



**UNIVERSIDADE ESTADUAL PAULISTA
“JÚLIO DE MESQUITA FILHO”
FACULDADE DE MEDICINA**

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**Avaliação da resposta inflamatória de
pacientes que desenvolveram COVID
longa.**

Dissertação apresentada ao Programa de Pós-Graduação em Patologia, da Faculdade de Medicina, Universidade Estadual Paulista “Júlio de Mesquita Filho”, Campus de Botucatu, como pré-requisito para obtenção do título de Mestre em Ciências, área de concentração em Patologia.

Orientadora: Profa. Dra. Luciane Alarcão Dias-Melicio

**Botucatu
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Palavras-chave: ACE2; COVID longa; Citocinas; SARS-CoV-2; TLR4.

Impacto potencial desta pesquisa

Impacto Científico: A geração de conhecimento na área de COVID Longa auxiliará nos avanços em pesquisa para que haja melhora na capacidade de tratamento e atenção aos pacientes. Encontrar um possível foco terapêutico à partir do entendimento da resposta de pacientes com COVID longa, é a proposta da pesquisa. Por conta do pouco conhecimento nesse campo, não se sabe ainda como tratar as sequelas que a COVID pode deixar, causando impactos de saúde no mundo todo.

Impacto Social: A pesquisa contribui com o bem-estar social, auxiliando no atendimento de saúde de qualidade, visando a diminuição da morbidade causada pela COVID e pelas suas sequelas na população. Abrir o precedente necessário para o teste de um imunobiológico específico pode avançar as condutas que visam melhoras dos sintomas dos pacientes.

Impacto Econômico: Apesar de não gerar renda direta, o avanço na área do tratamento da COVID Longa economizará grandes valores aos cofres dos sistemas de saúde. Isso aliviaria o uso de recursos, que assim podem ser dirigidos à outras causas.

Potential Impact of this Research

Scientific Impact: The generation of knowledge in the field of Long COVID will assist in advancements in research to improve the treatment capabilities and patient care. Identifying a possible therapeutic focus is the primary aim of this research. Due to the limited understanding in this field, it is still unknown how to effectively address the lingering effects that COVID can leave behind, causing health impacts worldwide.

Social Impact: The research contributes to social well-being by aiding in the provision of quality healthcare, aiming to reduce the morbidity caused by COVID and its lingering effects in the population. Creating the necessary precedent for testing a specific immunobiological could advance approaches that target symptom improvement in patients.

Economic Impact: While not directly generating income, progress in the field of Long COVID treatment could save significant amounts for healthcare systems. This would alleviate the burden on resources, which could then be directed towards other causes.

DEDICATÓRIA

À minha família: mãe, pai, irmão e esposa. Sem vocês eu não teria tido pelo que continuar.

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RESUMO

A COVID longa é uma doença debilitante, que ocorre em pelo menos 10% das pessoas infectadas pelo SARS-CoV-2. Muitos sintomas diferentes já foram identificados, mas a natureza da doença, sua frequência e principalmente sua etiologia e patogênese ainda não foram bem esclarecidos. Os estudos tem encontrado bastante heterogeneidade sobre os padrões de resposta e possíveis alterações encontradas na imunidade desses pacientes. Parte do problema se dá por conta da difícil seleção desses indivíduos, já que a sobreposição de outras doenças e de sequelas físicas pode dificultar e muito o diagnóstico correto da COVID longa. Por conta dessa dificuldade diagnóstica todos os pacientes do estudo tiveram casos leves e moderados de infecção inicial. Dessa forma, o presente trabalho visa esclarecer se existe diferenças nos padrões de resposta inflamatória desses pacientes, focando na produção de citocinas como IL-2, CXCL10 (IP-10), IL-1 β , TNF- α , CCL2 (MCP-1), IL-17A, IL-6, IL-10, IL-4, IFN- γ , IL-12p70, CXCL8 (IL-8), TGF- β 1; além de buscar identificar a frequência de subpopulações de leucócitos do sangue periférico e avaliação da expressão dos receptores ACE2 e TLR4 em monócitos, neutrófilos, linfócitos TCD4/CD8 e linfócitos B. Nossos resultados mostraram que os pacientes, além de terem aumento na expressão de TLR4 nos linfócitos B, também cursam com uma diminuição de pelo menos metade da população de linfócitos T CD4+ e aumento na população dos linfócitos T CD8+. Encontramos também aumento absoluto na população de monócitos não clássicos redução na expressão de ACE2 na membrana dos monócitos clássicos. Além disso caracterizamos nos pacientes, aumento da IL-6 e da IL17A e valores menores de IL-4, IL-2, IL-1 β , TNF- α , IL-10 e IL-12p70. Sendo assim há uma indicação compatível com a literatura atual, mostrando uma possível resposta inflamatória crônica, com diminuição dos CD4+ e aumento dos monócitos não clássicos. Esse tipo de resposta com IL17A e IL-6 elevada é diferente do padrão aceito atualmente de IL-1 β , IL-6 e TNF- α , mostrando que possivelmente pacientes com gravidades diferentes de infecção inicial podem ter diferentes padrões de citocinas quando cursam com sequelas.

Palavras-chave: SARS-CoV-2; COVID longa; citocinas; ACE2; TLR4.

ABSTRACT

Long COVID is a debilitating disease that occurs in at least 10% of individuals infected with SARS-CoV-2. Many different symptoms have been identified, but the nature of the disease, its frequency, and, most importantly, its etiology and pathogenesis have not yet been well elucidated. Studies have found significant heterogeneity in response patterns and potential alterations in the immunity of these patients. Part of the problem lies in the difficult selection of these individuals, as the overlap with other diseases and physical sequelae can greatly hinder the correct diagnosis of Long COVID. Due to this diagnostic challenge, all patients in the study had mild to moderate cases of initial infection. Thus, the present work aims to clarify whether there are differences in the inflammatory response patterns of these patients, focusing on the production of cytokines such as IL-2, CXCL10 (IP-10), IL-1 β , TNF- α , CCL2 (MCP-1), IL-17A, IL-6, IL-10, IL-4, IFN- γ , IL-12p70, CXCL8 (IL-8), TGF- β 1; as well as to identify the frequency of peripheral blood leukocyte subpopulations and evaluate the expression of ACE2 and TLR4 receptors in monocytes, neutrophils, TCD4/CD8 lymphocytes, and B lymphocytes. Our results showed that patients, in addition to an increase in TLR4 expression in B lymphocytes, also have a decrease of at least half of the CD4+ T lymphocyte population and an increase in the CD8+ T lymphocyte population. We also found an absolute increase in the non-classical monocyte population and a reduction in ACE2 expression on the membrane of classical monocytes. Furthermore, we characterized an increase in IL-6 and IL-17A and lower values of IL-4, IL-2, IL-1 β , TNF- α , IL-10, and IL-12p70 in patients. Thus, there is an indication consistent with the current literature, showing a possible chronic inflammatory response, with a decrease in CD4+ and an increase in non-classical monocytes. This type of response with elevated IL-17A and IL-6 is different from the currently accepted pattern of IL-1 β , IL-6, and TNF- α , indicating that patients with different severities of initial infection may have different cytokine patterns when experiencing sequelae.

Keywords: SARS-CoV-2; long COVID; cytokines; ACE2; TLR4.

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CAPÍTULO I – REVISÃO DA LITERATURA

Aspectos gerais da COVID-19

Desde o final de 2019 o mundo passou não só pela pandemia causada pelo SARS-CoV-2, agente etiológico da COVID-19, mas também por suas inúmeras consequências. Não é a primeira vez que um coronavírus (CoVs) surpreende os humanos e causa uma emergência sanitária. Tivemos epidemias com o SARS-CoV em 2002 e 2003 e a síndrome respiratória do Oriente Médio (MERS-CoV) em 2012¹. Esses coronavírus são vírus de RNA de fita simples, com sentido positivo e envelopados, variando de 26 a 32kb, tendo assim o maior genoma conhecido entre os vírus de RNA².

A COVID-19 tem sintomas similares aos da Influenza, sendo os mais comuns descritos como: febre, cansaço e tosse seca, podendo apresentar em alguns casos falta de ar, dor no corpo, dor de garganta e coriza³. Seu contágio ocorre através de partículas de fluidos corporais espalhadas pelo ar ou superfícies contaminadas com a presença do SARS-CoV-2. Desde o seu início, vem apresentando diferentes formas clínicas, variando de assintomática, leve, moderada, até formas mais severas e que levam os indivíduos a óbito⁴.

O vírus já infectou mais de 769.483.224 pessoas no mundo todo, com uma média de 0,9% de vítimas fatais (6.954.911 mortes). Importante ressaltar que esse valor de letalidade atual só foi possível graças a queda importante de casos graves desde o começo da vacinação⁵. O número de infectados ultrapassou, em muito, os casos de SARS-CoV com 8.000 pessoas infectadas e 800 mortos, e MERS-CoV com 2.500 casos e 800 mortos^{6,7}, demonstrando que o SARS-CoV-2 é muito mais infeccioso que os outros vírus da mesma família¹. No Brasil, estamos atualmente com 37.750.389 casos confirmados e 1,9% de vítimas fatais (705.054 mortes), seguindo as tendências mundiais, cálculos do meio de agosto de 2023^{5,8}.

O sistema imunológico exerce papel essencial no enfrentamento das infecções pelos coronavírus, induzindo a ativação das respostas imunológicas inata e adaptativa a fim de diminuir o foco infeccioso a partir do aumento do número das células fagocíticas, principalmente dos macrófagos^{9,10}, além do recrutamento de linfócitos e leucócitos para o enfrentamento viral e produção de

citocinas pró-inflamatórias como interferons IFNs e fatores de necrose tumoral TNFs, ativando as células NK e NKT^{1,11-13}.

Na resposta imune inata ocorre reconhecimento viral à partir do reconhecimento de padrões moleculares associados ao patógeno (PAMPs) e por receptores de reconhecimento de padrão (PRRs), que identificam os vírus de RNA^{14,15} à partir de diferentes receptores dos tipos Toll (TLR), RIG-I (RLR) e NOD (NLR)¹⁶. A fim de limitar a propagação do vírus, há ativação de vias de cascatas de sinalização de IRFs e NF- κ B (*nuclear factor kappa B*)¹, aumentando a expressão de citocinas pró-inflamatórias e o recrutamento de leucócitos para o local da infecção, além da expressão de IFN do tipo 1 que atua protegendo as células saudáveis através da indução da produção de proteínas antivirais^{2,15}.

Caso ocorra a desregulação dessa resposta, esta pode causar consequências patológicas graves aos tecidos pulmonares, cardíacos, renais e hepáticos, causando possivelmente além da pneumonia falências múltiplas em vários outros sistemas¹⁶. O quadro geral da resposta imune é semelhante ao que ocorre durante a SARS e a MERS, que apresentam alterações leucocitárias e uma tempestade de citocinas pró-inflamatórias, dando início a um quadro de sepse viral^{17,18}.

Os coronavírus apresentam proteínas que podem ser reconhecidas por diferentes PRRs, como o TLR-3 e o TLR4, ligando-se ao dsRNA durante sua replicação, ativando a resposta imunológica². Na infecção por SARS-CoV-2, nota-se também aumento do número de neutrófilos totais, linfócitos totais reduzidos e níveis séricos aumentados de IL-6 e de proteína C reativa, o que sugere uma forte resposta inflamatória. Isso é de fato evidenciado nos pacientes infectados com SARS-CoV-2, a partir dos níveis plasmáticos de citocinas da resposta inata, como IP-10, MCP-1, MIP-1A e IFN α , e os altos níveis de citocinas pró-inflamatórias, incluindo IL-2, IL-7, G-CSF, IP-10, MCP-1, MIP-1A e TNF- α ^{17,18}. Além disso, a mortalidade foi maior em pacientes que apresentavam níveis elevados de IL-6.¹⁸

Estudos também demonstram a participação da enzima conversora de angiotensina II (ACE2) como o receptor celular principal de entrada do SARS-CoV-2, desempenhando papel de células-alvo na infecção por esse vírus^{13,19,20}. Para a entrada viral, há a ligação da primeira subunidade da glicoproteína spike do vírus, S1, à ACE2 através do domínio de ligação ao receptor (RBD) enquanto

a S2 (a segunda subunidade), se fixa à serina protease 2 (TMPRSS2)^{21, 22}. A ECA2 está significativamente presente nas células epiteliais alveolares tipo II no pulmão e nas células do miocárdio, assim como na cavidade oral, indicando assim essa via como uma via de alto risco para a infecção^{13,20,23}.

A COVID Longa

Apesar dos grandes gastos e do grande impacto nos sistemas de saúde de todo o mundo, com os quadros agudos e graves, tornou-se preocupante também a influência do grupo de pessoas que após a remissão da doença ainda apresenta sintomas remanescentes importantes. A esse estado tem-se atribuído os nomes de COVID longa, síndrome pós COVID ou COVID crônica²⁴.

A definição mais reconhecida hoje é a adotada pela Organização Mundial de Saúde (OMS), que denomina a COVID longa como a continuação ou desenvolvimento de novos sintomas 3 meses após a infecção inicial pelo SARS-CoV-2, com esses sintomas tendo uma duração de pelo menos 2 meses e sem outra possível explicação²⁵.

A gama de sinais e sintomas apresentados é grande, acometendo os mais diferentes sistemas e tecidos. As sequelas encontradas na literatura atual englobam²⁶⁻³⁶:

1-) Sequelas Sistêmicas: como a fadiga, fraqueza, febre, suores, linfadenopatia, mal estar geral e tontura. 2-) Sequelas Cardiopulmonares: como falta de ar, dor no peito, tosse, expectoração, palpitações, rubor facial, hipertensão arterial sistêmica e outros. 3-) Sequelas do Sistema Respiratório Superior: como dor de garganta, congestão nasal, mudança de voz e outros. 4-) Sequelas do Sistema Gastrointestinal: como náusea e vômitos, diarreia, perda de apetite, dor de estômago, desconforto abdominal, perda de peso e sangue nas fezes. 5-) Sequelas da Musculatura Esquelética: como dor muscular, dor articular e perda de mobilidade, 6-) Sequelas do Sistema Neurológico: como dores de cabeça, tremores, convulsões, câimbras, bradicinesia, perda de coordenação, atrofia e perda de tônus muscular, alterações de marcha, perda e alterações do olfato (anosmia), perda e alterações do paladar (ageusia), perda auditiva, perda visual, disartria, parestesias, anestésias, neuralgia do trigêmeo, anormalidades nos

reflexos e outros. 7-) Sequelas Psicológicas e Sociais: como ansiedade, depressão, alterações do sono, transtorno de estresse pós traumático, disforia, perda de qualidade de vida e dependência de cuidados. 8-) Sequelas Neurocognitivas: como perda de memória, dificuldade de concentração, confusão, liberação frontal e outros déficits cognitivos. 9-) Outros: rash cutâneo, queda de cabelo, conjuntivite.

Esses sintomas persistem por até 6 meses ou mais após a infecção aguda. Enquanto pacientes mais graves tendem a ter mais complicações, mais de 30% dos casos que ainda mantêm sintomas por mais de 60 dias foram assintomáticos na infecção inicial³⁵ ou não precisaram ser hospitalizados³⁷.

Por conta das inúmeras apresentações clínicas possíveis da COVID longa, coube inicialmente uma discussão sobre como esses sintomas deveriam ser classificados, e o consenso atual é de que como pouco se sabe sobre como o organismo reage a esse quadro, deve-se permitir que os próprios pacientes relatem e expliquem seus sintomas e o quanto cada um deles impacta na sua vida.^{38,39}

Para exemplificar o possível tamanho do impacto econômico dessa síndrome, já foi mostrado que aproximadamente metade dos 3.762 pacientes com COVID longa, entrevistados em 56 países, ainda não conseguiam trabalhar turnos completos 6 meses após a infecção por conta principalmente da fadiga, da astenia e das disfunções cognitivas residuais⁴⁰.

Patogênese

As principais hipóteses atuais para a patogênese desse quadro mostram que há muitas possíveis causas, e que elas possivelmente se sobrepõem.

Uma delas mostra grandes quantidades circulantes remanescentes de pequenos trombos resistentes à fibrinólise^{41,42}. Esses estudos mostram grandes depósitos anômalos de amiloides e fibrina, que quando decompostos aumentam a quantidade sérica de SAA4 (amiloide sérico A) e Alfa2AP (alfa 2 antiplasmina), proteínas de fase aguda responsáveis por uma dissolução controlada de polímeros de fibrina⁴³⁻⁴⁵. Esses trombos tem se mostrado responsáveis por causarem obstrução dos capilares e as vezes até de vasos maiores, gerando

também destruição e disfunção endotelial⁴⁶⁻⁴⁸.

Há estudos que demonstram a persistência de biomarcadores relacionados ao processo de resposta à inflamação⁴⁹, principalmente seis deles: PRDX3, um antioxidante considerado marcador da resposta mitocondrial ao stress; CPS1, uma enzima mitocondrial do ciclo da ureia; o NDRG1, proteína conhecida por afetar o processo de diferenciação dos macrófagos⁵⁰ e maturação dos mastócitos⁵¹; o CTHRC1, substância responsável por reparação tecidual, mostrando que há agressão tecidual até nos casos mais leves; Cystatin C, um biomarcador controlado pela IL-10⁵²; e a redução da Progranulina, cuja função não é completamente conhecida, mas sabe-se que está associada a proteção em desordens neurodegenerativas⁵³. Essa desregulação imune⁵⁴⁻⁵⁷ pode vir acompanhada da reativação de outras doenças de base presentes, como pelo vírus Epstein-Barr ou pelo Herpes Virus 6^{58,59}.

Outra linha de estudos tem mostrado ainda que, podem ser encontrados reservatórios virais no organismo, com as proteínas virais e /ou RNA ainda presentes no plasma⁶⁰, ou igualmente distribuídas em vários tecidos do corpo, incluindo o sistema reprodutivo, cardiovascular, cérebro, músculos, olhos, linfonodos, tecido hepático, pulmonar, ou mesmo secreções, como fezes e urina⁶¹⁻⁶⁷, desde períodos médios, como 230 dias após o início dos sintomas⁵⁹⁻⁶² até mais longos, como 12 meses após o diagnóstico⁶⁸.

Uma outra possível hipótese seria o impacto do SARS-CoV-2 na microbiota intestinal. Estudos identificaram uma possível alteração na dinâmica do viroma⁶⁹, e mostraram a relação entre a disfunção da microbiota e a gravidade dos sintomas⁷⁰. Foi demonstrado também que o transplante fecal do conteúdo de pacientes acometidos, em camundongos saudáveis, fez com que os animais desenvolvessem sintomas similares a COVID longa⁷¹.

Aspectos Imunes na COVID Longa

Os estudos sobre desregulação imune na COVID longa têm tentado caracterizar padrões que possam explicar a presença dos diferentes sintomas persistentes. Inicialmente, foi notado que pacientes que não produziam ou tinham baixa produção de anticorpos durante a infecção aguda (incluindo baixa produção de IgG de base⁷² e baixos picos de IgG específico para a Spike⁷³)

tinham mais chance de desenvolver COVID longa^{72,73}. Foi associado também, que baixos níveis de anticorpos específicos para a doença eram inversamente proporcionais aos títulos de auto anticorpos⁷⁴. Esses baixos níveis de anticorpos protetores podem tornar os pacientes mais sujeitos a novos episódios da doença. Sabe-se já que há aumento no risco de surgimento de sequelas após o segundo ou terceiro episódio de infecção, mesmo que as pessoas tenham duas ou três doses de vacinas⁷⁵. Além do surgimento de novos sintomas, múltiplas infecções aumentam a chance do paciente de apresentar um quadro de sequelas similar ao da síndrome da fadiga crônica⁷⁶.

Alguns pacientes com COVID longa apresentam altos níveis de auto anticorpos. Os mais gerais tinham como alvo tecidos (tecido conectivo, componentes de matriz extracelular, fatores de coagulação e endotélio), órgãos e sistemas (pulmão, sistema nervoso central, pele e trato gastrointestinal) e proteínas imunomodulatórias (citocinas, quimiocinas, complemento e proteínas de superfície celular)⁷⁷. Quanto aos anticorpos mais específicos, foram encontrados autoanticorpos contra o receptor de ACE2⁷⁸, receptor β 2-adrenérgico, receptor M2 muscarínico, receptor de angiotensina 2 AT1 e receptor MAS de angiotensina 1-7⁷⁹.

Sobre as citocinas, encontrou-se aumento de IL-6, TNF, e IP10 nos pacientes acometidos^{80,81}. Outra quimiocina elevada é a CCL11, associada a perdas cognitivas e desordens psiquiátricas^{82,83}. Houve também aumento na expressão de interferon- β (IFN β) e de um interferon tipo 3 (IFN- λ 1)⁸⁴.

Uma dificuldade no estudo das citocinas é a sua cinética, podendo ser que padrões encontrados em alguns trabalhos sejam diferentes de outros devido ao período de tempo pós infecção em que são avaliados. Um exemplo comparado à COVID longa atualmente é o arquétipo da síndrome da fadiga crônica (também chamada encefalomielite miálgica) que mostra padrões de citocinas que começam a cair apenas de 2-3 anos após o começo dos sintomas^{85, 86}.

Quanto às populações celulares, foi detectado aumento no número de monócitos não clássicos, células B ativadas, células B duplo negativas⁸⁵, linfócitos T CD4+ secretores de IL-4 e IL-6 e diminuição de níveis de cortisol e células dendríticas 14 meses após a infecção original⁸⁶. Atualmente, um dos preditores mais confiáveis para a presença da COVID longa em um paciente é a

queda no cortisol sérico⁸⁵.

Alterações nas células T mostram número reduzido de CD4+ e CD8+ efetoras e alta exaustão das linhagens das células T^{54, 55}. Outro estudo mostrou diferenças entre padrões das células T dependendo da gravidade da infecção inicial. Enquanto pacientes que se recuperavam de infecções agudas mais graves apresentavam polarização para estados de senescência e exaustão de células T CD4+ e CD8+, perturbações de CD4+ Tregs, diminuição do número de populações naive, aumento de Granzima B e do IFN- γ , mostrando inflamação não resolvida; os pacientes que se recuperavam de infecções leves tinham aumento das naive e das CD4+ Tregs⁸⁷.

Outro grupo importante para ser estudado dentro da COVID longa são os pacientes imunossuprimidos devido ao padrão diferenciado de resposta imune. Entender como a doença se apresenta em pacientes que apresentam imunossupressão de respostas conhecidas poderia ajudar a elucidar a patogênese. No entanto os achados tem sido ambíguos e de difícil explicação. Enquanto um estudo demonstrou melhores desfechos gerais, sinalizando que crianças imunossuprimidas tinham menos chance de desenvolver COVID longa e as que acabavam desenvolvendo tinham sintomas mais leves e menos incapacitantes do que crianças imunocompetentes⁸⁸, o outro demonstrou piores desfechos, incluindo um caso de um paciente imunossuprimido cuja infecção longa foi responsável pelo acúmulo de mais de 40 mutações virais, demonstrando assim um ritmo de mutação maior *in vivo* nesses indivíduos quando comparado com a própria população geral, podendo ter um impacto maior na transmissão comunitária⁸⁹. Os Toll Like Receptors (TLR) foram identificados em humanos na década de 90^{90, 91}. Eles estão entre os receptores celulares mais estudados, sendo o TLR4 um dos mais importantes atualmente, devido sua capacidade de ativar boas respostas à vacinas, como na Hepatite B e HPV^{92,93}. Os TLRs são responsáveis por detectar patógenos invasores, iniciar a resposta imune inata e em sequência ativar a resposta imune adaptativa⁹⁴.

Na COVID-19 o TLR4, que inicialmente acreditava-se ter função protetora, passou a ser considerado fator de gravidade quando descoberta a capacidade da proteína *Spike* se ligar a ele⁹⁵ e iniciar a ativação de fatores de transcrição para NF- κ B, fator de regulação de interferon e ativador da proteína 1 (AP-1)⁹⁶. Essa ativação *in situ* dos macrófagos alveolares seria responsável por causar

uma inflamação local intensa e conseqüente destruição do tecido pulmonar^{97, 98}. Não só o TLR4 poderia ser causa imediata do nível de inflamação tecidual, mas poderia também ser responsável por aumentar a expressão de ACE2 na membrana celular, facilitando assim a entrada de novos vírus nas células⁹⁹.

Já na COVID longa uma hipótese atual é de que o TLR4 faria parte de um feedback em looping. A calprotectina S100A8/A9 estimularia cronicamente a sinalização TLR4/RAGE, expressando assim IL-1b, IL-6 e TNF α . A IL-6, ao e ligar ao seu receptor IL-6R, estimularia a produção de mais S100A8/A9 e a IL-1 estimularia o aumento de mRAGE e TLR-4 via NFk- β . Esse feedback IL-1b + IL-6 $\rightarrow$ IL-1R + IL-6R $\rightarrow$ TLR4/mRAGE + S100A8/A9 $\rightarrow$ IL-1b + IL-6 seria responsável pela manutenção da resposta inflamatória mesmo na ausência do vírus¹⁰⁰. Esse feedback alimentado pela IL-1b e pela IL-6 tem ditado o foco nos testes terapêuticos com inibidores dessas citocinas¹⁰⁰.

Justificativa e objetivos

As opções de diagnóstico e tratamento para a COVID longa atualmente são escassas. Poucas condutas conseguem surtir bons efeitos nos sintomas e incapacidades dos pacientes e além disso a heterogeneidade dos grupos faz com que o desenvolvimento de algo visando todos os diferentes sintomas seja muito difícil. Estudos buscando o melhor entendimento da resposta de pacientes que apresentam alguns acometimentos e outros não, são essenciais para a melhor elucidação dessas diferenças.

Não há ainda na literatura achados que tenham demonstrado um padrão único de resposta imunológica de cada indivíduo com o desenvolvimento da síndrome. O grande viés dos estudos atualmente é a inclusão de pacientes com qualquer tipo de sequela de COVID, sendo esses misturados com os grupo de pacientes com o diagnóstico correto de COVID longa. As duas são entidades diferentes, e sem extremo cuidado na seleção dos pacientes não são facilmente separadas. Diante disso, esse trabalho visa estudar as possíveis diferenças nas respostas de pacientes que apresentam sinais e sintomas de COVID longa e compará-las a pacientes que tiveram COVID e não apresentaram essas manifestações.

Uma seleção criteriosa foi então realizada, capaz de recrutar apenas

pacientes que apresentavam COVID longa e diferenciando dos pacientes que possam ter sequelas físicas responsáveis pelos seus sintomas. É importante o entendimento das diferenças nos quadros desses pacientes, para que seja possível encontrarmos alvos terapêuticos, melhorando assim a qualidade de vida desses indivíduos acometidos, diminuindo o impacto que a COVID longa pode deixar nos sistemas de saúde.

Sendo assim, nosso trabalho teve como objetivos:

- A avaliação de citocinas como IL-2, CXCL10 (IP-10), IL-1 β , TNF- α , CCL2 (MCP-1), IL-17A, IL-6, IL-10, IL-4, IFN- γ , IL-12p70, CXCL8 (IL-8), TGF- β 1, de todos os pacientes estudados, analisando os seus níveis das diferentes citocinas com a presença da doença.

- A identificação da frequência de subpopulações de leucócitos do sangue periférico e avaliação da expressão dos receptores ACE2 e TLR4 em monócitos, neutrófilos, linfócitos T CD4/CD8 e linfócitos B, nos diferentes grupos de pacientes estudados.

Referências

1. Cascella, M. et al. Features, Evaluation and Treatment Coronavirus (COVID-19). In: StatPearls. Treasure Island (FL): StatPearls Publishing, 2020.
2. Li G., Fan Y., Lai Y., Han T., Li Z., Zhou P. et al. Coronavirus infections and immune responses. *J Med Virol.* 2020 Apr; 92(4):424-432.
3. World Health Organization. Coronavirus. Disponível em: <https://www.who.int/health-topics/coronavirus>. Acesso em: agosto/2023.
4. Hu, Y.; Deng, H.; Huang, L.; Xia, L.; Zhou, X. Analysis of Characteristics in Death Patients with COVID-19 Pneumonia without Underlying Diseases. *Acad Radiol.* 2020 Apr 6. pii: S1076-6332(20)30164-1. doi: 10.1016/j.acra.2020.03.023.0
5. OMS - Coronavirus disease 2019 (COVID-19), Situation report – 156. Disponível em: https://www.who.int/publications/m/item/20230511_weekly_epi_update_156.pdf
6. De Wit, E. et al. SARS and MERS: recent insights into emerging coronaviruses. *Nature Reviews. Microbiology*, v. 14, n. 8, p. 523–534, 2016.
7. Yue, Y. et al. SARS-Coronavirus Open Reading Frame-3a drives multimodal necrotic cell death. *Cell Death & Disease*, v. 9, n. 9, p. 1–15, 5 set. 2018.
8. Ministério da Saúde. Coronavírus <https://www.gov.br/saude/pt-br/coronavirus/informes-diarios-covid-19/covid-19-situacao-epidemiologica-do-brasil-ate-a-se-18-de-2023>
9. Calich, V.; Vaz, C. *Imunologia*. 2 ed. Rio de Janeiro:Revinter, 17-57, 2009.
10. Saha, B.; Szabo, G. Innate Immune Cell Networking in Hepatitis C Virus Infection. *J Leukoc Biol.*, 2014 nov; 96(6):757-66.
11. Liu, Y. ; Yang, Y. ; Zhang, C. ; Huang, F. ; Wang, F. ; Yuan, J. et al. Clinical and biochemical indexes from 2019-nCoV infected patients linked to viral loads and lung injury. *Sci China Life Sci.* 2020 Mar;63(3):364-374
12. Cossarizza, A.; Biasi, S.; Guaraldi, G.; Girardis, M.; Mussini, C. SARS-CoV-2, the Virus that Causes COVID-19: Cytometry and the New Challenge for Global Health. *Cytometry A* (2020).
13. Hoffmann, M.; Kleine-Weber, H.; Schroeder, S.; Krüger, N.; Herrler, T.; Erichsen, S. et al. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* (2020).
14. Jensen, S.; Thomsen, A.R. Sensing of RNA viruses: A review of innate immune receptors involved in recognizing RNA virus invasion. *J. Virol.* 2012, 86, 2900–2910.
15. Nelemans, T.; Kikkert, M. Viral Innate Immune Evasion and the Pathogenesis of Emerging RNA Virus Infections. *Pathogenesis of Emerging Viral Infections. Viruses* 2019, 11(10), 961; <https://doi.org/10.3390/v11100961>.

16. Cao, X. Cao, X. COVID-19: immunopathology and its implications for therapy. *Nat Rev Immunol* (2020). <https://doi.org/10.1038/s41577-020-0308-3>.
17. Ruan, Q.; Yang, K.; Wang, W.; Jiang, L.; Song, J. Clinical predictors of mortality due to COVID-19 based on an analysis of data of 150 patients from Wuhan, China. *Intensive Care Med.* 2020 Mar 3. doi: 10.1007/s00134-020-05991-x.
18. Qin, C.; Zhou, L.; Hu, Z.; Zhang, S.; Yang, S.; Tao, Y.; Xie, C.; Ma, K.; Shang, K.; Wang, W.; Tian, D.S. Dysregulation of immune response in patients with COVID-19 in Wuhan, China. *Clin Infect Dis.* 2020 Mar 12. pii: ciaa248. doi: 10.1093/cid/ciaa248.
19. Zhou, P., Yang, X. L., Wang, X. G., Hu, B., Zhang, L., Zhang, W., Si, H. R., Zhu, Y., Li, B., Huang, C. L., Chen, H. D., Chen, J., Luo, Y., Guo, H., Jiang, R. D., Liu, M. Q., Chen, Y., Shen, X. R., Wang, X., Zheng, X. S., ... Shi, Z. L. (2020). A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature*, 579(7798), 270–273. <https://doi.org/10.1038/s41586-020-2012-7>.
20. Yu, Z., Zixian, Z., Yujia, W., Yueqing, Z., Yu, M., Wei, Z.. Single-cell RNA expression profiling of ACE2, the putative receptor of Wuhan 2019-nCov. *bioRxiv* 2020.01.26.919985
21. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, et al. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell.* Março de 2020;181(2):271–80.e8.
22. Hoffmann M, Kleine-Weber H, Krüger N, Müller M, Drosten C, Pöhlmann S. The novel coronavirus 2019 (2019-nCoV) uses the SARS-coronavirus receptor ACE2 and the cellular protease TMPRSS2 for entry into target cell. *Europe PMC.* 2020.
23. Xu, H., Zhong, L., Deng, J., Peng, J., Dan, H., Zeng, X., Li, T., & Chen, Q. (2020). High expression of ACE2 receptor of 2019-nCoV on the epithelial cells of oral mucosa. *International journal of oral science*, 12(1), 8.
24. Proal AD, VanElzakker MB. Long COVID or Post-acute Sequelae of COVID-19 (PASC): An Overview of Biological Factors That May Contribute to Persistent Symptoms. *Front Microbiol.* 2021 Jun 23;12:698169. doi: 10.3389/fmicb.2021.698169. PMID: 34248921; PMCID: PMC8260991.
25. World Health Organization. Post COVID-19 condition (Long COVID). Disponível em: <https://www.who.int/europe/news-room/fact-sheets/item/post-covid-19-condition>
26. Lopez-Leon S, Wegman-Ostrosky T, Perelman C, Sepulveda R, Rebolledo PA, Cuapio A, Villapol S. More than 50 long-term effects of COVID-19: a systematic review and meta-analysis. *Sci Rep.* 2021 Aug 9;11(1):16144. doi: 10.1038/s41598-021-95565-8. PMID: 34373540; PMCID: PMC8352980.
27. Silva Andrade B, Siqueira S, de Assis Soares WR, de Souza Rangel F, Santos NO, Dos Santos Freitas A, Ribeiro da Silveira P, Tiwari S, Alzahrani KJ, Góes-Neto A, Azevedo V, Ghosh P, Barh D. Long-COVID and Post-COVID Health Complications: An Up-to-Date Review on Clinical Conditions and Their Possible Molecular Mechanisms.

Viruses. 2021 Apr 18;13(4):700. doi: 10.3390/v13040700. PMID: 33919537; PMCID: PMC8072585.

28. Carfi A, Bernabei R, Landi F; Gemelli Against COVID-19 Post-Acute Care Study Group. Persistent Symptoms in Patients After Acute COVID-19. *JAMA*. 2020;324(6):603-605. doi:10.1001/jama.2020.12603

29. Rubin R. As Their Numbers Grow, COVID-19 "Long Haulers" Stump Experts. *JAMA*. 2020 Oct 13;324(14):1381-1383. doi: 10.1001/jama.2020.17709. PMID: 32965460.

30. Baig AM. Chronic COVID syndrome: Need for an appropriate medical terminology for long-COVID and COVID long-haulers. *J Med Virol*. 2021 May;93(5):2555-2556. doi: 10.1002/jmv.26624. Epub 2021 Mar 1. PMID: 33095459.

31. Wostyn P. COVID-19 and chronic fatigue syndrome: Is the worst yet to come? *Med Hypotheses*. 2021 Jan;146:110469. doi: 10.1016/j.mehy.2020.110469. Epub 2021 Jan 2. PMID: 33401106; PMCID: PMC7836544.

32. Mohabbat AB, Mohabbat NML, Wight EC. Fibromyalgia and Chronic Fatigue Syndrome in the Age of COVID-19. *Mayo Clin Proc Innov Qual Outcomes*. 2020 Dec;4(6):764-766. doi: 10.1016/j.mayocpiqo.2020.08.002. Epub 2020 Nov 13. PMID: 33204998; PMCID: PMC7661943.

33. Goldstein DS. The possible association between COVID-19 and postural tachycardia syndrome. *Heart Rhythm*. 2021 Apr;18(4):508-509. doi:10.1016/j.hrthm.2020.12.007. Epub 2020 Dec 11. PMID: 33316414; PMCID: PMC7729277.

34. Afrin LB, Weinstock LB, Molderings GJ. Covid-19 hyperinflammation and post-Covid-19 illness may be rooted in mast cell activation syndrome. *Int J Infect Dis*. 2020 Nov;100:327-332. doi: 10.1016/j.ijid.2020.09.016. Epub 2020 Sep 10. PMID: 32920235; PMCID: PMC7529115.

35. Huang C, Huang L, Wang Y, Li X, Ren L, Gu X, Kang L, Guo L, Liu M, Zhou X, Luo J, Huang Z, Tu S, Zhao Y, Chen L, Xu D, Li Y, Li C, Peng L, Li Y, Xie W, Cui D, Shang L, Fan G, Xu J, Wang G, Wang Y, Zhong J, Wang C, Wang J, Zhang D, Cao B. 6-month consequences of COVID-19 in patients discharged from hospital: a cohort study. *Lancet*. 2021 Jan 16;397(10270):220-232. doi: 10.1016/S0140-6736(20)32656-8. Epub 2021 Jan 8. PMID: 33428867; PMCID: PMC7833295.

36. Michelen M, Manoharan L, Elkheir N, et al. Characterising long COVID: a living systematic review. *BMJ Glob Health*. 2021;6(9):e005427. doi:10.1136/bmjgh-2021-005427

37. Tabacof L, Tosto-Mancuso J, Wood J, Cortes M, Kontorovich A, McCarthy D, Rizk D, Mohammadi N, Breyman E, Nasr L, Kellner C, Putrino D, Post-acute COVID-19 syndrome negatively impacts health and wellbeing despite less severe acute infection. doi: <https://doi.org/10.1101/2020.11.04.20226126>

38. Long COVID: let patients help define long-lasting COVID symptoms. *Nature*. 2020 Oct;586(7828):170. doi: 10.1038/d41586-020-02796-2. PMID: 33029005.

39. Alwan NA. Track COVID-19 sickness, not just positive tests and deaths. *Nature*. 2020 Aug;584(7820):170. doi: 10.1038/d41586-020-02335-z. PMID: 32782377.
40. Komaroff AL, Bateman L. Will COVID-19 Lead to Myalgic Encephalomyelitis/Chronic Fatigue Syndrome? *Front Med (Lausanne)*. 2021 Jan 18;7:606824. doi: 10.3389/fmed.2020.606824. PMID: 33537329; PMCID: PMC7848220.
41. Pretorius E, Venter C, Laubscher GJ, Lourens PJ, Steenkamp J, Kell DB. Prevalence of readily detected amyloid blood clots in 'unclothed' Type 2 Diabetes Mellitus and COVID-19 plasma: a preliminary report. *Cardiovasc Diabetol*. 2020 Nov 17;19(1):193. doi: 10.1186/s12933-020-01165-7. PMID: 33203441; PMCID: PMC7670290.
42. Venter C, Bezuidenhout JA, Laubscher GJ, Lourens PJ, Steenkamp J, Kell DB, Pretorius E. Erythrocyte, Platelet, Serum Ferritin, and P-Selectin Pathophysiology Implicated in Severe Hypercoagulation and Vascular Complications in COVID-19. *Int J Mol Sci*. 2020 Nov 3;21(21):8234. doi: 10.3390/ijms21218234. PMID: 33153161; PMCID: PMC7662625.
43. Schaller J, Gerber SS. The plasmin-antiplasmin system: structural and functional aspects. *Cell Mol Life Sci*. 2011 Mar;68(5):785-801. doi: 10.1007/s00018-010-0566-5. Epub 2010 Dec 7. PMID: 21136135.
44. Miszta A, Huskens D, Donkervoort D, Roberts MJM, Wolberg AS, de Laat B. Assessing Plasmin Generation in Health and Disease. *Int J Mol Sci*. 2021 Mar 9;22(5):2758. doi: 10.3390/ijms22052758. PMID: 33803235; PMCID: PMC7963172.
45. Pretorius E, Vlok M, Venter C, et al. Persistent clotting protein pathology in Long COVID/Post-Acute Sequelae of COVID-19 (PASC) is accompanied by increased levels of antiplasmin. *Cardiovasc Diabetol*. 2021;20(1):172. Published 2021 Aug 23. doi:10.1186/s12933-021-01359-7
46. Haffke, M. et al. Endothelial dysfunction and altered endothelial biomarkers in patients with post-COVID-19 syndrome and chronic fatigue syndrome (ME/CFS). *J. Transl Med*. 20, 138 (2022).
47. Charfeddine, S. Long COVID 19 syndrome: is it related to microcirculation and endothelial dysfunction? Insights from TUN-EndCOV study. *Front. Cardiovasc. Med*. <https://doi.org/10.3389/fcvm.2021.745758> (2021).
48. Pretorius, E. et al. Prevalence of symptoms, comorbidities, fibrin amyloid microclots and platelet pathology in individuals with Long COVID/post-acute sequelae of COVID-19 (PASC). *Cardiovasc. Diabetol*. 21, 148 (2022).
49. Doykov I, Hällqvist J, Gilmour KC, Grandjean L, Mills K, Heywood WE. 'The long tail of Covid-19' - The detection of a prolonged inflammatory response after a SARS-CoV-2 infection in asymptomatic and mildly affected patients. *F1000Res*. 2020 Nov 19;9:1349. doi: 10.12688/f1000research.27287.2. PMID: 33391730; PMCID: PMC7745182.
50. Watari K, Shibata T, Nabeshima H, Shinoda A, Fukunaga Y, Kawahara A, Karasuyama K, Fukushi J, Iwamoto Y, Kuwano M, Ono M. Impaired differentiation of macrophage lineage cells attenuates bone remodeling and inflammatory angiogenesis in

Ndrp1 deficient mice. *Sci Rep.* 2016 Jan 18;6:19470. doi: 10.1038/srep19470. PMID: 26778110; PMCID: PMC4726041.

51. Taketomi Y, Sunaga K, Tanaka S, Nakamura M, Arata S, Okuda T, Moon TC, Chang HW, Sugimoto Y, Kokame K, Miyata T, Murakami M, Kudo I. Impaired mast cell maturation and degranulation and attenuated allergic responses in Ndrp1-deficient mice. *J Immunol.* 2007 Jun 1;178(11):7042-53. doi: 10.4049/jimmunol.178.11.7042. PMID: 17513753.

52. Xu Y, Schnorrer P, Proietto A, Kowalski G, Febbraio MA, Acha-Orbea H, Dickins RA, Villadangos JA. IL-10 controls cystatin C synthesis and blood concentration in response to inflammation through regulation of IFN regulatory factor 8 expression. *J Immunol.* 2011 Mar 15;186(6):3666-73. doi: 10.4049/jimmunol.1001934. Epub 2011 Feb 7. PMID: 21300820.

53. Paushter DH, Du H, Feng T, Hu F. The lysosomal function of progranulin, a guardian against neurodegeneration. *Acta Neuropathol.* 2018 Jul;136(1):1-17. doi: 10.1007/s00401-018-1861-8. Epub 2018 May 9. PMID: 29744576; PMCID: PMC6117207.

54. Proal, A. D. & VanElzakker, M. B. Long COVID or post-acute sequelae of COVID-19 (PASC): an overview of biological factors that may contribute to persistent symptoms. *Front. Microbiol.* 12, 698169 (2021).

55. Klein, J. et al. Distinguishing features of Long COVID identified through immune profiling. Preprint at medRxiv <https://doi.org/10.1101/2022.08.09.22278592> (2022).

56. Glynne, P., Tahmasebi, N., Gant, V. & Gupta, R. Long COVID following mild SARS-CoV-2 infection: characteristic T cell alterations and response to antihistamines. *J. Investig. Med.* 70, 61–67 (2022).

57. Phetsouphanh, C. et al. Immunological dysfunction persists for 8 months following initial mild-to-moderate SARS-CoV-2 infection. *Nat. Immunol.* 23, 210–216 (2022).

58. Zubchenko, S., Kril, I., Nadizhko, O., Matsyura, O. & Chopyak, V. Herpesvirus infections and post-COVID-19 manifestations: a pilot observational study. *Rheumatol. Int.* <https://doi.org/10.1007/s00296-022-05146-9> (2022).

59. Peluso, M. J. et al. Evidence of recent Epstein-Barr virus reactivation in individuals experiencing Long COVID. Preprint at medRxiv <https://doi.org/10.1101/2022.06.21.22276660> (2022).

60. Zoe Swank, Yasmeen Senussi, Zachary Manickas-Hill, Xu G Yu, Jonathan Z Li, Galit Alter, David R Walt, Persistent Circulating Severe Acute Respiratory Syndrome Coronavirus 2 Spike Is Associated With Post-acute Coronavirus Disease 2019 Sequelae, *Clinical Infectious Diseases*, Volume 76, Issue 3, 1 February 2023, Pages e487–e490, <https://doi.org/10.1093/cid/ciac722>

61. Chertow D, Stein S, Ramelli A, et al. SARS-CoV-2 infection and persistence throughout the human body and brain. Published online December 20, 2021. <https://doi.org/10.21203/rs.3.rs-1139035/v1>

62. Tejerina, F. et al. Post-COVID-19 syndrome. SARS-CoV-2 RNA detection in plasma, stool, and urine in patients with persistent symptoms after COVID-19. *BMC Infect. Dis.* 22, 211 (2022).
63. Goh, D. et al. Persistence of residual SARS-CoV-2 viral antigen and RNA in tissues of patients with long COVID-19. Preprint at <https://www.researchsquare.com/article/rs-1379777/v1> (2022).
64. Ceulemans, L. J. et al. Persistence of SARS-CoV-2 RNA in lung tissue after mild COVID-19. *Lancet Respir. Med.* 9, e78–e79 (2021).
65. Menuchin-Lasowski, Y. et al. SARS-CoV-2 infects and replicates in photoreceptor and retinal ganglion cells of human retinal organoids. *Stem Cell Rep* 17, 789–803 (2022).
66. Cheung, C. C. L. et al. Residual SARS-CoV-2 viral antigens detected in GI and hepatic tissues from five recovered patients with COVID-19. *Gut* 71, 226–229 (2022).
67. Natarajan, A. et al. Gastrointestinal symptoms and fecal shedding of SARS-CoV-2 RNA suggest prolonged gastrointestinal infection. *Med* 3, 371–387.e9 (2022).
68. Swank, Z. et al. Persistent circulating severe acute respiratory syndrome coronavirus 2 spike is associated with post-acute coronavirus disease 2019 sequelae. *Clin. Infect. Dis.* <https://doi.org/10.1093/cid/ciac722> (2022).
69. Liu, Q. et al. Gut microbiota dynamics in a prospective cohort of patients with post-acute COVID-19 syndrome. *Gut* 71, 544–552 (2022).
70. Yeoh, Y. K. et al. Gut microbiota composition reflects disease severity and dysfunctional immune responses in patients with COVID-19. *Gut* 70, 698–706 (2021).
71. Mendes de Almeida, V. Gut microbiota from patients with mild COVID-19 cause alterations in mice that resemble post-COVID syndrome. *Res. Sq.* <https://doi.org/10.21203/rs.3.rs-1756189/v1> (2022).
72. Augustin, M. et al. Post-COVID syndrome in non-hospitalised patients with COVID-19: a longitudinal prospective cohort study. *Lancet Reg. Health Eur.* 6, 100122 (2021).
73. García-Abellán, J. et al. Antibody response to SARS-CoV-2 is associated with long-term clinical outcome in patients with COVID-19: a longitudinal study. *J. Clin. Immunol.* 41, 1490–1501 (2021).
74. Su, Y. et al. Multiple early factors anticipate post-acute COVID-19 sequelae. *Cell* 185, 881–895.e20 (2022).
75. Bowe, B., Xie, Y. & Al-Aly, Z. Acute and postacute sequelae associated with SARS-CoV-2 reinfection. *Nat. Med.* <https://doi.org/10.1038/s41591-022-02051-3> (2022).
76. Blomberg, J., Gottfries, C.-G., Elfaitouri, A., Rizwan, M. & Rosén, A. Infection elicited autoimmunity and myalgic encephalomyelitis/chronic fatigue syndrome: an explanatory model. *Front. Immunol.* 9, 229 (2018).
77. Wang, E. Y. et al. Diverse functional autoantibodies in patients with COVID-19. *Nature* 595, 283–288 (2021).

78. Arthur, J. M. et al. Development of ACE2 autoantibodies after SARS-CoV-2 infection. *PLoS ONE* 16, e0257016 (2021).
79. Wallukat, G. et al. Functional autoantibodies against G-protein coupled receptors in patients with persistent long-COVID-19 symptoms. *J. Transl Autoimmun.* 4, 100100 (2021).
80. Peluso, M. J. et al. Markers of immune activation and inflammation in individuals with postacute sequelae of severe acute respiratory syndrome coronavirus 2 infection. *J. Infect. Dis.* 224, 1839–1848 (2021)
81. Schultheiß, C. et al. From online data collection to identification of disease mechanisms: the IL-1 β , IL-6 and TNF- α cytokine triad is associated with post-acute sequelae of COVID-19 in a digital research cohort. SSRN <https://doi.org/10.2139/ssrn.3963839> (2021).
82. Teixeira AL, Gama CS, Rocha NP and Teixeira MM (2018) Revisiting the Role of Eotaxin-1/CCL11 in Psychiatric Disorders. *Front. Psychiatry* 9:241. doi: 10.3389/fpsy.2018.00241
83. Fernández-Castañeda, A. et al. Mild respiratory SARS-CoV-2 infection can cause multi-lineage cellular dysregulation and myelin loss in the brain. Preprint at bioRxiv <https://doi.org/10.1101/2022.01.07.475453> (2022).
84. Phetsouphanh, C. et al. Immunological dysfunction persists for 8 months following initial mild-to-moderate SARS-CoV-2 infection. *Nat. Immunol.* 23, 210–216 (2022).
85. Sachinidis A, Garyfallos A. Double Negative (DN) B cells: A connecting bridge between rheumatic diseases and COVID-19? *Mediterr J Rheumatol.* 2021 Sep 30;32(3):192-199. doi: 10.31138/mjr.32.3.192. PMID: 34964023; PMCID: PMC8693305.
86. Hornig, M. et al. Distinct plasma immune signatures in ME/CFS are present early in the course of illness. *Sci. Adv.* 1, e1400121 (2015).
87. Wiech M, Chroscicki P, Swatler J, Stepnik D, De Biasi S, Hampel M, Brewinska-Olchowik M, Maliszewska A, Sklinda K, Durlik M, Wierzba W, Cossarizza A and Piwocka K (2022) Remodeling of T Cell Dynamics During Long COVID Is Dependent on Severity of SARS-CoV-2 Infection. *Front. Immunol.* 13:886431. doi: 10.3389/fimmu.2022.886431
88. Kuczborska K, Buda P, Książyk J. Long-COVID in immunocompromised children. *Eur J Pediatr.* 2022 Sep;181(9):3501-3509. doi: 10.1007/s00431-022-04561-1. Epub 2022 Jul 14. PMID: 35834042; PMCID: PMC9281224.
89. Stanevich, O.V., Alekseeva, E.I., Sergeeva, M. et al. SARS-CoV-2 escape from cytotoxic T cells during long-term COVID-19. *Nat Commun* **14**, 149 (2023).
90. Medzhitov R, Preston-Hurlburt P, Janeway CA Jr. A human homologue of the *Drosophila* toll protein signals activation of adaptive immunity. *Nature* (1997) **388**:394–7. doi:10.1038/41131
91. Medzhitov R, Janeway CA Jr. Innate immunity: the virtues of a nonclonal system of recognition. *Cell* (1997) **91**:295–8. doi:10.1016/S0092-8674(00)80412-2

92. Ireton GC, Reed SG. Adjuvants containing natural and synthetic toll-like receptor 4 ligands. *Expert Rev Vaccines* (2013) **12**:793–807. doi:10.1586/14760584.2013.811204
93. Johnson DA. TLR4 agonists as vaccine adjuvants: a chemist's perspective. *Expert Rev Vaccines* (2013) **12**:711–3. doi:10.1586/14760584.2013.811189
94. Vaure e Liu, "A Comparative Review of Toll-Like Receptor 4 Expression and Functionality in Different Animal Species". *Frontiers in Immunology*, published: 10 July 2014 doi: 10.3389/fimmu.2014.00316
95. Choudhury A, Mukherjee S. In silico studies on the comparative characterization of the interactions of SARS-CoV-2 spike glycoprotein with ACE-2 receptor homologs and human TLRs. *J. Med. Virol.* 92(10), 2105–2113 (2020).
96. Zhao Y, Kuang M, Li J et al. SARS-CoV-2 spike protein interacts with and activates TLR4. *Cell Res.* 31(7), 818–820 (2021).
97. Choudhury A, Das NC, Patra R, Mukherjee S. In silico analyses on the comparative sensing of SARS-CoV-2 mRNA by the intracellular TLRs of humans. *J. Med. Virol.* 93(4), 2476–2486 (2021).
98. Mukherjee, "Toll-like receptor 4 in COVID-19: friend or foe?" *Future Virology* 10.2217/fvl-2021-0249 doi: 10.2217/fvl-2021-0249
99. Mohamed M. Aboudounya, Richard J. Heads, "COVID-19 and Toll-Like Receptor 4 (TLR4): SARS-CoV-2 May Bind and Activate TLR4 to Increase ACE2 Expression, Facilitating Entry and Causing Hyperinflammation", *Mediators of Inflammation*, vol. 2021, Article ID 8874339, 18 pages, 2021. <https://doi.org/10.1155/2021/8874339>
100. Holms RD. Long COVID (PASC) Is Maintained by a Self-Sustaining Pro-Inflammatory TLR4/RAGE-Loop of S100A8/A9 > TLR4/RAGE Signalling, Inducing Chronic Expression of IL-1b, IL-6 and TNFa: Anti-Inflammatory Ezrin Peptides as Potential Therapy. *Immuno.* 2022; 2(3):512-533. <https://doi.org/10.3390/immuno2030033>

CAPÍTULO II – ARTIGO CIENTÍFICO

**Submissão ao Cellular and Molecular
Immunology**

Evaluation of the Inflammatory Response in Long COVID Patients.

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Abstract

Long COVID is a complex and debilitating condition that occurs in at least 10% of individuals infected with SARS-CoV-2. Despite the estimated 65 million cases, it remains a poorly characterized disease, both clinically and etiologically. A significant challenge in its study is the considerable overlap with other conditions, making it exceedingly difficult to differentiate cases of Long COVID from cases exhibiting persistent symptoms due to physical sequelae rather than potential chronic viral infection. By meticulously selecting patients, the objective of this study was to assess aspects of immunity in individuals with Long COVID and compare them to those who had COVID-19 and did not develop sequelae. Our results showed that patients, in addition to having increased expression of TLR4 in B lymphocytes, also presented with a decrease of at least half of the population of CD4⁺ T lymphocytes and an increase in the population of CD8⁺ T lymphocytes. We also found an absolute increase in the population of non-classical monocytes and a significant loss in the expression of ACE2 on the membrane of classical monocytes. Moreover, we characterized an increase in IL-6 and IL-17A and lower values of IL-4, IL-2, IL-1 β , TNF- α , IL-10, and IL-12p70 in patients. Thus, there is an indication consistent with the current literature, showing a possible chronic inflammatory response, with a decrease in CD4⁺ and an increase in non-classical monocytes. This type of response with elevated IL-17A and IL-6 is different from the currently accepted pattern of IL-1 β , IL-6, and TNF- α , indicating that patients with different severities of initial infection may have different cytokine patterns when experiencing sequelae.

Keywords: SARS-CoV-2; long COVID; cytokines; ACE2; TLR4.

Abbreviations

COVID-19 (coronavirus disease 2019)

SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2)

ACE2 (angiotensin-converting enzyme 2)

TLR (toll-like receptor)

1. Introduction

Since the end of 2019, the world has undergone not only the pandemic caused by SARS-CoV-2, the etiological agent of COVID-19, but also its consequences. It is not the first time that a coronavirus (CoV) has caught humans off guard and caused a public health emergency. We had epidemics with SARS-CoV in 2002 and 2003, and Middle East Respiratory Syndrome (MERS-CoV) in 2012.¹⁻²

COVID-19 exhibits symptoms similar to influenza, with the most common being fever, fatigue, and dry cough. It may also present shortness of breath, body aches, sore throat, and runny nose in some cases. Its' transmission occurs through airborne particles of bodily fluids or surfaces contaminated with the presence of SARS-CoV-2.³⁻⁴ Since its onset, it has displayed varying clinical degrees, ranging from asymptomatic and mild to moderate forms, to more severe manifestations leading to fatalities.⁴

Despite the substantial expenditures and significant impact on healthcare systems worldwide due to acute and severe cases, it is equally important to assess the group of individuals who, even after disease remission, continue to experience persistent and significant residual symptoms. This state has been attributed a few different names, such as Long COVID, post-COVID syndrome, or chronic COVID. Two terms more recently used to characterize this group are PCC (post-COVID conditions) and PASC (Post-Acute Sequelae of SARS-CoV-2 infection). Although both are considered more scientific terms, neither fully distinguish between patients with sequelae and those with chronic infections.⁵ The most recognized definition today is that adopted by the WHO, which designates Long COVID as the continuation or development of new symptoms 3 months after the initial SARS-CoV-2 infection, with these symptoms persisting for at least 2 months and lacking other plausible explanations.⁶⁻¹⁷

These symptoms endure for up to 6 months or more after acute infection. While more severe patients tend to experience more complications, over 30% of cases with symptoms lasting beyond 60 days were either asymptomatic during initial infection¹⁶ or

did not require hospitalization.^{18.}

The current main hypotheses for the pathogenesis of this condition indicate multiple potential causes, all possibly overlapping. One hypothesis involves the presence of persistent, fibrinolysis-resistant small clots in blood circulation.^{19-26.} Studies have demonstrated the persistence of inflammation-related biomarkers.^{27-31.} This immune dysregulation³²⁻³⁵ may coincide with the reactivation of underlying conditions, such as Epstein-Barr or Herpes Virus 6 infections.^{36,37.} Other studies have shown the presence of viral reservoirs in the body, with viral proteins and/or RNA still detectable in plasma and other tissues.^{38-46.} Additionally, the impact of SARS-CoV-2 on the intestinal microbiota is considered a potential factor also.^{47-49.}

Studies on immune dysregulation in Long COVID have sought to characterize patterns that may account for the presence of persistent symptoms, examining both cytokine production patterns and the cellular populations and associated receptors.^{50-65.}

Regarding membrane receptors, ACE2 has been extensively studied and is recognized as the primary cellular receptor for SARS-CoV-2, facilitating virus-cell interactions and infection.^{66-68.} The spike glycoprotein's first subunit, S1, binds to ACE2 via the receptor-binding domain (RBD), while the second subunit, S2, binds to serine protease 2 (TMPRSS2).^{69,70} ACE2 is significantly present in type II alveolar cells in the lungs, myocardial cells, and the oral cavity, indicating those as high-risk pathways for infection.^{66,68,71.}

As for the TLR4 receptor, it transitioned from initially a beneficial role to a detrimental one when it was discovered that the spike protein can bind to it, initiating activation of transcription factors NF- κ B, interferon regulatory factor, and activator protein 1 (AP-1).^{73.} This in situ activation of alveolar macrophages is believed to trigger intense local inflammation and subsequent lung tissue destruction.^{74,75.} TLR4 might not only contribute to immediate tissue inflammation but also elevate ACE2 expression on cell membranes, thereby facilitating new virus entry.^{76.}

In Long COVID, a current hypothesis suggests that TLR4 is involved in a feedback loop ($IL-1\beta + IL-6 \rightarrow IL-1R + IL-6R \rightarrow TLR4/mRAGE + S100A8/A9 \rightarrow IL-1\beta + IL-6$) responsible for maintaining an inflammatory response even in the absence of the virus.^{77.} This feedback, driven by $IL-1\beta$ and $IL-6$, has guided therapeutic testing using cytokine inhibitors.^{77.}

A significant challenge in current studies is distinguishing between patients with any form of COVID sequelae and those definitively diagnosed with Long COVID. These two entities are distinct, and without meticulous patient selection, they cannot be easily separated.

Considering this, the present study aims to examine potential differences in

responses between patients exhibiting signs and symptoms of Long COVID and those without such manifestations. A rigorous selection process has been employed, recruiting only patients with the chronic form of COVID, excluding those whose symptoms could be attributed to physical sequelae.

It is imperative to evaluate and understand why certain patients present with this condition. This approach will allow the identification of therapeutic targets, thereby improving the quality of life for affected individuals and mitigating the impact of Long COVID on healthcare systems.

2. Material and Methods

2.1 Recruitment and Selection

Cases were selected in two ways: 1) through an online recruitment form distributed on social media platforms where patients could volunteer for the study; 2) among patients under medical follow-up at the Pulmonary Function Outpatient Clinic at the Hospital das Clínicas of the School of Medicine of Botucatu. The questionnaire aimed to identify patients with mild to moderate COVID-19 cases through online recruitment, while at the outpatient clinic, we aimed to identify severe patients (those who required hospitalization). Recruitment was conducted between January and December 2022.

Patients were invited and interviewed to collect data such as age, sex, date of acute infection, presence or absence of residual symptoms, duration and description of each symptom, number of vaccine doses at the time of infection, number of vaccine doses at the time of the interview, underlying medical conditions, and medications in use.

Patients who, during the interview, had any active infection or exhibited any physical alteration and/or concomitant underlying disease that could account for the presence of any of their symptoms were excluded. Controls were individuals who had COVID-19 and did not exhibit any residual signs or symptoms of the disease. A total of 20 individuals were selected, comprising 10 patients (1 male and 9 females, aged between 24 and 64 years) and 10 controls (4 males and 6 females, aged between 22 and 36 years). All recruits came through the online form since, due to the severity of the patients at the pulmonary function outpatient clinic, they all met one or more exclusion criteria.

The present study was approved by the Research Ethics Committee of the School of Medicine of Botucatu – UNESP (Ethics Approval Number: 5.219.411/2021), and all individuals signed the informed consent form before sample collection.

2.2 *Blood collection and material preparation*

A peripheral blood sample of 10 ml was collected from each individual and subsequently centrifuged for 10 minutes at 1500 rpm for separation. The plasma was then frozen at -80°C and stored for subsequent cytokine analysis. Erythrocytes were lysed with a hypotonic solution of 0.2% NaCl, and cell counting for cell adjustment (1x10⁶ cells/ml) was performed using a Neubauer chamber, with the sample stained with 0.04% Trypan Blue (dilution 1:10).

2.3 *Flow Cytometry Receptor Expression Assessment*

Following cell lysis and counting, the BD Cytotfix/Cytoperm™ Fixation/Permeabilization kit was employed for cellular permeabilization and fixation steps. The selected panel for the B lymphocyte analysis tube consisted of: CD19 – PerCPCy5.5, ACE2 – APC, and TLR4 – PE. The panel for Monocytes and Neutrophils analysis tube included: CD14 – PerCPCy5.5, CD16 – FITC, ACE2 – APC, and TLR4 – PE. Lastly, the panel for the T lymphocytes analysis tube included: CD3 – FITC, CD4 – PE Cy7, CD8 – PerCPCy5.5, ACE2 – APC, and TLR4 – PE.

At the end of each process, cells were washed twice with Wash Buffer diluted 1:10, as per kit instructions, and the final pellet of labeled cells was suspended in PBS with 2% PFA.

Samples were analyzed using a BD LSR Fortessa flow cytometer (Becton Dickinson®, Mountain View, CA, USA) at the Reproduction Laboratory of FMVZ, equipped with excitation lasers: blue 488-nm, 100 mW, and emission filters 530/30nm (FITC), 575/26nm (PE), 695/40nm (PerCPCy5.5), and 780/60 (PE Cy7); red 640-nm, 40 mW with a 660/20nm (APC) filter. Acquisition and analysis were performed using BD FACSDiva™ v6.1 software. Compensation beads (UltraComp eBeads™ Plus) and autofluorescence tubes were used as controls.

2.4 *Cytokines Quantification*

Cytokine quantification was conducted using the LEGENDplex™ Human Essential Immune Response Panel (13-plex) kit, which includes detection for IL-4, IL-2, CXCL10 (IP-10), IL-1β, TNF-α, CCL2 (MCP-1), IL-17A, IL-6, IL-10, IFN-γ, IL12p70,

CXCL8 (IL-8), and TGF- β 1. Plates with V-bottoms were utilized for detection, following the manufacturer's instructions for all steps. The acquisition was performed on a BD FACSCalibur™ flow cytometer at the Applied Biotechnology Laboratory (LBA – Hospital das Clínicas de Botucatu - HCFMB), and analysis was carried out using BioLegend's LEGENDplex™ analysis software, with results expressed in pg/ml.

2.5 Statistical Analysis

Group comparisons were conducted using mean and standard deviation, along with parametric tests including T-test, Poisson distribution, Negative Binomial distribution, and Gamma distribution based on the variance of each group. Data were analyzed using SAS Analytics Software, and graphs were generated using GraphPad Prism 6. Due to statistical age group differences, age was considered as a covariate in all analyses for correction. Statistical significance was considered at $p < 0.05$.

3. Results

3.1 General Characteristics of the Groups

The general characteristics of the groups, such as age, sex, and vaccine doses, are shown in **(Table 1)**.

In addition to these data, information about underlying conditions was collected. In the Long COVID group, two patients had Systemic Arterial Hypertension, one patient had Central Core Myopathy, Malignant Hyperthermia, and Celiac Disease, and one patient had Asthma and Antiphospholipid Antibody Syndrome. In the control group, one patient had systemic arterial hypertension, one patient had asthma, one patient had depressive and obsessive-compulsive disorders, and one patient had generalized anxiety disorder. There was no statistical significance in the comparison of chronic diseases between the groups ($p = 0.3766$).

Regarding medications used, two patients in the symptomatic group used common antihypertensives (combinations of an angiotensin receptor blocker and a thiazide diuretic), and one of them used a beta blocker for improving dysautonomia symptoms. In the control group, one patient used the same combination of antihypertensives, and two patients used controlled medications for mental health.

There was also no statistical significance in the comparison of medications between the groups (p. 0.7212).

The remaining symptoms were described according to **(Figure 1)**.

Table 1

Characteristics of patient and control groups for Long COVID.

	Long COVID (n = 10)	Controls (n = 10)	<i>p value</i>
Age (years)	39,3 (14,79)	27,4 (5,4)	0,004
Sex (women)	9 (90%)	6 (60%)	0,121
Vaccines at infection	1,8 (1,62)	2,6 (0,97)	0,569
Vaccines at interview	3,3 (0,48)	3,2 (0,42)	0,901

Tab.1 Data presented as absolute numbers, percentages (%), or means (standard deviation), as needed. Poisson and Gamma distribution.

Figure 1

Descriptive data on remaining symptoms in patients with Long COVID.

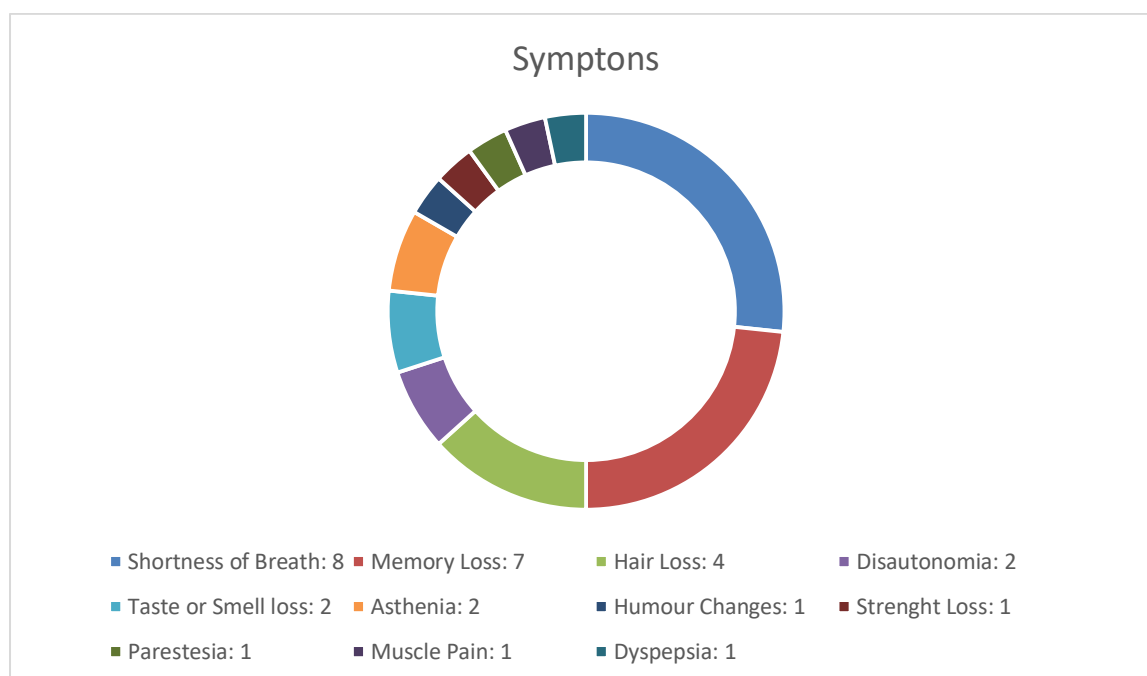


Fig. 1. Data in absolute numbers, showing how many complained of each symptom. (n=10)

3.2 Assessment of Cellular Subpopulations and ACE2 and TLR4 Expression.

ACE2 and TLR4 receptors were analyzed across all cellular subpopulations. The populations were analyzed as percentages of total leukocytes and, in the case of T lymphocytes, as a percentage of CD3+ population. The analysis and comparison of cellular populations and receptor expression are shown in the following figures: B lymphocytes (**Figure 2**), CD4+ T lymphocytes (**Figure 3**), CD8+ T lymphocytes (**Figure 4**), Classical Monocytes (**Figure 5**), Non-Classical Monocytes (**Figure 6**), and Neutrophils (**Figure 7**). On the left are the population and expression percentages, and on the right are the intensities of each receptor.

In the analysis of B lymphocytes (**Figure 2**), a significant increase in the percentage of cells expressing TLR4 was found in the Long COVID patient group. Although a slight increase in the mean fluorescence intensity of ACE2 was also observed in the control patient group, statistical significance was not identified.

Figure 2

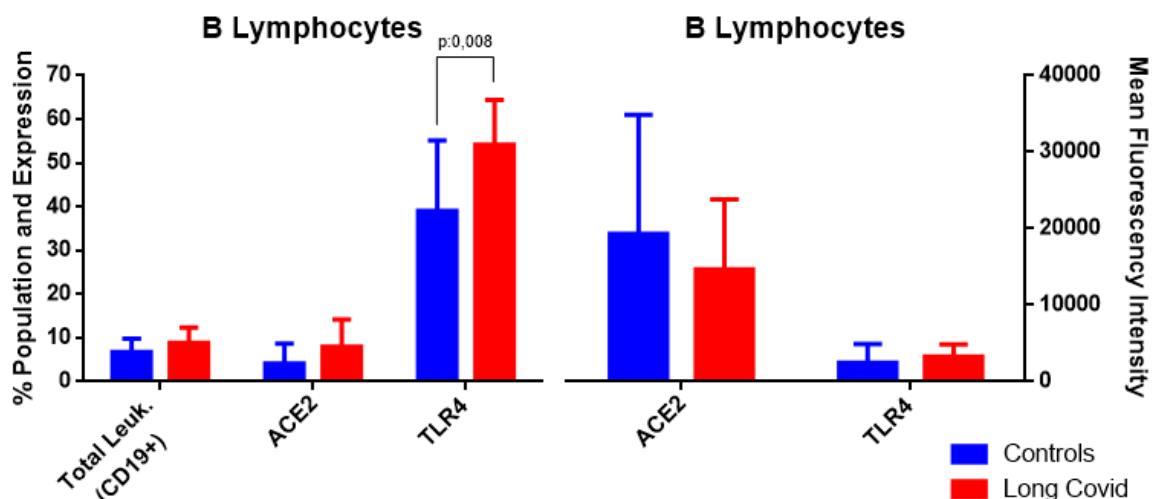


Fig.2. ACE2 and TLR4 expression in the B lymphocyte population. Data presented as percentage and mean fluorescence intensity of 20 tested individuals. Total Leukocytes (p. 0.0882), % ACE2 (p. 0.1496), % TLR4 (p. 0.0087). ACE2 intensity (p. 0.3141) and TLR4 intensity (p. 0.3333). Negative Binomial and Poisson distribution tests.

When evaluating CD4+ T lymphocytes (**Figure 3**), a decrease in this total subpopulation was found in the Long COVID patient group, with no difference in TLR4 expression between the groups. Regarding ACE2, no expression of this receptor was evident in the different groups.

Figure 3

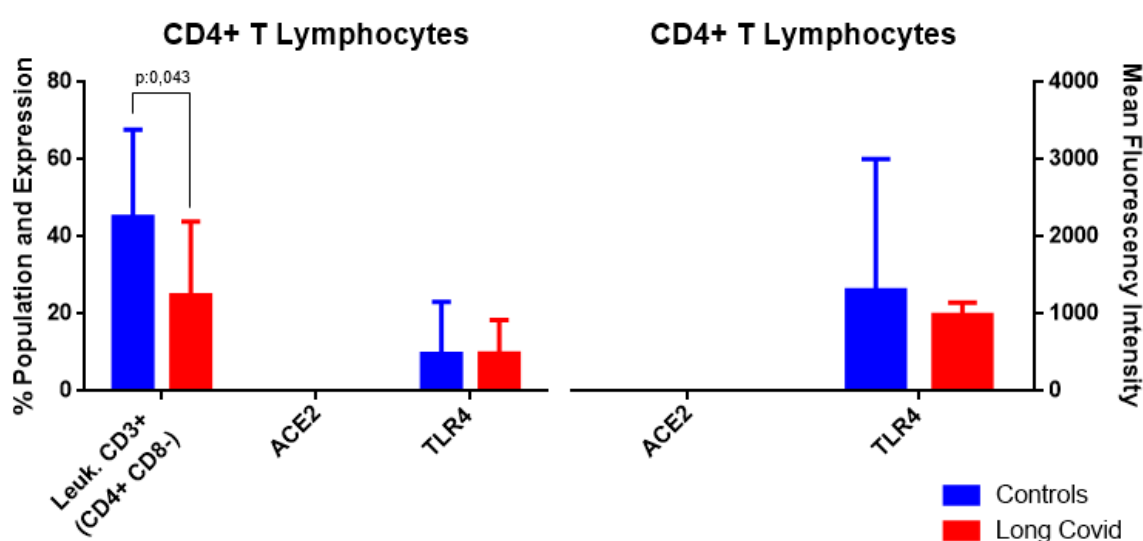


Fig.3. ACE2 and TLR4 expression in the CD4+ T lymphocyte population. Data presented as percentage and mean fluorescence intensity of 20 tested individuals. CD3+ Leukocytes (p. 0.0431), % TLR4 (p. 0.9865). TLR4 intensity (p. 0.2886). Negative Binomial and Poisson distribution tests.

As for CD8+ T lymphocytes (**Figure 4**), an increase in this absolute population was identified in Long COVID patients compared to controls. A small percentage of cells expressing ACE2 was identified in this cell group. However, no differences in the expression of ACE2 and TLR4 were identified in both groups.

Figure 4

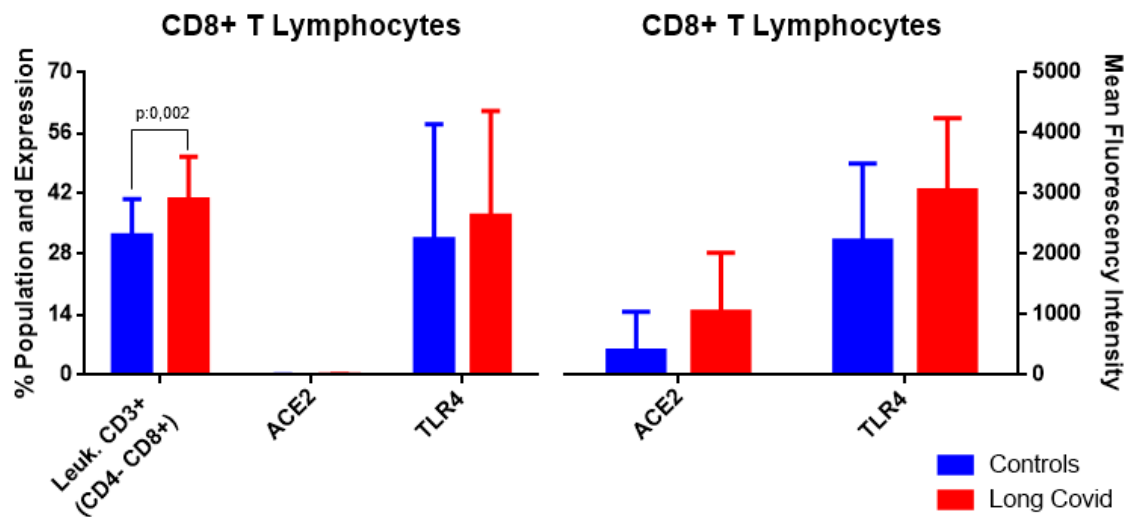


Fig.4. ACE2 and TLR4 expression in the CD8+ T lymphocyte population. Data presented as percentage and mean fluorescence intensity of 20 tested individuals. CD3+ Leukocytes (p. 0.0021), % ACE2 (p. 0.6023), % TLR4 (p. 0.6159). ACE2 intensity (p. 0.5394) and TLR4 intensity (p. 0.1008). Negative Binomial and Poisson distribution tests.

In the classical monocyte population (Figure 5), it was possible to characterize that the ACE2 expression intensity was higher in the control group. No differences were found in other comparisons related to TLR4.

Figure 5

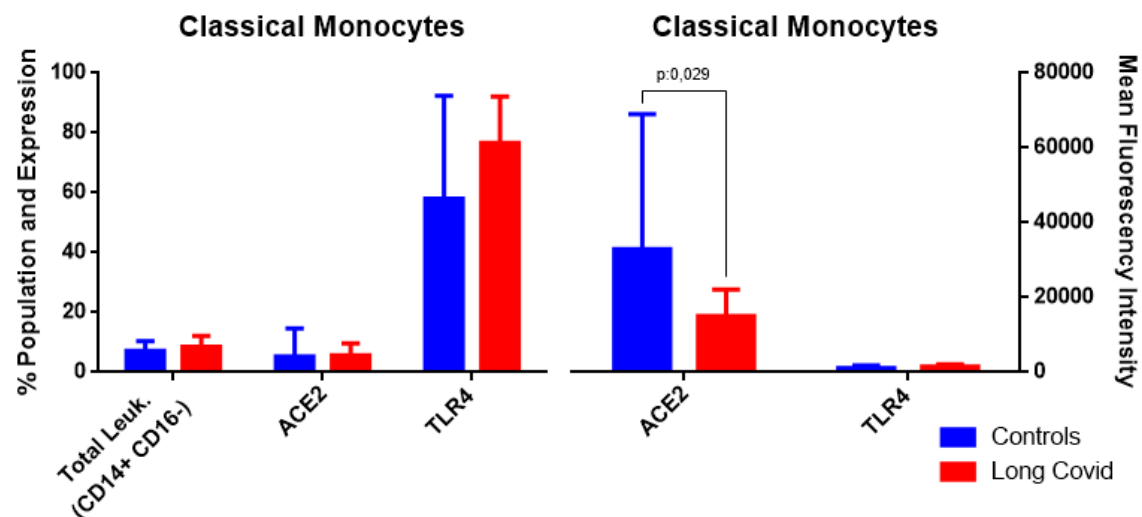


Fig.5. ACE2 and TLR4 expression in the Classical Monocyte population. Data presented as percentage and mean fluorescence intensity of 20 tested individuals. Total Leukocytes (p. 0.2867), % ACE2 (p. 0.9159), % TLR4 (p. 0.2565). ACE2 intensity (p. 0.0299) and TLR4 intensity (p. 0.1798). Negative Binomial and Poisson distribution tests.

Regarding non-classical monocytes (**Figure 6**), when evaluating total leukocytes, an increase in their proportion was characterized in the Long COVID patient group. When comparing the transition from classical to non-classical monocytes, a difference in ACE2 expression was found, showing a decrease in the % of cells capable of its

expression ($p < 0.001$) but without changes in its intensity ($p = 0.1025$). As for TLR4 expression, statistical significance was found, comparing both the % of expression ($p = 0.0104$) and intensity ($p = 0.0002$), showing an increase in non-classical monocytes for both analyses.

Figure 6

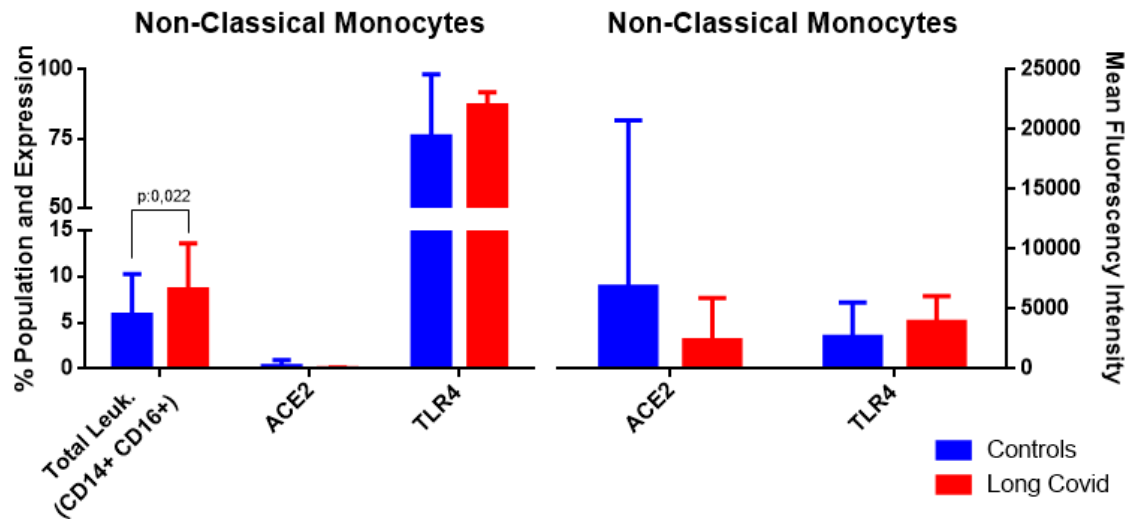


Fig.6. ACE2 and TLR4 expression in the Non-Classical Monocyte population. Data presented as percentage and mean fluorescence intensity of 20 tested individuals. Total Leukocytes ($p = 0.022$), % ACE2 ($p = 0.2666$), % TLR4 ($p = 0.1645$). ACE2 intensity ($p = 0.5843$) and TLR4 intensity ($p = 0.2343$). Negative Binomial and Poisson distribution tests.

Regarding neutrophils (**Figure 7**), similar to CD4+ T lymphocytes, we were unable to characterize the expression of ACE2 in either of the groups. In the TLR4 expression analysis, we also did not identify differences between the groups.

Figure 7

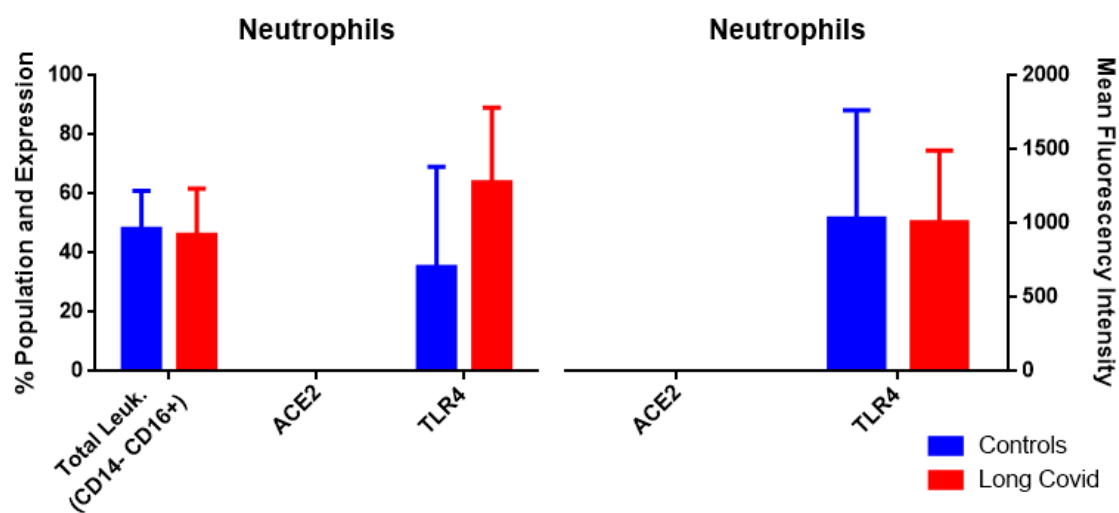


Fig.7. ACE2 and TLR4 expression in the Neutrophil population. Data presented as percentage and mean fluorescence intensity of 20 tested individuals. Total Leukocytes (p. 0.49), % TLR4 (p. 0.1267). TLR4 intensity (p. 0.9035). Negative Binomial and Poisson distribution tests.

3.3 Cytokines quantification

All cytokines (IL-4, IL-2, CXCL10 (IP-10), IL-1 β , TNF- α , CCL2 (MCP-1), IL-17A, IL-6, IL-10, IFN- γ , IL12p70, CXCL8 (IL-8), and TGF- β 1) were measured in both groups. They were divided into 3 groups for better visualization: cytokine levels higher in patients (**Figure 8**), cytokine levels higher in controls (**Figure 9**), and cytokines levels without differences between the two groups (**Figure 10**). Data are presented in concentration, with all results in pg/ml.

It was identified that in Long Covid patients, the levels of IL-6 and IL-17A were elevated when compared with control group (**Figure 8**).

Figure 8

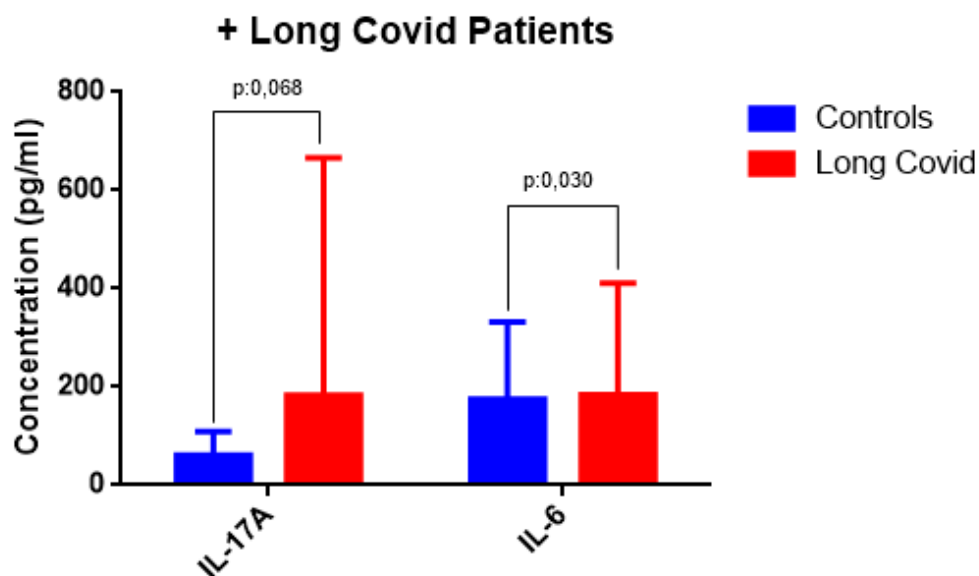


Fig.8. IL-17A and IL-6 concentrations. Data presented as means and standard deviations of values measured in 10 tested individuals on each group. IL-17A (p. 0.068) and IL-6 (p. 0.030). Gamma distribution test.

However, the levels of IL-4, IL-2, IL-1 β , TNF- α , IL-10, and IL-12p70 were decreased in Long Covid patient when compared with control groups (**Figure 9**).

Figure 9

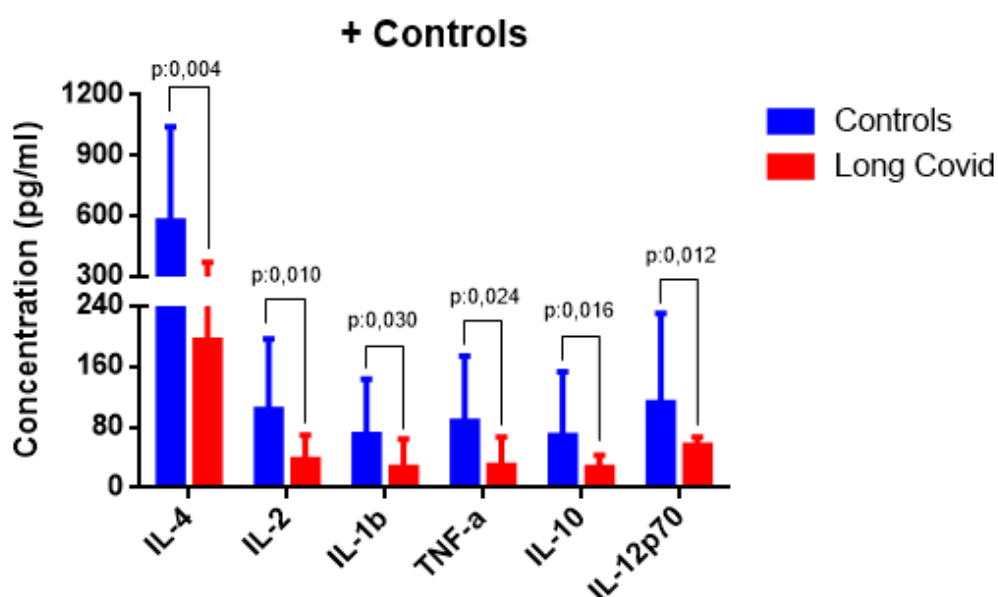


Fig.9. IL-4, IL-2, IL-1 β , TNF- α , IL-10, and IL-12p70 concentrations. Data presented as means and standard deviations of values measured in 10 tested individuals on each group. IL-4 (p. 0.004), IL-2 (p. 0.010), IL-1 β (p. 0.030), TNF- α (p. 0.024), IL-10 (p. 0.016), and IL-12p70 (p. 0.012). Gamma distribution test.

Lastly, it was not identified statistical difference regarding IP-10, MCP-1, IFN- γ , IL-8, and TGF- β 1 levels, between groups (**Figure 10**).

Figure 10

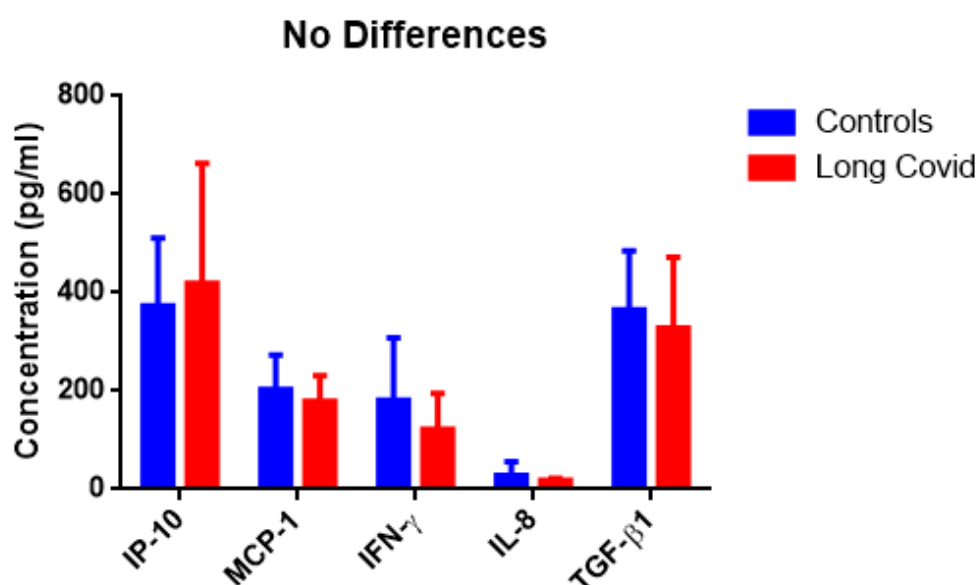


Fig.10. IP-10, MCP-1, IFN- γ , IL-8, and TGF- β 1 concentrations. Data presented as means and standard deviations of values measured in 10 tested individuals on each group. IP-10 (p. 0.4909), MCP-1 (p. 0.3525), IFN- γ (p. 0.1179), IL-8 (p. 0.0627), and TGF- β 1 (p. 0.4372). T-test and Gamma distribution test.

4. Discussion

ACE2 has been acknowledged as the primary binding receptor for the majority of coronaviruses since 2003^{78,79}. Its substantial presence in the upper respiratory epithelium⁸⁰ facilitates viral entry, allowing for a high viral replication rate and significant transmissibility during the early stages of COVID-19 infection (pre-symptomatic phase). Conversely, in the lower respiratory tract, the receptor is prevalent in type II alveolar cells, contributing to heightened tissue damage during the mid and late phases of infection.^{81,82} Although the relationship of this receptor in lung tissue is well understood, only a few studies have evaluated its expression in leukocytes, yielding conflicting outcomes. One study indicated that immune cells, including those in the spleen, thymus, lymph nodes, and bone marrow, do not express ACE2.⁸³ Nonetheless, some studies have demonstrated ACE2 expression in select cell subtypes.^{71,84,85} In our study, ACE2 expression was not detected in CD4+ T lymphocytes and neutrophils, and we believe these differences might be linked to the isolation site of these cells. While Li et al.'s study (2020)⁸⁴ isolated neutrophils from lung tissue, while our study isolated cells from the intravascular compartment. This variance in expression between tissue and vasculature suggests that ACE2 expression capability in certain immune cells is acquired post-migration and differentiation, which occurs after leaving their tissue of origin. Whether they start expressing ACE2 after migrating within vessels or if they hijack this receptor from endothelial cells during diapedesis could be a target for future research, given the demonstrated membrane fusion, especially during transcellular diapedesis.⁸⁶

Shen et al. (2022)⁸⁷ demonstrated that virus entry into T lymphocytes can occur independently of ACE2, and the entry route in these cells involves lymphocyte function-associated antigen 1 (LFA-1). This aligns with our findings of nearly complete absence of ACE2 expression in the lymphocyte population, with low expression observed only in the CD8+ T lymphocyte subset.

Regarding the findings of higher ACE2 expression in the non-classical monocyte group of control patients, two main hypotheses emerge. Firstly, the substantial ACE2 levels in control cells could assist in converting angiotensin 2 to angiotensin 1-7, potentially serving as an innate characteristic functioning as a protective factor against long COVID development. This protective mechanism of high ACE2 expression has already been demonstrated in acute COVID, and its low expression is associated with poor outcomes in the elderly and with comorbidities patients.^{88,89} Alternatively, chronic infection could reduce receptor levels due to internalization and consumption during viral cell entry. Both hypotheses are not

mutually exclusive and might collectively contribute to the observed outcome.

The currently accepted immune alterations in long COVID are closely linked to TLR4. Fontes et al. (2023)⁹⁰ demonstrated that cognitive deficit symptoms similar to long COVID could be neutralized in murine models by blocking TLR4. During initial infection, TLR4 also plays a crucial role, as cytokines associated with severe cases (IL-6 and TNF- α) are triggered through this receptor⁹¹. More recently, Holms et al. (2022)⁷⁷ suggested a potential feedback loop involving TLR4, which could sustain the inflammatory state in long COVID. Apart from inflammatory dysregulation, TLR4 present in B lymphocytes would be directly responsible for antibody production pathways⁹².

Our findings of increased TLR4 in B lymphocytes of patients bridge these concepts, suggesting that individuals with higher TLR4 expression might be susceptible to chronic COVID due to cytokine imbalance and possible elevated production of autoantibodies, contributing to certain symptoms in the clinical presentation. It could also contribute to the increase in cases of autoimmune diseases identified in these groups.

According to Sachinidis et al. (2021)⁶³ and Klein et al. (2022)³³, with our observed results of low CD4+ T lymphocytes, high CD8+ T lymphocyte numbers, and increased non-classical monocytes, we can readily support the hypotheses that long COVID patients are chronically inflamed and responding to possible viral reservoirs in the body.

Regarding cytokines, the increase in IL-6 found in this study has been extensively documented in the literature⁹³⁻⁹⁵. IL-6 elevation has been noted in the acute phase and persists for up to 7 months after initial infection⁹⁵. The association of increased IL-6 with elevated TLR-4 supports the feedback loop hypothesis sustaining inflammation, although statistical significance was not found for IL-1 β .

IL-17A has also been studied predominantly during the acute phase. The hypothesis of IL-17A's influence due to its potent inflammatory capacity has been postulated⁹⁶. Studies have shown a link between elevated IL-17A levels in the acute phase⁹⁷ and the development of pulmonary chronic symptoms⁹⁸. The substantial increase found in our study, coupled with increased TLR4 in B lymphocytes, suggests that the mechanism of autoantibody production might be responsible not only for the respiratory profile but also for the neurological profile of the disease, given that the number of patients with respiratory complaints was lower than those with neurological complaints.

Although no difference between neutrophil population was detected, the higher IL-17A levels also could induce more activation of these cells, which could result

in greater release of Neutrophil Extracellular Traps (NETs), since these structures are also involved with the pathogenesis of COVID⁹⁹. It has been shown that NETs may also have a role in the development of post COVID-19 syndromes, including immunotrombosis, neurological disorders, lung fibrosis and tumor growth⁹⁹. Notably, recent studies showed that NETs are persistent and may be involved in post COVID-19 syndrome pathology¹⁰⁰⁻¹⁰².

The decrease of IL-4 and IL-10 in long COVID patients further strengthens studies demonstrating similar results. Currently, conflicting results exist in the literature, with some showing increased levels of these cytokines in long COVID patients⁹⁷ and others indicating lower values⁹⁵. This finding suggests that the rigorous patient selection performed in our study may influence the pattern of these results. The decrease in these cytokines levels could impact the inflammation control, contributing to the chronic inflammatory response maintenance.

Regarding lower levels of IL-2, IL-1 β , IL-12 and TNF- α in Long COVID subjects, our results are discordant, since it was shown that the triad IL-1 β , IL-6 and TNF- α remain elevated in Long COVID – PASC¹⁰³. Our main hypothesis is that, because of our rigorous selection, all Long COVID patients had mild to moderate acute COVID cases and not a single severe patient was tested because of their confounding factors. This shows that possibly mild/moderate and severe acute cases have different long COVID cytokine profiles, explaining why a single drug is not being helpful in all cases. Generally, higher levels of these cytokines have been identified in long COVID patients compared to healthy individuals or recovered patients⁹⁸⁻⁹⁹. Another hypothesis to explain these results has been previously presented in studies of myalgic encephalopathy¹⁰⁴. These studies indicated potential differences in cytokine levels between tissues and serum due to the localized effect of cytokines. These cytokines, and IL-12p70, might also be subject to value alterations due to their kinetics¹⁰⁵. Phetsouphanh et al. (2022)¹⁰⁶ demonstrated entirely different profiles of these patterns in post-COVID patients at 4 and 8 months after initial infection.

It is imperative for more cohort studies to be conducted, employing rigorous patient selection criteria, that not only associate different clinical profiles of these patients with their laboratory profiles but also demonstrate the changes in these cytokines over time. Evidently, more than one clinical entity may exist within Long COVID, necessitating an individualized approach for different patient groups based on the duration of evolution and their persistent symptoms.

5. Conclusion

In summary, our data strongly suggest the persistence of a pro-inflammatory state in patients with persistent symptoms following the acute episode of COVID. The currently accepted triple pattern (IL-6, TNF-alpha, and IL-1 β) may not be the sole possible pattern among these patients, as our data also indicate IL-17A production + TLR4 higher expression in B lymphocytes, suggesting the possible relation to autoantibody production and neutrophil mediated inflammation.

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References

1. Cascella, M. et al. Features, Evaluation and Treatment Coronavirus (COVID-19). In: StatPearls. Treasure Island (FL): StatPearls Publishing, 2020
2. Li G., Fan Y., Lai Y., Han T., Li Z., Zhou P. et al. Coronavirus infections and immune responses. *J Med Virol.* 2020 Apr; 92(4):424-432.
3. World Health Organization. Coronavirus. Disponível em: <https://www.who.int/health-topics/coronavirus>. Access: 08/30/2023.
4. Hu, Y.; Deng, H.; Huang, L.; Xia, L.; Zhou, X. Analysis of Characteristics in Death Patients with COVID-19 Pneumonia without Underlying Diseases. *Acad Radiol.* 2020 Apr 6. pii: S1076-6332(20)30164-1. doi: 10.1016/j.acra.2020.03.023.0
5. Proal AD, VanElzakker MB. Long COVID or Post-acute Sequelae of COVID-19 (PASC): An Overview of Biological Factors That May Contribute to Persistent Symptoms. *Front Microbiol.* 2021 Jun 23;12:698169. doi: 10.3389/fmicb.2021.698169. PMID: 34248921; PMCID: PMC8260991.
6. World Health Organization. Post COVID-19 condition (Long COVID). Disponível em: <https://www.who.int/europe/news-room/fact-sheets/item/post-covid-19-condition>
7. Lopez-Leon S, Wegman-Ostrosky T, Perelman C, Sepulveda R, Rebolledo PA, Cuapio A, Villapol S. More than 50 long-term effects of COVID-19: a systematic review and meta-analysis. *Sci Rep.* 2021 Aug 9;11(1):16144. doi: 10.1038/s41598-021-95565-8. PMID: 34373540; PMCID: PMC8352980.
8. Silva Andrade B, Siqueira S, de Assis Soares WR, de Souza Rangel F, Santos NO, Dos Santos Freitas A, Ribeiro da Silveira P, Tiwari S, Alzahrani KJ, Góes-Neto A, Azevedo V, Ghosh P, Barh D. Long-COVID and Post-COVID Health Complications: An Up-to-Date Review on Clinical Conditions and Their Possible Molecular Mechanisms. *Viruses.* 2021 Apr 18;13(4):700. doi: 10.3390/v13040700. PMID: 33919537; PMCID: PMC8072585.
9. Carfi A, Bernabei R, Landi F; Gemelli Against COVID-19 Post-Acute Care Study Group. Persistent Symptoms in Patients After Acute COVID-19. *JAMA.* 2020;324(6):603-605. doi:10.1001/jama.2020.12603
10. Rubin R. As Their Numbers Grow, COVID-19 "Long Haulers" Stump Experts. *JAMA.* 2020 Oct 13;324(14):1381-1383. doi: 10.1001/jama.2020.17709. PMID: 32965460.
11. Baig AM. Chronic COVID syndrome: Need for an appropriate medical terminology for long-COVID and COVID long-haulers. *J Med Virol.* 2021 May;93(5):2555-2556. doi: 10.1002/jmv.26624. Epub 2021 Mar 1. PMID: 33095459.
12. Wostyn P. COVID-19 and chronic fatigue syndrome: Is the worst yet to come? *Med Hypotheses.* 2021 Jan;146:110469. doi: 10.1016/j.mehy.2020.110469. Epub 2021 Jan 2. PMID: 33401106; PMCID: PMC7836544.
13. Mohabbat AB, Mohabbat NML, Wight EC. Fibromyalgia and Chronic Fatigue Syndrome in the Age of COVID-19. *Mayo Clin Proc Innov Qual Outcomes.* 2020

- Dec;4(6):764-766. doi: 10.1016/j.mayocpiqo.2020.08.002. Epub 2020 Nov 13. PMID: 33204998; PMCID: PMC7661943.
14. Goldstein DS. The possible association between COVID-19 and postural tachycardia syndrome. *Heart Rhythm*. 2021 Apr;18(4):508-509. doi:10.1016/j.hrthm.2020.12.007. Epub 2020 Dec 11. PMID: 33316414; PMCID: PMC7729277.
 15. Afrin LB, Weinstock LB, Molderings GJ. Covid-19 hyperinflammation and post-Covid-19 illness may be rooted in mast cell activation syndrome. *Int J Infect Dis*. 2020 Nov;100:327-332. doi: 10.1016/j.ijid.2020.09.016. Epub 2020 Sep 10. PMID: 32920235; PMCID: PMC7529115.
 16. Huang C, Huang L, Wang Y, Li X, Ren L, Gu X, Kang L, Guo L, Liu M, Zhou X, Luo J, Huang Z, Tu S, Zhao Y, Chen L, Xu D, Li Y, Li C, Peng L, Li Y, Xie W, Cui D, Shang L, Fan G, Xu J, Wang G, Wang Y, Zhong J, Wang C, Wang J, Zhang D, Cao B. 6-month consequences of COVID-19 in patients discharged from hospital: a cohort study. *Lancet*. 2021 Jan 16;397(10270):220-232. doi: 10.1016/S0140-6736(20)32656-8. Epub 2021 Jan 8. PMID: 33428867; PMCID: PMC7833295.
 17. Michelen M, Manoharan L, Elkheir N, et al. Characterising long COVID: a living systematic review. *BMJ Glob Health*. 2021;6(9):e005427. doi:10.1136/bmjgh-2021-005427
 18. Tabacof L, Tosto-Mancuso J, Wood J, Cortes M, Kontorovich A, McCarthy D, Rizk D, Mohammadi N, Breyman E, Nasr L, Kellner C, Putrino D, Post-acute COVID-19 syndrome negatively impacts health and wellbeing despite less severe acute infection. doi: <https://doi.org/10.1101/2020.11.04.20226126>
 19. Pretorius E, Venter C, Laubscher GJ, Lourens PJ, Steenkamp J, Kell DB. Prevalence of readily detected amyloid blood clots in 'unclothed' Type 2 Diabetes Mellitus and COVID-19 plasma: a preliminary report. *Cardiovasc Diabetol*. 2020 Nov 17;19(1):193. doi: 10.1186/s12933-020-01165-7. PMID: 33203441; PMCID: PMC7670290.
 20. Venter C, Bezuidenhout JA, Laubscher GJ, Lourens PJ, Steenkamp J, Kell DB, Pretorius E. Erythrocyte, Platelet, Serum Ferritin, and P-Selectin Pathophysiology Implicated in Severe Hypercoagulation and Vascular Complications in COVID-19. *Int J Mol Sci*. 2020 Nov 3;21(21):8234. doi: 10.3390/ijms21218234. PMID: 33153161; PMCID: PMC7662625.
 21. Schaller J, Gerber SS. The plasmin-antiplasmin system: structural and functional aspects. *Cell Mol Life Sci*. 2011 Mar;68(5):785-801. doi: 10.1007/s00018-010-0566-5. Epub 2010 Dec 7. PMID: 21136135.
 22. Miszta A, Huskens D, Donkervoort D, Roberts MJM, Wolberg AS, de Laat B. Assessing Plasmin Generation in Health and Disease. *Int J Mol Sci*. 2021 Mar 9;22(5):2758. doi: 10.3390/ijms22052758. PMID: 33803235; PMCID: PMC7963172.
 23. Pretorius E, Vlok M, Venter C, et al. Persistent clotting protein pathology in Long COVID/Post-Acute Sequelae of COVID-19 (PASC) is accompanied by increased levels of antiplasmin. *Cardiovasc Diabetol*. 2021;20(1):172. Published 2021 Aug 23. doi:10.1186/s12933-021-01359-7

24. Haffke, M. et al. Endothelial dysfunction and altered endothelial biomarkers in patients with post-COVID-19 syndrome and chronic fatigue syndrome (ME/CFS). *J. Transl Med.* 20, 138 (2022).
25. Charfeddine, S. Long COVID 19 syndrome: is it related to microcirculation and endothelial dysfunction? Insights from TUN-EndCOV study. *Front. Cardiovasc. Med.* <https://doi.org/10.3389/fcvm.2021.745758> (2021).
26. Pretorius, E. et al. Prevalence of symptoms, comorbidities, fibrin amyloid microclots and platelet pathology in individuals with Long COVID/post-acute sequelae of COVID-19 (PASC). *Cardiovasc. Diabetol.* 21, 148 (2022).
27. Doykov I, Hällqvist J, Gilmour KC, Grandjean L, Mills K, Heywood WE. 'The long tail of Covid-19' - The detection of a prolonged inflammatory response after a SARS-CoV-2 infection in asymptomatic and mildly affected patients. *F1000Res.* 2020 Nov 19;9:1349. doi: 10.12688/f1000research.27287.2. PMID: 33391730; PMCID: PMC7745182.
28. Watari K, Shibata T, Nabeshima H, Shinoda A, Fukunaga Y, Kawahara A, Karasuyama K, Fukushi J, Iwamoto Y, Kuwano M, Ono M. Impaired differentiation of macrophage lineage cells attenuates bone remodeling and inflammatory angiogenesis in *Ndr1* deficient mice. *Sci Rep.* 2016 Jan 18;6:19470. doi: 10.1038/srep19470. PMID: 26778110; PMCID: PMC4726041.
29. Taketomi Y, Sunaga K, Tanaka S, Nakamura M, Arata S, Okuda T, Moon TC, Chang HW, Sugimoto Y, Kokame K, Miyata T, Murakami M, Kudo I. Impaired mast cell maturation and degranulation and attenuated allergic responses in *Ndr1*-deficient mice. *J Immunol.* 2007 Jun 1;178(11):7042-53. doi: 10.4049/jimmunol.178.11.7042. PMID: 17513753.
30. Xu Y, Schnorrer P, Proietto A, Kowalski G, Febbraio MA, Acha-Orbea H, Dickins RA, Villadangos JA. IL-10 controls cystatin C synthesis and blood concentration in response to inflammation through regulation of IFN regulatory factor 8 expression. *J Immunol.* 2011 Mar 15;186(6):3666-73. doi: 10.4049/jimmunol.1001934. Epub 2011 Feb 7. PMID: 21300820.
31. Paushter DH, Du H, Feng T, Hu F. The lysosomal function of progranulin, a guardian against neurodegeneration. *Acta Neuropathol.* 2018 Jul;136(1):1-17. doi: 10.1007/s00401-018-1861-8. Epub 2018 May 9. PMID: 29744576; PMCID: PMC6117207.
32. Proal, A. D. & VanElzakker, M. B. Long COVID or post-acute sequelae of COVID-19 (PASC): an overview of biological factors that may contribute to persistent symptoms. *Front. Microbiol.* 12, 698169 (2021).
33. Klein, J. et al. Distinguishing features of Long COVID identified through immune profiling. Preprint at medRxiv <https://doi.org/10.1101/2022.08.09.22278592> (2022).
34. Glynn, P., Tahmasebi, N., Gant, V. & Gupta, R. Long COVID following mild SARS-CoV-2 infection: characteristic T cell alterations and response to antihistamines. *J. Investig. Med.* 70, 61–67 (2022).
35. Phetsouphanh, C. et al. Immunological dysfunction persists for 8 months following initial mild-to-moderate SARS-CoV-2 infection. *Nat. Immunol.* 23, 210–216 (2022).

36. Zubchenko, S., Kril, I., Nadizhko, O., Matsyura, O. & Chopyak, V. Herpesvirus infections and post-COVID-19 manifestations: a pilot observational study. *Rheumatol. Int.* <https://doi.org/10.1007/s00296-022-05146-9> (2022).
37. Peluso, M. J. et al. Evidence of recent Epstein-Barr virus reactivation in individuals experiencing Long COVID. Preprint at medRxiv <https://doi.org/10.1101/2022.06.21.22276660> (2022).
38. Zoe Swank, Yasmeen Senussi, Zachary Manickas-Hill, Xu G Yu, Jonathan Z Li, Galit Alter, David R Walt, Persistent Circulating Severe Acute Respiratory Syndrome Coronavirus 2 Spike Is Associated With Post-acute Coronavirus Disease 2019 Sequelae, *Clinical Infectious Diseases*, Volume 76, Issue 3, 1 February 2023, Pages e487–e490, <https://doi.org/10.1093/cid/ciac722>
39. Chertow D, Stein S, Ramelli A, et al. SARS-CoV-2 infection and persistence throughout the human body and brain. Published online December 20, 2021. <https://doi.org/10.21203/rs.3.rs-1139035/v1>
40. Tejerina, F. et al. Post-COVID-19 syndrome. SARS-CoV-2 RNA detection in plasma, stool, and urine in patients with persistent symptoms after COVID-19. *BMC Infect. Dis.* 22, 211 (2022).
41. Goh, D. et al. Persistence of residual SARS-CoV-2 viral antigen and RNA in tissues of patients with long COVID-19. Preprint at <https://www.researchsquare.com/article/rs-1379777/v1> (2022).
42. Ceulemans, L. J. et al. Persistence of SARS-CoV-2 RNA in lung tissue after mild COVID-19. *Lancet Respir. Med.* 9, e78–e79 (2021).
43. Menuchin-Lasowski, Y. et al. SARS-CoV-2 infects and replicates in photoreceptor and retinal ganglion cells of human retinal organoids. *Stem Cell Rep* 17, 789–803 (2022).
44. Cheung, C. C. L. et al. Residual SARS-CoV-2 viral antigens detected in GI and hepatic tissues from five recovered patients with COVID-19. *Gut* 71, 226–229 (2022).
45. Natarajan, A. et al. Gastrointestinal symptoms and fecal shedding of SARS-CoV-2 RNA suggest prolonged gastrointestinal infection. *Med* 3, 371–387.e9 (2022).
46. Swank, Z. et al. Persistent circulating severe acute respiratory syndrome coronavirus 2 spike is associated with post-acute coronavirus disease 2019 sequelae. *Clin. Infect. Dis.* <https://doi.org/10.1093/cid/ciac722> (2022).
47. Liu, Q. et al. Gut microbiota dynamics in a prospective cohort of patients with post-acute COVID-19 syndrome. *Gut* 71, 544–552 (2022).
48. Yeoh, Y. K. et al. Gut microbiota composition reflects disease severity and dysfunctional immune responses in patients with COVID-19. *Gut* 70, 698–706 (2021).
49. Mendes de Almeida, V. Gut microbiota from patients with mild COVID-19 cause alterations in mice that resemble post-COVID syndrome. *Res. Sq.* <https://doi.org/10.21203/rs.3.rs-1756189/v1> (2022).
50. Augustin, M. et al. Post-COVID syndrome in non-hospitalised patients with COVID-19: a longitudinal prospective cohort study. *Lancet Reg. Health Eur.* 6, 100122 (2021).

51. García-Abellán, J. et al. Antibody response to SARS-CoV-2 is associated with long-term clinical outcome in patients with COVID-19: a longitudinal study. *J. Clin. Immunol.* 41, 1490–1501 (2021).
52. Su, Y. et al. Multiple early factors anticipate post-acute COVID-19 sequelae. *Cell* 185, 881–895.e20 (2022).
53. Bowe, B., Xie, Y. & Al-Aly, Z. Acute and postacute sequelae associated with SARS-CoV-2 reinfection. *Nat. Med.* <https://doi.org/10.1038/s41591-022-02051-3> (2022).
54. Blomberg, J., Gottfries, C.-G., Elfaitouri, A., Rizwan, M. & Rosén, A. Infection elicited autoimmunity and myalgic encephalomyelitis/chronic fatigue syndrome: an explanatory model. *Front. Immunol.* 9, 229 (2018).
55. Wang, E. Y. et al. Diverse functional autoantibodies in patients with COVID-19. *Nature* 595, 283–288 (2021).
56. Arthur, J. M. et al. Development of ACE2 autoantibodies after SARS-CoV-2 infection. *PLoS ONE* 16, e0257016 (2021).
57. Wallukat, G. et al. Functional autoantibodies against G-protein coupled receptors in patients with persistent long-COVID-19 symptoms. *J. Transl Autoimmun.* 4, 100100 (2021).
58. Peluso, M. J. et al. Markers of immune activation and inflammation in individuals with postacute sequelae of severe acute respiratory syndrome coronavirus 2 infection. *J. Infect. Dis.* 224, 1839–1848 (2021).
59. Schultheiß, C. et al. From online data collection to identification of disease mechanisms: the IL-1 β , IL-6 and TNF- α cytokine triad is associated with post-acute sequelae of COVID-19 in a digital research cohort. SSRN <https://doi.org/10.2139/ssrn.3963839> (2021).
60. Teixeira AL, Gama CS, Rocha NP and Teixeira MM (2018) Revisiting the Role of Eotaxin-1/CCL11 in Psychiatric Disorders. *Front. Psychiatry* 9:241. doi: 10.3389/fpsy.2018.00241
61. Fernández-Castañeda, A. et al. Mild respiratory SARS-CoV-2 infection can cause multi-lineage cellular dysregulation and myelin loss in the brain. Preprint at bioRxiv <https://doi.org/10.1101/2022.01.07.475453> (2022).
62. Phetsouphanh, C. et al. Immunological dysfunction persists for 8 months following initial mild-to-moderate SARS-CoV-2 infection. *Nat. Immunol.* 23, 210–216 (2022).
63. Sachinidis A, Garyfallos A. Double Negative (DN) B cells: A connecting bridge between rheumatic diseases and COVID-19? *Mediterr J Rheumatol.* 2021 Sep 30;32(3):192-199. doi: 10.31138/mjr.32.3.192. PMID: 34964023; PMCID: PMC8693305.
64. Hornig, M. et al. Distinct plasma immune signatures in ME/CFS are present early in the course of illness. *Sci. Adv.* 1, e1400121 (2015).
65. Wiech M, Chroscicki P, Swatler J, Stepnik D, De Biasi S, Hampel M, Brewinska-Olchowik M, Maliszewska A, Sklinda K, Durlik M, Wierzba W, Cossarizza A and Piwocka

- K (2022) Remodeling of T Cell Dynamics During Long COVID Is Dependent on Severity of SARS-CoV-2 Infection. *Front. Immunol.* 13:886431. doi: 10.3389/fimmu.2022.886431
66. Hoffmann, M.; Kleine-Weber, H.; Schroeder, S.; Krüger, N.; Herrler, T.; Erichsen, S. et al. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* (2020).
67. Zhou, P., Yang, X. L., Wang, X. G., Hu, B., Zhang, L., Zhang, W., Si, H. R., Zhu, Y., Li, B., Huang, C. L., Chen, H. D., Chen, J., Luo, Y., Guo, H., Jiang, R. D., Liu, M. Q., Chen, Y., Shen, X. R., Wang, X., Zheng, X. S., ... Shi, Z. L. (2020). A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature*, 579(7798), 270–273. <https://doi.org/10.1038/s41586-020-2012-7>.
68. Yu, Z., Zixian, Z., Yujia, W., Yueqing, Z., Yu, M., Wei, Z.. Single-cell RNA expression profiling of ACE2, the putative receptor of Wuhan 2019-nCoV. *bioRxiv* 2020.01.26.919985
69. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, et al. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell*. Março de 2020;181(2):271–80.e8.
70. Hoffmann M, Kleine-Weber H, Krüger N, Müller M, Drosten C, Pöhlmann S. The novel coronavirus 2019 (2019-nCoV) uses the SARS-coronavirus receptor ACE2 and the cellular protease TMPRSS2 for entry into target cell. *Europe PMC*. 2020.
71. Xu, H., Zhong, L., Deng, J., Peng, J., Dan, H., Zeng, X., Li, T., & Chen, Q. (2020). High expression of ACE2 receptor of 2019-nCoV on the epithelial cells of oral mucosa. *International journal of oral science*, 12(1), 8.
72. Choudhury A, Mukherjee S. In silico studies on the comparative characterization of the interactions of SARS-CoV-2 spike glycoprotein with ACE-2 receptor homologs and human TLRs. *J. Med. Virol.* 92(10), 2105–2113 (2020).
73. Zhao Y, Kuang M, Li J et al. SARS-CoV-2 spike protein interacts with and activates TLR4. *Cell Res.* 31(7), 818–820 (2021).
74. Choudhury A, Das NC, Patra R, Mukherjee S. In silico analyses on the comparative sensing of SARS-CoV-2 mRNA by the intracellular TLRs of humans. *J. Med. Virol.* 93(4), 2476–2486 (2021).
75. Mukherjee, "Toll-like receptor 4 in COVID-19: friend or foe?" *Future Virology* 10.2217/fvl-2021-0249 doi: 10.2217/fvl-2021-0249
76. Mohamed M. Aboudounya, Richard J. Heads, "COVID-19 and Toll-Like Receptor 4 (TLR4): SARS-CoV-2 May Bind and Activate TLR4 to Increase ACE2 Expression, Facilitating Entry and Causing Hyperinflammation", *Mediators of Inflammation*, vol. 2021, Article ID 8874339, 18 pages, 2021. <https://doi.org/10.1155/2021/8874339>
77. Holms RD. Long COVID (PASC) Is Maintained by a Self-Sustaining Pro-Inflammatory TLR4/RAGE-Loop of S100A8/A9 > TLR4/RAGE Signalling, Inducing Chronic Expression of IL-1b, IL-6 and TNFa: Anti-Inflammatory Ezrin Peptides as Potential Therapy. *Immuno*. 2022; 2(3):512-533. <https://doi.org/10.3390/immuno2030033>

78. Li, W. et al. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* 426, 450–454 (2003). This study identifies ACE2 as the receptor for SARS-CoV.
79. Hofmann, H. et al. Human coronavirus NL63 employs the severe acute respiratory syndrome coronavirus receptor for cellular entry. *Proc. Natl Acad. Sci. USA* 102, 7988–7993 (2005).
80. Lima RS, Rocha LPC, Moreira PR. Genetic and epigenetic control of ACE2 expression and its possible role in COVID-19. *Cell Biochemistry and Function*. 2021 Jun 1;39(6):713–26.
81. Hou, Y. J. et al. SARS-CoV-2 reverse genetics reveals a variable infection gradient in the respiratory tract. *Cell* 182, 429–446 (2020). This study shows that SARS-CoV-2 infection levels in COVID-19 autopsied lungs corresponds to a gradient of ACE2 expression in the upper and lower airways.
82. Ahn, J. H. et al. Nasal ciliated cells are primary targets for SARS-CoV-2 replication in early stage of COVID-19. *J. Clin. Invest.* <https://doi.org/10.1172/JCI148517> (2021).
83. Hamming I, Timens W, Bulthuis ML, Lely AT, Navis G, van Goor H. Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. *J Pathol.* 2004 Jun;203(2):631-7. doi: 10.1002/path.1570. PMID: 15141377; PMCID: PMC7167720.
84. Li G, He X, Zhang L, Ran Q, Wang J, Xiong A, et al. Assessing ACE2 expression patterns in lung tissues in the pathogenesis of COVID-19. *Journal of Autoimmunity*. 2020 Apr;112:102463.
85. Veras FP, Pontelli MC, Silva CM, Toller-Kawahisa JE, de Lima M, Nascimento DC, et al. SARS-CoV-2-triggered neutrophil extracellular traps mediate COVID-19 pathology. *The Journal of Experimental Medicine*. 2020 Sep 14;217(12):e20201129.
86. Filippi MD. Mechanism of Diapedesis: Importance of the Transcellular Route. *Adv Immunol.* 2016;129:25-53. doi: 10.1016/bs.ai.2015.09.001. Epub 2015 Oct 14. PMID: 26791857; PMCID: PMC4889131.
87. Shen XR, Geng R, Li Q, Chen Y, Li SF, Wang Q, Min J, Yang Y, Li B, Jiang RD, Wang X, Zheng XS, Zhu Y, Jia JK, Yang XL, Liu MQ, Gong QC, Zhang YL, Guan ZQ, Li HL, Zheng ZH, Shi ZL, Zhang HL, Peng K, Zhou P. ACE2-independent infection of T lymphocytes by SARS-CoV-2. *Signal Transduct Target Ther.* 2022 Mar 11;7(1):83. doi: 10.1038/s41392-022-00919-x. PMID: 35277473; PMCID: PMC8914143.
88. Viana SD, Nunes S, Reis F. ACE2 imbalance as a key player for the poor outcomes in COVID-19 patients with age-related comorbidities - Role of gut microbiota dysbiosis. *Ageing Res Rev.* 2020 Sep;62:101123. doi: 10.1016/j.arr.2020.101123. Epub 2020 Jul 16. PMID: 32683039; PMCID: PMC7365123.
89. Tavares CAM, Avelino-Silva TJ, Benard G, Cardozo FAM, Fernandes JR, Girardi ACC, Jacob Filho W. ACE2 Expression and Risk Factors for COVID-19 Severity in Patients with Advanced Age. *Arq Bras Cardiol.* 2020 Oct;115(4):701-707. English, Portuguese. doi: 10.36660/abc.20200487. PMID: 33111872; PMCID: PMC8386971.

90. Fontes-Dantas FL, Fernandes GG, Gutman EG, et al. SARS-CoV-2 Spike protein induces TLR4-mediated long-term cognitive dysfunction recapitulating post-COVID-19 syndrome in mice. *Cell Rep.* 2023;42(3):112189. doi:10.1016/j.celrep.2023.112189
91. Brandão SCS, Ramos JOX, Dompieri LT, Godoi ETAM, Figueiredo JL, Sarinho ESC, Chelvanambi S, Aikawa M. Is Toll-like receptor 4 involved in the severity of COVID-19 pathology in patients with cardiometabolic comorbidities? *Cytokine Growth Factor Rev.* 2021 Apr;58:102-110. doi: 10.1016/j.cytogfr.2020.09.002. Epub 2020 Sep 21. PMID: 32988728; PMCID: PMC7505161.
92. Schweighoffer E, Nys J, Vanes L, Smithers N, Tybulewicz VLJ. TLR4 signals in B lymphocytes are transduced via the B cell antigen receptor and SYK. *J Exp Med.* 2017 May 1;214(5):1269-1280. doi: 10.1084/jem.20161117. Epub 2017 Mar 29. PMID: 28356391; PMCID: PMC5413329.
93. Peluso M.J.Lu S.Tang A.F.et al.Markers of immune activation and inflammation in individuals with postacute sequelae of severe acute respiratory syndrome coronavirus 2 infection.*J Infect Dis.* 2021; 224: 1839-1848 <https://doi.org/10.1093/INFDIS/JIAB490>
94. Peluso M.J.Sans H.M.Forman C.A.et al.Plasma markers of neurologic injury and inflammation in people with self-reported neurologic postacute sequelae of SARS-CoV-2 infection.*Neurol Neuroimmunol Neuroinflamm.* 2022; 9e200003 <https://doi.org/10.1212/NXI.0000000000200003>
95. Patterson B.K.Guevara-Coto J.Yogendra R.et al.Immune-based prediction of COVID-19 severity and chronicity decoded using machine learning. *Front Immunol.* 2021; 12: 700782 <https://doi.org/10.3389/FIMMU.2021.700782/FULL>
96. Maione F, Casillo GM, Raucci F, Salvatore C, Ambrosini G, Costa L, Scarpa R, Caso F, Bucci M. Interleukin-17A (IL-17A): A silent amplifier of COVID-19. *Biomed Pharmacother.* 2021 Oct;142:111980. doi: 10.1016/j.biopha.2021.111980. Epub 2021 Jul 29. PMID: 34364043; PMCID: PMC8318692.
97. Queiroz M.A.F.Neves P.F.M.D.Lima S.S.et al.Cytokine profiles associated with acute COVID-19 and Long COVID-19 syndrome. *Front Cell Infect Microbiol.* 2022; 12: 1<https://doi.org/10.3389/FCIMB.2022.922422>
98. Wu J.Tang L.Ma Y.et al.Immunological profiling of COVID-19 patients with pulmonary sequelae.*mBio.* 2021; 12e0159921 <https://doi.org/10.1128/MBIO.01599-21>
99. Yuanfeng Zhu, † Xiaoli Chen, † and Xin Liucorresponding author *NETosis and Neutrophil Extracellular Traps in COVID-19: Immunothrombosis and Beyond. *Front Immunol.* 2022; 13: 838011.Published online 2022 Mar 2. doi: 10.3389/fimmu.2022.838011
100. Ng H, Havervall S, Rosell A, Aguilera K, Parv K, von Meijenfeldt FA, et al.. Circulating Markers of Neutrophil Extracellular Traps Are of Prognostic Value in Patients With COVID-19. *Arterioscler Thromb Vasc Biol* (2021) 41:988–94. doi: 10.1161/ATVBAHA.120.315267
101. Sawadogo SA, Dighero-Kemp B, Ouedraogo DD, Hensley L, Sakande J. How NETosis Could Drive “Post-COVID-19 Syndrome” Among Survivors. *Immunol Lett* (2020) 228:35–7. doi: 10.1016/j.imlet.2020.09.005
102. Huang C, Huang L, Wang Y, Li X, Ren L, Gu X, et al.. 6-Month Consequences of COVID-19 in Patients Discharged From Hospital: A Cohort Study. *Lancet* (2021) 397:220–32. doi: 10.1016/S0140-6736(20)32656-8

103. Schultheiß C, Willscher E, Paschold L, Gottschick C, Klee B, Glasauer S, et al.. From online data collection to identification of disease mechanisms: the IL-1 β , IL-6 and TNF- α cytokine triad is associated with post-acute sequelae of COVID-19 in a digital research cohort. SSRN Electron J [Internet]. (2021). doi: 10.2139/ssrn.3963839
104. VanElzakker, MB, Brumfield, SA, and Lara Mejia, PS. Neuroinflammation and cytokines in Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS): a critical review of research methods. *Front Neurol.* (2018) 9:1033. doi: 10.3389/fneur.2018.01033
105. Russell N. Ryan J. and Athena Akrami. A review of cytokine-based pathophysiology of Long COVID symptoms *Front Med (Lausanne)*. 2023; 10: 1011936. Published online 2023 Mar 31. doi: 10.3389/fmed.2023.1011936
106. Phetsouphanh, C., Darley, D.R., Wilson, D.B. et al. Immunological dysfunction persists for 8 months following initial mild-to-moderate SARS-CoV-2 infection. *Nat Immunol* 23, 210–216 (2022). <https://doi.org/10.1038/s41590-021-01113-x>