured in RPMI media (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin and streptomycin (both from Sigma-Aldrich) in 96-well plates for 24 h. Afterwards, the media plus FBS were removed; cells were washed with PBS and incubated with RPMI supplemented with 10% plasma of non-ICU ($n = 8$) and ICU ($n = 8$) COVID-19 patients for 15 h. Cell viability was measured using MTT (Sigma-Aldrich). After the treatment, MTT (20 µl, 5 mg/ml) was added, and incubations were continued for 4 h after that. The purple formazan was solubilized and the absorbance at 570 nm determined using a Spectramax M2 microplate reader (Molecular Devices). The evaluation of apoptosis rate was performed by using Annexin-V+ FITC following manufacturer's recommendations (Biolegend) in BD FACScalibur flow cytometer (Becton-Dickinson). THP-1 were identified and gated according to each FSC and SSC profile.

2.13 | Statistical analysis

The data normality was tested using the Kolgomorov–Smirnov test. Parametric data was presented as mean ± standard deviation (SD)/percentile 2.5–97. Participant characteristics, biochemical variables of oxidative stress, and IL-6 were compared among groups using one-way analysis of variance followed by Bonferroni post hoc test for multiple comparisons. Comparison between qualitative variables was performed using a χ^2 test. The evolution of CRP, oxidative stress variables and IL-6 were analyzed by a mixed generalized linear model followed by Bonferroni post hoc test correction for multiple comparisons. A p-value ≤ .05 were considered statistically significant. SPSS 22.0 software (IBM Inc.) was used for statistical analysis.

3 | RESULTS

In the present study, we investigated the redox status of non-ICU COVID-19 patients and ICU COVID-19 patients along hospitalization. First, we identified the symptoms and undergoing health conditions. Then, we compared oxidative stress markers and IL-6 concentration, as a hypercitokenemia indicator, in healthy individuals (control group), non-ICU COVID-19 patients, and ICU COVID-19 patients. Finally, we assessed the progression of the diseases evaluating C-reactive protein, redox biomarkers, and IL-6 at the moment of the hospital admission (T1), during hospitalization (T2), and at 0–72 h before the outcome (T3).

3.1 | Sociodemographic variables and the description of symptoms and underlying conditions of non-ICU and ICU patients

Sociodemographic characteristics are illustrated in Table 1. Briefly, 51 individuals were enrolled in our study and divided in healthy individuals, control group with negative PCR test for SARS-CoV-2 infection ($n = 12$), and patients hospitalized with COVID-19, which tested positive to SARS-CoV-2 infection ($n = 29$, 82.35%). Then, patients diagnosed with SARS-CoV-2 infection were divided according to the severity of the disease into non-ICU COVID-19 patients ($n = 18$, 27.45%) and ICU COVID-19 patients ($n = 11$, 54.9%). ICU patients were older than healthy individuals ($p < .05$), non-ICU and ICU patients demonstrated higher body mass and BMI compared to healthy individuals ($p < .05$). Mortality was observed only in the ICU group (46%).

The most common clinical symptoms observed included fever (non-ICU: 80%; ICU: 72.72%); respiratory failure (non-ICU: -; ICU: 80%); cough (non-ICU: 46.66%; ICU: 61.81%); dyspnea (non-ICU: 60%; ICU: 16.36%); body ache (non-ICU:26.6%; ICU: 21.81%); and sore throat (non-ICU: 40%; ICU: 18.8%);. Some underlying medical condition of the patients comprised hypertension (non-ICU: 25.4%; ICU: 29.7%); diabetes mellitus (non-ICU: 23.7%; ICU: 19.8%); neurological disease (non-ICU: 21%; ICU: 22.5%).

The comparison of healthy individuals, non-ICU COVID-19 patients, and ICU COVID-19 patients demonstrated no difference in the thiol content ($p = .4145$, Figure 1A) and in antioxidant status

TABLE 1 Sociodemographic and clinical characteristics of the patients

COVID-19			
	Healthy controls (*n* = 12)	**Non-ICU (*n* = 18)**	**ICU (*n* = 11)**
Age (years)	57.55 (38.71–76.29)	59.57 (49.41–69.72)	65.50 (61.60–69.39)[a]
Sex (female, %)	33.3	57.1	44.2
Body mass (kg)	61.75 (38.20–85.29)	86.76 (79.52–94.01)[a]	89.23 (81.23–96.45)[a]
BMI (kg/m^2)	23.66 (19.48–34.85)	29.56 (26.57–31.65)[a]	30.49 (27.83–33.15)[a]
Hospitalization (days)		7.33 (1–14.92)	20.53 (16.51–24.55)[a]
Death/mortality (%)		-	46.2
Symptoms (%)			
Fever		80.00%	72.72%
Respiratory failure			80.00%
Cough		46.66%	61.81%
Dyspnea		60.00%	16.36%
Body aches		26.66%	21.81%
Sore throat		40.00%	18.18%
Flu-like syndrome		13.33%	16.36%
Fatigue		20.00%	9.09%
Myalgia		20.00%	5.45%
Nausea or vomiting		20.00%	5.45%
Headache		13.33%	7.27%
Coryza		13.33%	7.27%
Diarrhea		6.66%	7.27%
Underlying medical conditions			
Hypertension		25.4	29.7
Diabetes mellitus		23.7	19.8
Neurological diseases		21	22.5
Cardiovascular diseases		7.8	12.7
Rheumatic diseases		1.2	1.2
Cancer		2.4	
Chronic kidney disease			2.4

Note: Data are presented as mean ± confidence intervals 95%.
Abbreviations: BMI, body mass index; ICU, intensive care unit.
[a]Statistical difference compared to healthy controls ($p < .05$).

($p = .3689$, Figure 1B). However, TAC concentration showed higher levels in the non-ICU COVID-19 patients and ICU COVID-19 patients compared to healthy individuals ($p < .001$, Figure 1C), while non-ICU COVID-19 patients had higher nitrite concentration compared to ICU COVID-19 patients ($p < .001$, Figure 1D). ROS concentration did not differ among the groups ($p = .2562$, Figure 1E). The non-ICU COVID-19 patients and ICU COVID-19 patients demonstrated higher protein oxidation ($p < .001$, Figure 1F) and TBARS concentration compared to healthy individuals ($p < .001$, Figure 1G). IL-6 concentration was higher in non-ICU ($p < .001$, Figure 1H) and ICU patients ($p < .05$, Figure 1H) compared to healthy individuals.

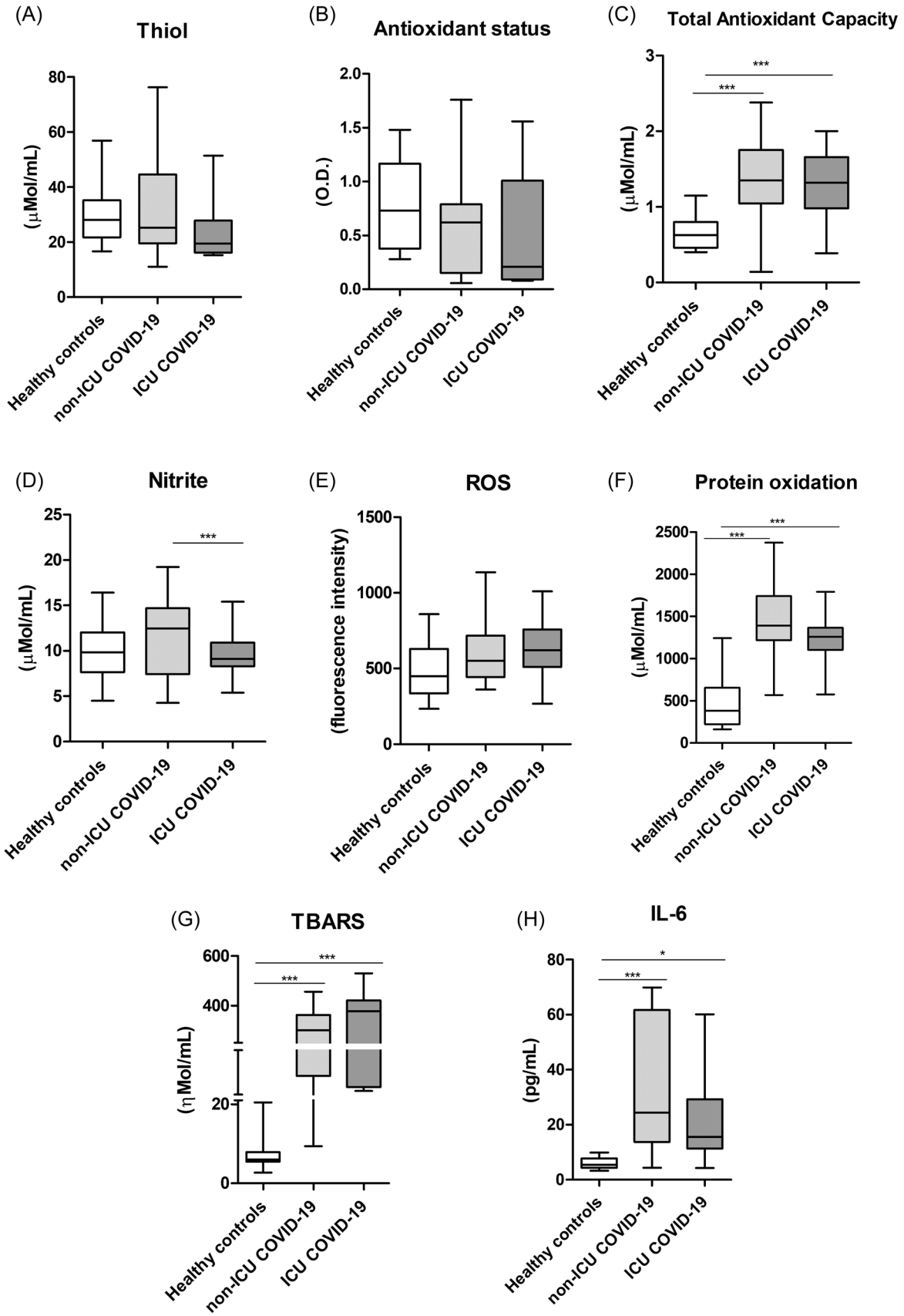

FIGURE 1 Biological markers of oxidative status and IL-6 in healthy individuals, non-ICU COVID-19 patients, and ICU COVID-19 patients. (A) Thiol concentration. (B) Antioxidant status. (C) TAC. (D) Nitrite concentration. (E) ROS concentration. (F) Protein oxidation (AOPP). (G) TBARS. (H) IL-6. Data are presented as mean ± SD. Difference among the groups were verified one way ANOVA followed by Bonferroni post hoc test. *$p < .05$; ***$p < .001$. ANOVA, analysis of variance; ICU, intensive care unit; ROS, reactive oxygen species; TAC, total antioxidant capacity.

3.2 | Redox status variables analyzed during the hospitalization period of non-ICU and ICU patients infected with SARS-CoV-2

The oxidative status of patients was also evaluated according to the progression of the hospitalization in both, non-ICU patients (open spheres) and ICU patients (solid spheres). As observed in Figure 2, lower C-reactive protein concentration was found at T3 compared to T1 in the non-ICU COVID-19 patients, while higher levels were detected at T1, T2, and T3 of ICU COVID-19 patients compared to the same periods in the non-ICU COVID-19 patients ($p < .01$, Figure 2A). The thiol content showed lower concentration in T3 of non-ICU group compared to T1, and ICU group at T1 had lower thiol concentration compared to non-ICU at T1 ($p < .05$, Figure 2B). The antioxidant status was higher at T1 and T3 in the ICU group compared to the same periods in the non-ICU group ($p < .05$, Figure 2C). Non-ICU COVID-19 patients demonstrated higher TAC concentration at T3 compared to T1, and the ICU COVID-19 patients at T3 had lower TAC concentration compared to T1 and the same period of non-ICU COVID-19 patients ($p < .05$, Figure 2D). The nitrite concentration was lower at T3 in ICU COVID-19 patients compared to non-ICU patients at the same period ($p < .05$, Figure 2E), and ICU patient demonstrated lower nitrite at T2 and T3 compared to T1 ($p < .01$, Figure 2E). Non-ICU group showed higher ROS content at T3 compared to T1, and the ICU group had higher ROS content at T2 and T3 compared to T1 of the same group, and higher ROS concentration in T3 of ICU patients compared to T3 of non-ICU ($p < .05$, Figure 3F). The protein oxidation concentration showed higher levels at T3 in ICU patients compared to T1 of the same group and to T3 of non-ICU group ($p < .01$, Figure 2G). TBARS concentration did not differ among groups ($p < .05$, Figure 2H). Non-ICU COVID-19 patients demonstrated higher IL-6 concentration at T3 compared to T1, and, in the ICU patients, the IL-6 was higher at T2 and T3 compared to T1, and T3 was higher compared to the T3 of non-ICU patients ($p < .05$, Figure 2I).

3.3 | Phagocytes generates higher amounts of ROS through changes in MMP

Granulocytes and monocytes produce higher ROS levels during infectious episodes such as viral infection. We firstly accessed the MMP and the ROS generation in granulocytes and monocytes in the peripheral blood of COVID-19 patients by flow cytometry. Both non-ICU and ICU COVID-19 patients presented a state of mitochondrial membrane depolarization in monocytes and granulocytes, identified by the decreases in Rhd-123 fluorescence intensity, compared to the controls ($p < .01$ for all comparisons). Furthermore, increased ROS production was identified in both immune cell types of COVID-19 patients compared to controls ($p < .05$). However, ICU COVID-19 patients presented higher ROS production by granulocytes and monocytes than non-ICU COVID-19 patients ($p < .01$ for both) (Figure 3).

3.4 | Plasma from severe COVID-19 patients induces decreases in cell viability and higher apoptosis rate in THP-1 monocytic cell line

Increased ROS generation are linked to cell death events in several conditions, such as acute infectious diseases. In this line, we incubated THP-1 monocytic cell line with the plasma obtained from non-ICU and ICU COVID-19 patients at T1 and T3 hospitalization periods. Plasma from both non-ICU and ICU COVID-19 groups collected at T3 reduced the cell viability of THP-1 cells ($p < .05$). On the other hand, increased annexin-V+ cells, indicating apoptosis, was found in THP-1 cells incubated with T3 plasma of non-ICU compared to T1 plasma from the same group ($p < .05$). Interestingly, T3 plasma of ICU COVID-19 group had the higher effects on cell viability ($p < .05$) and apoptosis rate ($p < .05$) than those observed in T3 plasma of non-ICU COVID-19 trial in THP-1 cells (Figure 4).

4 | DISCUSSION

Our study performed a follow-up of the redox status along the hospitalization of patients with different degrees of COVID-19 severity. This study showed the increase of antioxidant capacity, oxidation of protein, and TBARS levels in non-ICU COVID-19 and ICU COVID-19 patients compared to healthy individuals, as well as, higher nitrite concentration in non-ICU COVID-19 compared to ICU COVID-19 patients. Considering the progression of the hospitalization (T1, T2, and T3), it was observed an increase of protein oxidation at T3 of ICU patients, higher antioxidant status and lower TAC, reduced nitrite in T3 of ICU COVID-19, and higher ROS and IL-6 in both T2 and T3 of ICU COVID-19 patients. Furthermore, we showed that peripheral leukocytes produce a higher amount of ROS during severe SARS-CoV-2, with ICU COVID-19 patients presenting higher levels of ROS generation and decreased MMP in granulocytes and monocytes. Finally, the incubation of THP-1 monocytic cell line with plasma obtained from ICU-COVID-19 at T3 of hospitalization resulted in lower cell viability and higher apoptosis rate.

The symptoms and underlying conditions described in the patients enrolled in our study were observed either in mild or severe infection of SARS-CoV-2. The most frequent underlying conditions observed in the groups with COVID-19 were hypertension (28.57%), diabetes mellitus (21.42%), and neurological diseases (21.42%), which consists in group more susceptible to severe forms of SARS-CoV-2 infection.[3] The symptoms more frequently reported from the patients included fever (76.19%), cough (57.14%), and dyspnea (33.33%), while the patients with severe infection also reported respiratory failure, a common symptom from patients in this condition. Same symptoms were observed in studies analyzing SARS-CoV-2 infection.[2,18] Patients from infected groups were older and with higher body mass than noninfected group, these characteristics with the underlying condition are associated with oxidative stress, chronic inflammation, and metabolic alterations that could lead to low antioxidant

capacity, oxidative damage and a pro-inflammatory condition, contributing to the exacerbation of the pro-inflammatory cytokines release and a poor viral clearance increasing the severity of the infection.[19]

Patients with SARS-CoV-2 infection demonstrated high C-reactive protein levels especially in the beginning of the diseases. In the non-ICU groups, the levels of C-reactive protein decreased with the progression of the hospitalization until reach the reference value. The ICU group had higher levels than non-ICU, indicating a more severe infection. The C-reactive protein is a nonspecific acute phase reactant elevated in infection or inflammation and it is used as indicator of COVID-19 disease severity. A study of Stringer and colleagues identified that a simple threshold ≥40 mg/dl should be used within clinical practice to guide disease severity and likely disease progression, and recommended that CRP ≥ 40 mg/dl on admission may indicate an increased risk of disease progression and death, and warrants an enhanced level of discussion and clinical support.[20] In our study, the levels of C-reactive protein were 3.5-fold and 2-fold higher in the ICU patients and non-ICU patients, respectively, compared to the threshold.[20]

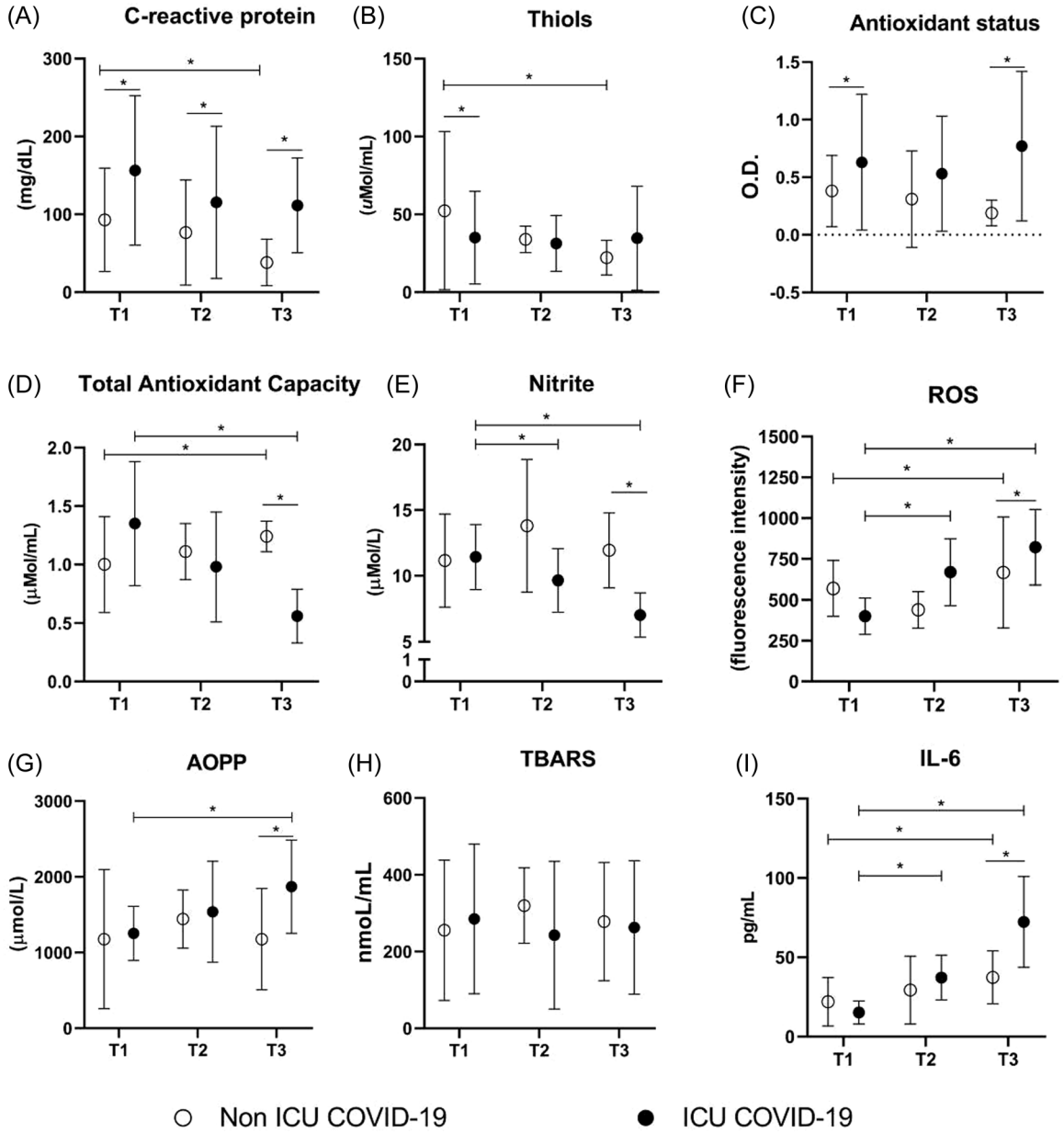

FIGURE 2 Biological markers of oxidative status and IL-6 in non-ICU COVID-19 patients (open spheres) and ICU COVID-19 patients (solid spheres) along the hospitalization (T1, T2, and T3). (A) C reactive protein. (B) Thiol concentration. (C) Antioxidant status. (D) Total Antioxidant Capacity. (E) Nitrite concentration. (F) ROS concentration. (G) Protein oxidation (AOPP). (H) TBARS. (I) Interleukin-6. Data are presented as mean ± SD. Difference among the groups were verified one way ANOVA followed by Bonferroni post hoc test. *$p < .05$. ANOVA, analysis of variance; ICU, intensive care unit; ROS, reactive oxygen species; TAC, total antioxidant capacity.

Our study showed an increase of ROS content at the end of the hospitalization in both groups, but with higher concentration in the ICU patients. Additionally, nitrite concentration and antioxidant capacity decreased along the hospitalization, while protein oxidation and IL-6 increased in ICU groups. ROS formation could be a physiological response of the organism once it had a role in signaling transduction, however, under pathological conditions ROS plays an essential function in the diseases processes.[21,22] The nitrite concentrations were higher in non-ICU patients compared to ICU, and during the hospitalization the levels in the ICU patients decreased. The nitrite can act as a precursor to form peroxinitrite, a free radical that induce oxidative damage.[23,24] Here, our results could indicate an increase in the oxidation processes that is related with reduced antioxidant capacity and nitrite concentration in the ICU group.[23,24]

Viral infections, including SARS-CoV-2, induce in the organism a range of oxidative and inflammatory response. The viral infection could trigger oxidative stress through the increase of reactive oxygen and nitrogen species (RONS), activation of immune response, and production of cytokines.[6,25] Alterations in enzyme function, as XO and NADPH oxidase, and mitochondrial dysfunction results in the increased production of superoxide and hydrogen peroxide, and reduced antioxidant defense.[26] Additionally, the bound between angiotensin-converting enzyme 2 and SARS-CoV-2 during viral invasion increases the binding of Ang II to angiotensin type 1 receptor, activating NADPH oxidase and induces the production of ROS mitochondrial that reacts with NO and reduce its bioavailability forming peroxynitrite.[23,27] The augment formation of RONS mediate signaling pathways responsible for the increased production of inflammatory cytokines.

Regarding antioxidant response, we measured thiol concentration, antioxidant status, and antioxidant capacity. Studies investigating oxidative stress markers in COVID-19 patients observed a depletion in antioxidant response together with increased production of ROS, pro-inflammatory cytokines, and oxidative damage.[6,8,11] Here, we showed higher antioxidant capacity in the non-ICU and ICU patients compared to healthy individuals, which might indicate the antioxidant response to the increased formation of ROS due to the infection. Additionally, when observed the hospitalization progression during T1, T2, and T3, the TAC increased in non-ICU and lowered in ICU groups on T3, indicating a recovery of the non-ICU patients and the progression of the infection and, possibly a consequent poor outcome of the ICU patients.

The results found corroborates to the oxidation markers evaluated, protein oxidation and lipid peroxidation (TBARS). TBARS concentration in non-ICU and ICU patients were higher than healthy individuals, showing that the SARS-CoV-2 infection cause an oxidative stress and inflammation capable of damage the membrane of the cells.[25] Furthermore, protein oxidation was higher in the infected patients compared to healthy individuals, and during the hospitalization it was observed an ascending concentration of protein oxidation in ICU patients, demonstrating protein damage, which might include DNA damage. So, the oxygen reactive species

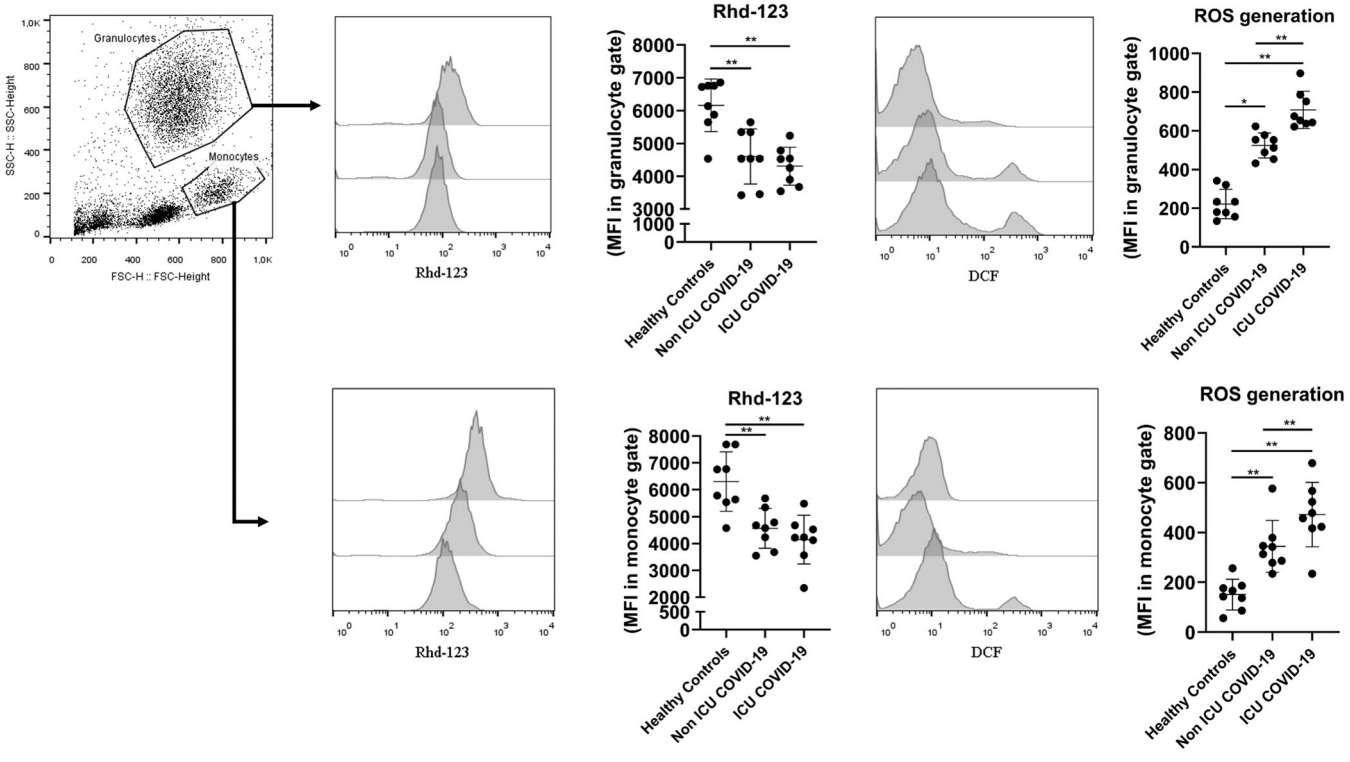

FIGURE 3 Mitochondrial membrane potential and ROS generation in granulocytes and monocytes in the peripheral blood of non-ICU and ICU COVID-19 patients. Data are presented as mean ± SD. Difference among the groups were verified one way ANOVA followed by Bonferroni post hoc test. *$p < .05$; **$p < .01$; ***$p < .001$. ANOVA, analysis of variance; ICU, intensive care unit; ROS, reactive oxygen species.

generated through the SARS-CoV-2 infection might result in the primary damage of membrane cells followed by the oxidation of proteins, DNA damage, and induce apoptosis.

Phagocytes are a greater source of ROS during viral and bacterial infections.[28] Here, we observed that non-ICU and ICU patients showed lower mitochondrial depolarization and higher ROS production, with higher alterations observed in severe cases of SARS-CoV-2 infection. In severe infections, the uncontrolled ROS production generated by innate immune cells leads to extensive cell damage and tissue injury which is associated with worse clinical outcomes. In this sense, excessive levels of ROS from neutrophils have been implicated in a cascade of biological events that drive pathological host response in COVID-19, including tissue damage, thrombosis and red blood cell dysfunction.[29] Furthermore, oxidative stress induced by neutrophils predict COVID-19-associated mortality,[30] and increased mitochondrial ROS production and impaired MMP in CD14+ monocytes were observed in severe COVID-19 patients who presented higher SARS-CoV-2 viral load.[9] The imbalance in redox state of innate immune cells leads to the activation of intracellular inflammatory pathways, mainly inflammasome, contributing to the COVID-19 hyperinflammation in severe cases.[31] In fact, several pro-oxidant genes were upregulated in mononuclear cells of severe COVID-19,[32] which confirms the redox state imbalance evoked in leukocytes during SARS-CoV-2 infection. Interestingly, the incubation of a THP-1 monocytic cell line with plasma obtained from non-ICU and ICU patients at T3 hospitalization period resulted in lower cell viability and only severe cases of COVID-19 induced higher apoptosis. Collectively, these results indicate that the redox imbalance induced during the SARS-CoV-2 infection impacts on cellular process, inducing cell damage and death.

The first phase of the SARS-CoV-2 infection is the viral replication and the innate immune response, followed by symptoms manifestation and higher levels of cytokines. Then, it is observed a severe systemic inflammatory syndrome with the progression of the disease.[33,34] So, the exacerbated response of the immune system against SARS-CoV-2 infection is characterized by a hypercytokinaemia state, an increase in cytokines release leading to a hyperinflammatory condition to contain the viral infection. The increase in cytokines release might be an indicator of the diseases severity. Here, we observed the augment of the IL-6 concentration along the hospitalization progression in non-ICU and ICU patients, however, higher levels were observed in the ICU group. IL-6 is mainly produce in response to pathogens by macrophages and T lymphocytes to control viral infection, and it had a great correlation with disease severity.[35] Previous studies showed that patients with severe form of the disease had higher IL-6 concentration compared to patients with mild or moderate forms, and IL-6 values was used to predict the risk of mortality in COVID-19 patients hospitalized, which is observed in our study that observed 46% of mortality in the ICU group.[36,37]

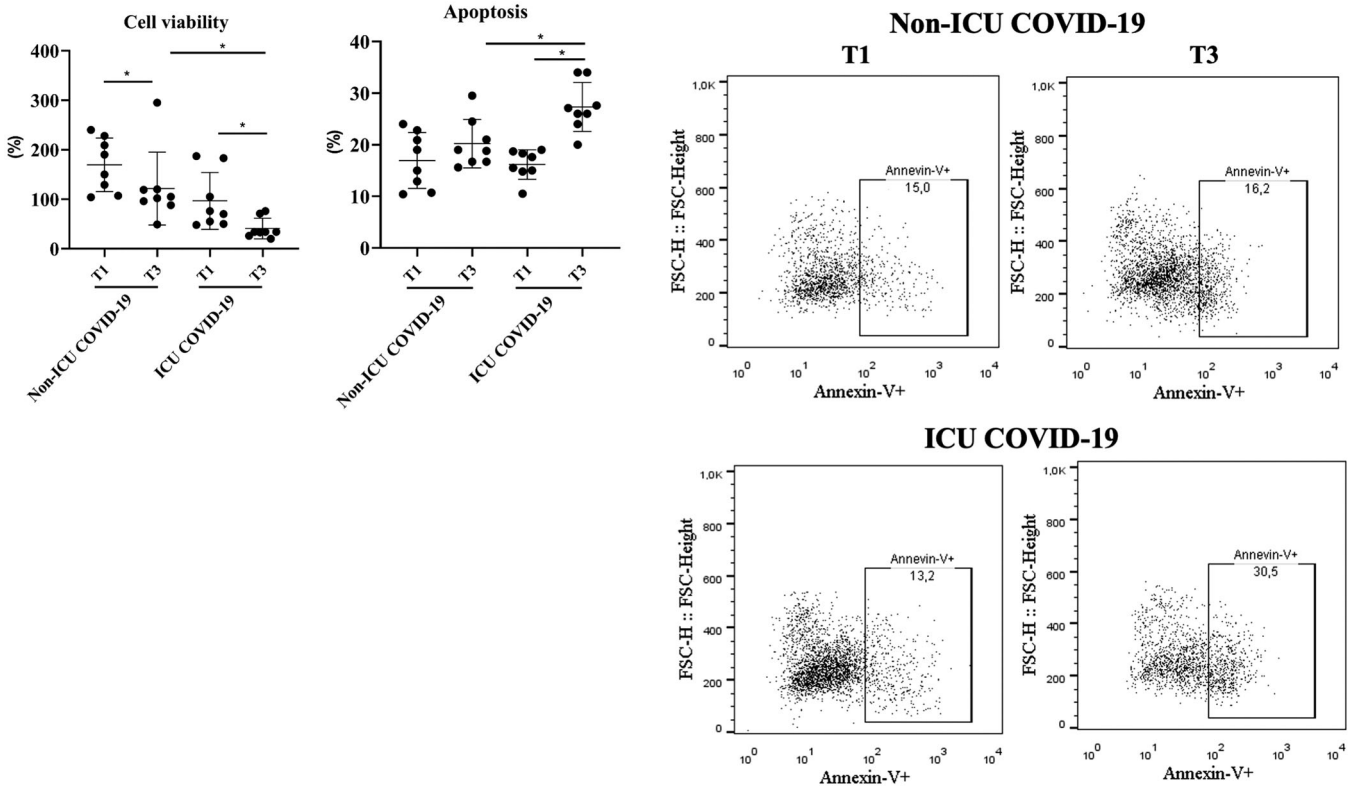

FIGURE 4 Cell viability and apoptosis rate in THP-1 monocytic cell line incubated with plasma from non-ICU and ICU COVID-19 patients. Data are presented as mean ± SD. Difference among the groups were verified one way ANOVA followed by Bonferroni post hoc test. *$p < .05$. ANOVA, analysis of variance; ICU, intensive care unit.

Oxidative stress had an important role in the pathogenesis of COVID-19, in the present study, we monitored the modification in redox mechanism caused by SARS-CoV-2 infection in the beginning of the disease and its progression in the hospitalization period. It would be critically important to use the oxidative and inflammatory parameters evaluated to COVID-19 monitoring. Here, we showed that there is an interesting dynamic in the biomarkers with a potential use in clinical management of the disease.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data will be available upon reasonable request.

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