UNIVERSIDADE FEDERAL DO AMAZONAS

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA

DOUTORADO EM BIOTECNOLOGIA

ALEXANDER LEONARDO SILVA JUNIOR

PERFIL IMUNOFENOTÍPICO E INFLAMATÓRIO DE PACIENTES GRAVES E CONVALESCENTES DA INFECÇÃO PELO SARS-CoV-2

MANAUS

2025

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Orientadora: Dra. Adriana Malheiro Alle Marie.

Coorientadores: Dr. Allyson Guimarães da Costa;

Dra. Susan Pereira Ribeiro.

MANAUS

2025

Ficha Catalográfica

Elaborada automaticamente de acordo com os dados fornecidos pelo(a) autor(a).

S586p Silva Junior, Alexander Leonardo
Perfil Imunofenotípico e Inflamatório de Pacientes Graves e Convalescentes da Infecção pelo SARS-CoV-2 / Alexander Leonardo Silva Junior. - 2025.
206 f. : il., color. ; 31 cm.

Orientador(a): Adriana Malheiro Alle Marie.
Coorientador(a): Allyson Guimarães da Costa.
Coorientador(a): Susan Pereira Ribeiro.
Tese (doutorado) - Universidade Federal do Amazonas, Programa de Pós-Graduação em Biotecnologia, Manaus, 2025.

1. COVID-19. 2. Inflamação. 3. Biomarcador. 4. Amazônia Brasileira. I. Marie, Adriana Malheiro Alle. II. Costa, Allyson Guimarães da. III. Susan Pereira Ribeiro. IV. Universidade Federal do Amazonas. Programa de Pós-Graduação em Biotecnologia. V. Título

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Aprovado em 14 de fevereiro de 2025.

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Dedico este trabalho a todos os sobreviventes da pandemia da COVID-19, àqueles que perderam entes queridos, e aos profissionais de saúde que saíam de suas casas todos os dias, sem saber se retornariam.

Este trabalho é para vocês.

AGRADECIMENTOS

Primeiramente agradeço a Deus por todas as vitórias e ensinamentos passados ao longo desses 27 anos de idade. Tudo culminou para este momento.

Agradeço, em segundo lugar, à minha família. Meus avós, Gaurino Gonçalves, Zuleika Amaral, minha mãe, Fabiane Amaral e minha irmã, Larissa Amaral, meu muito obrigado por estarem me acompanhando nesta caminhada, e terem feito a pessoa que sou atualmente. Ao meu pai, Alexander Leonardo, por ter me acolhido ao longo desta jornada, e me dado todo o apoio ao qual precisei.

Aos amigos desta longa trajetória, Thaynara Ogaya, Natália Manuela, Rosevelto Maia, Natasha Maranhão e Felipe Nogueira. Sou e sempre serei eternamente grato por toda mensagem de carinho, toda oração, disponibilidade nos momentos mais inusitados, e paciência nos meus áudios enormes no meio da madrugada.

À minha orientadora, Dra. Adriana Malheiro, por ter acreditado em mim, e me confiado esta enorme responsabilidade. Por não ter desistido, e dispor de tempo e paciência para me fornecer luz, nos momentos de escuridão.

Aos meus coorientadores, Dr. Allyson Costa, pelo auxílio prestado ao longo desses quatro anos. Obrigado pelos elogios, dedicação e cada puxão de orelha. À Dra. Susan Ribeiro e Dr. Rafick Sekaly, que me acolheram na Universidade de Emory, não tenho palavras para agradecer a experiência incrível que tive, e o quanto pude crescer profissionalmente!

Ao Dr. Nelson Fraiji, Dra. Myuki Crispim, Dra. Ester Sabino e Dr. Walter Neves, pela disponibilidade, parceria e dedicação em ler, conversar e investir neste projeto, bem como em mim.

Ao Dr. Olindo Filho e Dra. Andrea Teixeira, pela colaboração no Instituto René Rachou (Fiocruz/MG). Muito obrigado pelos momentos disponibilizados, pela paciência e cordialidade.

Agradeço à Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas (HEMOAM) por terem cedido a estrutura para o desenvolvimento deste projeto, e vários outros. Fico muito honrado em ter crescido dentro desta instituição, e colaborar com os

mais diversos projetos de pesquisa, sempre visando a promoção à saúde dos pacientes e doadores.

À Cláudia Abrahim, do laboratório de sorologia da Fundação HEMOAM, pela paciência, dedicação e cordialidade nos momentos em que precisei de uma conversa, ou uma dúvida. Muito obrigado por todo o ensinamento compartilhado.

Ao Hospital Universitário Getúlio Vargas (HUGV), por colaborar na obtenção das amostras dos pacientes com COVID-19, em meio à pandemia. Muito obrigado pela parceria!

À Universidade de Emory, que me acolheu e permitiu que parte deste projeto fosse desenvolvido lá. Às pessoas incríveis que conheci, e que foram fundamentais para a conclusão deste trabalho! Aos colegas que fiz no Laboratório de Patologia, Fernanda Coirada, Isabela Daher, Perla del Rio, Manini Majithia, Fernanda Bruno, Felipe ten Caten, Amanda Cabral, Maurício "Muchacho" Morales, David Viar, Valentino D'Onofrio meu eterno obrigado pela experiência incrível de ter conhecido vocês!

To Villa International friends, made for eternity, Maria José, Henri Poupy, Samanta Biswas, Isabella Bonfim, Nathalia Guimarães, João "Lukitas" Calandrini (guri) and all other friends around the world… Marcia Sbabo, Katie Archibald-Woodward and Rebecca Arayan, thank you so much for being so special to me!! Ah how lucky I am to have my soul touched by you all in this period! Hopefully, I will feel the pleasure to meet you guys again!!

À Stephany Dias, Lucas Oliveira, Nara Belezia, Jônatas Campelo, Henayara Trindade e Luiz Carlos, os alunos de graduação que me acompanharam neste, e outros projetos de pesquisa. Obrigado pela dedicação, paciência e companheirismo ao longo de todo este processo. Espero ter sido tão influente na vida de vocês, quanto vocês foram na minha.

À Universidade Federal do Amazonas (UFAM), pela imensa oportunidade em contribuir para a pesquisa e ciência brasileira. Por todos os cuidados e preocupações com cada aluno e aluna.

Ao Centro Universitário do Norte (UNINORTE), Faculdade Unyleya e Faculdade Estácio do Amazonas, por permitirem uma experiência sem igual dentro da docência, trabalho em equipe e desenvolvimento profissional. À Liga Acadêmica de Inovação em Análises Clínicas (LAINAC-UNINORTE), por serem alunos maravilhosos e muito dedicados, foi uma honra coordenar vocês em prol da saúde à população. Aos milhares de alunos que tive a honra de ser professor... Obrigado por tudo, de coração.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela bolsa e todo o aporte financeiro ao longo do desenvolvimento do projeto, no âmbito nacional e internacional, bem como minha jornada como pesquisador. À Fundação de Amparo à Pesquisa do Amazonas (FAPEAM) e Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) pelo suporte financeiro fornecido para o desenvolvimento deste, e de outros projetos do grupo de pesquisa.

A todos os outros integrantes dos vários grupos de pesquisa, que contribuíram de forma direta ou indireta neste trabalho, meu muito obrigado! A conclusão deste trabalho só foi possível pelo envolvimento e dedicação de cada um!

Por fim, e principalmente, a todos os participantes que aceitaram participar deste projeto. Vocês contribuíram para a pesquisa científica no Brasil em meio a uma pandemia. Isso não tem preço, e seremos eternamente gratos pela disponibilidade e cordialidade de todos!

Some men see things as they are and ask why. Others dream things that never were and ask why not.

George Bernard Shaw

RESUMO

Introdução: A COVID-19 é uma doença respiratória pulmonar aguda, transmissível por gotículas de ar liberadas por pacientes infectados. Muitos casos foram diagnosticados em 2020 e evoluíram para a forma grave, com altos números de óbito em todo o mundo, e já foi observado que parâmetros da resposta imunológica podem atuar como biomarcadores para desfecho clínico em indivíduos graves e internados. Entretanto, poucos estudos observaram indivíduos curados da infecção, sem interferentes, e menos ainda relacionam com a produção de anticorpos, o que salientou o desenvolvimento deste estudo para estabelecimento de novas estratégias imunológicas visando a melhoria de imunizantes e manejo de pacientes com COVID-19. **Objetivo:** Avaliar o perfil imunofenotípico e inflamatório de pacientes graves e convalescentes da infecção pelo SARS-CoV-2. **Metodologia:** Foram recrutados 51 doadores saudáveis, 12 pacientes diagnosticados com COVID-19 em estado leve, 6 moderados, 9 graves, e 138 convalescentes, que atingiram a cura clínica a cerca de 30 dias. Destes, 67 realizaram um acompanhamento longitudinal por mais dois meses. Dos participantes, foram coletados sangue total para análise por citometria de fluxo. O soro foi utilizado para quantificar o perfil de citocinas, quimiocinas e fatores de crescimento por Luminex, e dosagem de anticorpos anti-S e anti-N. Para análise de dados foram utilizados os softwares Graph PadPrism v. 9.0 e R Studio. Todas as análises foram consideradas com intervalo de confiança de 95% e valor de p significativo para $p < 0.05$. **Resultados:** Pacientes graves de COVID-19 demonstraram uma queda nos números de células NK e NKT, junto com monócitos inflamatórios e não clássicos. No soro, as concentrações de IFN-γ, CXCL10 e CXCL8 apresentaram potencial como biomarcadores imunológicos de caracterização clínica de pacientes com COVID-19. Dentro daqueles sob internação hospitalar, os que evoluíram a óbito apresentaram alta contagem de eosinófilos. Nos convalescentes, a sororeatividade para o anti-N decaiu ao longo dos três meses após a cura clínica, mas não para o anti-S. Marcadores, como a razão neutrófilo-linfócito, contagem de eosinófilos e IL-15, se mostraram altos durante toda a convalescença, embora tenha sido observada uma dinâmica inicial de produção celular. **Conclusão:** Com nossos achados, observamos que a exacerbação da imunidade inata na fase aguda pode aumentar o risco de óbito. Já um efeito reparador, pode colaborar na manutenção dos anticorpos IgG em convalescentes. Compreender a dinâmica imunológica envolvida na soroconversão permitirá o melhor manejo de pandemias futuras, causadas por agentes respiratórios virais, bem como estabelecer biomarcadores precisos de monitoramento e acompanhamento clínico de pacientes com COVID-19.

Palavras-chave: COVID-19; Inflamação; Biomarcador; Amazônia Brasileira.

ABSTRACT

Background: COVID-19 is an acute respiratory disease, transmissible by airdrops from infected patients. Many cases were diagnosed in 2020 and evolved to severe conditions, with high number of deaths around the world, and it was seen before that parameters from immune response can act as biomarkers to clinical outcomes in severe and hospitalized. However, few studies focused on individuals cured from infection, without interferents, and less were related to antibody production, what highlighted the development of this study to establish new immunological strategies aiming the improvement of vaccines and management of patients with COVID-19. **Aim:** To evaluate the immunophenotypic and inflammatory profile from severe and convalescent patients with SARS-CoV-2 infection. **Materials and methods:** 51 healthy donors were recruited, 12 patients with COVID-19 in mild condition, 6 moderates and 9 severe, and 138 convalescents, who reached clinical recovery approximately 30 days after. Among these, 67 participants had a monthly follow-up for two months. Whole blood was obtained from all participants to evaluate by flow cytometry. Serum samples were also collected to measure cytokines, chemokines, and growth factors using Luminex, and dosage of anti-S and anti-N antibodies. Data analysis was done in Graph PadPrism v.9 and R Studio. All analyses were performed with a 95% confidence interval and a p-value < 0.05. **Results:** Severe patients COVID-19 demonstrated a decrease in NK and NKT cells number, also with inflammatory and non-classical monocytes. In serum, the concentration of IFN-y, CXCL10 and CXCL8 had potential to act as immunological biomarkers of clinical decision to patients with COVID-19. Among those under hospital attendance, those who evolved to death had high amounts on eosinophils. In convalescents, serum reactivity to anti-N decayed throughout the three months after clinical recovery, but not to anti-S. Markers, such as neutrophil-lymphocyte ratio, eosinophil count and IL-15 were increased during all convalescence, despite it was seen an initial cellular dynamic. **Conclusion:** With our findings, we observed that exacerbation of innate immunity in acute phase can increase the risk of death. A reparative effect can collaborate with IgG antibodies maintenance in convalescents. Comprehend the immune dynamics in seroconversion will allow the better approach of future pandemics, caused by viral respiratory agents, as establish more precise biomarkers to monitor and follow patients with COVID-19.

Keywords: COVID-19; Inflammation; Biomarker; Brazilian Amazon.

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LISTA DE ABREVIATURAS E SIGLAS

SARS-CoV-2: Síndrome Respiratória Aguda Grave causada pelo Coronavírus tipo 2.

SARS-CoV: Síndrome Respiratória Aguda Grave causada por Coronavírus.

MERS-CoV: Síndrome Respiratória do Oriente Médio.

COVID-19: Doença do Coronavírus.

ssRNA+: RNA de fita simples com polaridade positiva.

S: Spike.

M: Membrana.

E: Envelope.

HE: Esterase de Hemaglutinina.

N: Nucleocapsídio.

OMS: Organização Mundial da Saúde.

ACE2: Enzima Conversora de Angiotensina 2.

RT-qPCR: Reação em Cadeia da Polimerase em Tempo Real pela Transcriptase Reversa.

cDNA: DNA complementar.

RNL: Relação Neutrófilo-Linfócito.

UTI: Unidade de Terapia Intensiva.

PF4: Fator Derivado de Plaqueta 4.

NETs: Redes Extracelulares de Neutrófilos.

IFN: Interferon.

PCR: Proteína C Reativa.

D30: 30 dias após cura clínica.

D60: 60 dias após cura clínica.

D90: 90 dias após cura clínica.

CEP-HEMOAM: Comitê de Ética em Pesquisa da Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas.

HEMOAM: Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas.

TCLE: Termo de Consentimento Livre e Esclarecido.

HUGV: Hospital Universitário Getúlio Vargas.

FVS-AM: Fundação de Vigilância em Saúde do Amazonas.

VCM: Volume Corpuscular Médio.

HCM: Hemoglobina Corpuscular Média.

CHCM: Concentração De Hemoglobina Corpuscular Média.

RDW: *Red Distribution Width*.

WBC: Contagem Global de Leucócitos.

CMIA: Testes de Imunoensaio de Micropartículas por Quimioluminescência.

FSC: *Forward Scatter*.

SSC: *Side Scatter*.

NK: *Natural Killer*.

DP: Desvio Padrão.

VPP: Valor Preditivo Positivo.

VPN: Valor Preditivo Negativo.

Sumário

1. INTRODUÇÃO

Em 2020 o mundo experienciou mais uma pandemia, com um número alarmante de casos e óbitos, causada por um novo tipo de coronavírus, causador da Síndrome Respiratória Aguda Grave (SARS-CoV-2). Por ser um vírus completamente novo, o sistema imunológico da população encontrava-se vulnerável, e isso, atrelado às poucas medidas empregadas na contenção de doenças respiratórias empregadas na época, favoreceu a rápida disseminação do vírus e infecção de milhões de pessoas.

O ano de 2021 apresentou o maior número de casos de COVID-19 diagnosticados no mundo inteiro, onde 1% evoluiu para óbito, e 3-20% foram internados em estado grave (BUSS et al., 2022; LAMERS; HAAGMANS, 2022). No Brasil, muitas medidas foram propostas, mas o painel epidemiológico apresentou variações com base nas regiões geográficas do país. Esse fator foi primordial para a posterior condição: desenvolvimento de novas variantes do vírus. Logo no início de 2021, houve o desenvolvimento de uma variante no Estado do Amazonas, que causou um caos nos setores públicos e privados voltados à saúde, devido à imensa quantidade de casos que necessitavam de internação (CASTRO et al., 2021; PRETE et al., 2022).

A resposta imunológica ao vírus na fase aguda foi um dos ramos muito estudados ao redor do mundo. Pacientes com o diagnóstico de COVID-19 classificados de forma grave apresentavam quadros mais intensos de febre, tosse, falta de ar, distúrbios intestinais, e em alguns casos, tromboses (ARCANJO et al., 2020; MARTENS et al., 2021; ZUO et al., 2021b). Biomarcadores imunológicos foram descritos, de forma tanto a prever um prognóstico, como também caracterizá-los. Foi observado que neutrofilia (BALZANELLI et al., 2021; CAI et al., 2021; LEPPKES et al., 2020), monocitopenia (RAJAMANICKAM et al., 2021; ZHANG et al., 2020c), alta relação neutrófilo-linfócito (ASGHAR et al., 2020; BALZANELLI et al., 2021; CAI et al., 2021; IMRAN et al., 2020; LEPPKES et al., 2020) e produção de mediadores inflamatórios levam a um quadro de lesão tecidual, responsável pelas mais diversas observações clínicas.

A gravidade em que o mundo se apresentou pôde ser observada entre os anos de 2020 e 2021. No entanto, ainda há uma escassez de estudos em indivíduos convalescentes. Estes são caracterizados pelo diagnóstico prévio do vírus, e que atingiram a cura clínica

(NG et al., 2021; RAJAMANICKAM et al., 2021). Tanto pacientes leves quanto graves, que não evoluíram a óbito, são considerados convalescentes, e pouco se sabe acerca da dinâmica imunológica neste quadro. Os marcadores de ativação celular e proteico ainda se apresentaram aumentados até cerca de 60 dias durante a convalescença, no entanto, há uma queda progressiva, junto com o aumento de neutrófilos imaturos, monócitos (KWIECIEŃ et al., 2021) e linfócitos (KIM et al., 2022).

A produção de anticorpos tem início ainda durante a fase aguda, para a maioria dos pacientes, embora ainda não se saiba os fatores que levam a uma produção eficaz de anticorpos na fase convalescente, nem quanto tempo perduram. Alguns estudos identificaram que pacientes sintomáticos apresentam maior concentração de anticorpos, do que os assintomáticos (SOKAL et al., 2021; WARDHANI et al., 2021; WU et al., 2021), enquanto outros descrevem uma correlação negativa da contagem de linfócitos com a concentração de anticorpos, sugerindo que quanto pior o perfil inflamatório na fase aguda, mais comprometido estará a produção de anticorpos na fase convalescente (WU et al., 2020).

Devido à baixa quantidade de estudos com pacientes convalescentes, advindos da infecção natural primária, bem como os que relacionam a dinâmica imunológica com a produção de anticorpos contra o do vírus, fez-se necessário compreender quais parâmetros da resposta imune intermedeiam a imunidade inata, bem como os presentes na imunidade adaptativa. Com nossos resultados, a dinâmica de biomarcadores poderá ser mais bem elucidada para potencializar a resposta imunológica após infecção, além de traçar estratégias para imunizantes e uso de plasma convalescente. Nossos dados contribuem para o entendimento da resposta imune nestes pacientes e poderão ajudar na consolidação de menores custos ao estado, melhora na qualidade de vida de pacientes infectados e flexibilidade quanto às medidas preventivas.

2. FUNDAMENTAÇÃO TEÓRICA

2.1. Aspectos etiológicos e genômicos do SARS-CoV-2

O novo coronavírus é um agente viral, descoberto no final de 2019 através de relatos de caso de pacientes com uma síndrome respiratória na província de Wuhan, localizada na China. Por apresentar similaridade genômica de 80% com o coronavírus 1, descrito em 2002, foi classificado dentro da mesma família taxonômica, pertencentes à ordem *Nidovirales,* e à família *Coronaviridae* (JAFARZADEH et al., 2020).

Os coronavírus apresentam similaridades genômicas e estruturais entre si. O primeiro coronavírus encontrado em humanos foi o causador da síndrome respiratória aguda grave (*Severe Acute Respiratory Syndrome* [SARS-CoV]), em 2002, seguido pelo da síndrome respiratória do oriente médio (*Middle East Respiratory Syndrome* [MERS-CoV]), em 2012, e por fim, o causador da doença do coronavírus (COVID-19), nomeado como SARS-CoV-2, descoberto em dezembro de 2019. Os agentes causadores do SARS foram descobertos na China, em províncias diferentes, enquanto que o MERS-CoV foi descoberto na Arábia Saudita (DOUSARI; MOGHADAM; SATARZADEH, 2020).

Vírus desta família podem ser classificados em quatro grandes grupos, denominados α, β, γ e δ coronavírus e são conhecidos por infectar mamíferos. Já foram relatados casos dos dois primeiros em humanos, sendo comum também em outros mamíferos, enquanto que os dois últimos são mais frequentes em aves, e alguns animais marinhos (DOUSARI; MOGHADAM; SATARZADEH, 2020; TURILLI; LUALDI; FASANO, 2022). Subtipos de α-coronavírus e β-coronavírus já foram encontrados em humanos, causadores de síndromes respiratórias, enquanto outros tipos virais, afetam principalmente os tratos superiores de hospedeiros imunocompetentes, e podem levar inclusive à complicações no trato gastrointestinal (CUI; LI; SHI, 2019; SINGH; YI, 2021)

Animais silvestres são os principais reservatórios selvagens dos coronavírus, sendo o morcego o principal, para o SARS-CoV e MERS-CoV. Acredita-se que estes dois coronavírus são oriundos de morcegos, devido à presença de sequências comuns encontrados no genoma, e junto com o SARS-CoV-2, compõem um subgênero dos β-coronavírus, os *Sarbecovirus* (CUI; LI; SHI, 2019). Cabe ressaltar que hospedeiros intermediários podem estar presentes, e acabar contribuindo para adaptação e disseminação do vírus em humanos, como ocorreu em camelos e dromedários,

hospedeiros intermediários para o MERS-CoV, enquanto para o SARS, temos o musang (ou cliveta de palmeira asiática) (CUI; LI; SHI, 2019; ZHANG; HOLMES, 2020).

Também acredita-se que o vírus possa ser proveniente do pangolin (*Manis javanica*), um animal importado ilegalmente no sul da China, devido à similaridade genômica e a presença de 6 mutações encontradas no gene que produz o epítopo RBD da proteína S, presente entre o agente causador da pandemia da COVID-19, e as variantes já descritas, capazes de infectar o pangolin (ZHANG; HOLMES, 2020).

Com o avanço da população humana sobre áreas silvestres, bem como consumo de animais crus, houve a adaptação de patógenos causadores de doenças endêmicas em animais para humanos, demonstrando os riscos associados a essa prática. Muitos coronavírus se adaptaram a humanos principalmente devido ao contato em mercados comunitários (CUI; LI; SHI, 2019). O mesmo ocorreu com o SARS-CoV-2, descoberto no mercado de Wuhan, China.

O SARS-CoV-2 é um vírus de RNA fita simples (ssRNA+), com polaridade positiva, 30 kb de extensão, 29.903 bp e apresenta uma estrutura envelopada com diâmetro de 80-160 nm (LIANG et al., 2020; SINGH; YI, 2021), pertencendo ao grupo IV, da classificação de Baltimore. Seu genoma apresenta 14 regiões de leitura aberta (ORFs), responsáveis por codificar proteínas não estruturais, utilizadas no processo de replicação. Dentro das principais proteínas, tem-se a tradução da proteína Spike (S), Membrana/Matriz (M), Glicoproteínas do Envelope (E), Esterase de Hemaglutinina (HE) e Nucleocapsídio (N). Outros coronavírus compartilham semelhança com as N, M, e os E, enquanto a HE está presente em apenas alguns β-coronavírus (SINGH; YI, 2021).

A proteína S é a mais estudada dentro da fisiopatologia do SARS-CoV-2 por ser a proteína que intermedeia a interação do vírus com seu receptor. Se trata de uma proteína que é compartilhada entre alguns vírus e principalmente entre os coronavírus. Varia de 1,160 a 1,400 aminoácidos, é localizada na superfície do vírus e intermedeia a interação do vírus com sua célula hospedeira. Dentro dos coronavírus, as regiões N-terminal e C-terminal a subunidade 1 estão envolvidas na adesão celular, enquanto a subunidade 2 está envolvida na infecção do vírus dentro da célula (HARRISON; LIN; WANG, 2020;

LIANG et al., 2020). Na figura 1 podemos observar o padrão genômico do SARS-CoV-2, junto com os sítios envolvidos na transcrição das proteínas virais.

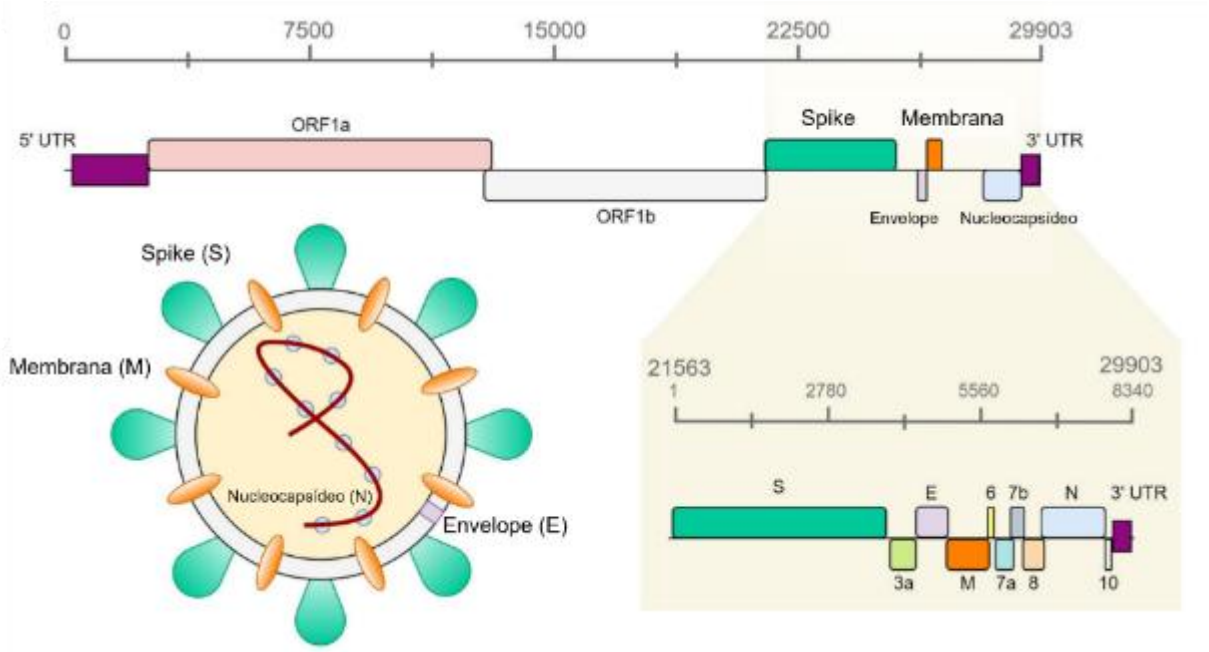


Figura 1: Estrutura genômica do SARS-CoV-2, com as ORFs e principais proteínas para o metabolismo viral.
Fonte: Adaptado de Singh & Yi, 2021.

Muitas mutações foram observadas nos coronavírus, o que facilitou a adaptação nas mais diversas espécies silvestres, entretanto, mutações não sinônimas, as quais ocorrem em baixa taxa, podem estar relacionadas à pressão da seleção natural que ocorre no meio ambiente. Essas características permitem a preservação e análise filogenética das variantes dos coronavírus, de forma a identificar as mutações sofridas ao longo dos anos. Estudos identificaram que a similaridade entre uma variante já presente em morcegos, RaTG13, e o novo coronavírus (SARS-CoV-2) é de 96%, com uma divergência temporal de 18 a 71 anos (SINGH; YI, 2021).

Ao longo da epidemia da COVID-19, novas variantes surgiram, levando ao aumento do número de casos de forma muito rápida, bem como mudanças no risco de desenvolvimento dos casos graves. Até o final de 2021, quatro variantes da COVID-19 haviam sido descritas pela Organização Mundial da Saúde (OMS), sendo classificadas e avaliadas quanto às características de transmissibilidade, gravidade e resposta imunológica. Conforme descrito no Quadro 1, pode-se observar que as quatro variantes

mais comuns apresentam mutações características, o que levou à melhor forma de transmissão e resistência ao sistema imunológico do hospedeiro (TAO et al., 2021; TURILLI; LUALDI; FASANO, 2022).

Classificação	Primeiro relato	Primeiro relato	Mutações de interesse
Alfa	Reino Unido	2020	**N501Y**, A570D, P681H, T716I, S982A, D1118H
Beta	África do Sul	2020	K417N, **E484K**, **N501Y**, **D614G**, A701V
Gamma	Brasil	2020	K417T, **E484K**, **N501Y**, **D614G**, H655Y
Delta	Índia	2020	L452R, T478K, **D614G**, P681R
Omicron	África do Sul e Botswana	2021	A67V, Δ69-70, T95I, G142D, Δ143-145, N211I, Δ212, ins215EPE, G339D, S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493R, G496S, Q498R, **N501Y**, Y505H, T547K, **D614G**, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K, L981F

Quadro 1: Padrão de mutações de interesse com base nas variantes genéticas do SARS-CoV-2. Em destaque estão as mutações descritas como relevantes para maior grau de infectividade ou evasão de anticorpos produzidos pelo sistema imunológico.
Fonte: Adaptado de Turilli, Lualdi e Fasano, 2022; Gómez, Perdiguero e Esteban, 2021.

Ao estudar as variantes, foi observado que diferem entre si quanto à transmissibilidade, resposta imunológica e gravidade. Já foi demonstrado que a pressão da seleção natural acabou por induzir à várias mutações no gene do SARS-CoV-2, principalmente na proteína Spike, entretanto, algumas regiões apresentaram maior pressão positiva do que outras (MARTIN et al., 2021). O principal problema observado se dá pelos testes de diagnósticos realizados e comercializados, bem como eficácia das vacinas em variantes diferentes (TURILLI; LUALDI; FASANO, 2022). Três mutações, no gene da proteína S, tomam destaque:

- **D641G:** Esta mutação leva ao maior grau de replicação dentro dos infectados, além de estar relacionada a um maior grau de infectividade e transmissibilidade. Foi descrita desde fevereiro de 2020, e apresentou maior prevalência nos pacientes identificados posteriormente. Além disso, vírions com essa mutação também apresentaram maior quantidade de proteína S na sua superfície (HARVEY et al., 2021; TAO et al., 2021).

- **N501Y:** Aumenta a afinidade da proteína S com o receptor Enzima Conversora de Angiotensina 2 (ACE2) e a taxa de replicação viral através da formação de mais uma ponte de hidrogênio. 14 mutações neste gene apareceram no final do ano de 2020, e cresceram para 30 em abril de 2021 devido à seleção natural positiva (GÓMEZ; PERDIGUERO; ESTEBAN, 2021; MARTIN et al., 2021).
- **E484K:** A mutação do aminoácido para K, Q ou P causa menor afinidade em anticorpos monoclonais e de convalescentes, no entanto apenas a troca para o aminoácido K causa queda significativa na neutralização (HARVEY et al., 2021). A mudança proteica (ácido glutâmico por lisina) também confere maior habilidade de adesão antigênica do RBD com o receptor celular, o que facilita o infectividade e replicação celular (GÓMEZ; PERDIGUERO; ESTEBAN, 2021; ISTIFLI et al., 2021).

A Figura 2 demonstra a localização dos principais epítopos da proteína Spike. As mutações ocorrem majoritariamente nestes epítopos e podem interferir tanto na capacidade de infectividade do vírus, bem como na resistência a anticorpos produzidos por indivíduos convalescentes, mas também vacinados. Cabe ressaltar que o processo de vacinação induz à produção de anticorpos, porém, com as novas variantes, pode haver escape viral devido à cepa viral utilizada para formulação da vacina (TAO et al., 2021).

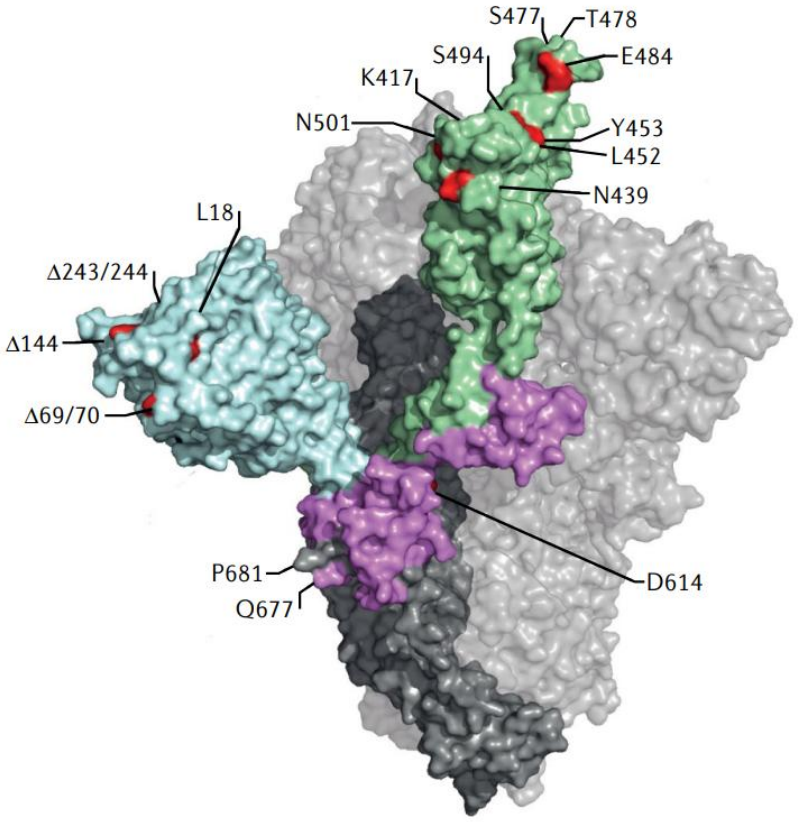


Figura 2: Principais epítopos da proteína Spike, com maior frequência de mutações gênicas entre as variantes.
Fonte: Tao et al., 2021.

Com o desenvolvimento das novas variantes, muitos picos de casos foram observados ao longo da pandemia, levando à dificuldade de tratamento eficaz para quadros leves e graves, além do grande número de óbitos em determinados locais. A compreensão do quadro epidemiológico permite destacar quais variantes são mais recorrentes, além de propor melhorias nos serviços de atenção à saúde. Tópico que será abordado mais à frente.

2.2. Forma de transmissão e epidemiologia da COVID-19

O SARS-CoV-2 é capaz de sobreviver em superfícies sob várias condições. Já foi observado que em temperatura ambiente, apresenta duração de 3 horas em aerossóis, 5 dias em metais e papeis, e 4 dias em madeiras, além de outras superfícies. Esses fatores contribuem para permanência e disseminação do vírus em superfícies, mesmo que inanimadas. Porém, a principal forma de disseminação se dá através da liberação de

fluídos biológicos, principalmente por gotículas, via nasocomial, bem como amostras fecais, urina, saliva e até mesmo por via intrauterina (KHAN et al., 2021).

Em um hospedeiro vivo, a principal forma de transmissão se dá pela entrada do SARS-CoV-2 nas vias respiratórias, através das gotículas liberadas no ambiente por infectados, ou até mesmo por contato com materiais contaminados. Por ser um patógeno de transmissão aérea, foi possível uma alta e rápida disseminação na China e no mundo, principalmente devido a fatores como ausência de medidas de controle eficazes, como imunização prévia ou barreiras, alto período de incubação (5-6 dias) e transmissão (14 dias) entre os infectados (HARRISON; LIN; WANG, 2020; KEVADIYA et al., 2021; KHAN et al., 2021).

O surto, que iniciou em 2019, logo se tornou uma pandemia em pouco mais de três meses. Em março de 2020, o vírus já estava presente em várias regiões do mundo, sendo declarado pela OMS o início da pandemia. Ao longo desse mesmo ano, vários casos foram observados no Brasil e no mundo, com alto número de internações e agravamento clínico dos pacientes infectados. Medidas de controle do vírus foram aplicadas, o que permitiu primeiramente a queda do número de casos dentro da população exposta, mas majoritariamente, o manejo de hospitais e centros de atenção à saúde suportarem o painel caótico de pacientes graves que necessitaram de atendimento médico (BUSS et al., 2022). No mundo, cerca de 1% dos casos evoluíram a óbito, e de 3-20% necessitaram de hospitalização, e grande parte destes ainda foram para a Unidade de Terapia Intensiva (UTI) devido aos fortes sintomas (LAMERS; HAAGMANS, 2022).

A variante alfa (B.1.1.7) foi a primeira a ser detectada, no Reino Unido, a partir de setembro de 2020, e apresenta a mutação N501Y, o que potencializou em sete vezes a afinidade do vírus à proteína ACE2 e resistência à neutralização dos anticorpos já produzidos. A variante beta (B.1.351) surgiu logo em seguida, em outubro de 2020, na África do Sul, com sete mutações e 1 deleção, localizadas nas proteínas S (K417N, E484K e N501Y), E, N, e nas regiões da ORF1a. Posteriormente, as variantes delta e gama surgiram em períodos próximos. Acredita-se que a primeira tenha surgido em dezembro de 2020, enquanto a segunda tenha surgido em janeiro de 2021, embora também seja estipulado que ambas se desenvolveram ao mesmo tempo, mas em localizações geográficas distintas. E por fim, a omicron, reportada na África do Sul em novembro de

2021. Na Figura 3 pode-se observar um acompanhamento longitudinal da porcentagem das variantes ao longo do período de 2020 até o início de 2022, demonstrando uma prevalência transitória da variante alfa, mas um predomínio da variante delta (ALEEM; SAMAD; SLENKER, 2022; GÓMEZ; PERDIGUERO; ESTEBAN, 2021; KOELLE et al., 2022).

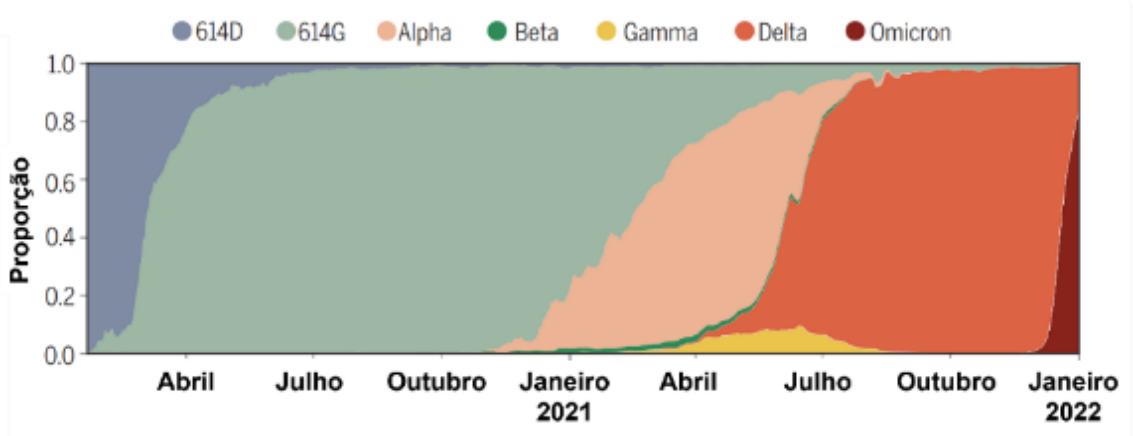


Figura 3: Padrão dos principais subtipos de SARS-CoV-2 que estavam circulando ao redor do mundo ao longo de 2020, até janeiro de 2022.
Fonte: Koelle et al., 2022.

A presença de novas variantes leva ao aumento do número de casos, principalmente das variantes que sofrem mutações em genes relacionados à infectividade e capacidade de evasão do sistema imunológico de um hospedeiro já sensibilizado, seja por contato prévio com o vírus, ou pela vacina. Esses fatores, atrelados à dependência de locais de assistência capacitados para realizar a identificação de casos, notificação, diagnóstico efetivo e preciso, torna a vigilância epidemiológica um desafio em vários países do mundo, principalmente aqueles com baixa renda (CASTRO et al., 2021).

No Brasil, o SARS-CoV-2 foi relatado pela primeira vez em 2020, porém o auge da pandemia se deu no Estado do Amazonas, entre novembro de 2020, e início de 2021, onde houve o desenvolvimento de uma nova variante, a gama (P.1). Com 12 aminoácidos diferentes no gene da proteína S da cepa original. Esta variante se mostrou mais agressiva e mais resistente, devido principalmente às mutações E484K e N501Y, no receptor RBD, que potencializaram a interação da proteína S com o receptor ACE2, além da primeira

mutação conferir resistência à interação de anticorpos neutralizantes à proteína S, tanto de indivíduos convalescentes, quanto de vacinados (IMAI et al., 2021).

Levaram 11 dias desde o diagnóstico do primeiro caso no Amazonas até a primeira morte relacionada à COVID-19. Mais adiante, na análise temporal, o estado apresentou o maior índice de mortalidade no país, sendo inclusive o dobro que a taxa por 100 mil habitantes do Brasil (CASTRO et al., 2021). Além disso, com a grande demanda de atendimento hospitalar, relacionado à falta de recurso público destinado à recepção dos pacientes diagnosticados com COVID-19 que necessitaram de internação, Manaus apresentou a maior taxa de mortalidade dentro de um estudo realizado em oito capitais brasileiras (5,3 por 1.000 habitantes) (PRETE et al., 2022). No período de um mês e meio, houve o desenvolvimento da P.1, e foi a linhagem de aproximadamente 75% dos casos de COVID-19 no Amazonas, atingindo níveis alarmantes na cidade (NAVECA et al., 2021).

O número de casos da COVID-19 no Brasil chegou a níveis alarmantes, principalmente no ano de 2021. Até março deste ano, o Brasil representava 9,5% dos casos mundiais de COVID-19, e 10,4% dos óbitos, sendo a população brasileira representativa de 2,7% da população mundial (CASTRO et al., 2021). Estima-se que até outubro de 2020, mais de 75% da população brasileira já havia sido infectada, o que está relacionado a problemas de vigilância, notificação dos casos, e baixa capacidade de testes de diagnóstico, principalmente em serviços de saúde privados.

Na Figura 4, é possível identificar as principais regiões brasileiras com maior número de casos durante o primeiro ano da pandemia, bem como sua distribuição a nível nacional. A regiões norte e nordeste apresentaram maior quantidade de casos por 100,000 habitantes, quando comparados ao restante do país, o que acredita-se que tenha sido devido à distribuição populacional e disponibilidade de recursos voltados ao atendimento hospitalar aos pacientes com COVID-19 (CASTRO et al., 2021).

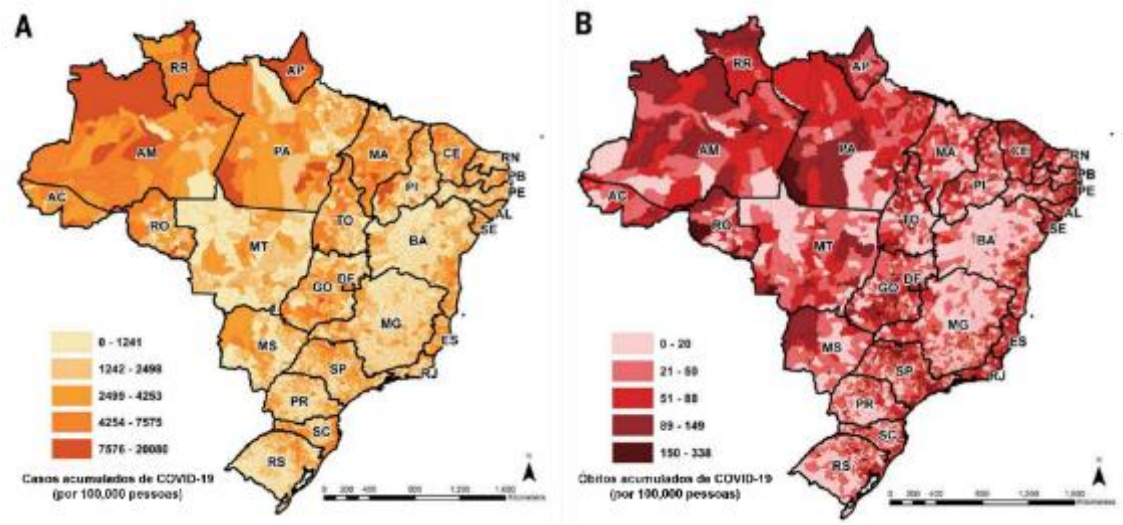


Figura 4: Padrão epidemiológico da COVID-19 no Brasil, representando em escala de cor o número de casos (A) e óbitos (B) de junho a outubro de 2020.
Fonte: Castro et al., 2021 (adaptado por Silva-Junior).

A soroprevalência da população apresentou um aumento exponencial em oito cidades brasileiras, quando avaliados doadores de sangue em diferentes idades e de ambos os gêneros, de 2020 a 2021. Aproximadamente 100% dos doadores de sangue avaliados apresentavam reatividade a anticorpos contra epítopos da COVID-19, principalmente em indivíduos mais jovens, com idade entre 25 e 44 anos, e do gênero masculino (PRETE et al., 2022). Acredita-se que a maior prevalência nessa faixa se dê por conta da exposição durante o período da pandemia, além das medidas preventivas e de restrição empregadas na cidade de Manaus. Até o final de 2022, novos casos de COVID-19, bem como novas variantes do vírus ainda continuam a aparecer, embora com os esforços dos profissionais de saúde, e efeitos da vacinação, a quantidade de internados e óbitos pela doença diminuiu drasticamente (BUSS et al., 2022).

2.3. Mecanismos de diagnóstico e fisiopatológicos do vírus em humanos

As ferramentas de diagnóstico são um ponto muito importante para a identificação dos casos de COVID-19. No início da pandemia, e até mesmo durante, muitos casos foram subdiagnosticados devido aos diagnóstico clínico-epidemiológico empregado nas cidades, e quadros de gripe ou outras doenças eram classificadas como infecção pelo SARS-CoV-2. Com o advento das novas tecnologias de diagnóstico,

específicas para o rastreio dos casos, foi possível a noção real das dimensões de distribuição do vírus dentro das populações.

Aplicação de testes rápidos/imunocromatográficos permitiram a testagem da maior parte dos indivíduos suspeitos, devido ao baixo custo, fácil realização, e sem a necessidade de pessoas com nível de treinamento especializado. Em contrapartida, situações como períodos de janela imunológica permitiam um alto número de pacientes falso-negativos. Cabe ressaltar que a detecção da maior parte dos testes rápidos utiliza o soro ou sangue total dos pacientes, para detectar IgM e/ou IgG, produzidos pelos pacientes. Já testes mais especializados, como o teste tempo real de reação em cadeia da polimerase com transcriptase reversa (RT-qPCR), que utiliza o material genético viral, coletado principalmente da fossa nasal, permite o diagnóstico mais preciso, além de captar o período de janela imunológica do indivíduo (KEVADIYA et al., 2021).

A figura 5 mostra o processo de diagnóstico empregado na detecção de pacientes infectados. Aqueles indivíduos com suspeita clínica de COVID-19, devem ser submetidos ao teste de RT-qPCR, para detecção viral, caso os sintomas tenham aparecido em aproximadamente 5-7 dias prévios ao teste. O diagnóstico molecular inicia com a coleta pelo swab realizado na fossa nasal ou na orofaringe, com posterior extração do RNA viral, produção do DNA complementar (cDNA), amplificação e posterior detecção. Pacientes infectados demonstram quantidades de amplificação proporcionais à carga viral, o que demonstra a reatividade no diagnóstico.

Infecções passadas podem ser detectadas pela presença de anticorpos anti-SARS-CoV-2. As vantagens incluem principalmente a flexibilidade do teste, rapidez, custo e período de diagnóstico. Pacientes que já foram previamente infectados pelo vírus podem apresentar reatividade nos testes por longos períodos (meses ou até mesmo anos). Os testes rápidos foram utilizados no início da pandemia para detecção IgM em pacientes com sintomas recentes, no entanto, apresentavam a limitação da janela imunológica.

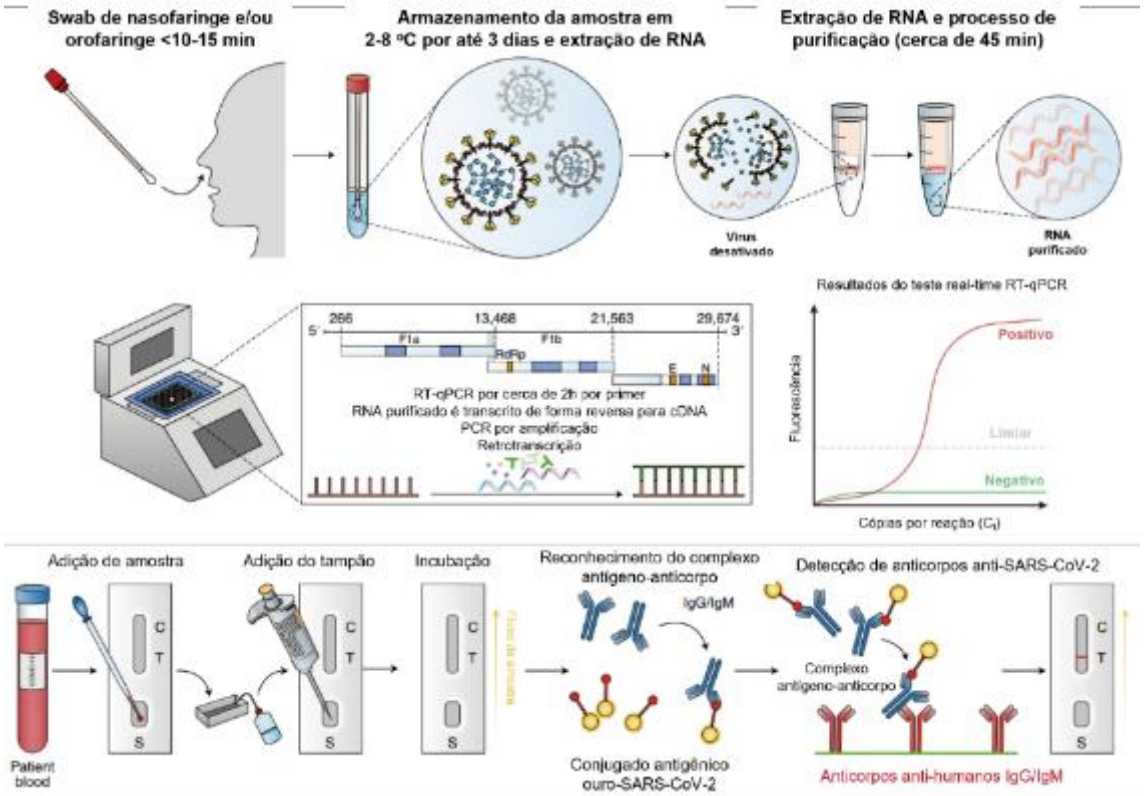


Figura 5: Ferramentas de diagnóstico da COVID-19. Diagnóstico molecular para identificação da carga viral em indivíduos infectados (topo). Detecção de anticorpos anti-SARS-CoV-2 IgM e/ou IgG para diagnóstico sorológico (base).
Fonte: Kevadiya et al., 2021 (adaptado por Silva-Junior).

Foi observado que a detecção dos anticorpos pode ocorrer em 50% dos infectados, caso testados em até 7 dias após infecção, e 100% para aqueles com 14 dias de infecção (KEVADIYA et al., 2021). O mais recomendado para casos suspeitos, é a realização de ambos os testes RT-qPCR e teste rápido, de forma a identificar a presença do vírus, mas também detectar os anticorpos circulantes, visto que o IgM tem um pico de produção 2 semanas após a infecção e tende a baixar, enquanto o IgG permanece alto por vários meses. Esse fator pode contribuir para evitar a recorrência de casos, bem como indicar proteção ao indivíduo.

Quadros novos de infecção iniciam com a entrada do vírus no organismo através das vias superiores, principalmente nas células ciliares da nasofaringe ou traqueia alveolares devido à alta expressão do receptor específico. O vírus utiliza a subunidade 1 da proteína S para se acoplar à ACE2, localizada na célula-alvo e a subunidade 2 para realizar a fusão viral e por fim, adentrar na célula (CEVIK et al., 2020; LAMERS; HAAGMANS, 2022; TURILLI; LUALDI; FASANO, 2022). Com a o reconhecimento

da ACE2, há clivagem da proteína S, e assim, a subunidade 2 é ativada, de forma a permitir que o material genético viral adentre na célula e inicie o processo de replicação (Figura 6) (LAMERS; HAAGMANS, 2022).

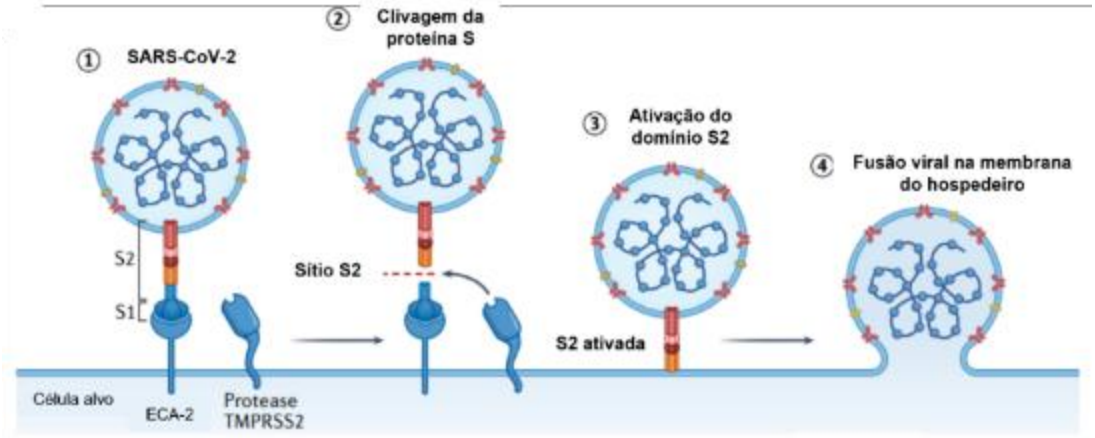


Figura 6: Processo de interação do vírus com a célula-alvo através do receptor ACE2, e entrada na célula.
Fonte: Lamers e Haagmans, 2022 (adaptado por Silva-Junior).

Outras vias de entrada podem ser utilizadas, embora não sejam tão exploradas, como a endossomal (via catepsina), neutropilina-1 e outras proteases (LAMERS; HAAGMANS, 2022).

Outras proteínas de interesse, no vírus, também são observadas, como a proteína M, que possui três regiões transmembranas, é a proteína em maior quantidade no vírion, e assim como a proteína E, são responsáveis por manter a estrutura morfológica do SARS-CoV-2, além também de contribuir no processo de fusão e entrada na célula hospedeira. Outras funções da proteína E estão inseridas no processo de patogênese, montagem e liberação do vírus. A proteína N fica localizada no nucleocapsídio do vírus, e atua na formação do complexo que se liga ao RNA, auxiliando no empacotamento do genoma viral. Por fim, a proteína HE interage com o ácido siálico nas glicoproteínas, e favorece a interação da proteína S, culminando na entrada do vírus na célula, bem como movimentação na mucosa (LIANG et al., 2020).

As etapas de interação do vírus com a célula incluem o processo de ligação no receptor ACE-2, por processos descritos anteriormente. Então, há liberação do genoma viral dentro da célula, e os processos de translação e proteólise, que irão favorecer a

replicação do RNA e por fim, a tradução das proteínas virais. Com isso, o complexo de Golgi faz o encapsulamento das partículas virais e posteriormente liberação do vírus nas vias aéreas, de forma que possa infectar outros tecidos e garantir a contaminação de indivíduos expostos, conforme demonstrado na Figura 7 (CEVIK et al., 2020).

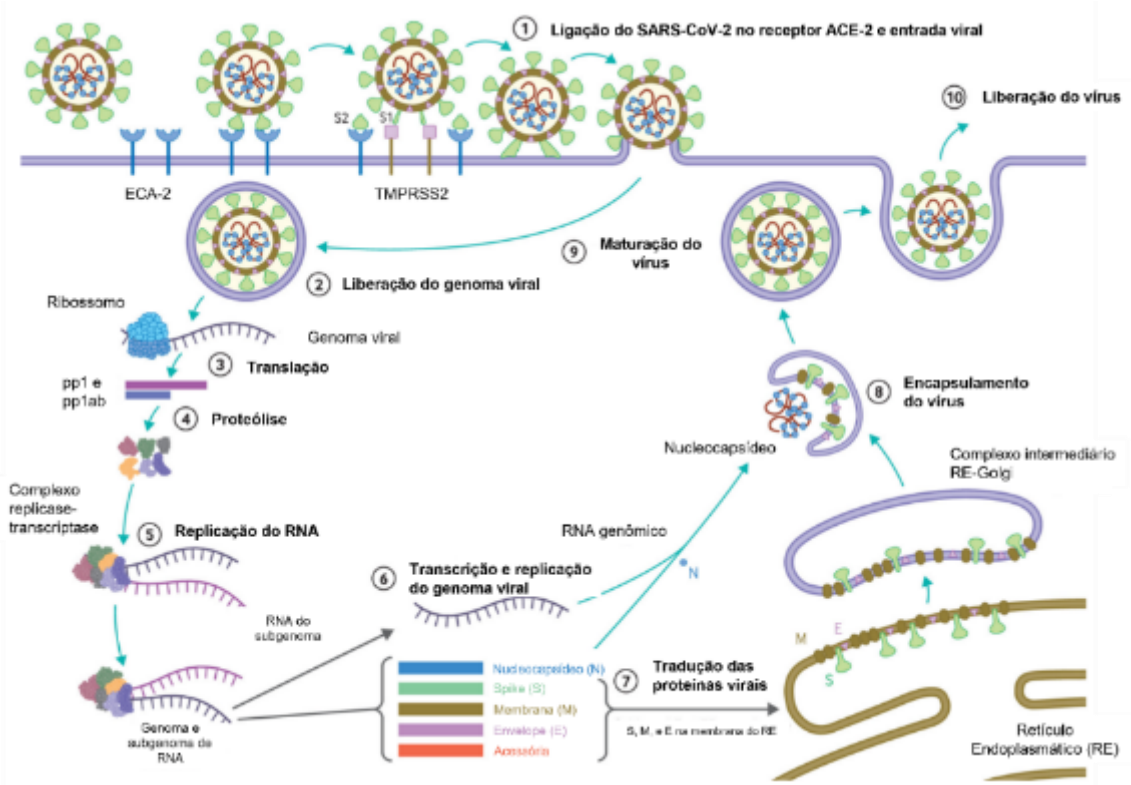


Figura 7: Mecanismo de entrada viral na célula-alvo e processo de replicação do vírus, até a formação de novas partículas virais.
Fonte: Cevik et al., 2020 (adaptado por Silva-Junior).

Com a entrada do vírus, há início da replicação viral e tradução das proteínas envolvidas no processo de replicação. A resposta imune inicia o processo de reconhecimento logo nas vias respiratórias através do reconhecimento dos padrões para que haja produção de interferons do tipo I e III, além das outras citocinas que por sua vez, irão interferir na replicação, bem como na ativação das células do sistema imune. As vias de transcrição serão ativadas tanto com o material genético do vírus, quanto pela ativação autócrina das citocinas produzidas (LAMERS; HAAGMANS, 2022).

Os primeiros locais a sofrerem com a infecção pelo SARS-CoV-2 são as vias respiratórias, e assim, o paciente apresenta sintomas sistêmicos como calafrios, fadiga e febre, no entanto, sintomas localizados em outros tecidos também podem ser observados,

como coração, sistema nervoso, muscular e digestivo (Quadro 2). Cabe ressaltar que nem todos os casos de COVID-19 irão evoluir para o comprometimento dos tecidos, mas também é importante salientar que indivíduos que possuem comorbidades ou doenças pré-existentes, como consequências da idade avançada, hipertensão, doenças cardiovasculares, diabetes, e obesidade, podem apresentar um perfil mais alarmante da doença (CEVIK et al., 2020; HARRISON; LIN; WANG, 2020; KHAN et al., 2021). O comprometimento de outros tecidos se dá possivelmente pela presença do receptor ACE2 em vários tecidos do organismo, além do pulmonar, como o tecido digestivo, renal, cardíaco e os adipócitos, havendo assim, dispersão do vírus para outros sítios de infecção através do vaso sanguíneo (CEVIK et al., 2020; KEVADIYA et al., 2021).

Tecido	**Sintomas**
Nasal	Rinite, faringite, rinorreia, anosmia (perda da sensibilidade de odores).
Garganta	Tosse seca (faringite).
Pulmão	Tosse, dispneia, expectoração, dor no peito, pneumonia, síndrome respiratória aguda grave.
Coração	Arritmia e dor no peito.
Neurológico	Tontura, dores de cabeça, perda de consciência.
Muscular	Mialgia e/ou artralgia, mal-estar, dor muscular no pescoço.
Digestivo	Diarreia, náuseas, vômitos e dor abdominal.

Quadro 2: Principais sinais e sintomas apresentados na COVID-19, com base nos tecidos comprometidos. Fonte: Harrison, Lin e Wang, 2020.

Quadros mais graves tendem a cursar com fadiga, diarreia e falta de ar, embora os que evoluem para óbito demonstram biomarcadores de gravidade, os quais incluem principalmente a interação do sistema imune através da produção de tempestade de citocinas e aumento de leucócitos (KHAN et al., 2021).

2.4. Resposta imunológica ao SARS-CoV-2 na fase aguda

O organismo humano é capaz de reconhecer partículas virais, e responder a elas. Durante o período de incubação, o paciente pode acabar apresentando um quadro assintomático, porém ainda com uma resposta imunológica (ABBAS; LICHTMAN;

PILLAI, 2018). Já foi observado que o paciente com COVID-19 pode apresentar diferentes perfis de resposta, saindo de um quadro leve a grave em poucos dias. Vários fatores estão atrelados a essa mudança, como presença de doenças pré-existentes, interações genéticas, predisposição e subtipo viral. Já foi observado que o vírus pode ser reconhecido no meio extracelular, ou intracelular, no período de replicação através, principalmente, das proteínas RIG-1 e MDA5, que reconhece RNAs dupla fita e induz a produção de interferons e consequente ativação das células do sistema imune (HARRISON; LIN; WANG, 2020; LAMERS; HAAGMANS, 2022).

O padrão de resposta imunológica ao vírus ainda é um campo que necessita de mais informações, uma vez que uma grande parcela de pacientes apresenta um quadro variável em relação aos valores quantitativos dos leucócitos. Os neutrófilos são uma população leucocitária, com grande participação na resposta imune inata, responsáveis pelo combate direto de patógenos e células infectadas, mediado principalmente por fatores como fagocitose, liberação de grânulos, e em alguns casos, apresentação de antígeno à imunidade adaptativa. Valores quantitativos de neutrófilos na doença ativa se mostraram com alto potencial para um preditor de gravidade (LEPPKES et al., 2020; MAN et al., 2021; RODRIGUEZ et al., 2020).

Sabe-se que a participação dos neutrófilos possui um fator primordial na atividade da doença uma vez que a relação com os linfócitos, descritas como a relação neutrófilo-linfócito (RNL) é um parâmetro que pode inclusive ser utilizado no momento da admissão do paciente para estratificá-lo e atuar como prognóstico (CAI et al., 2021; KWIECIEŃ et al., 2021; MARTENS et al., 2021). Foi observado um perfil imunofenotípico caracterizado por neutrófilos imaturos (CD10+CD16low) e com baixa capacidade de adesão (CD11b+) no sangue periférico de pacientes com quadros mais graves (LOURDA et al., 2021; REYES et al., 2021), bem como em pacientes na Unidade de Terapia Intensiva (UTI). Acredita-se que estes fatores estejam ligados à produção das citocinas e fatores de crescimento durante a tempestade de citocinas presentes em condições graves, bem como a um desvio à esquerda devido à atuação local e sistêmica dos neutrófilos, e recrutamento pela medula óssea, mediado por CXCL1, CXCL2, CXCL3, CXCL5, CXCL8, CXCL10, CCL2, CCL20, IL-6 e TNF (DIDANGELOS, 2020;

GEBREMESKEL et al., 2021; KWIECIEŃ et al., 2021; METZEMAEKERS et al., 2021; PARACKOVA et al., 2020).

A condição de hipóxia apresentada pelo paciente durante quadros agudos, ativa fatores de transcrição responsáveis pela indução de citocinas inflamatórias, como IL-1β, IL-6, e CXCL8, embora também tenham sido encontrados níveis elevados de fator derivado de plaquetas 4 (do inglês *Platelet-derived Factor* 4 [PF4]) e CCL5, todos capazes de ativar neutrófilos e induzir mecanismos capazes de agravar a condição clínica do paciente com COVID-19 (GEBREMESKEL et al., 2021; HARRISON; LIN; WANG, 2020; MCELVANEY et al., 2020; MIDDLETON et al., 2020). Além da ativação dos neutrófilos, também há maior produção de redes extracelulares de neutrófilos (NETs), por neutrófilos maduros. Esse componente é capaz de induzir a um estado inflamatório através da ativação da via do inflamassoma e consequente produção de interferons (IFN) do tipo I (AYMONNIER et al., 2022), o que posteriormente pode levar à ativação do sistema imune adaptativo, lesão endotelial e trombose (METZEMAEKERS et al., 2021; MIDDLETON et al., 2020).

Os monócitos, também pertencem à imunidade inata, e assim como os neutrófilos, também realizam o processo de fagocitose e apresentação de antígeno, porém apresentam um papel mais central nestes mecanismos, além de induzir a inflamação local e sistêmica. A participação dos monócitos é algo marcante ao longo da infecção e eliminação do vírus (KWIECIEŃ et al., 2021), com redução após eliminação do vírus, enquanto outros estudos demonstraram resultados contrários, bem como no fluido broncoalveolar (KIM et al., 2022). A migração dos monócitos para o tecido parece ser induzida pela tempestade de citocinas, principalmente IL-6, CXCL10, CCL2, CCL3, CCL4, CCL7 e TNF-α, o que aumenta a permeabilidade dos monócitos ao endotélio e assim, a infiltração pulmonar, mas também o aumento do número de grânulos intracelulares e maior lesão tecidual, o que é um dos fatores agravantes para a inflamação local e consequente destruição dos alvéolos pulmonares (HARRISON; LIN; WANG, 2020; MARTENS et al., 2021).

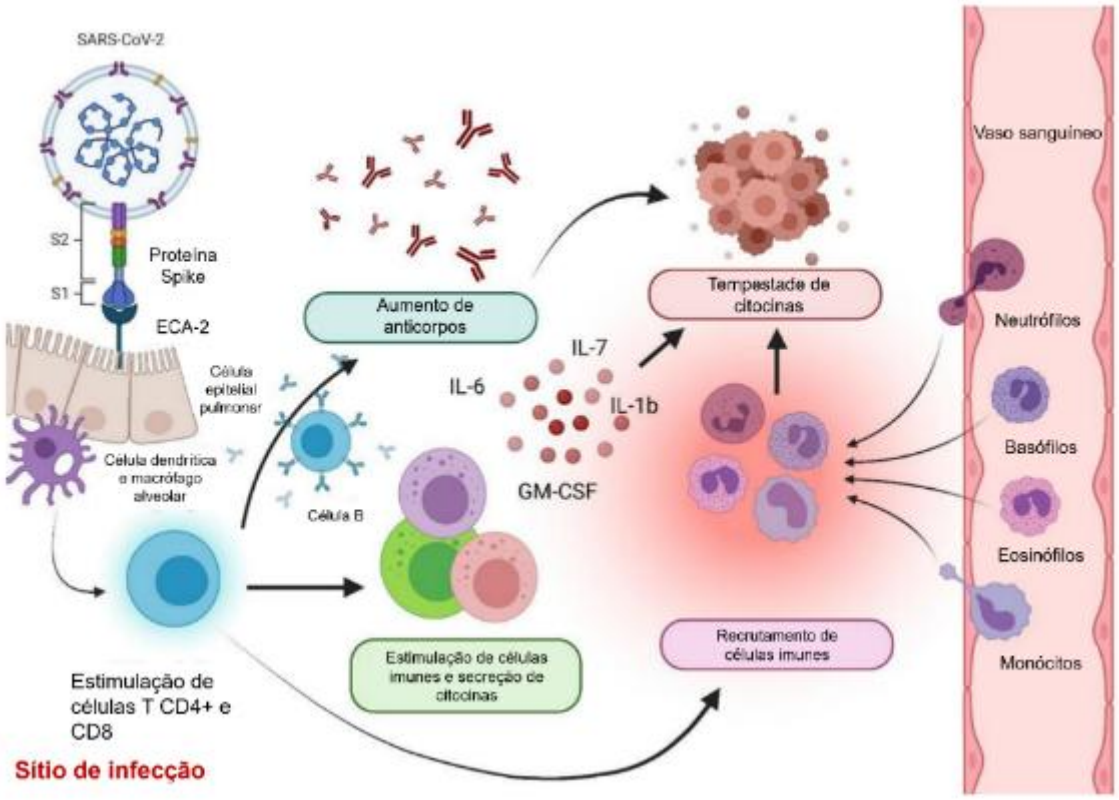


Figura 8: Participação da resposta imunológica em pacientes infectados pelo vírus SARS-CoV-2.
Fonte: Chavda et al., 2021 (adaptado por Silva-Junior).

Marcadores de ativação de monócitos (sCD14 e sCD163) já foram relacionados à gravidade dos pacientes, principalmente por apresentarem uma correlação positiva com a IL-6 e a proteína C reativa (PCR) (HASAN et al., 2021; ZINGAROPOLI et al., 2021). A participação dos monócitos/macrófagos no tecido pulmonar é marcante devido à participação na tempestade de citocinas, com posterior redução quantitativa, aumento de grânulos citoplasmáticos e expressão de moléculas de adesão membranares (MARTENS et al., 2021; ZHOU et al., 2020). A dinâmica da resposta imune necessita de maior compreensão dos mecanismos que levam à uma polarização da resposta, uma vez que macrófagos M1, embora sejam mais inflamatórios, são mais suscetíveis à infecção pelo vírus devido ao alto pH que apresentam, em comparação aos macrófagos M2 (KNOLL; SCHULTZE; SCHULTE-SCHREPPING, 2021; LV et al., 2021).

A participação de outras populações celulares, como eosinófilos, também foi reportada como em baixa quantidade nos indivíduos admitidos na UTI, sendo inclusive sugerido como um marcador de pior prognóstico (GEORGAKOPOULOU et al., 2021;

GLICKMAN et al., 2021; GONZÁLEZ et al., 2021; KWIECIEŃ et al., 2021; TAN et al., 2021; YAN et al., 2021). Em pacientes graves, a interferência na produção da IL-5 pode ser um fator relacionado à eosinopenia (MARTENS et al., 2021). Já em pacientes com um bom curso clínico, a contagem de eosinófilos tende a aumentar cerca de 20 dias após admissão ao hospital, e sua permanência foi relacionada à redução na taxa de mortalidade (FRAISSÉ et al., 2020; GEBREMESKEL et al., 2021; GEORGAKOPOULOU et al., 2021; GONZÁLEZ et al., 2021; XIE et al., 2021). Vários fatores foram sugeridos à eosinopenia, como produção de corticosteroides pela glândula adrenal, o que interfere na liberação de eosinófilos pela medula óssea, bem como aumenta a migração para o tecido; dano medular causado pela COVID-19, embora ainda não totalmente esclarecido; e/ou maior migração dos leucócitos para o tecido pulmonar, e assim, quadro de eosinopenia na corrente sanguínea (XIE et al., 2021). Além disso, também pode estar associado à infecção do vírus neste perfil celular (WEI et al., 2020).

Os eosinófilos também foram relacionados a quadros de pneumonia eosinofílica, caracterizados pela observação de mais de 25% de eosinófilos no tecido pulmonar. Condição esta observada devido à migração de eosinófilos ao pulmão em indivíduos graves que evoluíram a óbito, processo mediado por citocinas e mediadores inflamatórios (KIM et al., 2022). Embora não se saiba ao certo qual a funcionalidade dos eosinófilos na resposta antiviral, foi observado um perfil ativado, por meio da baixa expressão de CD15, CD66b e CD193, e aumento de CD62L e CD147. A expressão de CD69 se mostrou como marcador associado à produção de moléculas inflamatórias, e ao óbito, enquanto CD66b, CD11b, CD11a e CD24 permitiram caracterizar os pacientes em estágio moderado (LOURDA et al., 2021).

Muitos estudos avaliam a resposta imunológica frente à COVID-19, em indivíduos infectados pelo vírus. Muitos fatores estão ligados ao perfil de resposta, bem como à capacidade do sistema imune de produzir citocinas e outros mediadores inflamatórios. Respostas mais exacerbadas foram observadas em indivíduos mais velhos, assim como com comorbidades, como diabetes (IMRAN et al., 2020; LIU et al., 2020a; MORADI et al., 2021a) e asma (EGUÍLUZ-GRACIA et al., 2018; NIESSEN et al., 2021). Em contrapartida, poucos estudos avaliam o paciente após a cura clínica, no estágio de convalescença.

2.5. Convalescença: o que sabemos até agora?

A fase convalescente se refere a um estágio após a COVID-19, com a melhora no quadro clínico, no entanto, os estudos divergem quanto ao período de análise realizado em indivíduos convalescentes da COVID-19. A compreensão do sistema imune neste estado é primordial para melhora clínica e identificação de novos biomarcadores relacionados ao melhor ou pior prognóstico dentro da imunidade adaptativa (NG et al., 2021; RAJAMANICKAM et al., 2021).

Com o desenvolvimento da fase convalescente, tem-se uma participação maior de neutrófilos ativados (CD14/CD11b+) aproximadamente 30 dias após a cura clínica (CHAO et al., 2021; KWIECIEŃ et al., 2021), com redução de neutrófilos ativados (CD64+) (SEERY et al., 2021) e a produção de NETs semanas após o teste positivo (MIDDLETON et al., 2020; PARACKOVA et al., 2020). Ainda na fase convalescente, foi observada maior prevalência de granulócitos imaturos, provavelmente devido à baixa funcionalidade dos neutrófilos, bem como à intensa necessidade de produção celular pela medula óssea (KIM et al., 2021; KWIECIEŃ et al., 2021).

A análise quantitativa dos monócitos demonstrou retorno à normalidade após a fase aguda, independente do estágio. Condições críticas foram caracterizadas por um alto número de monócitos, que posteriormente decaiu para níveis normais na convalescença, enquanto que a monocitopenia em pacientes graves apresentou melhora significativa (NEELAND et al., 2021; QIN et al., 2021; RAJAMANICKAM et al., 2021). Em contrapartida, a participação das subpopulações de monócitos apresentou uma melhora quantitativa apenas cerca de 150 dias após a cura clínica, demonstrando uma possível atuação reparadora dos monócitos/macrófagos dentro da convalescença da COVID-19 (NEELAND et al., 2021; RAJAMANICKAM et al., 2021).

Em relação aos linfócitos, há uma melhora rápida dos linfócitos em pacientes leves e moderados, com predomínio de citocinas de perfil Th1, embora apresente melhora nos valores das células T auxiliares, citotóxicas e de memória, esta ocorre em um longo período de tempo (SHUWA et al., 2021; ZHANG et al., 2020c). Células de memória foliculares específicas para a proteína S apresentaram queda nos primeiros 4 meses após o *clearance* viral, mas ainda houveram níveis detectáveis tanto da proteína S quanto da N 6 meses após (FERRERAS et al., 2021; WHEATLEY et al., 2021; ZUO et al., 2021a).

A participação de outras células também se apresentam como potenciais biomarcadores, visto que a maior parte da interação da resposta ocorre via sistema imune inato, e a redução leva a um melhor prognóstico, tanto dos eosinófilos (KIM et al., 2022; KWIECIEŃ et al., 2021; VITTE et al., 2020), basófilos (RODRIGUEZ et al., 2020) e células dendríticas (PARACKOVA et al., 2020; WINHEIM et al., 2021). No entanto, mais estudos se fazem necessários para compreender a dinâmica imunológica que ocorre no indivíduo na fase aguda, e na fase convalescente, para melhor compreender os mecanismos envolvidos na produção de anticorpos, melhor clínica dos pacientes, e melhor grau de imunização da população com histórico de COVID-19.

2.6. Relevância

A pandemia causada pelo SARS-CoV-2 levou a um alto número de casos e óbitos ao longo do mundo. Sua disseminação começou no final do ano de 2019, e devido à inexperiência imunológica da população, houve fácil e rápida disseminação do vírus. Desde então, o enorme montante de casos veio diminuindo, no entanto ainda são observadas internações hospitalares e óbitos advindos das complicações causadas durante a fase aguda da doença.

O período pandêmico foi marcado por um caso na saúde pública do Brasil, principalmente na cidade de Manaus-AM, devido ao desenvolvimento da nova variante, o que levou ao aumento alarmante do número de casos na região nortista, bem como a intensa necessidade de oxigênio para os pacientes acometidos. Com o perfil epidemiológico traçado, várias medidas de contenção foram aderidas, de forma a evitar a dispersão do vírus, no entanto, muitos óbitos ainda ocorreram até a implantação de novos imunizantes contra o vírus.

Com a entrada do vírus no hospedeiro humano, há infecção principalmente das células pulmonares, causando quadros de febre, tosse e falta de ar, que podem ser desde leves até graves. A atuação do sistema imunológico é marcante, e muitos estudos trouxeram novas perspectivas nos pacientes infectados pelo vírus, e com SARS, tanto ao analisar células, quanto moléculas. Em contrapartida, poucos estudos observam a presença de biomarcadores imunológicos dentro do contexto convalescente dos pacientes.

O estado de convalescença é marcado pelo período após infecção pelo vírus, e resolução do quadro, de forma que o paciente apresente melhor clínica do sintoma, e um resultado de diagnóstico viral negativo ou não reagente. Entender a dinâmica imunológica por trás da resolução da infecção, tanto na fase aguda, quanto na fase de transição para a resposta imune adaptativa tem tomado um eixo chave na compreensão dos mecanismos imunológicos, bem como na predição de novos marcadores.

Com isso, devido à escassez de estudos sobre os pacientes convalescentes, ligado à necessidade de se entender a dinâmica envolvida na fase aguda, e as mudanças para a fase convalescente, demonstram a necessidade em compreender os mecanismos celulares da resposta imune, incluindo as subpopulações de células, bem como a produção de mediadores inflamatórios, pró-inflamatórios, e principalmente a produção de anticorpos contra as proteínas virais, de forma a melhorar medidas de prevenção e acompanhamento daqueles pacientes com o SARS-CoV-2, mas principalmente entender a sinergia na fase convalescente.

3. OBJETIVOS

3.1. Geral

Avaliar o perfil imunofenotípico e inflamatório de pacientes infectados e convalescentes da infecção pelo SARS-CoV-2.

3.2. Específicos

- Caracterizar o perfil sociodemográfico, celular circulante e de memória de pacientes leves, moderados, graves e convalescentes da infecção pelo SARS-CoV-2;
- Quantificar citocinas, quimiocinas e fatores de crescimento em pacientes leves, moderados, graves e convalescentes da infecção pelo SARS-CoV-2;
- Identificar marcadores imunológicos e laboratoriais alto produtores de pacientes leves, moderados e graves com COVID-19;
- Descrever biomarcadores imunológicos e laboratoriais para fim de decisão para desfechos clínicos de pacientes com COVID-19 hospitalizados;
- Determinar a relação de marcadores imunológicos no início da fase de convalescença que impactam na produção de anticorpos neutralizantes contra SARS-CoV-2;
- Destacar os principais marcadores laboratoriais envolvidos na fase convalescente da COVID-19 até 90 dias após a cura clínica.

4. METODOLOGIA

4.1. Tipo de pesquisa

Este é um estudo que se divide em descritivo longitudinal e exploratório. Para o descritivo foram feitas coletas de materiais biológicos de três grupos: 1) Candidatos aptos à doação de sangue, sem histórico de COVID-19 e sem nenhuma doença aparente, como grupo de doadores saudáveis, coletados previamente à pandemia da COVID-19; 2) Pacientes infectados pelo SARS-CoV-2, diagnosticados por RT-qPCR, com sintomas clínicos leves, e que não estavam internados em hospital para tratamento dos sintomas da COVID-19; 3) Pacientes infectados pelo SARS-CoV-2, diagnosticados por RT-qPCR, e internados em hospital de referência devido aos sinais e sintomas graves. Para o quarto grupo foi feito um acompanhamento longitudinal: 4) Indivíduos convalescentes da COVID-19, aptos à doação de sangue, e que atingiram a cura clínica há aproximadamente 30 dias.

Os indivíduos do grupo convalescente foram avaliados de forma longitudinal, com coletas biológicas por mais dois meses, descritos neste trabalho como D30 (30 dias após a cura clínica), D60 (60 dias após a cura clínica) e D90 (90 dias após a cura clínica).

4.2. Aspectos éticos

Este projeto faz parte de um projeto maior, intitulado “Estudo de Biomarcadores Imunológicos em Pacientes Convalescentes da Infecção pelo Vírus SARS-CoV-2 (COVID-19)”, submetido e aprovado pelo Comitê de Ética em Pesquisa da Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas (CEP-HEMOAM) sob o número do parecer: 4.126.784. Todos os participantes aceitaram participar do projeto através da assinatura do Termo de Consentimento Livre e Esclarecido (TCLE), segundo recomendações da Resolução N° 466/2012, e a Declaração de Helsinki.

4.3. População amostral

Este projeto foi composto por indivíduos recrutados por demanda espontânea, onde foram incluídos 51 candidatos aptos à doação de sangue como grupo de indivíduos saudáveis. As amostras foram coletadas em outro projeto de pesquisa, previamente aprovado pelo CEP-HEMOAM, e conduzido anos antes da pandemia da COVID-19.

Para o grupo de pacientes com a infecção pelo SARS-CoV-2 foram incluídos 46 pacientes. Estes pacientes foram divididos em dois grupos: 1) 28 pacientes que necessitaram de internação hospitalar, recrutados e incluídos pelo Hospital Universitário Getúlio Vargas (HUGV); 2) 18 pacientes que não necessitaram de internação hospitalar, mas aceitaram participar do projeto de pesquisa na fase sintomática.

Além disso, foram incluídos 139 indivíduos por conveniência, que atingiram a cura clínica da COVID-19 a aproximadamente 30 dias, como grupo convalescente. Esses pacientes foram recrutados para doação de plasma convalescente, e foram acompanhados de forma longitudinal, com uma coleta do sangue periférico ao longo do período de três meses, como citado anteriormente.

4.4. Critérios de elegibilidade

4.4.1. Critérios de inclusão

Em ambos os grupos de doadores saudáveis e de pacientes convalescentes foram incluídos indivíduos de ambos os gêneros, maiores de 18 anos e que aceitaram participar do projeto através da assinatura do TCLE. Para o grupo de doadores de sangue saudáveis, foram incluídos indivíduos aptos à doação de sangue e que passaram pelos critérios clínicos e laboratoriais estabelecidos pelo Ministério da Saúde. Todos os incluídos de ambos os grupos testaram negativo para HIV, HBV, HCV, Sífilis, Doença de Chagas e HTLV, além de não apresentarem nenhum sintoma no momento da doação de sangue.

Para o grupo de pacientes convalescentes, foram incluídos aqueles que além de atenderem aos critérios para doação de sangue, também tiveram o diagnóstico de infecção pelo SARS-CoV-2 por RT-PCR, com quadro sintomático, mas que declararam ausência dos sintomas a aproximadamente 30 dias, e apresentavam um resultado de RT-PCR negativo no momento da coleta.

Para os indivíduos com COVID-19, foram incluídos aqueles com o diagnóstico por RT-PCR, com a fase sintomática da doença ainda em atividade. Foram incluídos indivíduos que necessitaram de internação hospitalar no Hospital HUGV, bem como aqueles que não necessitaram de atendimento hospitalar.

4.4.2. Critérios de não inclusão

Para indivíduos que participaram do grupo convalescente, não foram incluídos aqueles que faziam uso de medicamentos inibidores da Enzima Conversora de Angiotensina, ou com anticorpos anti-eritrocitários.

Independente do grupo, não foram incluídos gestantes, puérperas, indígenas ou menores de idade.

4.4.3. Critérios de exclusão

Foram excluídos pacientes que não possuíam amostra biológica suficiente para realização dos procedimentos metodológicos. Do grupo convalescente, foram excluídos aqueles com a doença sintomática no momento da inclusão, que ainda possuíam o teste de RT-PCR para SARS-CoV-2 positivos no momento da inclusão, e ou aqueles com reatividade sorológica para algum patógeno de importância transfusional. Além disso, ainda do grupo convalescente, foram excluídos das análises longitudinais, aqueles que não possuíam os três acompanhamentos.

4.5. Recrutamento, coleta de dados sociodemográficos e amostras biológicas

Para o grupo de indivíduos saudáveis, os doadores foram recrutados durante o processo de doação de sangue, após atenderem aos critérios clínicos para doação. As amostras biológicas deste grupo foram obtidas antes do primeiro relato de caso de SARS-CoV-2 no final de 2019, garantindo a confiabilidade como grupo de doadores saudáveis. Para este grupo foi aplicado um questionário para coleta dos dados sociodemográficos, e posteriormente foram coletados 8 mL do sangue periférico, distribuídos em um tubo de EDTA K2 (BD Vacutainer® EDTA K2) e um tubo com gel separador (Gel BD SST® II Advance).

A lista de pacientes diagnosticados com COVID-19 por RT-PCR foi obtida da Fundação de Vigilância em Saúde (FVS-AM). Os pacientes eram contatados por telefone, e convidados a participar do estudo após a contagem de aproximadamente 30 dias da pausa dos sintomas. Caso aceitassem, se dirigiam à Fundação HEMOAM, onde o projeto era explicado, assinavam o TCLE. A participação destes pacientes seguia com a aplicação de um questionário para coleta de dados sociodemográficos, e uma coleta de 8 mL de sangue, distribuídos em um tubo de EDTA e um tubo com gel separador do sangue periférico.

Os pacientes internados no HUGV foram abordados e convidados a participar do estudo, onde foram coletados 8 mL distribuídos em um tubo de EDTA e um tubo com gel separador do sangue periférico para exames de rotina, e que foram aproveitados para realização dos testes da pesquisa, desde que utilizados em até 12 horas após a coleta. Os dados epidemiológicos foram coletados de prontuários físicos e/ou eletrônicos e armazenados em tabelas do Microsoft Excel 2010.

Indivíduos do grupo de pacientes diagnosticados com COVID-19, mas que não necessitaram de internação hospitalar foram recrutados da lista de indivíduos reportados pela FVS-AM. Caso aceitassem participar do estudo, a coleta era realizada em um local isolado, garantindo todos os cuidados com a biossegurança.

Foram coletados dados epidemiológicos referentes à data de nascimento, cidade e estado de nascimento, profissão, raça, peso, altura, endereço e gênero. Quanto às variáveis clínicas, foram coletados os dados de tipagem sanguínea ABO e RhD, data de início e término dos sintomas, se internaram em hospital, com data de entrada e alta, se fizeram uso de ventilação mecânica, quais medicamentos tomam rotineiramente, bem como se tomaram alguma vacina contra a COVID-19. Os dados de vacinação eram coletados em cada visita.

4.6. Análise do hemograma

O hemograma de todas as amostras foi realizado com o sangue total, coletado no tubo de EDTA, no laboratório de hematologia da Fundação HEMOAM em no máximo 48 horas após a coleta. Foi utilizado o contador hematológico automático do setor para esta finalidade, ADVIA 2120i (Siemens, USA). Os parâmetros utilizados para análise foram os dados hematimétricos de contagem global de hemácias, hematócrito, hemoglobina, volume corpuscular médio (VCM), hemoglobina corpuscular média (HCM), concentração de hemoglobina corpuscular média (CHCM), *red distribution width* (RDW), contagem global de leucócitos (WBC), e os valores absolutos de neutrófilos, monócitos, linfócitos, eosinófilos, basófilos e plaquetas.

4.7. Análise da produção de anticorpos

Foi realizada a avaliação qualitativa e quantitativa de anticorpos de classe IgM e IgG contra as proteínas do SARS-CoV-2 no soro dos participantes. Primeiramente,

como critério de inclusão, foi realizado um teste imunocromatográfico, qualitativo contra o SARS-CoV-2, para detecção de anticorpos de classe IgM e/ou IgG, utilizando uma gota de sangue total (20 µL), obtido por punção no dedo anelar, ou 10 µL obtido do soro. A gota era adicionada ao teste do kit COVID-19 IgG/IgM ECO Teste da ECO Diagnóstica (Lote 202009032), e considerado positivo ao menor sinal de reatividade nas linhas teste "M" e/ou "G", analisada por pelo menos dois integrantes da equipe. A presença da barra do controle era um critério para que o teste pudesse ser levado em consideração, e todo o protocolo foi seguido conforme recomendações do fabricante. Dados de sensibilidade e especificidade do teste estão descritos nos Anexos.

Posteriormente, o soro obtido nos tubos com gel separador foi empregado para a confecção dos testes de imunoensaio de micropartículas por quimioluminescência (CMIA). Para o teste qualitativo, foram utilizados 200 µL para detecção de anticorpos de classe IgG com o kit SARS-CoV-2 IgG ARCHITEC (Abbott), onde o resultado foi expresso como Index (S/C), e utilizado para classificar em positivo (Index $\geq$ 1.4) ou negativo (Index < 1.4). O valor quantitativo expresso em Index, e o resultado qualitativo foram armazenados para fins analíticos. Todos os procedimentos foram seguidos conforme recomendações do fabricante. O resultado de concordância percentual positiva após o início dos sintomas indicado pelo fabricante está descrito nos Anexos.

Para o teste qualitativo e quantitativo, foram utilizados 200 µL para detecção de anticorpos de classe IgG pela técnica de CMIA com o kit SARS-CoV-2 IgG II Quant ARCHITECT (Abbott), onde o resultado do ensaio foi obtido de forma qualitativa através do valor de corte de 50.0 AU/mL, e quantitativa, expresso pela concentração em AU/mL. O resultado qualitativo, e o resultado quantitativo foram utilizados para análise dos dados. O protocolo foi seguido conforme recomendações do fabricante. O resultado de concordância percentual positiva por dias após o início dos sintomas e PCR positivo indicado pelo fabricante está descrito nos Anexos.

4.8. Avaliação das populações celulares por imunofenotipagem

O sangue total coletado no tubo de EDTA foi utilizado para imunofenotipagem, utilizando anticorpos monoclonais conjugados a fluorocromos, capazes de emitir diferentes feixes de fluorescência quando interagem com a proteína-alvo.

Para a marcação dos linfócitos T, foi empregado primeiramente o anticorpo monoclonal anti-CD3, com posterior uso dos linfócitos T citotóxicos ativados (CD3CD8+CD69+) e não ativados (CD3+CD8+CD69-). Para os linfócitos B, foram marcados os linfócitos B convencionais (CD19+) e B1 (CD19+CD5+). Os monócitos foram segregados quanto ao subtipo clássico ($CD14^{high}CD16^{-}$), intermediário ($CD14^{high}CD16^{+}$) e não clássico ($CD14^{low}CD16^{+}$). As células dendríticas foram segregadas em convencionais (CD14-CD11c+) e plasmacitóides (CD14-CD123+). A expressão de CD3 foi utilizada para segregar as células NK em NK (CD3-CD56+CD16+) e NKT (CD3+CD56+CD16+). No Quadro 3 estão dispostas as marcações empregadas nos diferentes tubos, bem como os anticorpos utilizados e os respectivos fluorocromos.

Células	Anticorpo (s)	Fluorocromo
Linfócitos T	Anti-CD3	APC
	Anti-CD8	PE
	Anti-CD69	PERCP
	Anti-CD4	FITC
Linfócito B	Anti-CD5	FICT
	Anti-CD19	PE
Monócitos	Anti-CD16	FITC
	Anti-CD14	APC
	Anti-HLA-DR	PE
Célula Dendrítica	Anti-CD123	FITC
	Anti-CD14	APC
	Anti-CD11c	PE
Células NK	Anti-CD3	APC
	Anti-CD16	FITC
	Anti-CD56	PE
	Anti-CD69	PERCP

Quadro 3: Painel de marcadores imunofenotípicos com as células identificadas por imunofenotipagem e citometria de fluxo. Constam os anticorpos utilizados para clusterizar as populações, bem como os fluorocromos conjugados.

A marcação com imunofenotipagem foi analisada pela técnica de citometria de fluxo, no laboratório de Marcadores Celulares da Fundação HEMOAM. Foi utilizado o citômetro FACSCanto II para aquisição das amostras, e posteriormente analisadas pelo software FlowJo v.10.8, para construção das *gates* e quantificação da porcentagem dos perfis celulares.

Os leucócitos foram separados primeiramente com base nos parâmetros de *Forward Scatter* (FSC) e *Side Scatter* (SSC), onde posteriormente foram eliminadas as

hemácias não lisadas, com base no pequeno tamanho e complexidade. Posteriormente, foi empregada uma *gate* diferente para cada subpopulação de célula, conforme as figuras 9 e 10.

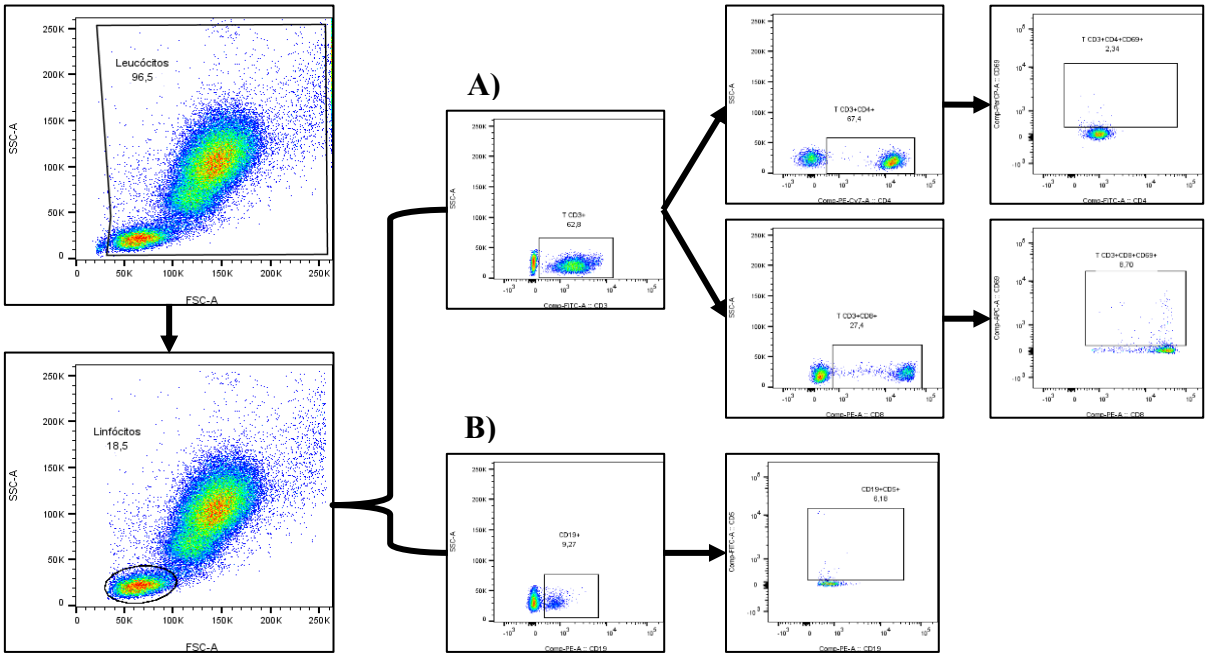


Figura 9: Análise imunofenotípica das células da imunidade adaptativa, com a delimitação dos leucócitos e linfócitos no software FlowJo v.8.10. **A)** Painel de marcação para linfócitos T (CD3+), T auxiliares (CD3+CD4+), T citotóxicos (CD3+CD8+) e ativados (CD69+). **B)** Painel de marcação para linfócitos B (CD19+) e B1 (CD19+CD5+).

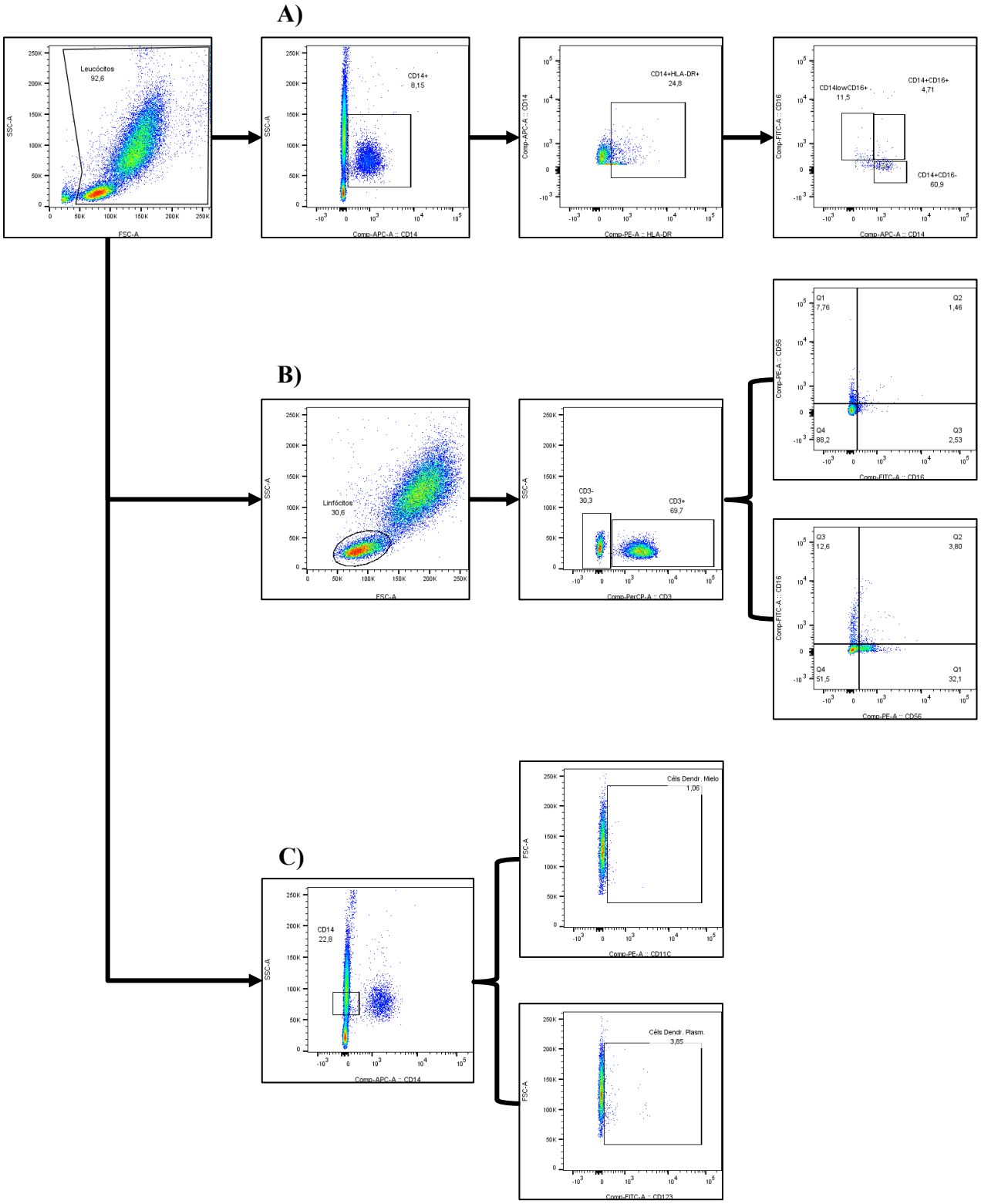


Figura 10: Análise imunofenotípica das células da imunidade inata, com delimitação dos leucócitos no software FlowJo v.8.10. Estratégia utilizada para identificação e quantificação de **A)** monócitos ativados (CD14+HLA-DR+) e suas subpopulações clássico ($CD14^{high}CD16^{-}$), intermediário ($CD14^{high}CD16^{+}$) e não clássico ($CD14^{low}CD16^{+}$); **B)** Células NK (CD3-CD16+CD56+) e NKT (CD3+CD16+CD56+); **C)** Células dendríticas convencionais (CD14-CD11c+) e plasmacitóides (CD14-CD123+).

4.9. Quantificação das moléculas

Para quantificação das moléculas inflamatórias, foi utilizado o soro, obtido na amostra com gel separador, onde foram dosadas as citocinas: Eotaxin, IL-1β, IL-1ra, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-15, IL-17A, IFN-γ, TNF-α, IP-10, MCP-1 (MCAF), MIP-1α, MIP-1β, RANTES, FGF basic, G-CSF, GM-CSF, PDGF-BB e VEGF.

Essas moléculas foram dosadas pela técnica de Luminex, que emprega *beads* magnéticas com diferentes intensidades de fluorescência, conjugadas a anticorpos específicos para cada molécula analisada. Para esta finalidade, foi utilizado o kit *Bio-Plex Pro-Human Cytokine Standard 27-Plex* da Bio-Rad, seguindo as recomendações do fabricante. A aquisição dos resultados obtidos foi realizada no equipamento Luminex 200 System, e a análise da fluorescência para obtenção dos resultados quantitativos foi analisado pelo software *Bioplex Manager*. Os valores das concentrações foram utilizados para o armazenamento dos dados e posterior análise estatística. Este procedimento foi realizado no Instituto René Rachou, na Fundação Oswaldo Cruz de Minas Gerais.

4.10. Análise de dados

Os dados qualitativos e quantitativos foram tabulados e armazenados no programa Microsoft Excel 2010. Posteriormente, foi utilizado o software GraphPad Prism v.5.0 (San Diego, CA, USA) para análise da normalidade pelo teste de Shapiro-Wilk. O resultado obtido foi utilizado para escolha do plano estatístico posterior.

Para a primeira análise, comparando os dados quantitativos dos indivíduos do grupo saudável, pacientes com COVID-19 leve, moderados e graves, foi utilizado o teste de Kruskal-Wallis, com pós-teste de Múltiplas Comparações de Dunn. Já para a análise pareada de todos os indivíduos convalescentes, foram isolados os 67 doadores que realizaram o acompanhamento ao longo de todos os três meses, e para esta análise, foram utilizados os testes pareados. O resultado do teste quantitativo apresentou distribuição não Gaussiana, e portanto, foram empregados testes não paramétricos nas análises. Os indivíduos vacinados não foram utilizados nas análises gerais. Para todas as análises, foi empregado o intervalo de confiança de 95% e valor de p significativo quando $p < 0,05$.

A análise de correlação dos parâmetros hematológicos com a produção quantitativa de anticorpos foi realizada pelo teste de correlação de Spearman, considerando correlações positivas e negativas, quando $p < 0,05$. As análises foram realizadas em dois softwares: GraphPad Prism v. 9.0 e R Studio, conforme descrito nos Resultados.

A análise de modelo misto foi empregada para comparar os grupos leve, moderado e grave, dos pacientes com COVID-19, e utilizando o grupo de indivíduos saudáveis como referência. Com base no valor do logFC, os parâmetros eram segregados quanto reguladores positivos ($logFC > 0$) ou negativos ($logFC < 0$). Foram utilizados parâmetros significativos, quando o valor de p ajustado < 0.05. Para essa análise, os dados de gênero e idade foram empregados como cofatores de interferência. Essa análise foi realizada no R Studio, com protocolos e pipelines de análise padronizados e validados.

4.11. Riscos e benefícios

Este estudo apresentou o risco de desconforto e produção de hematoma ou equimose (mancha arroxeada), no local da coleta do sangue periférico a todos os participantes durante a coleta do material biológico. Para evitar ou reduzir este risco, a coleta foi realizada por profissionais treinados da Fundação HEMOAM, dispostos a dar o devido suporte em intercorrências durante e após o processo de coleta sanguínea. Também houve o risco de exposição de resultados laboratoriais. Para reduzir este risco, foi atribuído código aos participantes, não sendo possível identificá-los por ninguém, além dos coordenadores do estudo.

Os resultados obtidos poderão auxiliar em novas formas de diagnóstico da COVID-19, bem como aumentar a compreensão do perfil imunológico de pacientes com a doença em atividade, mesmo que em contextos clínicos diferentes, além de monitorar o perfil de resposta humoral no período de três meses após a cura clínica. Estes dados poderão nortear estudos futuros para identificação de novos alvos terapêuticos para tratamento e diagnóstico.

5. RESULTADOS

Os resultados deste estudo serão divididos em duas partes principais, com base nos resultados e grupos. Os capítulos serão distribuídos em:

- 5.1: Resultados da análise dos pacientes diagnosticados com COVID-19, que estavam internados no hospital HUGV, durante o recrutamento hospitalar. Estes dados estão descritos no manuscrito presente no capítulo 8.3 desta tese.
- 5.2: Resultados da análise de pacientes convalescentes da COVID-19, que estavam sem sintomas a pelo menos 30 dias. Estes dados estão presentes no artigo presente no capítulo 8.2, e publicados na revista *Scientific Reports* em 2024 (DOI: https://doi.org/10.1038/s41598-024-71419-x).
- No apêndice, também há um artigo de revisão narrativa, com materiais publicados acerca da resposta imunológica na fase aguda e convalescentes da COVID-19. Este artigo foi publicado na revista *Immuno* em 2023 (DOI: https://doi.org/10.3390/immuno3010007).

5.1. Análise de marcadores imunológicos em doadores de sangue saudáveis e pacientes com COVID-19 leve e hospitalizados

5.1.1. Distribuição sociodemográfica dos indivíduos saudáveis e pacientes com COVID-19

O grupo de 51 doadores de sangue foi composto por 36 homens (70.6%) e 15 mulheres (29.4%), com idade média de 32 anos (DP ± 12 anos). Já o grupo de pacientes com COVID-19 leve foi composto por 9 homens (75%) e 3 mulheres (25%), com média de idade de 39 anos (DP ± 8.9 anos). Dos 15 pacientes que necessitaram de atendimento hospitalar, foram subdivididos em: 6 com COVID-19 moderada, sendo 2 homens (33%) e 4 mulheres (67%) com idade média de 46 anos (DP ± 23.8 anos) que não necessitaram de ventilação mecânica; e 9 com COVID-19 graves, distribuídos em 5 (56%) homens e 4 (44%) mulheres, com idade média de 59 anos (DP ± 21 anos) que necessitaram de ventilação mecânica durante a estadia no hospital. A análise de dados pelo teste de Qui-quadrado mostrou diferença significativa na distribuição de gênero entre os grupos ($p < 0.0001$), como mostrado na Figura 11.

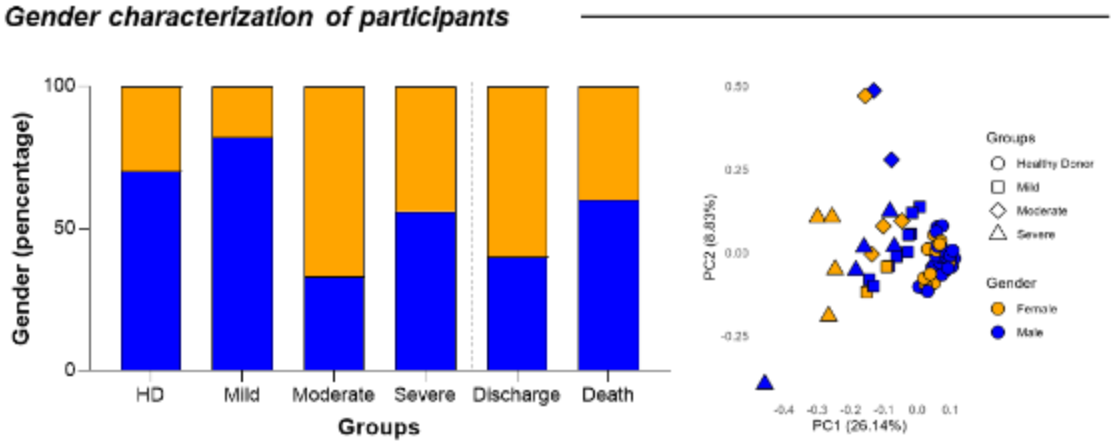


Figura 11: Distribuição do gênero entre os grupos de indivíduos saudáveis e pacientes com COVID-19. Análise realizada pelo teste exato de Fisher com os valores percentuais de cada grupo, distribuídos entre homens e mulheres (esquerda); PCA categorizado pelos grupos de indivíduos saudáveis (n = 51), leves (n = 12), moderados (n = 6) e graves (n = 9), e coloridos pelo gênero dos participantes (direita).

Por outro lado, observamos que pacientes graves apresentaram uma idade maior que o grupo de indivíduos saudáveis ($p < 0.0001$) e leves ($p = 0.006$), mas não houve

diferença entre o grupo leve. O PCA demonstrou uma segregação conforme idade, entre os grupos, embora não foi possível delimitar os limites dos clusters, é possível observar que aqueles participantes mais jovens são mais facilmente segregados daqueles com idade mais avançada (Figura 12).

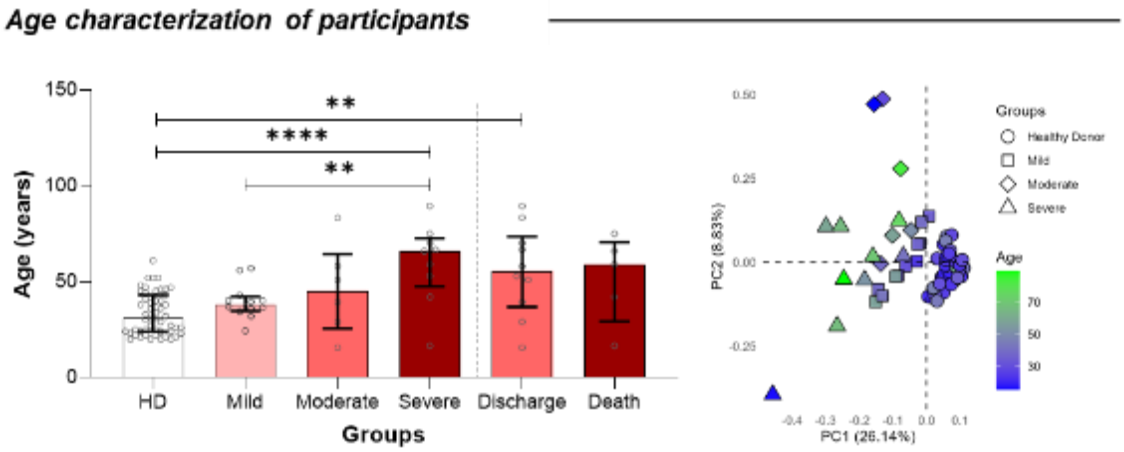


Figura 12: Distribuição da idade entre os grupos de indivíduos saudáveis e pacientes com COVID-19. Comparação das idades pelo Teste de Kruskal-Wallis e Mann-Whitney (esquerda); PCA categorizado pelos grupos e colorido pela escala de idade dos participantes (direita).

Dos 15 participantes hospitalizados, 2 (13.3%) não tinham histórico prévio de comorbidades. 7 (46.6%) tinham diabetes [3/7 foram a óbito], 6 (40%) tinham hipertensão [2/6] foram a óbito, 6 (40%) tinham alguma neoplasia [2/6 foram a óbito]. Quanto ao desfecho clínico, os 15 pacientes hospitalizados foram subdivididos em Alta (n = 10, 4 [40%] homens e 6 [60%] mulheres), a qual ocorreu em média 33 dias (± 24) dias após internação. Apenas 5 (50%) necessitaram de CTI e 5 (40%) necessitaram de ventilação mecânica. Por outro lado, os outros 5 [3 [60%] homens e 2 [40%] mulheres] pacientes que evoluíram à Óbito em uma média de 72 dias (± 44) após admissão hospitalar. A análise estatística demonstrou diferença na proporção do gênero ($p < 0.0001$) e idade (Doadores Saudáveis vs Alta; $p = 0.002$), como demonstrado na Figura 12.

5.1.2. A inflamação causada pela COVID-19 é marcada pela relação de marcadores inflamatórios e pró-inflamatórios

Os pacientes graves apresentaram níveis baixos de RBC, hemoglobina, hematócrito, mas também um aumento de WBC e contagem de neutrófilos, conforme

Tabela 1. Embora a contagem de linfócitos totais tenha caído drasticamente nos pacientes leves, não houve diferença entre o grupo moderado e grave. A Razão Neutrófilo-Linfócito (do inglês *Neutrophil-Lymphocyte Ratio* [NLR]), um importante marcador de progressão clínica, mostrou que o grupo moderado obteve um NLR de 36.42 $x10^3$/µL [IQR = 31.8-48.5], maior que o dos indivíduos saudáveis (1.89 $x10^3$/µL [1.5-2.3], $p < 0.0001$) e leves (2.16 $x10^3$/µL [IQR = 1.3-3.1], $p = 0.005$).

Hematological parameters (median [IQR])	Healthy Donor (n = 51)	Mild (n = 12)	Moderate (n = 6)	Severe (n = 9)	p value
RBC ($x10^6$/µL)	5.03 [4.6-5.4]	4.94 [4.4-5.4]	3.47 [2.4-4.4]	3.66 [3-4.4]	***< 0.001***^{b,c,d,e}
Hemoglobin (g/dL)	14.9 [13.6-16]	14.65 [13.7-15.7]	10.15 [6.5-12.2]	10.1 [8.5-12.7]	***< 0.001***^{b,c,d,e}
Hematocrit (%)	44.7 [40.6-47.4]	44.1 [40.7-48]	30.05 [19.6-37.3]	32.9 [25.7-39.5]	***< 0.001***^{b,c,d,e}
MCV (fL)	87.8 [84.7-90.4]	91.35 [87.1-94.1]	86.75 [78.8-91.8]	89.8 [84.3-92.6]	0.1773
MHC (pg)	29.7 [28.8-30.7]	30.4 [28.9-30.9]	28.7 [25.7-32]	28.4 [27.8-29.3]	0.0624
MCHC (g/dL)	34.1 [33.1-34.6]	32.55 [32.2-33.3]	33 [32.5-34.3]	32.3 [31.5-33.7]	***0.0015***^{a,c}
RDW (%)	13.7 [13.1-14]	14.25 [13.8-15]	13.85 [12.3-15.2]	13.7 [11.8-17.2]	0.0843
WBC ($x10^6$/µL)	6.33 [5.2-7]	5.92 [4.1-6.6]	8.9 [8.6-9]	12.26 [9.2-14.5]	***< 0.001***^{b,c,d,e}
Neutrophil ($x10^3$/µL)	3.34 [2.8-4.2]	3.63 [2.6-4.5]	8.3 [7.9-8.3]	10.19 [6.4-11.7]	***< 0.001***^{b,c,d,e}
Lymphocyte ($x10^3$/µL)	1.85 [1.6-2.2]	1.62 [1-2.2]	0.23 [0.2-0.3]	1.35 [1.2-2]	***< 0.001***^{b,d}
Monocyte ($x10^3$/µL)	0.38 [0.3-0.4]	0.39 [0.4-0.5]	0.43 [0.4-0.5]	0.61 [0.5-1]	***0.0014***^{c}
Eosinophil ($x10^3$/µL)	0.2 [0.1-0.4]	0.08 [0-0.2]	0.03 [0-0]	0.26 [0.1-0.5]	***< 0.001***^{a,b,e,f}
Basophil ($x10^3$/µL)	0.03 [0-0.1]	0.03 [0-0]	0.04 [0-0]	0.02 [0-0.1]	0.63
LUC (%)	1.7 [1.4-2.4]	1.65 [1.2-2.3]	0.9 [0.8-1]	1.6 [1.3-2.1]	***0.0105***^{b}
NLR ($x10^3$/µL)	1.89 [1.5-2.3]	2.16 [1.3-3.1]	36.42 [31.8-48.5]	6.67 [4.7-9.9]	***< 0.001***^{b,c,d,e}
Platelets ($x10^3$/µL)	243 [210-282]	205 [168.8-278.3]	152.9 [106.2-242.4]	224.7 [147.8-360.3]	0.1284
MVP (fL)	8.1 [7.2-8.6]	8.25 [7.9-9.8]	7.45 [6.3-8.7]	7.6 [7-8.8]	0.2997

Tabela 1: Parâmetros hematológicos de indivíduos saudáveis e grupos de pacientes com COVID-19. Análise estatística foi realizada com teste de Kruskal-Wallis e Múltiplas Comparações de Dunn. Para todas as comparações, foi utilizado intervalo de confiança de 95% e valor de p significante quando $p < 0.05$. [a]Diferença significativa entre HD vs Mild; [b]Diferença significativa entre HD vs Moderate; [c]Diferença significativa entre HD vs Severe; [d]Diferença significativa entre Mild vs Moderate; [e]Diferença significativa entre Mild vs Severe; [f]Diferença significativa entre Moderate vs Severe.
RBC: Hemácias; MCV: Volume Corpuscular Médio; MHC: Hemoglobina Corpuscular Média; RDW: Ratio Distribution Width; WBC: White blood count; NLR: Neutrophil-Lymphocyte ratio; MVP: Volume Plaquetário Médio.

A infecção aguda demonstrou um perfil muito similar entre os indivíduos leves e graves, baseado no perfil imunológico. Por outro lado, é perceptível que há um aumento de marcadores imunológicos, quando comparados a indivíduos saudáveis. O perfil celular demonstrou um aumento de monócitos intermediários e células T citotóxicas ativadas, mas também uma queda nos níveis de monócitos clássicos e não clássicos, células NK, NKT e linfócitos T helper (Figura 13).

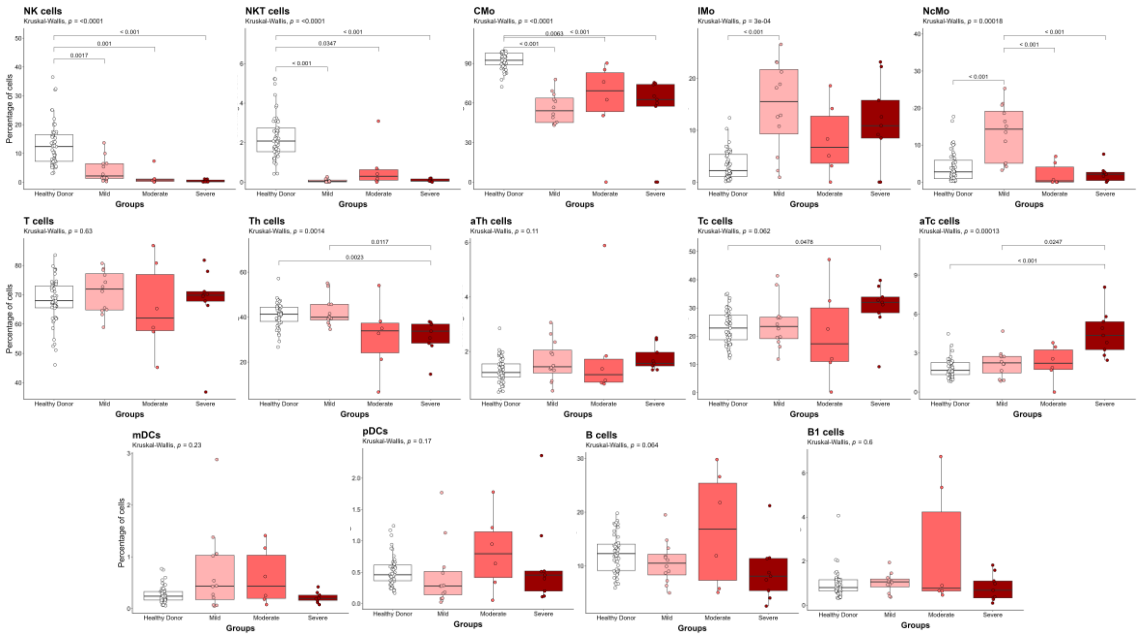


Figura 13: Perfil celular do grupo de indivíduos saudáveis e pacientes com COVID-19 leve e grave. NK: Natural Killer; NKT: Natural Killer T; CMo: Monócitos Clássicos; Imo: Monócitos Intermediários; NcMo: Monócitos não clássicos; Th: T helper; aTh: T helper ativado; Tc: T citotóxico; aTc: T citotóxico ativado; mDCs: Células Dendríticas Mieloides; pDCs: Células Dendríticas Plasmacitoides.

Quanto aos marcadores solúveis, na fase aguda da COVID-19, observamos que os grupos apresentaram aumento de CCL11, CXCL8, CCL4, CXCL10, IL-1β, IL-6, TNF-α, IL-12, IFN-g, IL-17, IL-4, IL-9, IL-10, IL-13, IL-7, VEGF, GM-CSF e G-CSF, bem como queda nos níveis de CCL5 e IL-15 (Figura 14).

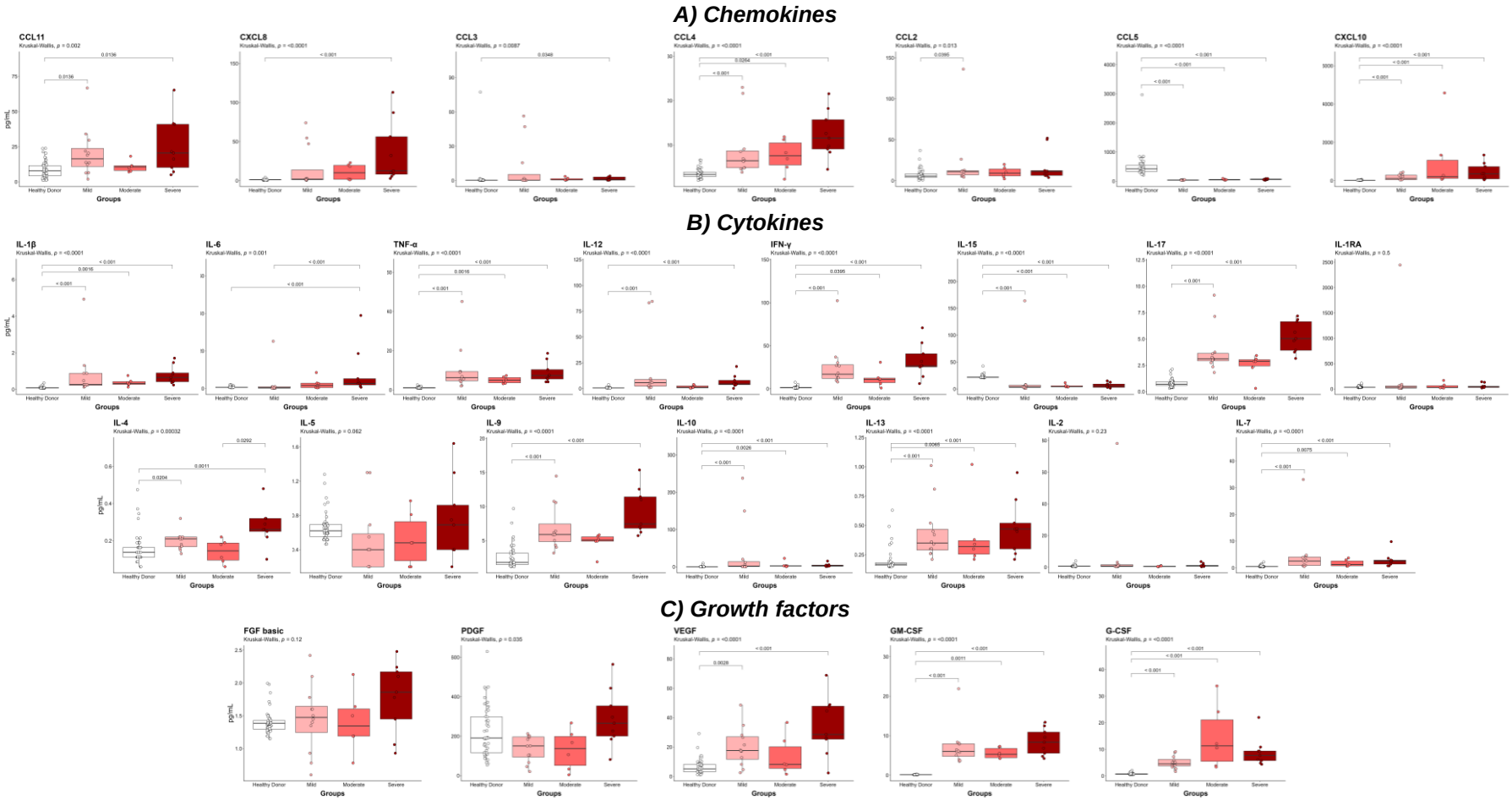


Figura 14: Perfil molecular solúvel de pacientes com COVID-19 leves, moderados e graves.

5.1.3. Os marcadores inflamatórios são os principais contribuintes para a condição grave, enquanto a moderada é influenciada por marcadores de proliferação

Embora o PCA tenha demonstrado uma segregação apenas entre o grupo de doadores saudáveis e os outros grupos de pacientes com COVID-19, foi observado que, com base nos marcadores imunológicos, os grupos de pacientes leve, moderato e grave apresentaram um perfil muito similar (Figura 15A). Baseado na contribuição dos marcadores para o perfil clínico, os três grupos de COVID-19 compartilham os mesmos marcadores, mas conforme a gravidade aumenta, os marcadores inflamatórios GM-CSF, G-CSF, CXCL10, IFN- γ, IL-7, TNF-α, IL-1β, VEGF e IL-17 são os principais marcadores que contribuem para essa gravidade (Figura 15B), como observado no heatmap (Figura 15C).

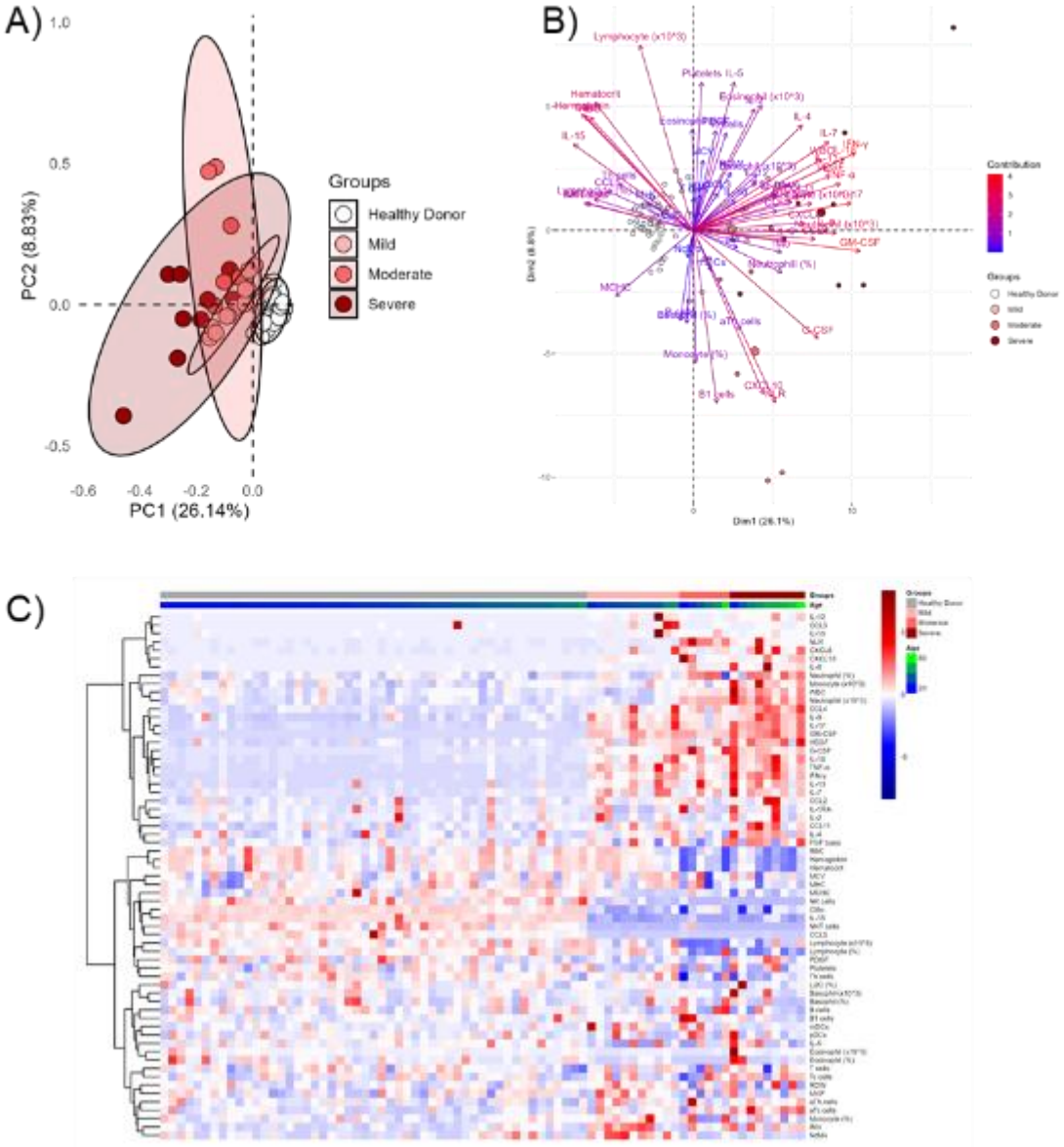


Figura 15: A inflamação aguda são os principais contribuintes para a condição grave em pacientes com COVID-19 grave. (A) PCA segregando por grupo; (B) PCA com os contribuintes da fase aguda, com as cores dos pontos representando os participantes e as setas representando a contribuição; (C) Heatmap com todos os parâmetros clusterizados nas linhas e os grupos organizados nas colunas.

Do grupo de doadores saudáveis para condição de COVID-19 leve (sintomáticos sem necessidade de hospitalização), foi observado que GM-CSF, IFN-γ, IL-12, IL-10 e monócitos intermediários tiveram o maior FC, enquanto células NKT, CCL5 e IL-15 tiveram o maior decaimento. Do grupo leve para moderado (hospitalizados sem necessidade de ventilação mecânica), NLR, células NKT, CXCL8, IL-6 e G-CSF foram os que apresentaram o maior crescimento, enquanto monócitos não clássicos, eosinófilos (%) e linfócitos totais tiveram o maior decaimento. Ainda, aqueles pacientes em

atendimento hospitalar, e que necessitaram de ventilação mecânica, transicionando do grupo moderado para o grupo grave, apresentaram um aumento na contagem de eosinófilos (% e total) na circulação, bem como monócitos não clássicos, contagem total de linfócitos e IL-12 (Figura 16). Estes marcadores sugerem sua capacidade como biomarcadores da progressão da doença.

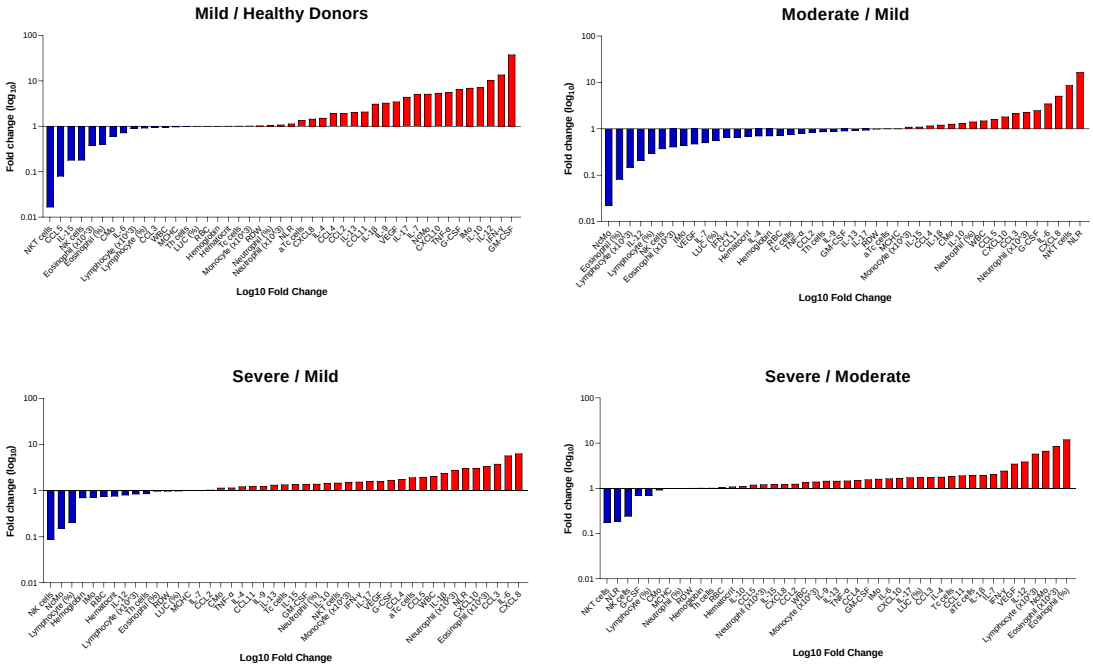


Figura 16: Análise de Fold Change (FC) com as médias de cada parâmetro dos grupos, isoladas pelo pós-teste de Dunn e organizadas com base no resultado do FC.

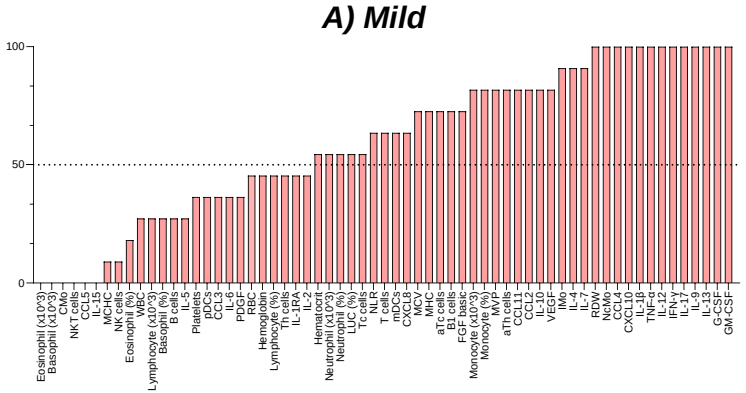
5.1.4. Análise de alto produtores nos grupos com COVID-19

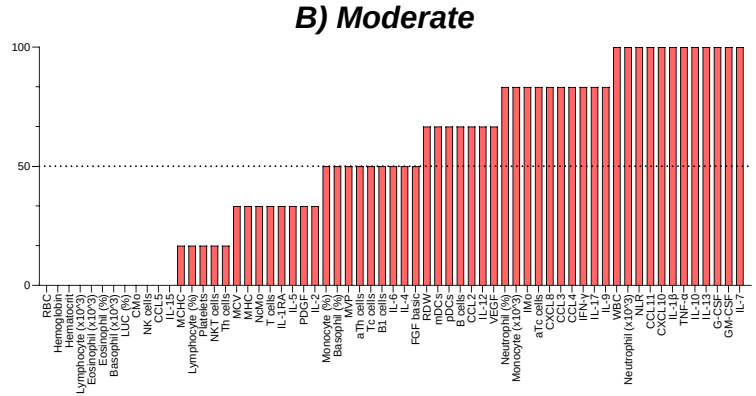
Posteriormente, os grupos de pacientes com COVID-19 foram isolados e calculada a mediana global. Com isso, de cada grupo, foi calculada a quantidade de participantes que apresentaram valores acima da mediana global. Posteriormente, calculados o percentual desses participantes por grupo, e organizados com base no percentual.

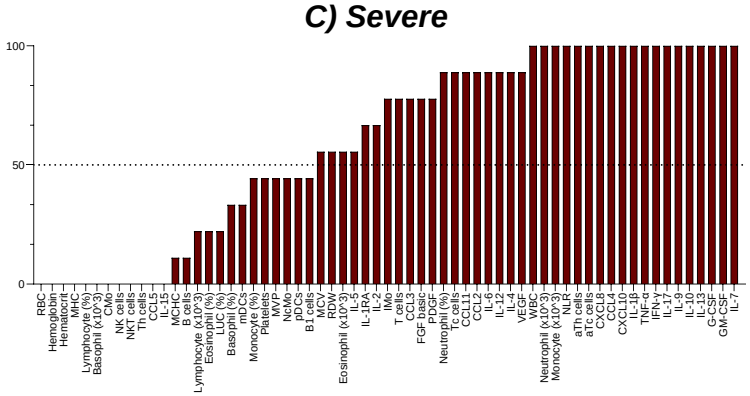
Com isso, foi identificado que apenas o grupo leve apresentou alta produção de 10 marcadores, sendo eles: IMo, hematócrito, hemoglobina, NcMo, linfócitos B1, linfócitos Th, linfócitos T, contagem percentual de linfócitos, RDW e RBC. Já o grupo moderado apresentou alta produção da contagem de basófilos, CHCM e células NKT. Pacientes graves de COVID-19 apresentaram alta produção de 19 marcadores, descritos

na Figura 17, no entanto, é notória a produção de marcadores inflamatórios e relacionados à migração celular.

A forma leve da doença e aqueles hospitalizados (mas sem ventilação mecânica) possuem um perfil mediado, além dos marcadores descritos anteriormente, de células dendríticas mieloides, HCM, Células NK, VPM, contagem relativa de basófilos e monócitos, e células B. Enquanto a condição de hospitalizado compartilha marcadores inflamatórios, como neutrófilos, CXCL8, IL-10, NLR, G-CSF, CCL5, IL-6, WBC, IL-5 e IL-15, além de outros (Figura 17D).







D) Intersections report of high producers

Groups	Inters.	Elements
Mild / Moderate	7	mDCs, MHC, NK cells, MVP, Basophil (%), Monocyte (%), B cells
Mild / Severe	9	IL-7, Eosinophil (x10^3), LUC (%), MCV, IL-12, Lymphocyte (x10^3), CCL2, CCL11, Eosinophil (%)
Moderate / Severe	15	pDCs, Neutrophil (x10^3), CXCL8, cMo, CXCL10, IL-10, CCL3, Neutrophil (%), NLR, G-CSF, CCL5, IL-6, WBC, IL-5, IL-15
Mild	10	IMo, Hematocrit, Hemoglobin, NcMo, B1 cells, Th cells, T cells, Lymphocyte (%), RDW, RBC
Moderate	3	Basophil (x10^3), MCHC, NKT cells
Severe	19	FGF basic, Monocyte (x10^3), IL-9, IL-13, IFN-γ, Platelets, IL-1β, aTc cells, GM-CSF, aTh cells, VEGF, IL-4, TNF-α, CCL4, IL-17, IL-2, IL-1RA, PDGF, Tc cells

Figura 17: Análise de alto produtores nos grupos de pacientes com COVID-19. Altos produtores utilizando o percentil 50 nos grupos Mild (A), Moderate (B) e Severe (C). A intersecção de cada grupo e dos conjuntos foi descrita no relatório (D).

As matrizes de correlação foram construídas isolando cada grupo. O grupo de doadores saudáveis mostrou correlações fracas entre os parâmetros, enquanto o grupo leve apresentou correlações mais fortes, variando entre positivas e negativas. Os pacientes hospitalizados tiveram poucas correlações, quando comparadas ao grupo leve, o que pode estar relacionado ao atendimento hospitalar (Figura 18).

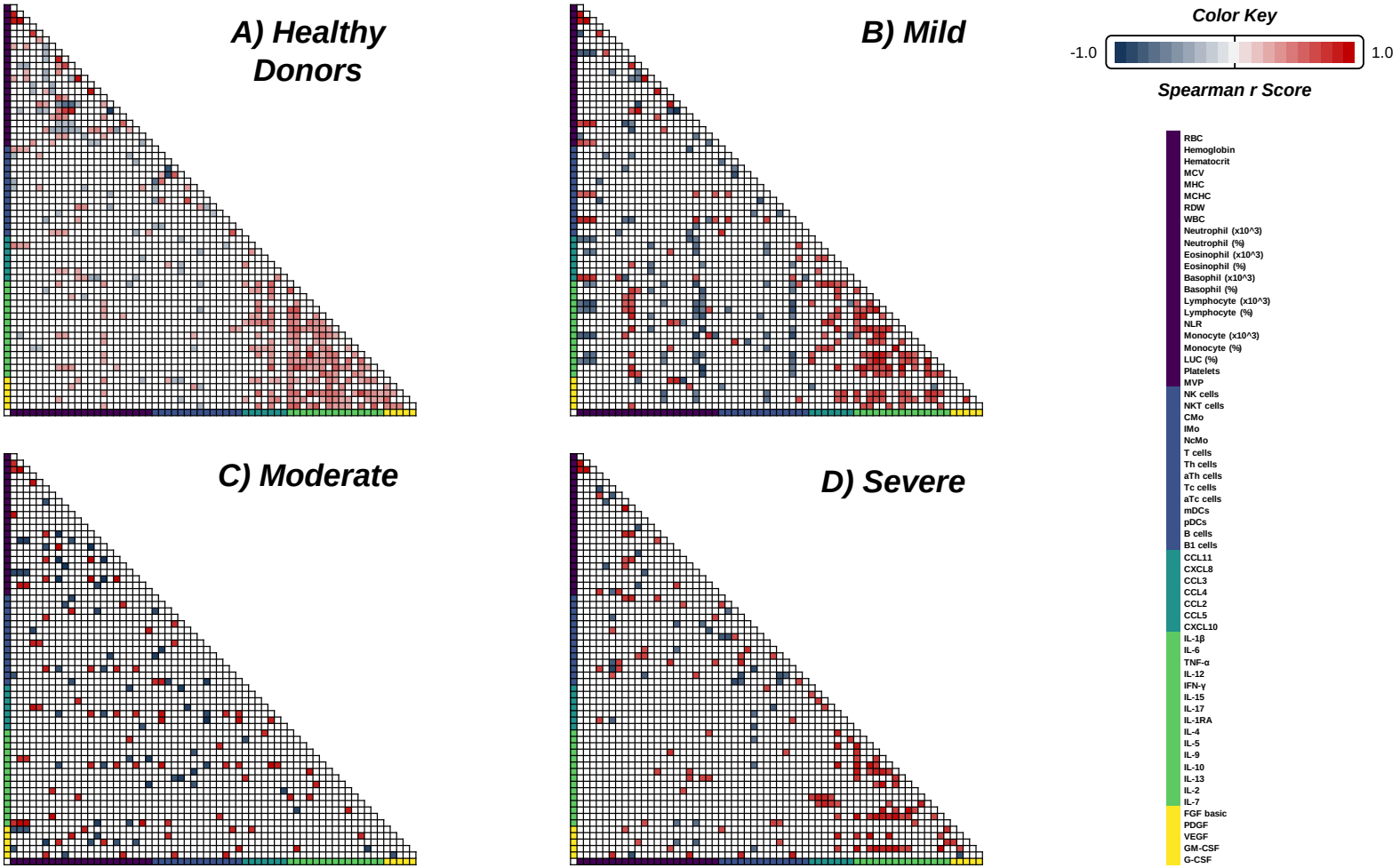


Figura 18: Matrizes de correlação de todos os parâmetros nos indivíduos saudáveis (A) e pacientes com COVID-19 leve (B), moderado (C) e graves (D). As correlações foram construídas utilizando os índices de correlação de Spearman (r). Correlações significativas ($p < 0.05$ foram plotadas, e os valores de r foram escalados em uma escala azul-vermelha, variando de -1.0 para 1.0. Os parâmetros (contagem sanguínea, perfil fenotípico, quimiocinas, citocinas e fatores de crescimento foram classificados com base em escalas de cor.

5.1.5. Contagem de eosinófilos aumenta naqueles pacientes hospitalizados com COVID-19 com pior desfecho clínico

O grupo de indivíduos saudáveis foi utilizado como referência no modelo de análise mista, junto com idade e gênero, como cofatores. O gênero não mostrou influência em nenhum parâmetro analisado, no entanto, a idade mostrou uma relação com a contagem de eosinófilos totais (logFC = -0.028, p = 0.0154) e eosinófilos (%) (logFC = -0.147, p = 0.0163). O teste de correlação de Spearman foi realizado para identificar a relação de ambos os parâmetros com a idade. Quando agrupados por todos os parâmetros, a idade apresentou uma correlação negativa apenas com eosinófilos (%) (r = -0.31, p = 0.0067), mas perdeu significância quando estratificado por grupos (Figura 19). Mesmo assim, nós realizamos as análises mistas considerando os dados demográficos dos pacientes.

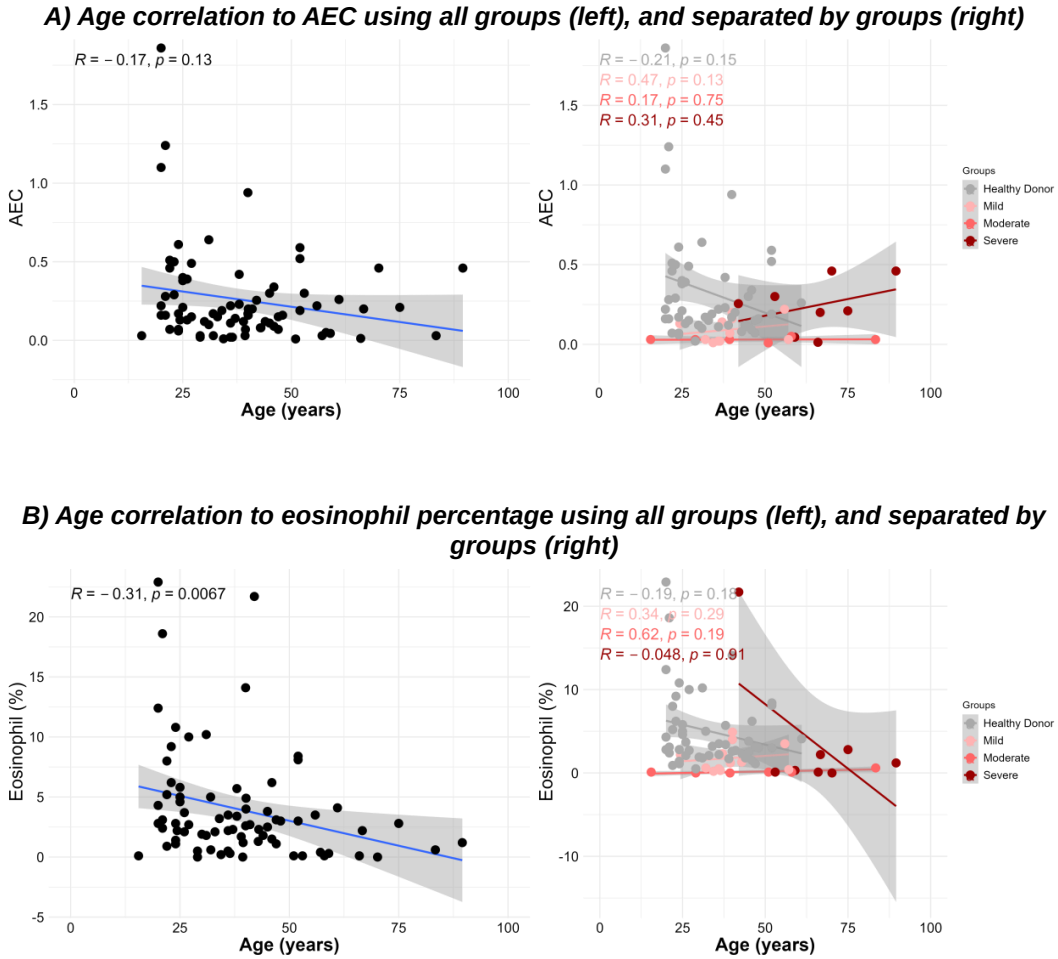


Figura 19: Correlação entre idade e produção dos linfócitos T auxiliares. A) Correlação utilizando todos os grupos juntos; B) Correlações segregadas por grupo.

O grupo leve apresentou maior logFC nas citocinas, principalmente inflamatórias e pro-inflamatórias, IFN-γ (logFC = 18.4, $p < 0.001$), IL-10 (logFC = 16.7, $p = 0.012$), VEGF (logFC = 11.4, $p = 0.002$) e IL-12 (logFC = 10.8, $p = 0.003$), e menor logFC foi observado em CCL5 (logFC = -443.3, $p < 0.001$), CMo (logFC = -36.6, $p < 0.001$) and IL-15 (logFC = -17.9, $p < 0.01$). Um perfil similar foi observado em pacientes moderados, com maior logFC em CXCL10 (logFC = 1,160, $p < 0.01$) e NLR (logFC = 40.9, $p < 0.01$). O grupo grave também apresentou alto logFC em CXCL10 (logFC = 692, $p = 0.002$), seguido por PDGF (logFC = 103, $p = 0.043$) e IL-1RA (logFC = 39.1, $p = 0.001$). (Figura 20).

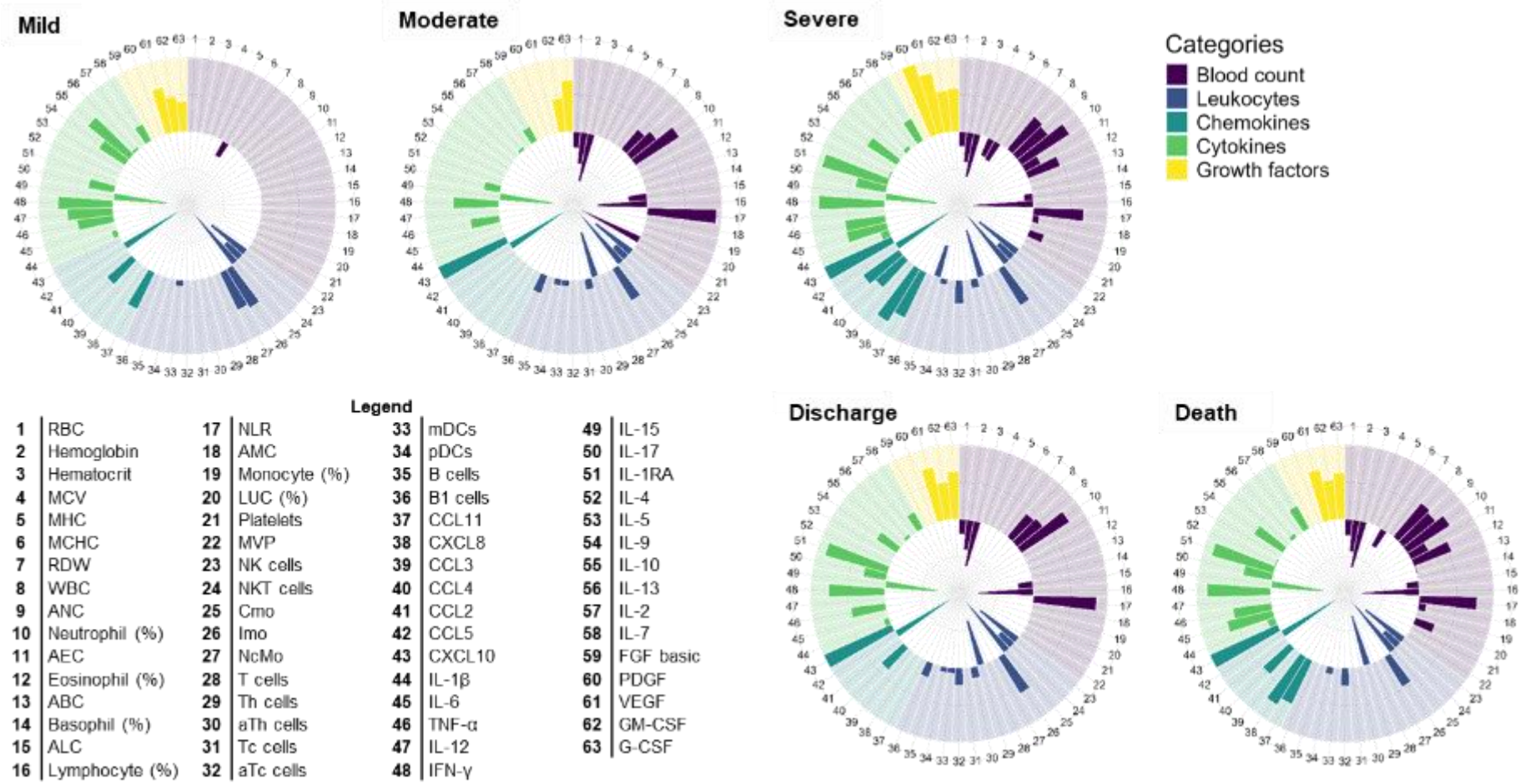
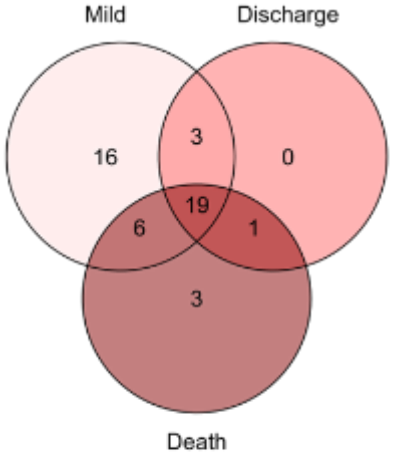
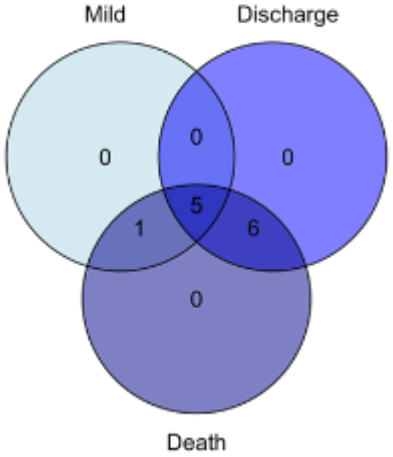


Figura 20: Seleção dos principais marcadores imunológicos para alta e baixa regulação com base nas condições, e usando como base o grupo saudável. Gráficos de radar demonstrando o FC significante de cada grupo.

Posteriormente, os grupos moderado e grave foram divididos com base no desfecho hospitalar, em Alta e Óbito, referente aos pacientes que evoluíram para alta (n = 10) ou óbito (n = 5), respectivamente. Embora compartilhem um perfil similar entre os parâmetros de logFC, foi construído um Diagrama de Venn para identificar parâmetros compartilhados entre o grupo leve e os desfechos. Nossos resultados demonstraram que os marcadores aqui observados não são capazes de identificar aqueles pacientes hospitalizados que irão evoluir para alta hospitalar. No entanto, aqueles que evoluem para óbito tiveram um aumento da contagem total de eosinófilos, eosinófilos percentuais e LUC (%) (Figura 21). Hospitalização, independente do desfecho, foi marcada com alta contagem de WBC e queda de linfócitos totais, hemoglobina, linfócitos (%), RBC, hematócrito e células T helper.



Groups	Inters.	Elements
Mild / Death	6	CCL11, CXCL8, IL-4, Monocyte (x10^3), IL-6, Basophil(x10^3)
Mild / Discharge	3	mDCs, aTh cells, B1cells
Discharge / Death	1	WBC
Mild / Discharge / Death	19	GM-CSF, IL-17, TNF-α, IFN-γ, IMo, IL-9, IL-7, CCL4, IL-1β, VEGF, IL-13, G-CSF, CXCL10, aTc cells, Neutrophil (%), IL-1RA, NLR, Neutrophil (x10^3), pDCs
Mild	**16**	**NcMo, IL-12, IL-10, RDW, MVP, Monocyte (%), CCL3, MCV, CCL2, T cells, IL-2, FGF basic, Th cells, Tc cells, Hematocrit, MHC**
Death	**3**	**Eosinophil (x10^3), Eosinophil (%), LUC (%)**



Groups	Inters.	Elements
Mild / Death	1	MCHC
Discharge / Death	6	Lymphocyte (x10^3), Hemoglobin, Lymphocyte (%), RBC, Hematocrit, Th cells
Mild / Discharge / Death	5	IL-15, CMo, NKT cells, N Kcells, CCL5

Figura 21: Caracterização por grupo e influência dos parâmetros imunológicos, comparados a indivíduos saudáveis. A influência foi caracterizada por cor para influência positiva (logFC > 0, cor vermelha) ou negativa (logFC < 0, cor azul).

5.2. Perfil imunológico em doadores de sangue saudáveis e pacientes convalescentes da COVID-19

5.2.1. Perfil sociodemográfico

51 doadores de sangue saudáveis foram incluídos nesta abordagem do estudo. A média de idade foi de 32.39 anos (DP ± 11.63), com 36 (70.6%) homens e 15 (29.4%) mulheres. A maior parte dos participantes eram pardos (n = 45 [88.3%]), seguido por 4 (7.8%) caucasianos e 2 (3.9%) afro-americanos. Cinco (9.8%) eram do tipo sanguíneo A positivo, 27 (52.9%) eram O positivo, e 19 (37.3%) eram O negativo.

A média de idade do grupo convalescente foi de 39.94 anos (DP ± 11.56), com 51 (82.3%) homens e 11 (17.7%) mulheres. Quanto à etnicidade, 42 (67.8%) weram pardos, 18 (29%) eram caucasianos, e apenas 2 (3.2%) eram afro-americanos. Quanto ao tipo sanguíneo, 17 eram do tipo A (14 [22.6%] positivo e 3 [4.9%] negativo), 2 eram do tipo B (1 [1.6%] positivo e 1 [1.6%] negativo), 2 eram do tipo AB (ambos positivos [3.2%]), e 41 eram do tipo O (40 [64.5%] positivo e 1 [1.6%] negativo). Grande parte eram sobrepeso ou obesos (n = 23 [37.1%] cada), seguido por 15 (24.2%) com IMC normal e apenas um [1.6%] tinha baixo IMC. Análise estatística demonstrou diferença significativa apenas em idade (p = 0.0009) e tipo sanguíneo (p < 0.0001) entre ambos os grupos, no entanto, acreditamos que a diferença no tipo sanguíneo ocorra devido à alta prevalência dos tipos O e A na população estudada (Tabela 2).

Sociodemographic data	**Healthy donors (n = 51)**	**Convalescents (n = 62)**	**p value**
Age, mean ± SD	32.39 ± 11.63	39.94 ± 11.56	***0.0009***
Gender			
Male, n (%)	36 (70.6)	51 (82.3)	0.1794
Female, n (%)	15 (29.4)	11 (17.7)	
Ethnicity			
Caucasians, n (%)	4 (7.8)	18 (29)	***0.0182***
Admixed, n (%)	45 (88.3)	42 (67.8)	
African Americans, n (%)	2 (3.9)	2 (3.2)	
Blood type			
A+ / A-, n (%)	5 (9.8) / 0 (0)	14 (22.6) / 3 (4.9)	***< 0.0001***
B+ / B-, n (%)	0 (0) / 0 (0)	1 (1.6) / 1 (1.6)	
AB+ / AB-, n (%)	0 (0) / 0 (0)	2 (3.2) / 0 (0)	
O+ /O-, n (%)	27 (52.9) / 19 (37.3)	40 (64.5) / 1 (1.6)	
Body Mass Index (kg/m^2)			
Low (<18,5), n (%)	1 (2.0)	1 (1.6)	0.6504
Normal (18,5 – 24,9), n (%)	17 (33.3)	15 (24.2)	

Overweigh (25 – 29,9), n (%)	19 (37.3)	23 (37.1)
Obesity (>30), n (%)	14 (27.4)	23 (37.1)

Tabela 2: Dados sociodemográficos de doadores saudáveis e pacientes convalescentes.
SD: Desvio Padrão; Pos: Positivo; Neg: Negativo. Teste de Qui-Quadrado foi utilizado isolando os grupos sanguíneos (A, B, AB e O), e subgrupos (positivo ou negativo) e incluindo apenas aqueles com observação > 1 para questões estatísticas.

Uma vez que foi observada diferença entre idade, etnicidade e tipo sanguíneo entre os grupos HD e Convalescente (D30), nós realizamos uma análise dos parâmetros imunológicos segregando nossos grupos baseado no tipo sanguíneo e idade. Nenhuma diferença foi observada na comparação entre os grupos com relação à tipagem sanguínea entre os grupos HD e Convalescença. O padrão observado nas observações gerais não parece mudar quando relacionado a nenhuma dessas três características, e portanto, acreditamos que nenhuma teve alguma ou pouca interferência no sistema imunológico dos nossos participantes. A análise do heatmap e PCA segregados por esses parâmetros está mostrado na Figura 22 para idade (22A), etnicidade (22B) e tipo sanguíneo (22C).

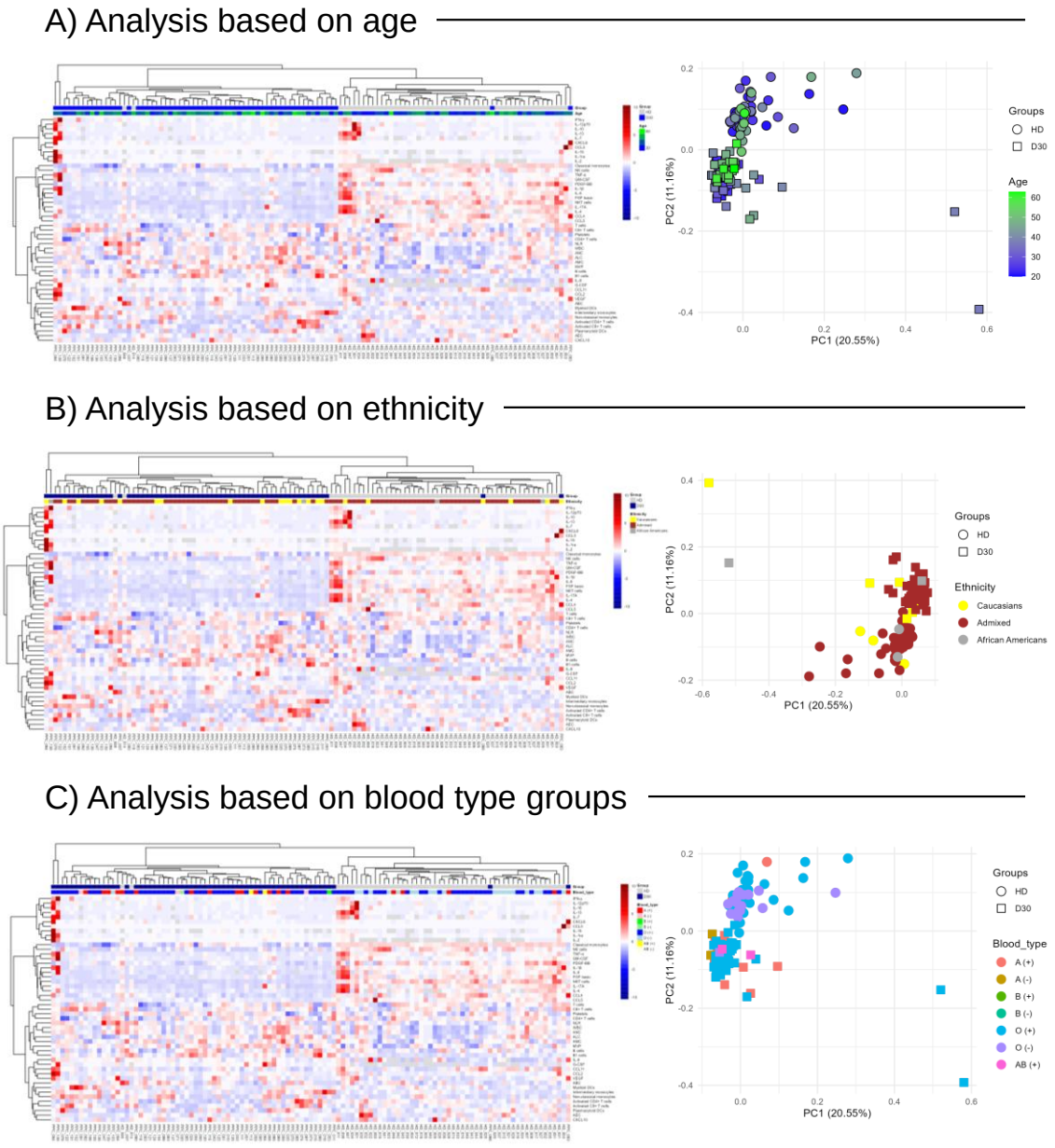
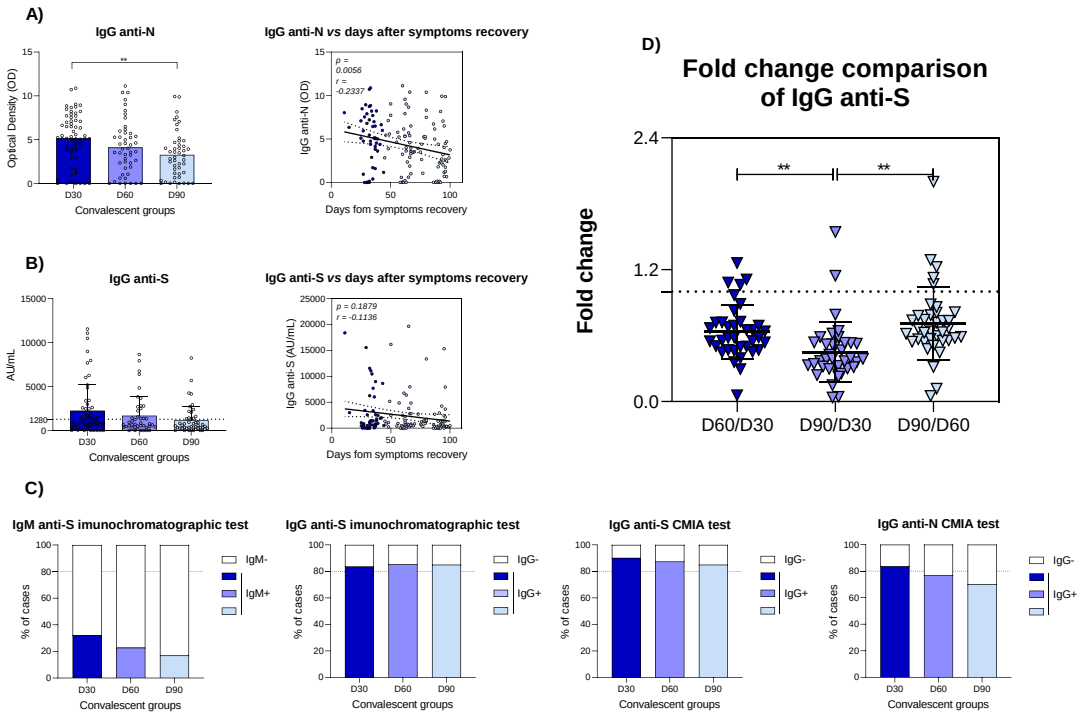


Figura 22: Caracterização de todos os parâmetros quando às informações sociodemográficas (idade (A) e etnicidade (B)) e tipo sanguíneo (C) de todos os participantes. Valores numéricos foram utilizados na construção de ambos heatmap e PCA.

Ao analisar os dados clínicos do grupo de convalescente, 5 dos 62 pacientes (8.1%) necessitaram de hospitalização e ventilação mecânica. A média do tempo de estadia no hospital foi de 15.82 dias (DP ± 10.49), variando de 1 a 51 dias.

5.2.2. Dinâmica dos anticorpos na convalescença

A concentração de anticorpos foi analisada nos grupos convalescentes e comparados com o número de dias após a pausa dos sintomas. IgG anti-nucleocapsídeo (anti-N) demonstrou uma queda significativa (p = 0.0017) de 30 a 90 dias após a cura clínica, o que foi confirmada posteriormente pela análise de correlação. Esta análise revelou uma correlação negativa e significante redução na concentração de anti-N com o aumento do número de dias após o final dos sintomas (p = 0.0056), como demonstrado na Figura 23A. Embora a anti-Spike (anti-S) IgG também tenha decaído, a concentração não mostrou diferenças significativas na análise comparativa e nem na correlação (Figura 23B). Isso sugere que a presença do anticorpo anti-S no soro persiste, enquanto anticorpos anti-N decai lentamente após a cura clínica.



Groups	D30	D60	D90	D30	D60	D90	D30	D60	D90	D30	D60	D90
Positive, n (%)	20 (32.3)	11 (22.9)	8 (17)	52 (83.9)	41 (85.4)	40 (85.1)	56 (90.3)	42 (87.5)	40 (85.1)	52 (83.9)	37 (77.1)	33 (70.2)
Negative, n (%)	42 (67.7)	37 (77.1)	39 (83)	10 (16.1)	7 (14.6)	7 (14.9)	6 (9.7)	6 (12.5)	7 (14.9)	10 (16.1)	11 (22.9)	14 (29.8)

Figura 23: Análise da produção sorológica de anticorpos durante a convalescença. A) Comparação da concentração de IgG anti-nucleocapsídeo (OD) e correlação da concentração do anti-N com os dias após os sintomas. B) Comparação da concentração de IgG anti-Spike (AU/mL) e correlação entre a concentração e dias após os sintomas. O cutt-off de 1.280 AU/mL foi marcado para demonstrar os participantes elegíveis à doação de plasma convalescente no D30 (n=30/62 [48.4%]), D60 (n=20/48 [41.7%]) e D90 (n=14/47 [29.8%]); C) Porcentagem de participantes com resultado qualitativo (pos/neg) de anticorpo ao longo do período estudado, baseado no teste imunocromatográfico e teste quimioluminescente (CMIA) para anti-S

e anti-N, com valores absolutos e relativos na tabela abaixo; D) Comparação do Fold Change de anticorpo IgG anti-S no D60/D30, D90/D30 e D90/D60, usando o resultado quantitativo dos pacientes com todo o follow-up (n = 45). Análise estatística foi feita com One-Way ANOVa seguido pelo pós-teste de Turkey, considerando significativo quando $p < 0.05$. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Deve-se notar que a imunidade permanece ativa por alguns dias após o clearance viral e alguns pacientes fizeram a soroconversão de um estado positivo (no D30) para um negativo (em ambos D60 e D90) durante o período do estudo, para ambos anti-N e anti-S, considerando o limiar de positividade do fabricante.

O soro de 20, dos 62 participantes (32.3%) com diagnóstico prévio de COVID-19 e período sintomático testou positivo para IgM no D30 usando o teste imunocromatográfico. Este número decaiu para 11/48 (22.9%) no D60 e 8/47 (17%) no D90. Os testes IgG apresentaram alta sensibilidade. Todos os três testes (ambos imunocromatográfico e quimioluminescência [CMIA] anti-S, e CMIA anti-N) detectaram mais de 80% de sororeatividade nos indivíduos convalescentes no d30. Os grupos D60 e D90 ainda apresentaram uma sororeatividade acima de 80% para detecção de anticorpos anti-S, enquanto a detecção de anti-N caiu para 70.2% no D90 (Figura 23C).

A comparação do fold change entre os dias mostrou que a concentração do anti-S muda gradualmente de um mês para o próximo, no entanto, há uma diferença significativa para o terceiro mês. Um padrão similar foi observado quando comparado do D30 para o D60 e D60 para D90, indicando que a concentração do anti-S do D30 para o D60 não variou significativamente comparado com a variação do D60 para o D90. No entanto, houve uma queda na concentração de anticorpo do D30 para o D90 (Figura 23D).

A Organização Mundial da Saúde lançou uma lista com testes aprovados para detecção de anticorpos IgG anti-S, capazes de identificar potenciais candidatos à doação de sangue convalescente. Os valores qualitativos e quantitativos do teste CMIA foi aprovado para monitorar potenciais doadores de sangue elegíveis quanto à concentração de anticorpos (> 1.280 AU/mL). Entre os indivíduos convalescentes que aceitaram participar, apenas 30/62 (48.4%) tiveram concentração suficiente no D30. No D60, apenas 20/48 (41.7%) permaneceram elegíveis, e posteriormente caiu para 14/47 (29.8%) no D90 (Figura 23B).

5.2.3. O perfil inflamatório é mediado por células de memória e monócitos patrulhadores

A contagem de células NK e NKT apresentou uma queda significativa após a COVID-19 e não demonstrou sinais de aumento nem apenas após 90 dias após cura clínica (Figura 24A e B). Embora a contagem de monócitos tenha aumentado nos pacientes no D30, comparados com os doadores saudáveis, esse aumento parece ser devido ao aumento de monócitos inflamatórios e patrulhadores. A contagem absoluta de monócitos (AMC) decaiu no segundo mês após a cura clínica (Tabela 3), mas a contagem de monócitos patrulhadores continuou a decair ao longo do tempo, atingindo concentrações maiores no D90 (Figura 24C-E).

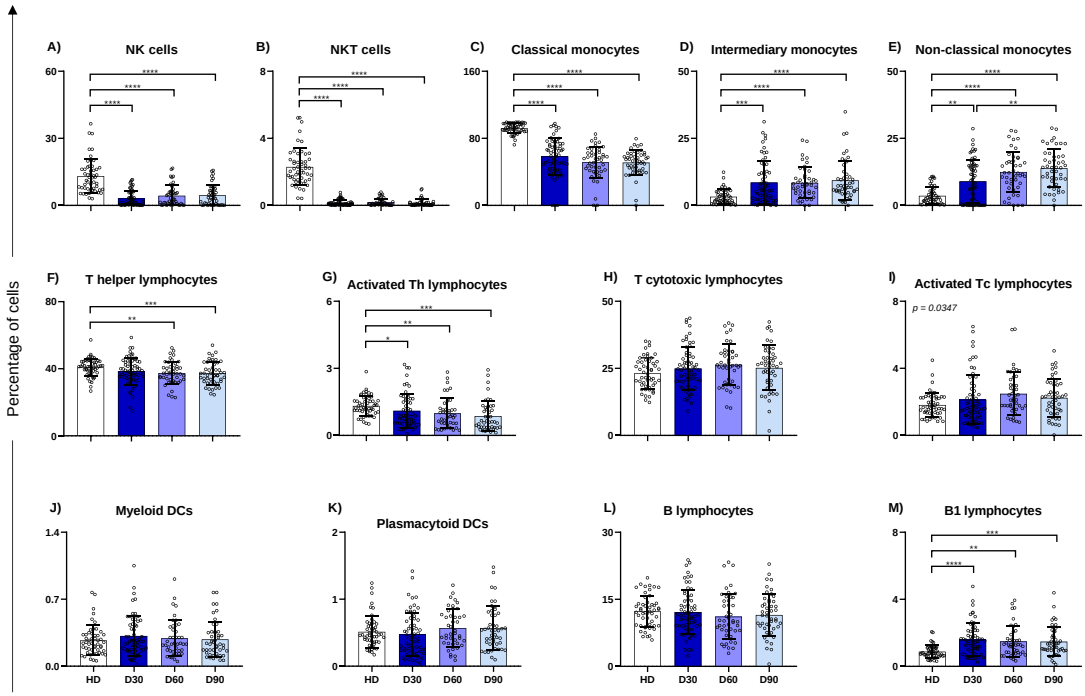


Figura 24: Análise fenotípica das células imunes, comparando os grupos HD, D30, D60 e D90. O resultado está expresso como mediana e intervalo interquartil do percentual de células. Os gráficos de barra representam as análises de: A) Células NK; B) Células NKT; C) Monócitos clássicos; D) Monócitos inflamatórios; E) Monócitos patrulhadores; F) Linfócitos T helper; G) Linfócitos T helper ativados; H) Linfócitos T ciotóxicos; I) Linfócitos T citotóxicos ativados; J) Células dendríticas plasmacitoides; K) Células dendríticas mieloides; L) Linfócitos B; e M) Linfócitos B1.
A análise dos dados foi realizada com o teste de Kruskal-Wallis e pós teste de Dunn, considerando significativo quando $p < 0.05$. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Variables, median [IQR]	HD n = 51	D30 n = 62	D60 n = 48	D90 n = 47	p value
RBC (×10^6 /µL)	4.99 [4.59 - 5.40]	4.90 [4.56-5.20]	4.89 [4.47-5.15]	4.89 [4.71-5.29]	0.4676
Hemoglobin (g/dL)	14.90 [13.60-16.00]	14.40 [13.38-15.03]	14.35 [13.23-14.90]	14.40 [13.50-15.10]	0.0602
Hematocrit (%)	44.70 [40.6-47.4]	42.60 [40.45-45.45]	42.75 [39.13-44.50]	43.90 [41.50-46.20]	0.0660
MCV (fL)	87.80 [84.70-90.40]	88.15 [84.85-90.80]	87.90 [84.35-91.08]	89.10 [85.30-91.40]	0.7292
MCH (pg)	29.70 [28.8-30.70]	29.60 [28.38-30.60]	29.85 [28.10-30.70]	29.80 [27.90-30.80]	0.8607
MCHC (g/dL)	34.05 [33.10-34.60]	33.70 [32.78-34.450]	33.59 [32.75-34.48]	33.40 [32.60-34.10]	0.1667
RDW (%)	13.70 [13.10-14.00]	14.40 [13.75-14.95]	14.40 [13.90-15.08]	14.35 [13.68-15.03]	***<0.0001***^{a,b,c}
WBC (×10^6 /µL)	6.33 [5.17-6.95]	6.78 [5.87-7.51]	6.34 [5.75-7.57]	6.65 [5.61-7.97]	0.2906
Neutrophil (x10^3 /µL)	3.34 [2.8-4.17]	4.00 [3.21-4.65]	3.80 [3.14-4.61]	3.79 [3.04-4.84]	0.0757
Lymphocyte (x10^3 /µL)	1.85 [1.59-2.18]	1.98 [1.61-2.22]	1.93 [1.6-2.3]	1.98 [1.61-2.23]	0.8081
Monocyte (x10^3 /µL)	0.38 [0.28-0.42]	0.43 [0.37-0.48]	0.37 [0.33-0.42]	0.38 [0.28-0.43]	***0.0028***^{a,d,e}
Basophil (x10^3 /µL)	0.03 [0.02-0.05]	0.04 [0.02-0.06]	0.03 [0.02-0.04]	0.03 [0.02-0.05]	0.2906
Eosinophil (x10^3 /µL)	0.19 [0.12-0.38]	0.19 [0.13-0.26]	0.18 [0.13-0.26]	0.16 [0.12-0.26]	0.6799
Platelet count (x10^3 /µL)	243.0 [210-282]	259.5 [212-290]	245.0 [215-266]	245.00 [209-272]	0.7717

Tabela 3: Parâmetros laboratoriais de doadores saudáveis e pacientes convalescentes no D30, D60 e D90. [a]Diferença significativa entre HD vs D30; [b]Diferença significativa entre HD vs D60; [c]Diferença significativa entre HD vs D90; [d]Diferença significativa entre D30 vs D60; [e]Diferença significativa entre D30 vs D90; [f]Diferença significativa entre D60 vs D90.
A análise dos dados foi realizada com o teste de Kruskal-Wallis e pós teste de Dunn, considerando significativo quando $p < 0.05$ e destacado os significantes em negrito.

Por outro lado, ambos linfócitos T helper e T helper ativados decaíras conforme a convalescença progrediu (Figura 24 F e G). A contagem de linfócitos T citotóxicos não variou significativamente entre os grupos analisados, mas a mediana de linfócitos T citotóxicos ativados demonstrou diferença significativa no teste de Kruskal-Wallis, no entanto, não foi possível determinar onde estava a diferença (Figura 24H e I).

A subpopulação circulante de células dendríticas não apresentou diferença entre os doadores saudáveis e indivíduos convalescentes, nem entre os subgrupos da convalescença (Figura 24J e K).

Embora os linfócitos B não apresentaram diferença, foi observado um aumento nos linfócitos B1 durante a convalescença, o que aparenta persistir ao longo do trimestre analisado (figura 24L e M).

5.2.4. A convalescença é marcada por mediadores regulatórios de reparação tecidual

A fase convalescente parece ser marcada por altos níveis de VEGF, G-CSF, IL-2, IL-9, e CXCL10, mas também baixa concentração de FGF basic, PDGF-BB, GM-CSF, IL-1β, IL-6, TNF-α, IFN-γ, IL-4, IL-5, IL-17A, IL-10, CXCL8, CCL3, CCL4 e CCL5 (Figura 25). CCL5 apresentou queda no D30, mas parece aumentar a concentração no soro até o D90, atuando como possível marcador precoce de normalidade sistêmica.

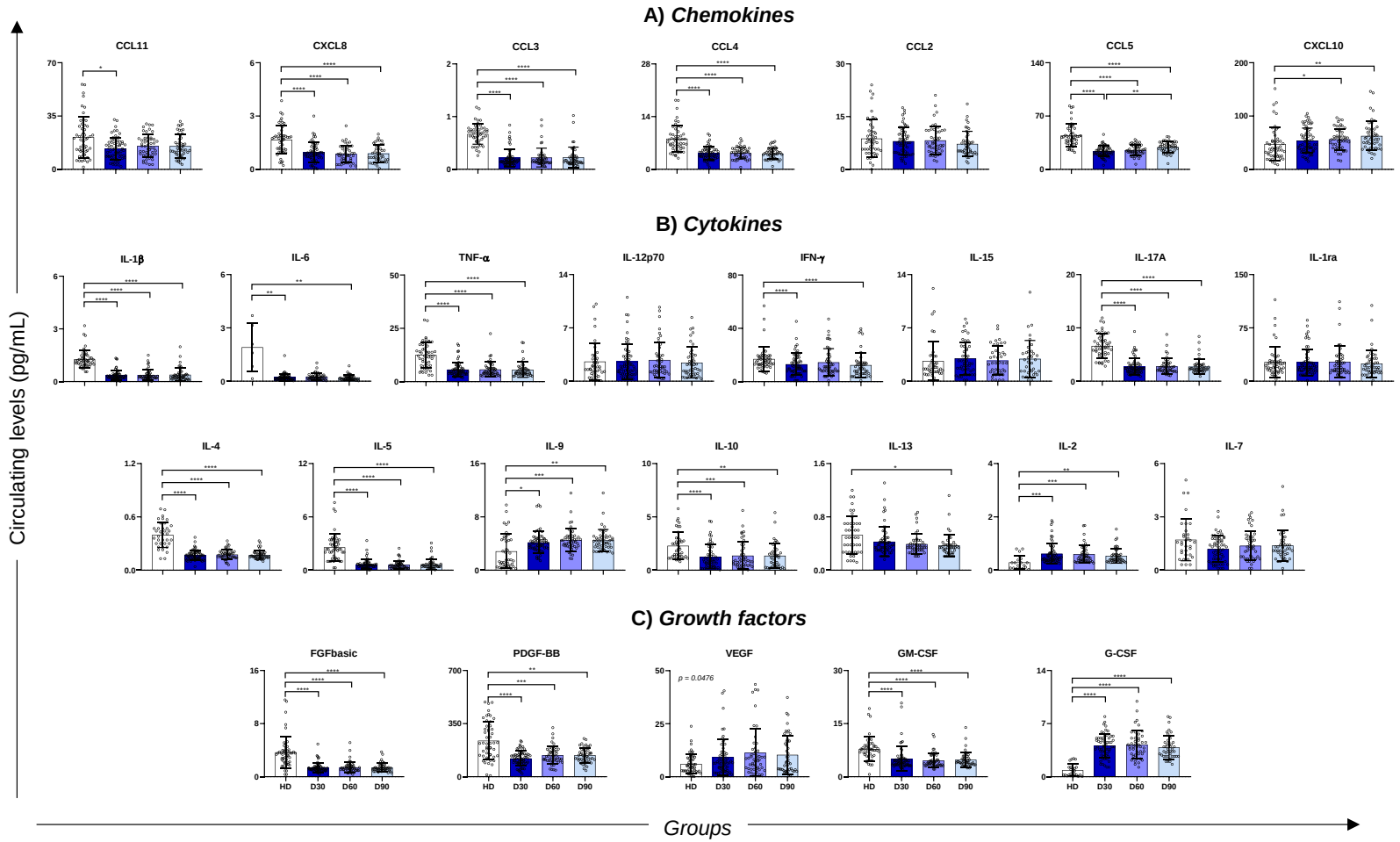


Figura 25: Nível de moléculas solúveis circulantes comparando os grupos HD, D30, D60 e D90. O resultado está expresso como mediana e intervalo interquartil em pg/mL Nível circulante de quimiocinas (A), citocinas (B) e fatores de crescimento (C).
A análise dos dados foi realizada com o teste de Kruskal-Wallis e pós teste de Dunn, considerando significativo quando $p < 0.05$. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

5.2.5. A dinâmica de marcadores tende a normalizar conforme a convalescença progride

A matriz de correlação demonstra que os indivíduos saudáveis têm menores interações entre citocinas, e mais interações com células circulantes e suas subpopulações, enquanto aqueles na convalescença exibem uma dinâmica de moléculas mais intensa. Pacientes no estágio inicial da convalescença, representados pelo D30, demonstraram maior número de interações entre as moléculas, principalmente citocinas.

O número de correlações aparenta decair conforma a convalescença progride até o estágio final observado em nosso estudo. Embora ainda é possível observar que os anticorpos anti-S apresentam correlação positiva com a contagem absoluta de eosinófilos (AEC) e CXCL10 no D30 (em conjunto com anticorpos anti-N) e uma correlação negativa com monócitos patrulhadores.

No D60, a imunomodulação foi relacionada a moléculas inflamatórias como IL-6 e CXCL8, e correlacionada negativamente com a contagem de linfócitos B. No D90, a participação parece ser guiada por células dendríticas plasmacitoides e níveis de CXCL8 (Figura 26 A e B). A AEC mostrou correlação positiva e significativa com ambos os anticorpos anti-N e anti-S no D30 e D90 (Figura 26), o que pode estar relacionado à sua funcionalidade.

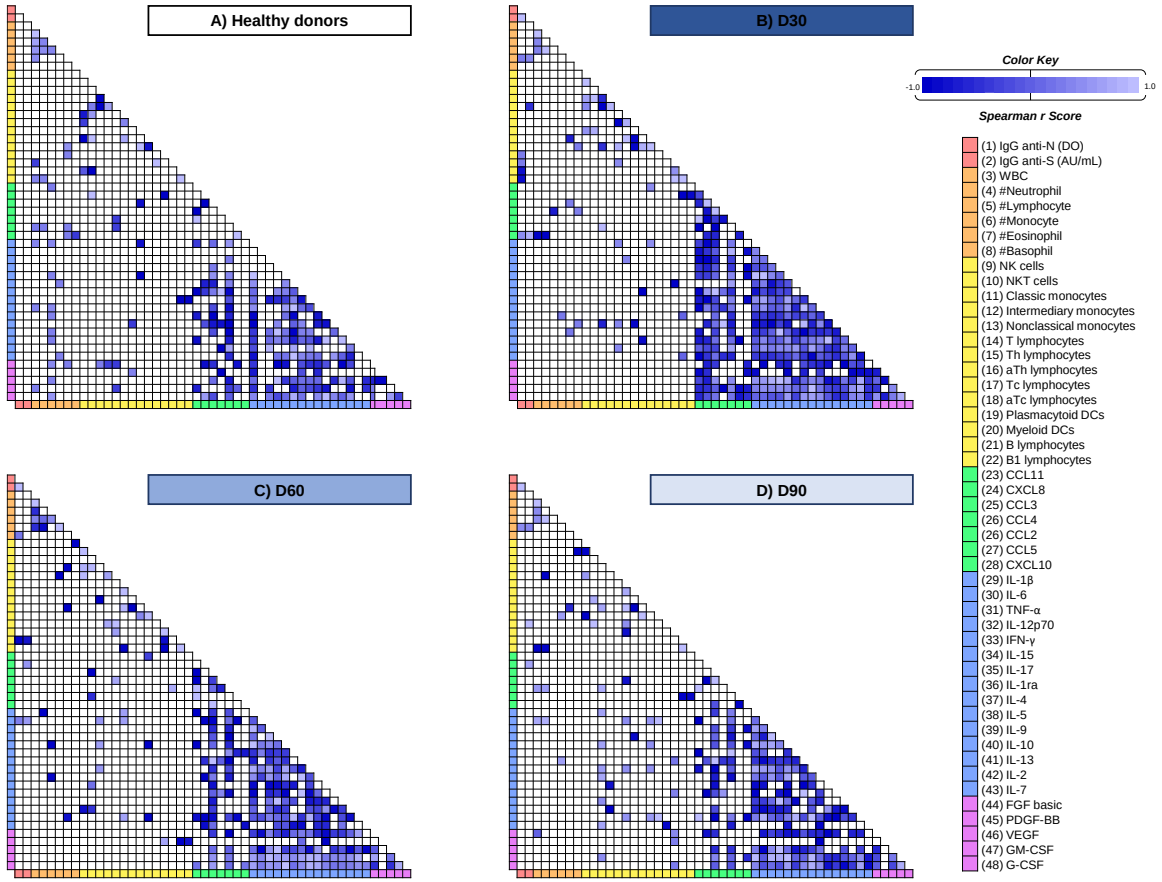


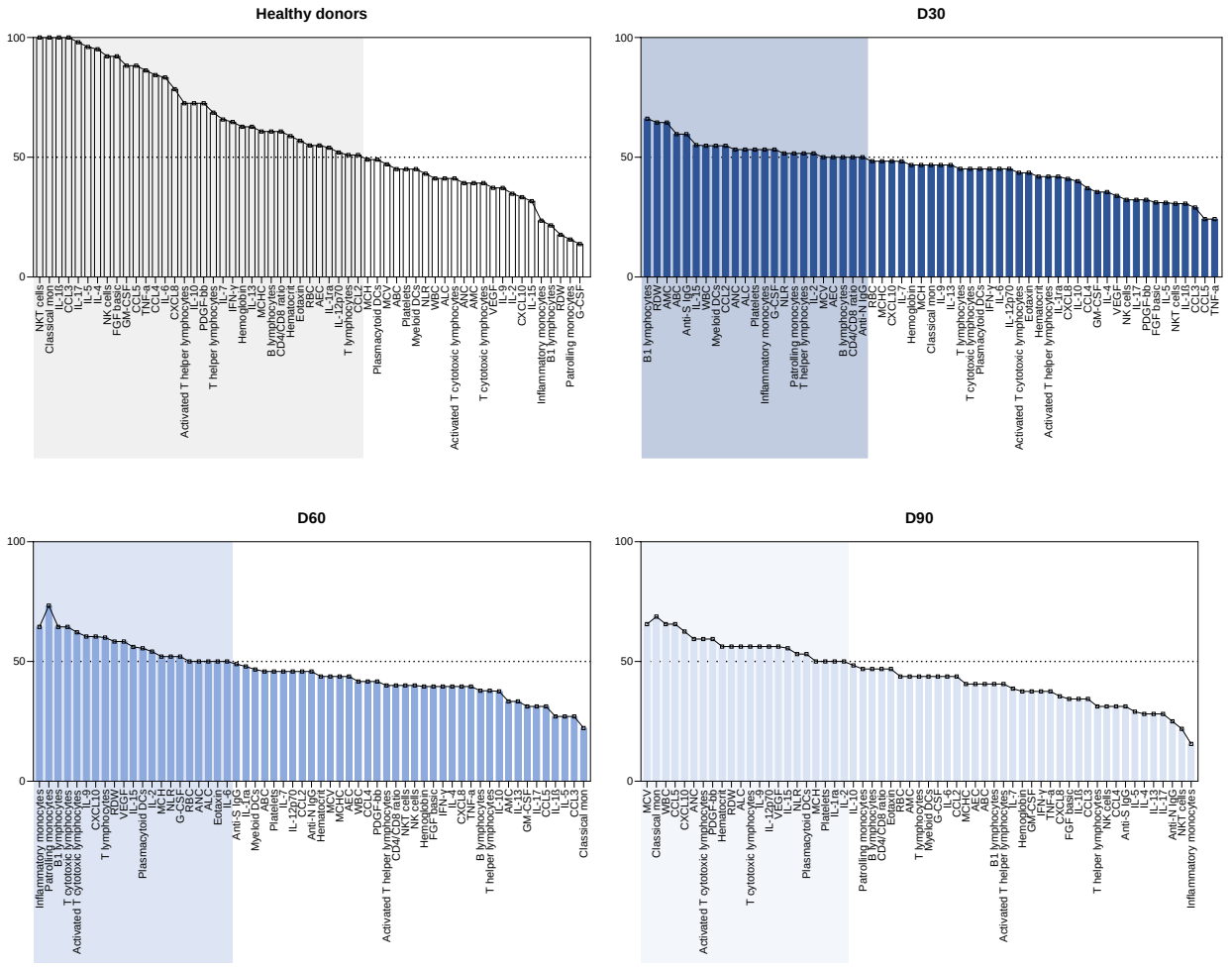
Figura 26: Matriz de correlação de biomarcadores indicando diferença no padrão de doadores saudáveis (A) e convalescentes no D30 (B), D60 (C) e D90 (D). As correlações são baseadas no índice de correlação de Spearman (r). A correlação foi significativa quando $p < 0.05$ entre todos os marcadores analisados. A escala de azul, variando de -1.0 a 1.0, demonstra a força da correlação, como representado na imagem.
WBC: White Blood Count; ANC: Absolute Neutrophil Count; ALC: Absolute Lymphocyte Count; AMC: Absolute Monocyte Count; AEC: Absolute Eosinophil Count; ABC: Absolute Basophil Count; NK: Natural killer; Chemokines: CXCL8, CXCL10, CCL3, CCL4, CCL2, CCL5 and CCL11; Cytokines: IL-1β, IL-1ra, IL-6, TNF-α, IL-12p70, IFN-γ, IL-2, IL-7, IL-9, IL-15, IL-4, IL-5, IL-13, IL-17A, IL-10; Growth factors: VEGF, FGF basic, PDGF-BB, GM-CSF, G-CSF. HD: Healthy donors.

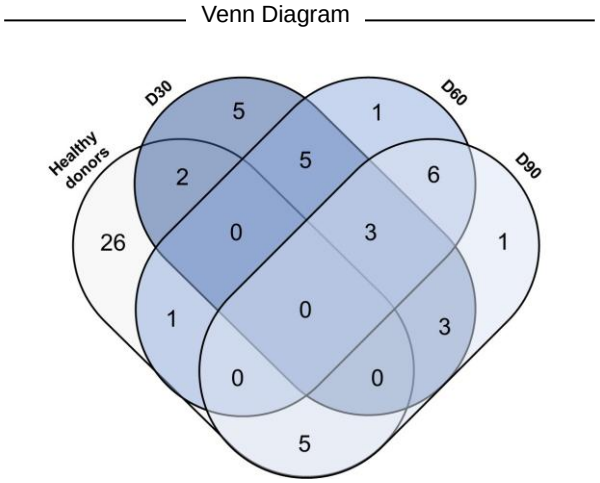
5.2.6. Marcadores inflamatórios ainda podem ser utilizados para caracterizar as fases iniciais da convalescença

A análise de biomarcadores e o diagrama de Venn demonstram que indivíduos convalescentes são caracterizados por altos níveis de IL-15, NLR e RDW. No entanto, quando segregados quanto ao estágio da convalescença e inspecionados por biomarcadores em cada tempo, nós observamos que no início da convalescença, 30 dias após a cura clínica, altos níveis de monócitos, basófilos, plaquetas, células dendrítitcas mieloides e anticorpos anti-S também foram observados.

Adiante, a imunomodulação do primeiro (D30) para o segundo mês (D60), pode ser caracterizada pelo aumento de monócitos inflamatórios e patrulhadores, linfócitos B1, G-CSF, IL-2 e razão IFN-γ/IL4. Embora os linfócitos B1 estejam elevados na circulação, apenas o D30 foi marcado com aumento de anticorpos anti-S.

Estágios posteriores da convalescença, observados no nosso estudo, foram marcados por ambos os linfócitos T citotóxicos ativados e totais, células dendríticas plasmacitoides, VEGF, IL-9 e CXCL10. Este perfil sugere um processo reparativo, o que pode estar relacionado à intensa lesão causada pela imunidade durante a fase aguda. Nenhum dos grupos convalescentes se apresentou como alto produtor de anticorpos antti-N (Figura 27).





Venn Diagram Report

Groups	Intersections	Elements
D30 / D60 / D90	3	NLR, RDW, IL-15
HD / D30	2	CCL2, T helper lymphocytes
HD / D60	1	T lymphocytes
HD / D90	5	Hematocrit, Classical monocytes, CCL5, PDGF-bb, IL-12p70
D30 / D60	5	Inflammatory monocytes, G-CSF, IL-2, B1 lymphocytes, Patrolling monocytes
D30 / D90	3	ANC, WBC, ALC
D60 / D90	6	IL-9, CXCL10, Activated T cytotoxic lymphocytes, VEGF, T cytotoxic lymphocytes, Plasmacytoid DCs
HD	26	FGF basic, IL-13, CXCL8, IFN-γ, IL-1β, GM-CSF, Hemoglobin, NK cells, IL-10, Eotaxin, IL-7, IL-4, CCL3, MCHC, B lymphocytes, TNF-α, AEC, CCL4, IL-6, IL-17, NKT cells, IL-1ra, Activated T helper lymphocytes, IL-5, CD4/CD8 ratio, RBC
D30	**5**	**Platelets, AMC, ABC, Anti-S IgG, Myeloid, DCs**
D60	**1**	**MCH**
D90	**1**	**MCV**

Figura 27: Assinatura de biomarcadores dos grupos representados pelo diagrama de Venn. A) Frequência dos indivíduos com os biomarcadores acima do cut-off. B) Diagrama de Venn representando os grupos, intersecções e elementos, sugerindo potenciais marcadores para imunomodulação na convalescença. A mediana global para cada parâmetro foi calculada e usado para caracterizar os participantes como baixo (<50%) ou alto (>50%) produtores. HD: Healthy donors.

6. Discussão

O mundo enfrentou um problema de saúde pública, que se tornou uma pandemia desde 2020. Entre as principais características que contribuíram para a rápida disseminação do SARS-CoV-2 e desenvolvimento de condições críticas extremas, muito estudos destacaram a ausência de imunidade e presença de fatores de risco

Ao analisarmos os pacientes com COVID-19, observamos que aqueles com a condição mais grave apresentaram uma idade significativamente maior, o que pode se dar devido às características imunológicas de senescência (HOU et al., 2022; HU et al., 2021; MODERBACHER et al., 2020).

As mudanças hematológicas observadas em nossos resultados são sustentadas pelo perfil fisiopatológico da doença. Eventos trombóticos apresentados em pacientes com COVID-19, e reportados em estudos anteriores, demonstrou que a suplementação de oxigênio induz o aumento dos níveis de fibrinogênio e posterior agregação eritrocitária, bem como viscosidade sanguínea. Esses agregados podem impactar na disponibilidade de eritrócitos circulantes no vaso sanguíneo, mas juntos com o dano na membrana e a alta expressão de fosfatidilserina, há aumento da remoção de eritrócitos pelo baço (KLEI et al., 2017; NADER et al., 2022). Estes fatores levam a uma cascata de eventos onde a diminuição de eritrócitos no sangue causa uma condição de hipóxia no paciente (BERZUINI et al., 2021), o que corrobora com nossos achados nos nossos pacientes graves.

Quanto ao perfil inflamatório descrito nos nossos pacientes, o envolvimento do neutrófilo em pacientes graves foi alto, o mesmo observado por outros autores (BALZANELLI et al., 2021; IMRAN et al., 2020; LOURDA et al., 2021; MCELVANEY et al., 2020; MORADI et al., 2021a; ZHANG et al., 2020d; ZHAO et al., 2020). Um efeito do desvio à esquerda foi observado, provavelmente devido à dinâmica imunológica relacionada à produção celular da medula óssea. Muitos neutrófilos circulantes, durante a fase aguda da COVID-19, apresentam um perfil imaturo (CD10+), e já foram correlacionados com marcadores inflamatórios como CXCL8, CXCL10, CCL3, CCL4, IL-6 e IL-1RA (CARISSIMO et al., 2020; METZEMAEKERS et al., 2021; PARACKOVA et al., 2020; WILK et al., 2020).

A função desses neutrófilos imaturos ainda não é bem elucidado se apresentam um perfil regulatório ou pró-inflamatório, mas quando associados à contagem de

linfócitos (representado pelo NLR), nossos dados demonstram uma queda significante com base na gravidade. Para estratégias e análises futuras, compreender os mecanismos por trás da neutrofilia e linfopenia, o perfil proliferativo e de recrutamento para o local da inflamação pode melhorar as abordagens terapêuticas e condições agudas em pacientes com uma primo-infecção, como observada no SARS-CoV-2 em nossos pacientes.

O perfil fenotípico mostrou uma contagem baixa de células NK e NKT em pacientes graves, como já descrito na literatura (KIM et al., 2022; TAGHILOO et al., 2021; ZHANG et al., 2020c), e também relacionados ao comprometimento da citotoxicidade aguda (ANTONIOLI et al., 2020; LEEM et al., 2020). Entre os fatores relacionados a esse desbalanceamento, problemas no eixo IL-15/IL-15RA foram sugeridos como componentes importantes na exaustão funcional das células NK, bem como senescência e um controle mais eficiente da infecção viral (FLAMENT et al., 2021; ZHANG et al., 2020d; ZHANG; HOLMES, 2020)

Assim como observado nas células NK/NKT, as subpopulações de monócitos também estavam diminuídas no sangue, conforme houve o aumento da gravidade. Essas células participam no reconhecimento viral e eliminação, incluindo células infectadas, e embora o SARS-CoV-2 possa infectar monócitos, devido à expressão do receptor viral ACE2, um aumento na granularidade e permeabilidade das células endoteliais já foi reportado (KIM et al., 2022; MARTENS et al., 2021; ZHANG et al., 2020c; ZHOU et al., 2020), o que pode acabar justificando a queda observada nos nossos grupos moderado e grave.

Foi sugerido anteriormente que essa redução no sangue periférico, junto com a de células Th, não se dá por conta da ausência de produção ou estímulo, mas sim devido à tempestade de citocinas e excesso de estimulação para migrar da circulação para o tecido (DIDANGELOS, 2020; GEBREMESKEL et al., 2021), e para isso, observamos o perfil solúvel de moléculas nos nossos pacientes.

Nossos achados mostram um aumento na concentração sérica de CCL11, CXCL8, CCL4 e CXCL10 no grupo grave, o que pode estar relacionado à expressão de moléculas de adesão em leucócitos circulantes (GEBREMESKEL et al., 2021; MORADI et al., 2021a; ZHANG et al., 2020d). Estes fatores cogiram na hipótese salientada anteriormente. A CXCL10 é conhecida como um forte indutor de ativação e recrutamento de linfócitos, eosinófilos, monócitos e células NK (JING; VASSILIOU; GANEA, 2003;

YOUNG; LEE; SONG, 2009), as mesmas encontradas diminuídas em nossos achados, bem como com alta produção de CCL11, CXCL8 e CCL4, que potencializam a inflamação e degranulação de granulócitos (BELPERIO et al., 2000; BYSTRY et al., 2001; REN et al., 2010; SMITH; HUMPHRIES, 2009).

O perfil inflamatório parece ser mediado por IL-1β, IL-6, TNF-α, IL-12, IFN-γ, IL-17, IL-4, IL-9, IL-10, IL-13, IL-7, VEGF, GM-CSF e G-CSF, o que salienta a dinâmica da tempestade de citocinas em nossos pacientes. Estes marcadores foram demonstrados anteriormente como potenciais para manutenção da doença e progressão para modular a imunidade, ativação celular, recrutamento de células e proliferação (KNOLL; SCHULTZE; SCHULTE-SCHREPPING, 2021; KUMAR et al., 2021; QIN et al., 2021; THEOBALD et al., 2021).

Embora os eosinófilos foram descritos como marcadores de melhora na progressão da doença (GEORGAKOPOULOU et al., 2021; GLICKMAN et al., 2021; GONZÁLEZ et al., 2021; KWIECIEŃ et al., 2021; LIU et al., 2021; TAN et al., 2021; VITTE et al., 2020; YAN et al., 2021), nossos achados demonstram diferente. Essa relação do aumento na contagem de eosinófilos e melhor prognóstico já foi sugerido por outros autores, no entanto nós sugerimos que nossos pacientes estavam também com um processo de degranulação, o que poderia explicar o aumento na gravidade e contribuição para piores desfechos. Já foi observado que eosinófilos ativados (CD69+) foram correlacionados a marcadores inflamatórios, e contribuem para infiltração tecidual nos pulmões, degranulação, coagulação e metabolização da matriz extracelular (LOURDA et al., 2021). Determinar a funcionalidade do eixo IL-4/IL-5 pode contribuir na compreensão da dinâmica dos eosinófilos e outros granulócitos durante a fase aguda da doença e o dano tecidual apresentado.

Quanto aos achados nos pacientes convalescentes, nós conduzimos um estudo prospectivo de indivíduos convalescentes, recrutados 30 dias após a cura clínica, e acompanhados até 90 dias após a cura clínica. Nestes grupos, os procedimentos realizados também foram os mesmos descritos para aqueles pacientes com COVID-19, descrito anteriormente.

Nossos achados, quanto à dinâmica de anticorpos demonstrou que anticorpos anti-S IgG persistem no sangue circulante por mais tempo que anticorpos de classe IgM, como esperado. Em nosso estudo, apenas 32% dos participantes tinham IgM detectável

no D30, o que é menor do que o reportado na literatura (DAN et al., 2021). Ao mesmo tempo, mais de 80% dos convalescentes apresentaram IgG anti-S detectável, o que corrobora com outros estudos (BOŠNJAK et al., 2020; DAN et al., 2021; XIANG et al., 2021). É importante salientar que o anti-S IgG demonstrou maior estabilidade, quando comparado com o anti-N durante a convalescença. O pico da produção de anticorpo ocorre aproximadamente 10 a 20 dias após o início dos sintomas e parece ser influenciado primariamente por anticorpos IgG de classes 1 e 3 (KIM et al., 2021, 2022). Alguns estudos reportaram que a persistências de células B de memória e células circulantes Tfh possuem um papel importante na produção de anticorpor anti-S, enquanto que a produção de anti-N e anti-RBD apresentam uma queda significativa (CHANSAENROJ et al., 2022; JUNG et al., 2021; NELSON et al., 2022; PRETE et al., 2022; SOKAL et al., 2021).

A dinâmica imunológica durante a convalescença envolveu maior participação de monócitos inflamatórios e patrulhadores. A contagem de monócitos totais normalizou conforme houve a progressão da convalescença, mas as subpopulações ativadas pareceram aumentar, incluindo um aumento dos monócitos patrulhadores. Na literatura, ambos patrulhadores e inflamatórios foram encontrados diminuídos durante a convalescença, e estabilizaram sua concentração após aproximadamente cinco meses (KWIECIEŃ et al., 2021; RAJAMANICKAM et al., 2021; ZHANG et al., 2020b).

Esta diferença pode estar relacionada à ativação de monócitos, uma vez que já foi previamente demonstrado que a expressão de HLA-DR aumenta durante a convalescença (NEELAND et al., 2021; QIN et al., 2021; RAJAMANICKAM et al., 2021). No entanto, há poucos relatos quanto à quais subpopulações expressão esse marcador durante a fase convalescente. O maior controle do perfil inflamatório e reparatório pode ser uma das razões para esse aumento, uma vez que o aumento de ambas as subpopulações persiste por mais dois meses após o *clearance* viral.

Embora nossos resultados demonstraram que a convalescença pode ser marcada por citotoxicidade, aparenta ser regulada por linfócitos T. Células NK e NKT apresentaram uma queda notável, o que foi descrito anteriormente (ANTONIOLI et al., 2020; LEEM et al., 2020; TAGHILOO et al., 2021) e pode estar relacionada à produção de outras células envolvidas no processo de reparação tecidual. Este mesmo padrão foi observado durante a fase aguda, tanto nos nossos resultados, como na literatura também, uma vez que a contagem de células NK foi relacionada com a gravidade da doença e

comprometimento da citotoxicidade (TAGHILOO et al., 2021). Curiosamente, ambas células dendríticas mieloide e plasmacitoide não apresentaram diferença, indicando que seu papel no combate antiviral ocorre de forma rápida e estímulo-dependente.

O perfil molecular demonstrou que um padrão inflamatório ainda predomina frente a um perfil antinflamatório. No entanto, ambos os perfis foram reduzidos, quando comparados aos indivíduos saudáveis. A dinâmica imunológica durante a convalescença é caracterizada por baixa produção de mediadores inflamatórios, e fatores reparadores, bem como intensa regulação mediada pelo aumento da concentração de CXCL10, IL-2 e G-CSF. A concentração de anti-N diminuiu por linfócitos B no início da convalescença, mas no D60, parece que essa população celular também atua na queda da produção de anticorpos anti-S, característica que já não é vista no D90. Algumas células imunológicas ainda estiveram presentes no D60, mas com uma queda considerável na concentração de anticorpos, observado na análise de *fold change*, provavelmente devido à queda no estímulo inflamatório. Foi demonstrado que células de memória tendem a persistir mesmo com a redução na produção de anticorpos, demonstrando reatividade à maioria dos agentes antivirais mesmo após seis meses (DAN et al., 2021; SOSA-HERNÁNDEZ et al., 2020).

Durante todo o período de convalescença, houve um aumento no nível de NLR. Nossa avaliação dos pacientes agudos, bem como estudos prévios, sugeriram esse marcador como potencial para prognóstico em pacientes agudos (ASGHAR et al., 2020; LEPPKES et al., 2020; MAN et al., 2021; RODRIGUEZ et al., 2020; TAJ et al., 2021), e nossos achados demonstram que este permanece elevado por períodos elevados, até mesmo na convalescença.

Todos os três meses da convalescença foi caracterizado pela alta produção de IL-15. Na fase inicial, também houve o envolvimento de G-CSF e IL-2, enquanto o último estágio foi marcado principalmente pela produção de IL-9 e CXCL10. Conforme salientado anteriormente, o desbalanço do eixo IL-15/IL-15RA é um fator crucial nos processos biológicos das células NK e NKT, o que contribui para o rápido processo de controle da infecção mediado por citotoxicidade e resposta de anticorpos durante a convalescença (FERRERAS et al., 2021; FLAMENT et al., 2021; MASSELLI; VITALE, 2021; NOTARBARTOLO et al., 2021; ZHANG et al., 2020c, 2020d).

Moléculas como G-CSF, IL-2 e IFN-γ estão envolvidas na tempestade de citocinas durante a fase aguda da COVID-19, e como observado em nosso estudo, este perfil de proliferação persiste na convalescença, mediado principalmente por G-CSF e IL-2. Isso pode estar relacionado à funcionalidade de reparo e migração de granulócitos do vaso sanguíneo para o tecido lesionado (KWIECIEŃ et al., 2021). A dinâmica subsequente da IL-9 e CXCL10 no período D60/D90 também foi descrito em processos de alergia crônica e estão associados ao envolvimento de mastócitos e indução da adesão de linfócitos ao endotélio e inibição da medula óssea. A CXCL10, embora previamente relacionada a desfechos a curto prazo e possível biomarcador de melhora clínica de indivíduos com COVID-19, sua participação na convalescença pode estar associada a propriedades antinflamatórias (WU et al., 2021).

Nós observamos que nos grupos convalescentes, o D30 foi o período ótimo para coleta de plasma convalescente. Menos de 50% dos nossos participantes tiveram uma concentração de anticorpos suficientes para doação de plasma, o que decai ainda mais no D90. Enquanto muitos estudos foram em avaliar a eficácia do uso de plasma convalescente em pacientes com COVID-19 aguda, poucos estudos avaliam os fatores relacionados na dinâmica imunológica e produção de anticorpos, particularmente relacionado na obtenção de plasma convalescente (NELSON et al., 2022; ZHOU et al., 2020). No entanto, foi observada uma necessidade emergente de melhorar a qualidade na obtenção de plasma para estocagem, considerando a possibilidade de futuras pandemias e o desenvolvimento de casos graves.

7. Conclusão

Neste estudo, trouxemos dados referente à dinâmica imunológica de indivíduos saudáveis, recrutados antes da pandemia da COVID-19, dados de pacientes com COVID-19 com estágio leve (sem internação hospitalar), moderado (hospitalizados sem ventilação mecânica) e grave (hospitalizados sob ventilação mecânica). Também foram incluídos indivíduos convalescentes da infecção vírus, considerados aqueles curados da infecção, e habilitados para doação de sangue 30, 60 e 90 dias após a cura clínica.

Nossos resultados demonstraram que na fase aguda, o perfil inflamatório é preponderante, e a tempestade de citocinas pode acabar por ser um fator crucial no aumento da gravidade. Nós demonstramos a potencialidade de moléculas como IFN-γ, IL-1β, GM-CSF, IL-17, e TNF-α de atuarem como preditores de desfecho clínico dos pacientes agudos. Mas além disso, também sugerimos que a contagem de eosinófilos, monócitos não clássicos e contagem de linfócitos também possuem um grande potencial como biomarcador, mas desta vez, relacionado à necessidade do uso de ventilação mecânica naqueles pacientes hospitalizados. Os eosinófilos também parecem participar na progressão da doença, e um pior desfecho, quando pacientes já se encontram em uma condição grave. Embora ainda não tão bem estabelecido, desvendar os mecanismos por trás desta, e de outras células no processo de lesão tecidual pode propor novos mecanismos de acompanhamento e tratamento de futuros casos de doenças pulmonares.

Quanto à fase convalescente, nós identificamos a rápida queda de anti-N IgG após o *clearance* viral, enquanto a variação de anti-S IgG ocorre de forma mais lenta nos três primeiros meses. Isso demonstra a melhor habilidade do anti-S para fins terapêuticos e de diagnóstico. Também identificamos que após a infecção, um perfil inflamatório ainda está presente, mediado principalmente por monócitos inflamatórios e patrulhadores, bem como linfócitos B1, com imunomodulação mediada por G-CSF, IL-2 e IL-15 na convalescença. A inflamação ainda está presente nos estágios iniciais, mas por aproximadamente 60 dias, o perfil parece começar a mudar para uma característica mais proliferativa, mediada por IL-9 e CXCL10. Nossos dados contribuem na pesquisa de biomarcadores envolvidos no processo de produção de anticorpos, e avaliação de células e moléculas circulantes em indivíduos convalescentes. Conhecer a principal função dos marcadores aqui descritos poderão sugerir melhores medidas de manejo após infecção pelo SARS-CoV-2.

8. Apêndice

8.1. Artigo 1: "*Immune Dynamics Involved in Acute and Convalescent COVID-19 Patients*", publicado na revista Immuno

immuno

MDPI

Review

Immune Dynamics Involved in Acute and Convalescent COVID-19 Patients

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Abstract: COVID-19 is a viral disease that has caused millions of deaths around the world since 2020. Many strategies have been developed to manage patients in critical conditions; however, comprehension of the immune system is a key factor in viral clearance, tissue repairment, and adaptive immunity stimulus. Participation of immunity has been identified as a major factor, along with biomarkers, prediction of clinical outcomes, and antibody production after infection. Immune cells have been proposed not only as a hallmark of severity, but also as a predictor of clinical outcomes, while dynamics of inflammatory molecules can also induce worse consequences for acute patients. For convalescent patients, mild disease was related to higher antibody production, although the factors related to the specific antibodies based on a diversity of antigens were not clear. COVID-19 was explored over time; however, the study of immunological predictors of outcomes is still lacking discussion, especially in convalescent patients. Here, we propose a review using previously published studies to identify immunological markers of COVID-19 outcomes and their relation to antibody production to further contribute to the clinical and laboratorial management of patients.

Keywords: SARS-CoV-2; inflammation; antibodies; adaptive immunity; immune hallmarks

Citation: Silva-Junior, A.L.; Oliveira, L.d.S.; Belezia, N.C.T.; Tarragô, A.M.; Costa, A.G.d.; Malheiro, A. Immune Dynamics Involved in Acute and Convalescent COVID-19 Patients. *Immuno* **2023**, *3*, 86–111. https://doi.org/10.3390/immuno3010007

Academic Editor: Stefano Aquaro

Received: 9 February 2023
Revised: 8 March 2023
Accepted: 15 March 2023
Published: 17 March 2023

1. Background

Coronavirus disease 2019 (COVID-19) is an infectious disease caused by a betacoronavirus, reported as the pathogen of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). It was first described in 2019, related to transmission from wild animals, in the province of Wuhan, China. Cases rapidly increased around the world due to its easy transmission via aerosol; however, other fluids, such as urine and saliva, as well as surfaces (paper, wood, and metal) for more than 4 days [1–4], were suggested as transmission routes, but conclusive evidence is still needed.

After exposure to the virus, the respiratory tract is the main tissue affected by angiotensin-converting enzyme (ACE2) expression, the human's receptor to the viral spike protein. After binding, SARS-CoV-2 enters the target cell, thereby establishing infection. The human first line of defense plays an important role in immune recognition and management of the disease. The viral protein receptor is expressed mainly in pulmonary, cardiac, renal, and lipid-rich tissues, and symptoms are represented by cough and fever; in severe cases, symptoms may evolve to pneumonia and death. Dynamics of extrinsic factors, such as age and comorbidities [5–7], together with intrinsic factors, such as immune response, have been

demonstrated as key factors in a patient's clinical outcome during the acute phase, in their post-COVID-19 symptoms, and in their protection from new variants [8–10].

The innate and adaptive immune responses contribute to viral clearance and to the production of specific antibodies. Many studies have evaluated and characterized the immunity imbalance in patients with active disease, showing a hyperinflammatory response in severe patients, led by a cytokine storm that causes more disadvantages than benefits in the resolution of the disease [11–16]. However, few papers have proposed evaluating those patients who had a favorable outcome, known as convalescent patients.

Many individuals may present long-term symptoms, named post-acute COVID-19 (starting 3–12 weeks from the acute phase) and post-COVID-19 syndrome (or long COVID, starting more than 12 weeks from the acute phase), which highlights the damage caused by the acute phase, as well as the risk of adverse effects and death [17,18]. Leukocytes are responsible for acute dynamics, as well as the production of markers of immunity. Although leukocytes play many different roles against SARS-CoV-2, they are also a key factor in tissue repair and convalescence [19,20]. Higher levels of total leukocytes are described, but the immune cell and soluble protein profile changes during infection (active COVID-19) between mild and severe patients, especially to predict the consequences in convalescence [21–23].

Here, we propose a concise review of immune aspects in acute COVID-19 patients, as well as contribute to the understanding of the immune dynamics during active disease and the contribution to the convalescent stage. Comprehending the process involved can promote better clinical guidelines, identify better hallmarks, and improve the patient's quality of life.

2. An Overview of Immunology in the Acute Phase of COVID-19

The establishment of a SARS-CoV-2 infection triggers the host's immune response to recognition, inflammation, and viral clearance. Previous studies have highlighted the prevalence of neutrophils and monocytes as the first immune cells to migrate to the infectious site through the stimulus of chemokines and the expression of adhesion molecules by endothelial cells, acting as a key factor in innate immunity [24,25]. The recognition of microorganisms and infected cells is mediated by pattern recognition receptors, especially Toll-like receptors (TLRs), NOD-like receptors (NLRs), and others related to intermediate intracellular mechanisms of activation that contribute to the effects observed during the immunological response.

2.1. NETosis Plays a Pivotal Role in COVID-19 Pneumonia Severity

Neutrophils act mainly by phagocytosis, which occurs via the inclusion and digestion of components into intracellular organelles, mediated mainly by enzymes. In addition, neutrophils produce inflammatory mediators, such as reactive oxygen species (ROS), which contribute not only to the activation of other immune cells, but also to cell recruitment to the local site of infection [21,26]. Although cytokines and other proteins have been described in terms of neutrophil interaction in the immune system, their relationship with COVID-19 severity remains scarcely known. Neutrophils play an important role in innate and adaptive responses since it is the first cell to reach the inflammatory site, where they can recognize the pathogen, digest it, and promote an immune response through the release of inflammatory cytokines [27].

The absolute neutrophil count (ANC) has been reported as an important severity mediator among COVID-19 patients, along with lymphocyte count. Patients who required intensive care unit (ICU) admission for pneumonia caused by COVID-19 or who developed the severe form of the disease had higher values of ANC and/or neutrophil-to-lymphocyte-ratio (NLR) [6,10,24,25,28–35]. NLR determination is an easy and cost-effective test to perform in clinical practice, and it has shown a significant improvement in the stratification of COVID-19 patients at hospital admission [21,30,36,37], as well as its potential as a

prognostic factor for COVID-19 outcome [34,37–40], especially in those with comorbidities, such as type 2 diabetes, hypertension, and ischemic heart disease [25,32,41].

Relative values expressed as a percentage of neutrophils, related to absolute leucocyte count, did not demonstrate a significant difference in acute patients, even among individuals with confirmed SARS-CoV-2 infection and those patients exposed [21,42], emerging as a non-recommended parameter in clinical and laboratory COVID-19 management.

The acute phase is marked by inflammatory and inhibitory cell surface markers, such as CD63, CD64, CD117, and CXCR3, driven mainly by CXCL8 and G-CSF [42–46]. The participation of immature neutrophils marked by $CD10^{+}$ and $CD16^{low}$ is prominent in the severe form, with acute respiratory distress syndrome [28,47] and ICU patients close to discharge, when compared to moderate and mild patients, driven by G-CSF [43]. Due to the urgency of inflammatory mediators, the cell phenotype profile shows a 'shift to the left', with the presence of immature neutrophils, although it is unknown whether they are immunosuppressive or pro-inflammatory. A positive correlation of immature neutrophils was seen with inflammatory markers of IL-6, IL-1ra, CXCL8, CXCL10, CCL3, CCL4, and vascular endothelial growth factor [12,43,48,49].

CD11b, another important neutrophil marker related to adhesion to alveolar macrophages [50], was demonstrated to be controversial under COVID-19 disease activity [12,27,51], but was associated with prolonged viral replication, being significantly reduced in those with a poor outcome [51]. From the acute to convalescent stage, this marker was shown not to suffer a significant difference [42].

The expression of activated markers and adhesion molecules is important for a better understanding of neutrophil physiology and further therapeutic strategies. Thus, immature neutrophils demonstrated greater participation in COVID-19 disease, potentially due to their regulatory function, the tissue healing process, and the low expression of adhesion markers, which contribute to their maintenance in peripheral blood [12,49,52].

Severe stages of the disease are characterized by hypoxemia, which was demonstrated to activate transcriptional factor HIF-1α (hypoxia-inducible factor 1α), responsible for further production of an inflammatory profile, guided mainly by IL-1β, IL-6, and IL-8 (CXCL8) [29,53] (Figure 1). Other molecules, such as platelet-derived factor 4 (PF4) and CCL5 chemokine, can trigger neutrophil activation and promote mechanisms that may aggravate the condition of COVID-19 patients [54].

The participation of chemokines and cytokines is still not clear; however, their participation can recruit neutrophils to local activity. When infected by SARS-CoV-2, the lungs express chemokines CXCL1, CXCL2, CXCL3, CXCL5, CXCL8, and CCL20 that induce neutrophil chemotaxis from blood vessels to the lungs [53,55]. Furthermore, cytokines and chemokines in COVID-19 patients are guided mainly by the inflammatory process, involving CCL2, CXCL10, CXCL8, IL-6, and tumor necrosis factor (TNF) [53]. These molecules are important acute inflammatory mediators, which drive chemotaxis, increase adhesion molecules, and induce positive immune regulation from neutrophils and other cells [53].

Novel neutrophil mechanisms have been an important focus of research. Neutrophil extracellular traps (NETs), nets composed mainly of histones and proteins (lactoferrin, cathepsins, elastase, and myeloperoxidase), along with cytoskeleton components and other plasmatic proteins, are among the major mechanisms of neutrophils to maintain homeostasis [56,57]. NET release and ROS production are based on the neutrophil maturation level. Immature granulocytes are reportedly lower during viral clearance, when compared to normal individuals, which suggests that it might reflect the recruitment of mature and efficient neutrophils to leave the circulation and migrate to the tissue [21].

NETs also have the property of activating inflammasome complex NLRP3, which is responsible for type I interferon production and inflammatory cytokines [58]. Additionally, there is a stimulus of adaptive immune response, endothelial injury, thrombosis mediated by the immune system, and occlusion of small vessels, which may worsen pneumonia experienced by COVID-19 patients [43]. On the other hand, the capture of microorganisms

and immune cells allows the maintenance of inflammatory damage and downregulates it, thus reducing the consequences of exacerbated inflammation.

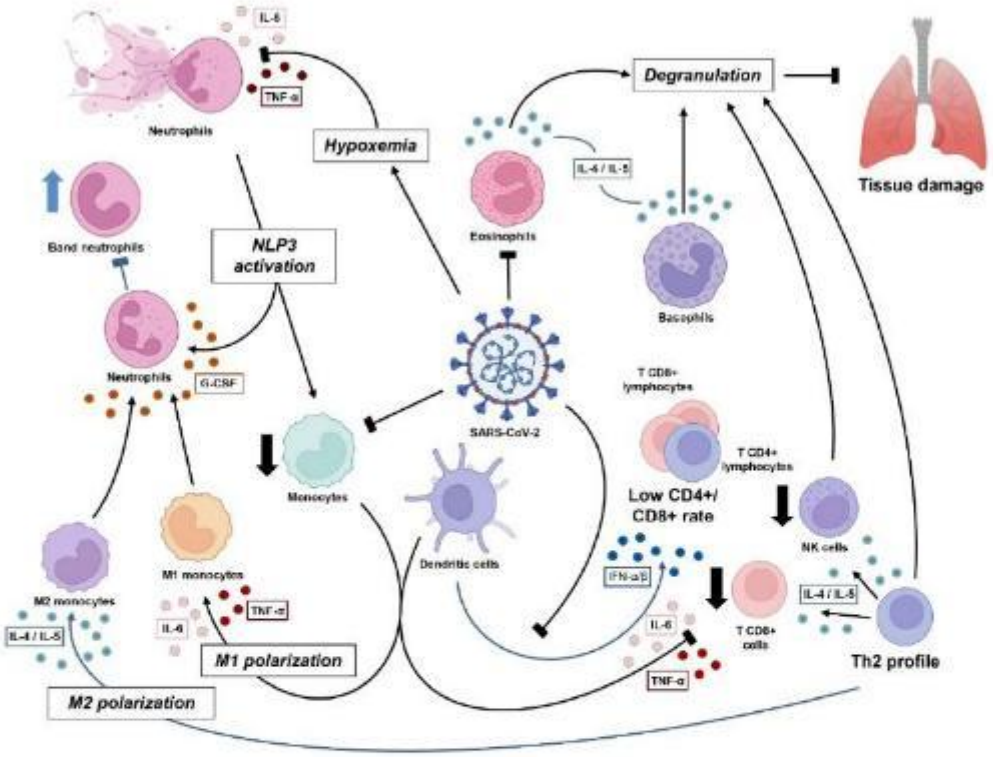

Figure 1. Dynamics of immune cell response against SARS-CoV-2 in acute phase. The virus induces pulmonary damage, which further causes hypoxemia. Both infection and tissue damage lead to inflammatory mediators, mainly interleukin-1β (IL-1β), CXCL8, and IL-6, which further cause NETosis and promote positive inflammatory feedback via inflammasome activation in both neutrophils and monocytes. Subsequently, critical patients experience low monocyte count, in terms of both T $CD8^+$ and natural killer (NK) cells, with M1 polarization (driven by cytokine storm), all related to a worse clinical condition. Instead, when the antiviral stage is established, there is sustained type I interferon (IFN) by dendritic cells, contributing to the Th2 profile (IL-4 and IL-5), which induces the degranulation process by lymphocytes (CD8+ and NK), eosinophils, and basophils, and an M2 polarization, which results in a better prognosis. The release of granules has good and bad effects, whereby they can damage viral and human infected cells, but may also damage normal tissue, characterizing the respiratory syndrome. Macrophages produce granulocyte colony-stimulating factor (G-CSF), which acts in bone marrow to mature leukocyte progenitors. This may lead to a 'shift to the left' and improve viral response.

Several studies have described the formation of NETs in COVID-19 patients, with increased rates of markers in patients who evolved severe complications, such as thrombosis and death, or in intubated compared to non-intubated patients and convalescents [43,54,57], even though patients with a mild form of COVID-19 express a different type of immune

response. NET components myeloperoxidase (MPO)-DNA, neutrophil elastase (NE)-DNA, and citrullinated histones H3 exhibited no significant difference in mild and ICU patients, but they were increased when compared to healthy individuals [12,34,59]. In contrast, higher values of neutrophil elastase were found among ICU patients on entrance, with a median reduction after 7 days and further discharge [43]. Ng et al. [60] suggested a positive correlation of NET production, poor outcome, and inflammatory markers, such as WBC, ANC, and inflammatory cytokines, in addition to increased rates of thrombosis in the COVID-19 group.

A higher concentration of NETs in the respiratory tract than in circulating plasma was also observed, and a direct correlation index to clinical illness severity score was established [54]. It is important to note that NET release induces direct and indirect effects in a systemic manner. Activation of the complement system due to the presence of tissue factor, along with the formation of aggregates in the pulmonary compartment, worsens the clinical condition of pneumonia [56,61].

The thrombosis effects caused by NETs can cause injury not only to the pulmonary tract, but also to the endothelial, renal, liver, and cardiac systems, and they may be involved in further organ failure. Individuals with previous chronic disease, characterized as groups at risk of COVID-19, may experience an increase in endothelial injury due to the intense activity of immune cells, cytokine storm, and inflammatory mediator production [44,54,56]. The participation of thrombotic factors, such as D-dimer and von Willebrand factor, has been reported as being more intense in severe COVID-19 patients; although few studies have described the direct correlation of NETosis and thrombotic molecules, a relationship among NET, platelet aggregates, and the state of thrombosis has already been proposed [44,54,60].

2.2. *Improvement of Severe Cases Is Marked by Eosinophilia*

Eosinophils are polymorphonuclear cells that play a role in innate immunity; they are characterized by granules extremely rich in inflammatory mediators, such as cationic proteins, peroxidase, hydrolase, and lysophospholipase. This lineage has been extensively studied in terms of parasite response and allergic diseases [62]. This cell profile also presents PRRs and produces cytokines, nitric oxide, and proteins, which contribute to viral clearance [63].

There is an important relationship between cytokines and eosinophil production. It is already known that IL-4 promotes the expression of adhesion molecules that further contribute to eosinophil adhesion to the endothelium, while IL-5 induces degranulation. Both cytokines are produced mainly by mast cells, basophils, and Th2 lymphocytes, which are extremely important in allergic responses. The degranulation process is commonly used in parasites due to membrane damage, but it can also damage the host tissue [62].

Eosinopenia (<40/mm^3) was reported in patients admitted to the ICU, where it was suggested as a prognostic factor for poor outcomes [7,8,21,63–67]. In severe patients, CD8$^+$ T cells contribute to eosinophil proliferation via the production of IL-5; however, due to exhaustion on the first cell, the IL-5 level may suffer interference, which might be a cause of eosinopenia at the beginning of the disease course [36]. Around 20 days after hospital admission, the absolute eosinophil count (AEC) usually exceeds 1500/mm^3 in patients ready for discharge. This eosinophilia commonly lasts around 5 days and is correlated with a reduced mortality rate [8,53,64,68,69].

Asian race/ethnicity with eosinophilia was a predictor for a shorter hospital stay, while other races/ethnicities showed no significant difference [63]. Other factors that have been proposed to contribute to no resolution of AEC are higher age, alcohol abuse, tobacco use, hypertension, diabetes mellitus, chronic pulmonary disease, chronic kidney failure, comorbidities, previous use of corticoids, and initial symptoms of normal cough, dyspnea, arthromyalgia, asthenia, and saturation >95% [8]. Some studies with asthmatic patients presented the same pattern, in addition to reporting the protective aspect of eosinophilia and a Th2 cytokine profile on disclosure of COVID-19 patients with asthma [63,70].

This cell lineage was also seemingly connected to severe symptoms, with a few patients with normal AEC experiencing fever, fatigue, shortness of breath, and inflammatory infiltrates at hospital admission, in addition to increased rates of aggravation [69], related to the natural killer (NK) T-cell response and further eosinophilic lung inflammation [26].

Sustained eosinopenia was found in severe cases and in patients with cytokine storm syndrome [28,36], and three hypotheses have been suggested [69]: (1) production of corticosteroids by the adrenal during an acute response, which blocks the release of eosinophils from bone marrow and induces migration of eosinophils to the tissue, culminating in reduced eosinophils in circulation; (2) COVID-19 may cause damage to the bone marrow, which would also impact eosinophil count (hypothesis not fully elucidated); (3) upregulation of Th1 and Th2 cytokines by viral clearance promotes leukocyte migration to pulmonary tissue, resulting in a lower availability in peripheral blood. Although not widespread, eosinopenia might also be related to the infection of eosinophils by SARS-CoV-2, as previously demonstrated [9]. These issues can be highlighted, especially as the eosinophil count increases at the same rate as clinical improvement and viral load reduction.

Both eosinophils and neutrophils were found in the bronchoalveolar fluid extracted from patients with severe COVID-19, in addition to eosinophil cationic proteins, which confirms the importance of eosinophils in the local immune response against SARS-CoV-2. The number of eosinophils in the pulmonary tract can cause similar inflammation to acute eosinophilic pneumonia, with a previous observation of >25% of eosinophils in the lungs [26]. Although it is known that COVID-19 is the agent responsible for leukocyte recruitment to the pulmonary tissue, the role of eosinophils in viral clearance is not fully understood.

The surface markers on COVID-19 patients demonstrate an activate profile characterized by a lower expression of CD15, CD66b, and CD193 and a higher expression of CD62L, CD69, and CD147, compared to noninfected individuals. A comparison between moderate and severe patients revealed $CD69^+$ eosinophils in the latter, which might be related to decreased outcome, whereas CD66b, CD11b, CD11a, and CD24 are present in eosinophil membranes in moderate patients, influencing clinical management [28].

Activated eosinophils ($CD69^+$) exhibit a positive correlation with soluble inflammatory molecules in severe patients, such as IFN-γ, CCL2, CCL7, and CCL8. They play a key role in lung tissue infiltration, degranulation of neutrophils, clotting factor activation, molecule recognition, and extracellular matrix metabolization [28].

2.3. *Granulocytes and Monocytes Management in Viral Clearance*

COVID-19 pathology is guided by a Th2 cytokine profile, which is directly connected to the participation of eosinophils, basophils, and the local inflammatory response. The absolute basophil count (ABC) was found at lower levels during the initial course of the disease, while showing recovery over the course of illness [22]. An absence of ABC recovery was present in severe patients who required mechanical ventilation and who evolved to a fatal outcome, possibly being a biomarker for a poor outcome [51]. A negative relationship between basophil count and both severe and hospitalized COVID-19 patients demonstrates the importance of basophils in local viral clearance [71].

The evaluation of basophils during disease activity demonstrated an increased rate of $CD131^+$ (IL-3 membrane receptor) cells, CD11b, CD63, and CXCR4 [28] but a low expression of GM-CSF and IL-5 receptors [51]. Furthermore, the involvement of thrombotic events in severe cases, as well as immunomodulatory effects during the acute and chronic responses, highlights the important role of basophils in immunity against SARS-CoV-2 [71]. Basophils are involved in the hypersensitivity response, production of mucus, vaso-constriction, inflammation, and tissue damage, but more studies must be conducted to evaluate their effect in acute COVID-19 [62].

Monocytes, on the other hand, represent a subpopulation of leukocytes from the same precursor as neutrophils. They are known as agranulocytes, with a main function related to the recognition of pathogens and cell products by PRRs and subsequent phagocytosis.

The stimulus activates intercellular mechanisms that contribute to cytokine storm and migration to tissue.

These cells participate in both innate and adaptive immunity, acting in COVID-19 viral clearance or as antigen-presenting cells to combat the virus or induce antibody production, respectively. Monocytes are classified into three specific subtypes according to their cell surface proteins: classical ($CD14^{++}CD16^{-}$), inflammatory ($CD14^{++}CD16^{+}$), and patrolling ($CD14^{+}CD16^{++}$). It is important to highlight that other markers can also be used, such as chemokine receptors and cytokine production [72].

Monocytes also contribute to an interesting aspect of COVID-19 physiopathology, as they were previously shown to express ACE2, thus being influenced by the virus [73]. It was shown that prolonged viral infection (more than 10 days of positive RT-PCR tests from admission) can reduce ACE2 mRNA, as well as levels of soluble ACE2, instead quickly returning to normal in patients with a negative RT-PCR in less than 10 days [74].

The absolute monocyte count (AMC) is a low-cost measurement, and monocytosis/monocytopenia was previously related to hospital discharge. Those with higher AMC spent fewer days in hospital (15 days), while those with lower levels of AMC remained for a prolonged period (40 days) [73]. In addition to its functionality, low rates of monocytes were found in severe and in mechanical ventilation patients [21,40,51,75], further associated with age [33] and the presence of atypical and vacuolated monocytes [73]. It was suggested that these morphological changes come from the process of monocyte infection, as also observed in visceral leishmaniasis, but not other viral diseases [73].

Severe disease is marked by a lower rate of AMC when compared to mild cases [40,75,76]. Monocyte soluble markers show that, during acute COVID-19, there is a higher secretion of sCD14 and sCD163 when compared to normal individuals. sCD163 is correlated with the time elapsed from hospital admission, whereas sCD14 is correlated with several laboratory parameters, including IL-6 and C reactive protein (CRP). Accordingly, a few differences were observed between ICU and non-ICU patients, but patrolling monocytes produce less sCD163 and more sCD14 [23,49,77–79]. Corticoids were suggested as a factor interfering with monocyte activation and inflammatory pathways, although the CD163 receptor was increased in all subtypes of monocytes in severe conditions [10,75,80–82].

The participation of monocytes/macrophages in pulmonary inflammatory diseases has been reported, especially classical monocytes in asthma [83,84]. Some studies have reported higher levels of monocytes in peripheral blood with a further reduction in convalescent stage, whereas others have reported the opposite [21,85,86], with similar observations in bronchoalveolar fluid [26]. A delay in interferon signaling leads to the infiltration of monocytes into the pulmonary tract, thus inducing the production of inflammatory mediators that drive the response to cytokine storm, which results in positive feedback to leukocytes, contributing to tissue damage and regulation of antiviral cytokines. Coronaviruses mediate antiviral cytokines via translational mechanisms that are usually involved in viral clearance, such as type I interferon, which acts as an escape mechanism [87].

During cytokine storm syndrome, monocytes tend to reduce their quantitative circulating value, but increase the granularity and permeability of endothelial cells [36,85]. This might result in the expression of adhesion molecules and migration to other tissues. Although it is well established that cytokine storm is the main event that influences a worse prognosis, monocytes (and even dendritic cells) were suggested to not be the main producers of proinflammatory cytokines [88].

The infection stage involves the participation of $CD16^{+}$ monocytes, in contrast to a healthy status [73,79]. However, among COVID-19 subgroups, mild and severe stages presented increased rates of inflammation ($CD16^{+}$) and presenting ability ($HLA\text{-}DR^{+}$) when compared to critical patients [49,51,75,76,81]. Winheim et al. [89] demonstrated that, within the subpopulation of activated monocytes, there was participation mainly by classical monocytes. This inflammatory profile was demonstrated to be guided by higher levels of TNF-α, IL-6, IL-10, IL-2, IL-4, IL-13, IL-18, CCL3, CCL4, and CCL2, although only IL-6 had a significant positive relationship with $CD16^{+}$ monocytes and a negative relationship with

HLA-DR$^+$ monocytes [19,76,90–93]. This suggests that proinflammatory monocytes drive IL-6 production, which may be related to the proinflammatory state and low HLA-DR production in critical patients (Figure 1).

We must highlight that both extra- and intracellular mechanisms play an important role in SARS-CoV-2 clearance, especially inflammasome activation, as mentioned before, in neutrophils and monocytes. Interactions among viral RNA [94], NETs [43,58], and the dysregulation of calcium concentration [95] influence NLRP3 activation, mediated by the viral envelope protein. Upon stimulus, this induces the maturation of proinflammatory cytokines IL-1β and IL-18, which further stimulates IL-6 and TNF, promoting inflammation in the lungs. These events worsen disease progression, representing the major mediators of cytokine storm, and cause systemic dysfunctions, such as macrophage recruitment and leukocyte degranulation [96]. Moreover, severe patients experience an increased rate of activation of the NLRP3 and TXNIP inflammasome pathways [79].

The expression of surface markers demonstrates a predominance of M1 macrophages in critical patients, when compared to noncritical patients, due to the increased expression of CD80, higher production of IL-6, TNF-α, and TGF-β cytokines (although *M*1 macrophages are not the main source of these cytokines), and lower expression of MHC-II [73,82,88,97]. It is important to note that *M*1 macrophages show increased odds of tissue migration due to their rheologic properties, but also fewer acid granules, which contribute to viral RNA perseverance in the cell. *M*2 macrophages, otherwise, present more acid granules, which lead to viral RNA instability and further degradation [90,98]. Even though alveolar *M*2 macrophages (CD206$^+$) are also present during the immune response, when compared to the healthy status, the cytokine profile barely varied, with an IL-4/IL-13 balance to induce *M*2 macrophages [73,99]; however, a decrease in CD86$^+$ expression and MHC-II [88] was observed.

Dendritic cells (DCs) play an important role in viral clearance, antigen presentation from innate to adaptive immunity, and the capture of apoptotic/necrotic cells [100]. Immature DCs show an increased ability to recognize antigens, while mature DCs are important producers of IL-12, IL-1β, type I and type II IFN, IL-4, IL-10, and TNF-α. [101]. Their function in COVID-19 disease remains unclear, and their participation is mainly guided by inflammatory status. Some studies demonstrated a lower expression of c-KIT$^+$ in cDC1 and of plasmacytoid DCs in mild/moderate patients, together with a significant reduction in DC count. Moderate patients were marked by a higher expression of CD38. However, with the progression of disease severity, a lower participation of the inflammatory DC3 subset (CD163$^-$ CD14$^-$) was reported, with an increase in the c-KIT receptor [49,81,102,103].

Patients both with and without neurological symptoms 4 weeks after disease onset demonstrate increased rates of DC density and mature DCs, when compared to a healthy status. However, differences were not observed between infected groups [104]. These data suggest long-term participation of DCs, whether the patient is symptomatic or not. Cell activation and inflammatory status are compromised by increased age, and senescence is a common factor of immunity, which, when related to COVID-19, seems to play a key role in response, potentially reflecting why newer patients experience disease differently [101].

During viral infection, plasmacytoid DCs (pDC) are important producers of type I IFN (especially IFN-α) via recognition of RNA by TLR7/8, once there is participation of the PD-L1$^+$CD80$^-$ DC population; however, their levels are diminished with severity [49]. Even though a phenotypic profile is prominent in asymptomatic patients, hospitalized patients display a phenotype characterized by PD-L1$^+$CD80$^+$. It is important to highlight the higher expression of CD86 in asymptomatic patients [49], demonstrating their greater ability to stimulate DCs, whereas hospitalized patients had a higher expression of CD80 [12,103] and lower HLA-DR [105].

During viral infections (including viruses other than coronavirus), viral escape can occur through interference with type I IFN production via antagonism of transcriptional factors. This was established by some studies describing a higher concentration of IFN-α and IFN-genes at the beginning of disease, with a subsequent reduction, even in patients

that experience a severe form, in comparison to mild patients [12,106,107]. IFN-α plays an important role in dendritic cell functionality during acute disease, even in convalescence. A normal level is not restored for more than 6 months after infection, and a positive correlation index between IFN-α cytokine and pDCs has been demonstrated, which might be related to the decrease in P1-pDCs in hospitalized patients [106]. Costimulatory molecules CD80 (B7-1) and CD86 (B7-2), expressed mainly by DCs, are capable of activating T cells and participating in the SARS-CoV-2 response. A lower circulating level of mDC and pDC $CD86^+$ has been reported in the acute phase; however, their efficiency in activating T lymphocytes must be explored, as their dynamics in acute disease interfere with antibody titers during convalescence [106,108].

2.4. Lymphocytes: When Adaptive Immunity Takes Place

Lymphocytes are important cells related to antiviral regulation, maintenance of homeostasis, and inflammatory responses. They are commonly divided into T and B lymphocytes according to their functionality and cell surface marker expression; however, both classes participate in innate and adaptive immunity involving soluble proteins (mainly cytokines, chemokines, and antibodies) through cell-to-cell interactions [109].

The absolute lymphocyte count (ALC) parameter, whether alone or combined with neutrophil count, had a greater ability to estimate a worsening prognosis when low [110], especially in ICU patients [111,112] and in those who evolved to death [40]. The mechanisms that drive lymphopenia are still not clear, but some concerns have been raised, such as (1) lymphocyte infection by the virus, (2) cell migration from blood to tissue, and (3) damage to lymphoid organs (and further production of lymphocytes) [113,114].

Due to the intense participation of cytotoxic T ($CD8^+$) cells, the $CD4^+/CD8^+$ ratio is lower in diagnosed patients and those under ventilation therapy [49,51], when compared to healthy individuals, guided by inflammatory TNF-α and IFN-γ cytokines as major contributors [41,111]. This suggests an inflammation process related to antiviral activity, especially due to the expression of suppressor marker CD57 and adhesion molecules (CD38 and HLA-DR) on cytotoxic lymphocytes [6,49]. It would be reasonable to think that a side-effect can be seen in terms of tissue damage, as inflammation induces degranulation activity from T $CD8^+$ lymphocytes and can be harmful to regular tissues [115–118].

T lymphocytes can be divided into different subtypes, according to cytokine production and functionality. The cytokine environment is characterized by the main populations of Th1 (mediated by INF-γ), Th2 (IL-4 and IL-13), Th17 (IL-17A and IL-17F), Treg (IL-10, IL-35, and TGF-β), and Th follicular (IL-21). Other T lymphocyte subpopulations also participate, such as Th9 (IL-9) and Th22 (IL-22), but their role during COVID-19 disease is not well described [119]. Cytokine storm, known as the key factor underlying disease severity in COVID-19, is guided by IL-1β, IL-2, IL-6, IL-7, IL-8, IL-10, G-CSF, GM-CSF, CXCL10, CCL2, CCL3, IFN-γ, and TNF-α [118,120–122]. However, few molecules have been related to disease outcome, such as the perseverance of higher IL-1ra and chemokine (CXCL10, HGF, CCL3, CCL7, and MIG) concentrations in plasma associated with a worse outcome, whereas CXCL10 and CCL7 were shown to predict COVID-19 improvement [121].

Previous studies described an increase in T helper cells, albeit slowly, in mild disease, as well as in Treg cells ($CD4^+CD25^{high}$), B lymphocytes, and NKT cells [6,41,51,116]. The severity score presented a negative relationship with both $CD8^+$ and $CD4^+$ lymphocytes, as well as NK cells [5,118,123–125], which may be related to their degranulation ability. All subtypes (naïve, memory, and effector T $CD8^+$ and $CD4^+$ cells) had increased rates of both apoptosis and migration characteristics, suggesting an inflammatory functionality and local repairment [126]. In mild patients, the immune response is based on the nonconventional Th1 cell lineage; however, in patients who evolve to a severe condition, the Th2 profile also participates [26]. Regardless of symptoms and severity, lymphocytes express CD38, CD39, CD69, CTLA-4, HLA-DR, Ki-67, and PD-1 markers, potentialized mainly by T helper and cytotoxic memory cells [6,85,127]. This profile induces acute and local damage over the course of the disease, and some parameters may decrease with disease severity [128].

In moderate-to-severe patients, not only were T effector cells found to express granulysin, but NKT ($CD160^+$) cells also produce more granzymes A, B, and H to solve viral infection, which persists for a longer time in critical patients [82,129]. A severe condition was marked by a significantly lower count of NK ($CD56^+$ and $CD16^+$) cells [5], compromising cytotoxicity [115,130]. T $CD8^+$ memory cells and effector cells ($CD45RO^+$ and $CCR7^-$) are influenced by the IL-15/IL-15RA axis [116,131,132], PD-1, and inhibitory receptors (NKG2A and NKG2D) during severity, which play a key role in the functional exhaustion of NK cells, senescence, and apoptosis [115,118,120,126,133]. Imbalance of the IL-15 axis may induce a significant reduction in NKT γδ cells ($CD160^+$), which was previously proposed to promote rapid control of the disease via direct cytotoxicity, as well as induce cytotoxicity mediated by antibodies, similar to regular NK cells [79,126,129,131]. The increased rates of cytotoxic T cells and type I cytokines were described as biomarkers of a poor outcome in convalescence, which may be guided by the higher release of enzymes, inducing cellular damage in tissues [127,134].

During SARS-CoV-2 infection, a significant proportion of T γδ cells can be identified, when compared to healthy individuals, but no differences were observed between patients under ventilation and SARS groups [48,52,107,126,135,136]. Instead, these cells presented a higher expression of $CD4^+$ and $CD25^+$ markers [137], although mild patients also had increased levels of TCR γδ naïve and central memory cells compared to severe patients [135]. Mucosal-associated invariant T cells (MAIT) did not differ among COVID-19^+ patients, but correlated positively with patient's age and negatively with severity, whereby those with pneumonia, hypoxia, and ICU had lower levels [49,107,126,136], which were increased among patients who were discharged in <15 days, along with an increase in iNKT cells [107]. However, MAIT cells with the expression of the CD8 marker were increased in patients in the ICU, together with IFN-γ and granzyme B production. COVID-19 patients show higher $CD69^+$ expression on MAIT cells, which is further correlated with CRP, IL-18, and IFN-α levels, suggesting an influence on inflammatory markers [52,79,107]. Once these cells are in the minority in blood, their participation in the inflammatory status still requires further elucidation. Their reduction in severity might be related to the intense production of other innate immune cells with a more active functionality in inflammation than nonconventional T cells, although cytotoxicity is stimulated, even in minority cells.

Many studies have raised concerns about laboratorial and clinical markers, especially related to prognosis. Few bioinformatic studies have addressed this issue using acute patients; however, it remains unclear whether other hallmarks can be proposed, characterizing the various clinical profiles that patients may experience (asymptomatic, mild, severe, ICU, and convalescence). Considering the completeness of data, science faces a need to comprehend the mechanisms involved in immune dynamics underlying significant clinical changes. These questions must be addressed in future studies on coronavirus so as to construct a bridge between basic and clinical studies.

3. What Do We Know about Convalescence so Far

Convalescence is known as the stage after COVID-19 clinical recovery. This group is formed by patients who were diagnosed with COVID-19 (whether symptomatic or not) but did not evolve to death. Comprehending the immune system's behavior during the acute phase plays an important role in recognizing the key points related to clinical improvement, as well as in identifying novel hallmarks related to a better or poorer outcome, and identifying the dynamics in adaptive immunity [60,75].

In convalescents, a significant increase in activated neutrophil count ($CD45/CD11b^+$) 28 days after clinical recovery was reported [21,27], although a reduction in $CD64^+$ neutrophils [45] and NETs was observed a few weeks [12,54] to months [60] after a positive SARS-CoV-2 RT-PCR test.

Activation markers on neutrophils have been assessed, and a lower metabolic function with few genetic materials has been observed in COVID-19 acute patients, compared to healthy individuals. However, this is even lower in convalescence, together with

higher rates of immature granulocytes, which might be related to a lower number of well-functioning neutrophils, as well as urgent granulopoiesis [21]. It was suggested that, after disease, there is an increased circulation of nonreactive neutrophils; however, after a period of time, the reactivity function re-emerges [21,82]. The neutrophil population seems to be controlled quantitatively, according to Rodriguez et al. [39], although evolution of the inflammatory profile from mild and severe patients to convalescence was not determined. This profile may be a consequence of the cytokine storm from SARS-CoV-2 infection to the convalescent period, especially related to the reduction in IFN-α levels over time since symptom onset [79].

Immature granulocytes are significantly increased during acute COVID-19, reaching even higher levels in convalescence [21], albeit not from the neutrophil lineage [49]. This might be due to the intense immune response to help fight against the virus, whereas, in convalescence, there is an urgent need for the immune replacement of functional cells.

Eosinophils tend to be reduced in bronchoalveolar fluid during the convalescent stage, as indicated by immature cell markers $CD45^+/CD24^+/CD16^{low}$ [26], whereas they tend to be increased in blood vessels [9,21,22]. A direct relationship between AEC and ALC was described, which could be a reasonable field to comprehend immune factors associated with a transient stage of acute complications and adaptive response [138].

Although not yet fully understood, convalescent patients (±14 days after clinical recovery) with a higher titer of antibodies also express a higher mean AEC and level of immature granulocytes [138]. Even though Vitte et al. [66] evaluated convalescent patients 28 days after symptom onset, where the median eosinophil count increased to a greater level than in patients in the mild group, the level only decreased in 3/19 severe patients, with the remainder presenting a better outcome. Eosinophils demonstrate an important correlation with inflammatory markers during all disease pathologies, considering specific markers of innate immunity following symptom onset, when considering the evolution of dendritic cells, T lymphocytes and monocytes, until the recovery stage [39].

Several studies have proposed a concise evaluation of eosinophils in convalescence; however, despite being classically known as elements of innate immunity, they were also demonstrated to participate in adaptive immunity. There is an urgent need to comprehend the biological mechanisms underlying this cell's behavior to improve therapeutic strategies.

The production of IL-6 and IL-4 by basophils in convalescence are associated with higher production rates of antibody against SARS-CoV-2. However, patients admitted to the ICU had a slight reduction in IL-6 levels, when compared to those who were not [39]. Although the mechanisms underlying the relationship among basophils, viral clearance, and antibody production are not fully understood, this cell lineage plays a pivotal role in viral load and local repairment.

During recovery (1–10 days after admission), the AMC tends to return to normality, and the level of activated monocytes is reduced [75]. Lower counts observed during the critical stage tend to increase in mild and severe conditions (characterized by a higher number of monocytes) (Figure 2), along with a reduced expression of inflammatory markers (CD38, sCD14, CRP, CD163, and soluble tissue factor) and an increased expression of $HLA\text{-}DR^+$ [42,75,76]. The convalescent stage, characterized by a stabilization of symptoms, has been described with a significantly increased level of monocytes, when compared to COVID-19-infected and healthy patients [21]. However, the participation of subtypes is still controversial [75,76,126]. Monocyte stabilization (and its subtypes) was demonstrated to occur only 151 days after infection [42,75].

On the other hand, macrophages are influenced by antibody production against SARS-CoV-2 epitopes. Polarization to M1 macrophages is also observed in convalescents, especially stimulated by IFN-γ, resulting in a further production of CXCL8 and CCL2, which are both important chemokines for monocyte migration to tissue [79,91,98,139]. IFN-γ was described to play an important role in monocyte activity, with delayed IFN production (during acute disease) potentially driving a late inflammatory response against the virus [73]. Although inflammation is mainly driven by *M*1 macrophages, *M*2 macrophages

may also contribute with proinflammatory cytokines when antibodies are produced. IgG anti-SARS-CoV-2 is recognized by a complex of receptors, but inflammation is driven by FcγRIIa recognition. Typically, this receptor, together with other FcγRs, is largely expressed in macrophages; when stimulated, it contributes to a cytokine storm through Il-1β, IL-6, TNF-α, and CXCL8 production [140]. This highlights that, depending on the stimulus of adaptive immunity and further antibody titer in acute conditions, the immune dynamics may worsen disease progression (Table 1).

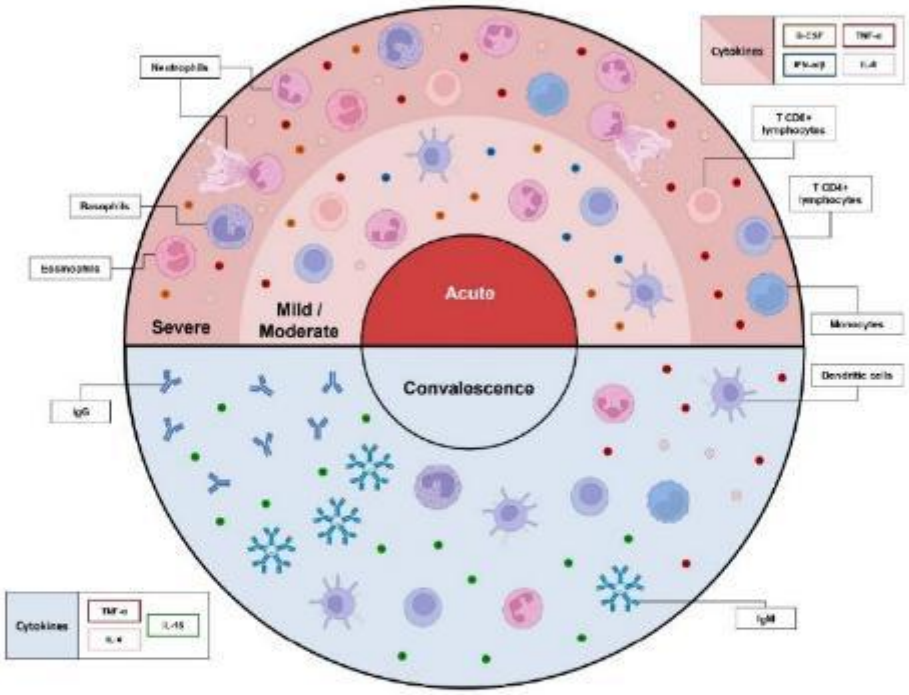

Figure 2. Transient immunological response in mild/moderate, severe, and convalescent patients with cellular and molecular profile. Severe patients experience intensive immune dynamics, driven by cytokine storm (TNF-α, IL-6, IFN-γ, and CCL2), which may worsen clinical condition, as well as compromise adaptive immunity. With clinical improvement, the Th2 profile becomes more prominent, with participation of IL-10, IL-2, IL-4, IL-13, IL-15, IL-18, CCL3, CCL4, and CCL2; however, if a Th1 profile persists, a poor outcome can be seen. Those with a mild/moderate profile have more antiviral properties (IFN-α and IL-15RA), with a higher proportion of T $CD8^+$ lymphocytes, neutrophils (especially bands), and dendritic cells. Regulatory and anti-inflammatory mechanisms prevent severe conditions, but may also interfere during convalescence. This stage is marked by an improvement in monocyte count (for those severe) and a change in cytokines to a repairment profile. In convalescent patients, higher antibody IgM titers are seen during the first months after infection, while IgG may survive for years, potentiating the prevention of new infections. Adaptive cell surveillance prevails over innate immunity; however, local damage to tissues may cause further complications or even increase the risk of death.

Table 1. Major functions of innate immune cells and their subtypes during COVID-19 disease and convalescence. Phenotypic and functional dynamics are described, which may contribute to laboratorial and clinical aspects of SARS-CoV-2 infection, as well as participation during convalescence.

Immune Cells		COVID-19	Performance in Convalescents	References
Neutrophils		• Important marker of prognosis when calculated with ALC on neutrophil-to-lymphocyte ratio (NLR) • Stimulus for NET release • Increase in thrombosis risk and organ damage in severe cases • Immature neutrophil ($CD16^+$) participation	• Reduction in NET production • Senescent neutrophil appearance • Low expression of activation markers on cell surface	[17,23,27,44,47,54,59]
Eosinophils		• Protective against severe outcome in ICU patients • Increased rate is directly proportional to patient's clinical improvement • Lower in severe patients and with cytokine storm syndrome • Activated neutrophils ($CD69^+$) related to poor outcome	• Contribution to higher titer of antibodies • Low rate of infected eosinophils by SARS-CoV-2	[9,29,39,69,95,138]
Basophils		• High products of inflammatory mediators IL-36	• Indirect contribution to antibody production through IL-4 and IL-6 production	[31]
Monocytes	Classical	• Lower	• Higher than in acute phase • Higher expression of HLA-DR	[11,23,73,75,76,88]
	Inflammatory	• Higher level • Associated with IL-6, TNF-α, and inflammatory markers in critical patients	• Reduced levels • Higher control of inflammatory status	
	Patrolling	• May be used to differentiate mild and severe patients	• Stabilization of blood count 10 to 150 days after symptoms onset	

Table 1. Cont.

Immune Cells		COVID-19	Performance in Convalescents	References
Macrophages	M1	• Inflammatory profile ($CD80^+$) • Guided by IL-6 and TNF-α • Less interference on viral replication, when compared to M2 macrophages, due to fewer acid granules • Higher expression of CD11b and $CD11c$, and lower expression of CD68 and CD169	• Increased participation by IFN-γ • Intense production of CXCL8 and CCL2	[88,91,98,139]
	M2	• Alveolar macrophages ($CD206^+$) show higher dimension, together with M1 • Interference with viral replication due to acid granules • Higher expression of CD163, $CD11c$, and CD169, and lower expression of CD68	• Recognition of IgG anti-SARS-CoV-2 occurs by FcγRIIa and induces pro-inflammatory molecules (IL-6, IL-1b, TNF-α, and CXCL8).	[73,90,98,140]
Dendritic cells (DCs)	Plasmacytoids	• Lower in infected patients • Proportional to a worse prognosis • Reduced ability to produce IFN-α and TNF-α • Moderate expression of CD38	• Increased, when compared to severe patients • Suggested increase after diagnosis	[70,77,91,93,105]
	Monocyte-derived/myeloid DCs	• Lower in severe patients • Low HLA-DR expression in severe patients • Poor functional alterations • Increased expression of CD11b and $CD11c$, especially on $CD1c^+$ mDC	No material found	[12,86,98,139]

The pDC and myeloid DC (mDC) counts during the acute phase are lowered in circulating blood, when compared to a convalescent and healthy status, related to the patient's age and potentially the migration of these cells from blood to lymph nodes [12,42,51,77,85,86,106,134]. Lower counts are seen in severe when compared to mild patients [12]. However, when compared to the convalescence stage, the total DC count remains lower when compared to normal individuals. Among subpopulations, only transitional DC had an increased rate [89], whereas $CD1c^+$ mDC was reduced, and $CD141^+$ and $CD16^+$ mDC exhibited no difference from healthy patients.

The participation of DCs is yet to be unraveled, but the damage occurring in severe patients prevails during convalescence. The cytokine storm experienced by some patients allows a greater effect of the negative IL-6/DC axis and further disease progression [106].

Convalescence is also marked by an improvement in ALC, which occurs rapidly in mild/moderate patients, compared to critical patients [82]. Furthermore, a Th1 profile is maintained, with higher prevalence of MAIT cells ($CXCR3^+$), in those who had mild disease [136]. The reactive ability of MAIT cells ($CD69^+$) remains for a few days after symptom onset, but reduce after 1 month [79]. Few studies evaluated Tγδ cells in convalescence, but a nonsignificant difference was demonstrated among healthy, infected, and convalescent patients [49,126]. Those with a severe form presented greater granzyme K production in convalescence, as well as a strong decrease in $CD56^{low}CD16^+$ effector cells [116]. Those who presented a better outcome had normalization of helper, cytotoxic, and memory cells, but a perseverance of nonconventional Th1 cells, as well as an increase in IFN-γ, TNF-α, and IL-10 cytokines. There is typically an improvement in parameters, but a long time is needed to return to normality [127,129].

Memory and follicular T cells and antibodies specific to spike proteins are low in the first 4 months after viral clearance in mild patients [132,141]; however, a T-cell response has been observed even after 6 months toward the spike, nucleocapsid, and M proteins [142]. Although B cells are stimulated following infection, few studies have explored the components underlying this activation. An increase in circulating B lymphocytes ($CD19^+CD10^+$) was shown to better drive adaptive immunity in symptomatic and RT-PCR-negative patients than in severe RT-PCR-positive patients [6,117,127,143] (Table 2).

Table 2. Participation of phenotypic and functional lymphocyte cells during COVID-19 disease and convalescence.

	Immune Cells	COVID-19	Performance in Convalescents	References
T lymphocytes	Helper ($CD4^+$)	• Negative correlation with prognosis • Contribution to cytotoxicity by T $CD8^+$ cells • Regulation of a Th1 polarized response and B-cell proliferation	• Specificity to spike, M, N, and RBD antigens for periods over 5 months	[6,128,132,144–147]
	Cytotoxic ($CD8+$)	• Increase in diagnosed patients (despite clinical presentation) • Lower in severe patients • Reduced frequency of memory and effector T $CD8^+$ cells ($CD45RO^+$ and $CCR7^-$) • Activated cytotoxic T cells (HLA-DR) do not show significant variation	• Severe patients show perseverance on production of granzymes, which might be related to long-COVID-19Specificity to viral antigens 4 weeks after clinical improvement	[5,20,118,116,132,147]
	B lymphocytes	• Increased circulation of B lymphocytes $CD19^+$ in severe conditions	• Reactive memory phenotype against S, RBD, and N antigens • Low seroreactivity rate to most antigens after periods over 6 months, but good (although reduced) seroreactivity rate to S antigen	[148,149]

Atypical memory B cells were the first lineage of adaptive immunity described, and their perseverance might indicate a worse outcome. Instead, immature transitional cells are related to clinical improvement and a higher surveillance rate. In convalescence, a higher participation of classical memory cells has been found, with a more prominent involvement of immature transitional B cells [150]. Immune reactivity to SARS-CoV-2 was observed even 5 months after infection, especially to spike, RBD, and N proteins [132,148].

Nonconventional T cells demonstrated reactivity and expansion even 3 months after the first SARS-CoV (in 2003) infection, which was further correlated to IgG anti-SARS-CoV [151]. Analyses have demonstrated that antibodies participate in immune defense, even though an increase in effector cells suggests a better clinical outcome in those patients diagnosed with COVID-19 [135]; nonregulated production of antibodies may cause a hyperactive immune response and lead to further immune cell infiltration into the lungs [140].

The majority of B lymphocytes are characterized as memory cells [116] and $CD27^{-}$ IgD^{-} [127], which mainly produce IgM a few weeks after viral clearance, as well as IL-10. Plasmablast participation is transient during the convalescence of patients who experience mild disease, but sustained in severe cases [39,48,89,116]. Transitional cells ($CD24^{high}CD38^{high}$) markedly increased in the transition from severe disease to convalescence, whereas mild and moderate patients showed no significant difference. Ki-67, an important protein for cell proliferation, is increased in acute patients, when compared to convalescence, showing a positive correlation with T helper cells [85,127], which might be due to the intense stimulus during the acute phase, with a further regulation process in convalescence [143]. In contrast, a discussion of viral infectivity must be established, as some studies described the participation of higher viral titers in severe patients, as well as the ability to effectively induce both innate and adaptive immunity [132].

Few data are available on adaptive immunity and the factors associated with the convalescent stage; furthermore, controversial data have been presented, hindering comprehension of the main component contributing to higher or lower production of antibodies. It is clear, however, that anti-spike lymphocytes have a low survival rate when compared to other SARS-CoV-2 antigens, whereas more studies must be conducted to determine the influence of dendritic cell activation and cytokine involvement to unravel this issue. These factors may contribute to immune efficacy during the acute stage and impair adaptive immunity activation. We recognize that this process may contribute to not only treatment protocols, but also the availability of neutralizing antibodies in convalescence.

Specific antibodies against COVID-19 proteins are key factors influencing immune protection. Many studies have found that those convalescent patients who experienced a symptomatic stage of COVID-19 have a higher rate of antibody production [152]. Those with fever, cough dyspnea, and pneumonia are 50 times more likely to produce higher antibody titers [138,153]. The presentation of higher titers of antibodies in severe patients has been discussed; although some studies have proposed that the severity of symptoms is related to a higher viral load and the availability of viral antigens to induce the immune system, only the anti-SARS-CoV-2 spike/RBD region antibody is increased in severe patients [85,132].

The antibody response is an important step in immunity that actively participates in cellular response, complement activation, and immunity protection [82,153]. One of the functionalities of antibodies includes the activation of the classical pathway of the complement system, thus mediating the damage to infected tissue and release of anaphylatoxins. Although aimed at viral clearance, it was identified that the immune complex from the membrane attack complex is fixed on lung vessels, potentially causing irreversible pulmonary damage in fatal COVID-19 [82].

A negative correlation has been found between lymphocyte count and antibody titers, suggesting that an intense acute inflammatory response may compromise antibody production during the convalescent stage [152]. Patients in the acute phase that produced higher IFN-γ levels were demonstrated to have higher antibody titers and greater lymphocyte activation, suggesting that adaptive immunity is IFN-γ-dependent [151]. However, viral

load during the acute phase was also highlighted as a possible interfering with antibody production [124].

Detectable IgM antibodies are found in less than 60% of convalescent patients 4 weeks after COVID-19, while IgG is detectable in more than 75% [148]. Moreover, no seroreactivity has been reported in convalescents who experienced reinfection [10]. Anti-nucleocapside IgG1 and IgG3 were found to increase along recovery, whether in critical or noncritical cases, reaching their peak 10 to 20 days post symptoms. In contrast, IgG2 and IgG4 were poorly detected [26,82].

The specificity of antibodies against different epitopes has been described. The main antibodies studied are against nucleocapsid (N), spike (S), and RBD proteins, in which a rapid decrease in IgG anti-N and anti-RBD has been observed, whereas anti-S is seemingly more stable in serum, which is further correlated to memory B cells and circulating Tfh cell survival up to 10 months [124,153–155]. All antibodies, despite reducing over time, exhibit an increase in their neutralization ability and their ability to bind to B lymphocytes [153,156]. Somatic mutations and defects in the germinal center at the beginning of convalescence were demonstrated to be related to antibody titers [156].

IgA has a serum conversion around 70% in noncritical patients and 100% in critical patients [82]. Some studies have suggested that IgA levels (specifically anti-RBD) tend to remain detectable in serum due to antigen presentation by follicular DCs and long-lived antigens [153,156]. In mild cases, IgA anti-N demonstrates a transient increase, with a peak 15 to 20 days post symptoms, whereas critical patients, despite a reduction, exhibited more stability until around 40 days [26,82].

Different strategies have been proposed to induce adaptive immunity. By 2022, many vaccines were developed; despite convalescence or vaccination, it has been observed that previously exposed patients still have a low risk of contracting COVID-19 [157,158]. These concerns, together with vaccine efficacy, remain to be addressed due to several issues: (1) individual immunization rate; (2) seroconversion; and (3) completeness of vaccination strategy. We do recognize that the immune response during the acute and convalescent stages of disease has an established profile, while the analysis of vaccinated and reinfected patients must also be fully reviewed to determine the factors predicting severity of new cases and to improve quality of life.

4. Conclusions

SARS-CoV-2 has infected millions of people around the world, being responsible for many deaths, as well as sequelae in those who survived. Many issues of immunity have been raised, mainly the challenge in responding to a new virus, the disbalance in immune response, the cytokine storm, and the hyperinflammation dynamics in severe patients, which can compromise further activation of the adaptive system. The pathway taken by the immune system in the acute phase is not only directly related to the outcome, but its effects can also be seen during the convalescence phase.

Although the participation of cells acts as a hallmark of disease progression and severity, neutrophils, lymphocytes, and eosinophils have been revealed as good predictors of clinical outcome in reaching convalescence. A remarkable 'shift to the left' is clear, which contributes to chemotaxis and impaired functionality in convalescence. Type I IFN and IL-15 are related to guide immunity, as well as thrombotic events, orchestrated by NETs and basophils.

The dynamics in the acute phase are coordinated by granulocytes and enzymes, alleviated by increased monocytes in convalescence. However, this functionality is situation-specific, and the participation of macrophages in tissues after viral clearance remains unclear.

We recognize that this review focused only on people infected with SARS-CoV-2 and not other diseases, such as HIV, lupus, diabetes, and immune disorders, which could change the immune profile and, thus, the patient's severity score. The dynamics of immunity interfere with the convalescent stage, and several questions have been raised since the emergence of SARS-CoV, related to viral biology, human immune response, and treatment

strategies; the appearance of SARS-CoV-2 has resulted in further questions. Several studies have proposed that convalescence, with no distinction of time, is a checkpoint for many cells and molecules.

Comprehending whether immune factors induce a potent response to mild and severe COVID-19, especially in convalescents, is essential for proposing novel treatments, increasing quality of life, and improving disease prognosis. Long immunization is now a priority to prevent the severity of new cases of COVID-19, as well as reduce the mortality rate of other wild viruses. Public policies and new strategies must be addressed under this framework, due to the chaotic situation experienced by several countries.

Author Contributions: Conceptualization, A.L.S.-J. and A.M.; investigation, A.L.S.-J., L.d.S.O. and N.C.T.B.; writing—original draft preparation, A.L.S.-J., L.d.S.O. and N.C.T.B.; writing—review and editing, A.L.S.-J., A.M.T., A.G.d.C. and A.M.; supervision, A.G.d.C. and A.M.; project administration, A.L.S.-J.; funding acquisition, A.M. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by the Fundação de Amparo à Pesquisa do Estado do Amazonas (FAPEAM) (Pró-Estado Program (#002/2008, #007/2018, and #005/2019), the POSGRAD Program (#005/2022), the PCTI-EMERGESAÚDE/AM—Chamada II Program (#006/2020)), the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)/Consolidação doutorado. A.L.S.-J., L.d.S.O. and N.C.T.B. have fellowships from CAPES and FAPEAM (Ph.D. and SI students). A.G.d.C. and A.M. are level 2 research fellows from CNPq. A.G.d.C. is a research fellow from FAPEAM (PRODOC Program #003/2022). The funders had no role in study design, decision to publish, or preparation of the manuscript.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data used to support the findings of this study are included within the article.

Acknowledgments: The authors would like to extend their gratitude to all laboratory staff for their support of this research.

Conflicts of Interest: The authors declare no conflict of interest.

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8.2. Artigo 2: "*Immunologic mediators profile in COVID-19 convalescence*", publicado na revista Scientific Reports.

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Immunologic mediators profile in COVID-19 convalescence

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SARS-CoV-2 caused the pandemic situation experienced since the beginning of 2020, and many countries faced the rapid spread and severe form of the disease. Mechanisms of interaction between the virus and the host were observed during acute phase, but few data are available when related to immunity dynamics in convalescents. We conducted a longitudinal study, with 51 healthy donors and 62 COVID-19 convalescent patients, which these had a 2-month follow-up after symptoms recovery. Venous blood sample was obtained from all participants to measure blood count, subpopulations of monocytes, lymphocytes, natural killer cells and dendritic cells. Serum was used to measure cytokines, chemokines, growth factors, anti-N IgG and anti-S IgG/IgM antibodies. Statistic was performed by Kruskal–Wallis test, and linear regression with days post symptoms and antibody titers. All analysis had confidence interval of 95%. Less than 35% of convalescents were anti-S IgM+, while more than 80% were IgG+ in D30. Anti-N IgG decreased along time, with loss of seroreactivity of 13%. Eosinophil count played a distinct role on both antibodies during all study, and the convalescence was orchestrated by higher neutrophil-to-lymphocyte ratio and IL-15, but initial stages were marked by increase in myeloid DCs, B1 lymphocytes, inflammatory and patrolling monocytes, G-CSF and IL-2. Later convalescence seemed to change to cytotoxicity mediated by T lymphocytes, plasmacytoid DCs, VEGF, IL-9 and CXCL10. Anti-S IgG antibodies showed the longest perseverance and may be a better option for diagnosis. The inflammatory pattern is yet present on initial stage of convalescence, but quickly shifts to a reparative dynamic. Meanwhile eosinophils seem to play a role on anti-N levels in convalescence, although may not be the major causative agent. We must highlight the importance of immunological markers on acute clinical outcomes, but their comprehension to potentialize adaptive system must be explored to improve immunizations and further preventive policies.

Keywords Severe acute respiratory syndrome (SARS), Immune hallmarks, Antibody, Brazil

COVID-19 is a global viral disease caused by the Betacoronavirus known as Severe Acute Respiratory Syndrome Coronavirus- 2 (SARS-CoV-2). It primarily affects the lungs, leading to both local and systemic complications. Among individuals with no prior immunity, or those with comorbidities associated to worse outcomes, an

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increased risk of death has been observed[1–3]. The incubation period I typically around 5 days, with transmission possible within a 14-day period through the respiratory tract. This facilitates its rapid spread from an infected individual, even if asymptomatic, to an uninfected person[4–6].

Main symptoms include dry cough, dyspnea, pneumonia, and in severe cases, respiratory syndrome[4,6,7]. Among infected, 3–20% require hospitalization, with most going to the ICU, and 1% die from complications in the acute phase[8,9]. Studies described human body's viral response to understand the dynamics of cells and proteins, to prevent recurrence and reduce risks during acute phases[10–14].

Innate immune cells have been proposed as markers of severity during acute disease[11,15,16]. However, by 2022, many people had already been exposed to the virus, and the convalescence stage became a central topic of discussion. This stage refers to the clinical improvement after COVID-19, and there is still a lack of discussion regarding the immune factors that coordinate antibody and memory cell production[17,18]. Numerous studies have evaluated the interaction between cells, cytokines, chemokines, and growth factors, but their correlation with antibody production has not been fully elucidated yet[19–22].

The World Health Organization (WHO) has proposed that individuals who have recovered from SARS-CoV-2 may donate plasma for a convalescent plasma therapy, which consists of transfusion of plasma enriched with antibodies from cured individuals into patients infected with SARS-CoV-2 and with severe form of the disease[23]. This approach is based on passive immunization, where antibodies specific to viral epitopes are transferred from convalescent individuals to acute patients. However, this procedure is still under study, and certain criteria must be met. Males are the primary population to plasma donation, once woman may present specific antibodies that can enhance transfusion reaction acute lung injury. Despite gender, the donor also must have sufficient concentration of antibodies in the serum[24,25]. This treatment is experimental, but there is not enough studies to standardize a protocol and with higher. Understand the major mechanisms related to antibody production in convalescent patients from COVID-19 can promote new therapeutic strategies to prompt higher antibody titers and so, reduce the severity of population and improve treatment to those with immunocompromising diseases.

Our aim was to conduct a longitudinal study with COVID-19 convalescent patients who had recovered from the acute phase at 30, 60, and 90 days. Understanding the involvement of cells and soluble proteins in antibody surveillance will contribute to developing further strategies for patient follow-up after the acute disease, provide important insights into convalescent progression, improve vaccination approaches, and ultimately enhance the quality of life for patients.

Results

Sociodemographic data

Fifty-one healthy donors were enrolled in this study. The mean age was 32.39 years (SD ± 11.63), with 36 (70.6%) males and 15 (29.4%) females. The majority were of admixed ethnicity (n = 45 [88.3%]), followed by 4 (7.8%) Caucasians and 2 (3.9%) African Americans. Five (9.8%) had blood type A positive, 27 (52.9%) had blood type O positive, and 19 (37.3%) had blood type O negative.

The mean age of the convalescent group was 39.94 years (SD ± 11.56), with 51 (82.3%) males and 11 (17.7%) females. In terms of ethnicity, 42 (67.8%) were admixed, 18 (29%) were Caucasians, and only 2 (3.2%) were African Americans. Regarding blood type, 17 were type A (14 [22.6%] positive and 3 [4.9%] negative), 2 were type B (1 [1.6%] positive and 1 [1.6%] negative), 2 were type AB (both positive [3.2%]), and 41 were type O (40 [64.5%] positive and 1 [1.6%] negative). The majority were overweight or obese (n = 23 [37.1%] each), followed by 15 (24.2%) with a normal BMI, and only one [1.6%] had a low BMI. Statistical analysis showed significant difference only in age ($p = 0.0009$) and blood type ($p < 0.0001$) between both groups, however we believe the blood type difference is related to higher prevalence of O and A types in the population studied (Table 1).

Once there was seen difference among age, ethnicity and blood type between HD and Convalescent (D30) groups, we conducted an analysis of immunological parameters segregating our groups based on blood type and age. Any difference was seen in the comparison of blood groups inside HD or Convalescent group. The pattern seen in the general observation seemed not to change when related to any of these three characteristics, and so, we believe that none of them had any or few interferences in the immune system in our participants. Heatmap and PCA analysis segregated by these parameters are shown in Fig. S1 for age (S1A), ethnicity (S1B) and blood type (S1C).

Analyzing the clinical data from the convalescent group, 5 out of 62 patients (8.1%) required hospitalization and mechanical ventilation. The mean hospital length of stay (LOS) was 15.82 days (SD ± 10.49), ranging from 1 to 51 days.

Antibody dynamic in convalescence

The antibody concentration was evaluated in the convalescent groups and compared to the number of days after the end of symptoms. Anti-nucleocapsid (anti-N) IgG showed a significant decrease ($p = 0.0017$) from 30 to 90 days after clinical recovery, which was further confirmed by correlation analysis. The analysis revealed a negative and significant reduction in anti-N concentration with an increase in the number of days after the end of symptoms ($p = 0.0056$), as shown in Fig. 1A. Although anti-Spike (anti-S) IgG also decreased, the concentration levels showed no significant difference, even in the correlation analysis (Fig. 1B). This suggests that the presence of anti-S antibodies in the serum persists, while anti-N antibodies decrease slowly after recovery (Fig. 1B). It should be noted that immunity remains active for a few days after viral clearance, and some patients seroconverted from a positive state (at D30) to a negative state (at both D60 and D90) during the study period for both anti-N and anti-S antibodies, using the manufacturer's cut-off.

Serum samples from 20 out of 62 participants (32.3%) with a previous diagnosis of COVID-19 and a symptomatic period tested positive for IgM using an immunochromatographic test at D30. This number decreased

Sociodemographic data	Healthy donors (n = 51)	Convalescents (n = 62)	p value
Age, mean ± SD	32.39 ± 11.63	39.94 ± 11.56	**0.0009**
Gender			
Male, n (%)	36 (70.6)	51 (82.3)	0.1794
Female, n (%)	15 (29.4)	11 (17.7)	
Ethnicity			
Caucasians, n (%)	4 (7.8)	18 (29)	**0.0182**
Admixed, n (%)	45 (88.3)	42 (67.8)	
African Americans, n (%)	2 (3.9)	2 (3.2)	
Blood type			
A+/A−, n (%)	5 (9.8)/0 (0)	14 (22.6)/3 (4.9)	**< 0.0001**
B+/B−, n (%)	0 (0)/0 (0)	1 (1.6)/1 (1.6)	
AB+/AB−, n (%)	0 (0)/0 (0)	2 (3.2)/0 (0)	
O+/O−, n (%)	27 (52.9)/19 (37.3)	40 (64.5)/1 (1.6)	
Body mass index (kg/m^2)			
Low (< 18.5), n (%)	1 (2.0)	1 (1.6)	0.6504
Normal (18.5–24.9), n (%)	17 (33.3)	15 (24.2)	
Overweigh (25–29.9), n (%)	19 (37.3)	23 (37.1)	
Obesity (> 30), n (%)	14 (27.4)	23 (37.1)	

Table 1. Sociodemographic data of healthy donors and convalescent patients. *SD* standard deviation, *Pos* positive, *Neg* negative. Chi-square test was performed isolating blood groups (A, B, AB and O), and subgroups (positive or negative), and including only those with observation > 1 for statistical purposes. Significant values are in bold.

to 11 out of 48 participants (22.9%) at D60, and 8 out of 47 participants (17%) at D90. IgG detection tests showed higher sensitivity. All three tests (both anti-S immunochromatographic and CMIA, and anti-N CMIA) detected more than 80% seroreactivity in convalescent individuals at D30. The D60 and D90 groups still had a seroconversion rate higher than 80% for anti-S antibody detection, while anti-N antibody detection decreased to 70.2% at D90 (Fig. 1C).

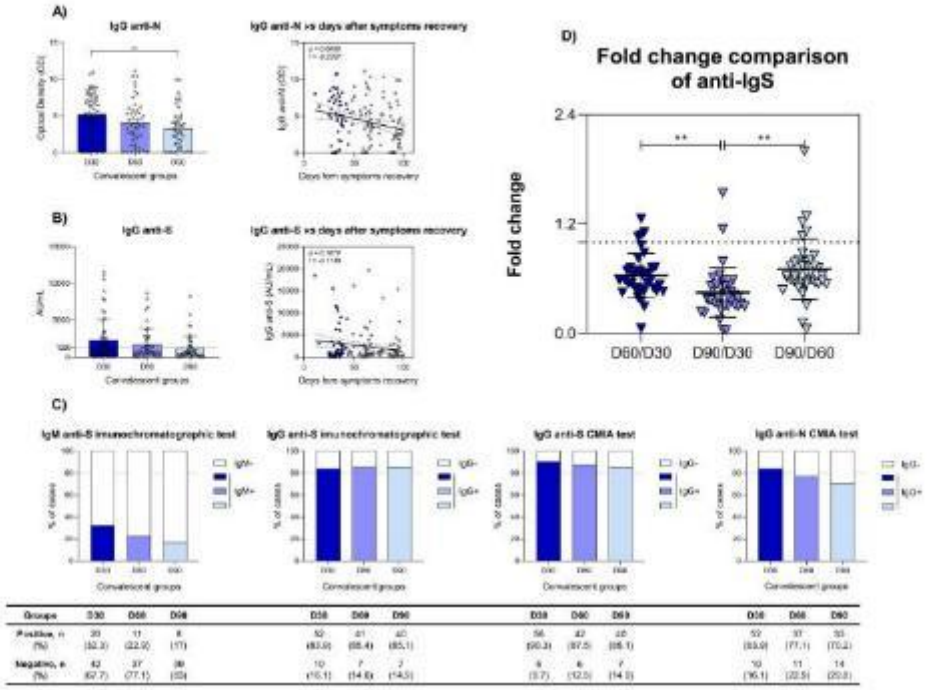
The fold change comparison shows that the antibody concentration changes gradually from 1 month to the next, but there is a more significant difference at the third month. A similar pattern was observed when comparing D30 to D60 and D60 to D90, indicating that the antibody concentration from D30 to D60 did not vary significantly compared to the change from D60 to D90. However, there was an overall decrease in antibody concentration from D30 to D90 (Fig. 1D).

WHO released a list of approved tests to detect anti-S IgG antibodies, which can help identify potential convalescent blood donors. The qualitative and quantitative CMIA test has been approved for monitoring potential blood donors who meet the eligibility criteria for antibody concentration (> 1.280 AU/mL). Among all the convalescent individuals who agreed to participate, only 30 out of 62 (48.4%) had a sufficient concentration at D30. By D60, only 20 out of 48 participants (41.7%) remained eligible, and this number further decreased to 14 out of 47 participants (29.8%) at D90 (Fig. 1B).

Inflammatory profile is mediated by memory cells and patrolling monocytes

Cell counts and subpopulations were evaluated over time using blood count and flow cytometry, respectively. NK and NKT cells were significantly reduced after COVID-19 and showed no significant signs of recovery even after 90 days (Fig. 2A,B). Although the monocyte count increased in D30 patients compared to healthy controls, this increase appeared to be driven by inflammatory and patrolling monocytes. The absolute monocyte count (AMC) decreased in the second month after symptom resolution (Table 2), but patrolling monocytes continued to increase over time, reaching even higher levels at D90 (Fig. 2C–E). On the other hand, both total T helper cells and activated T helper cells decreased as convalescence progressed (Fig. 2F,G). Total T cytotoxic lymphocytes did not vary significantly between the analyzed groups, but the median of activated T cytotoxic lymphocytes showed a significant difference, although statistical analysis could not determine which groups differed (Fig. 2H,I). The subpopulation of circulating dendritic cells showed no difference between healthy donors and convalescent individuals, nor between convalescent subgroups (Fig. 2J,K). Although B lymphocytes showed no difference, an increase in B1 lymphocytes was observed during convalescence, which appeared to persist throughout the 3-month period analyzed (Fig. 2L,M).

To evaluate the involvement of cytokines, chemokines, and growth factors in convalescent individuals, we quantified these soluble proteins and compared them to the healthy donor group. The convalescent stage appeared to be characterized by higher levels of VEGF, G-CSF, IL-2, IL-9, and CXCL10, but also lower serum concentrations of FGF basic, PDGF-BB, GM-CSF, IL-1β, IL-6, TNF-α, IFN-γ, IL-4, IL-5, IL-17A, IL-10, CXCL8, CCL3, CCL4, and CCL5 (Fig. 3). CCL5 were significantly low at D30 but appeared to increase until D90, serving as early markers of return to normality.



Groups	D30	D60	D90	D30	D60	D90	D30	D60	D90	D30	D60	D90
Positive, n (%)	30 (32.3)	11 (22.9)	8 (17)	52 (83.9)	41 (68.4)	40 (85.1)	56 (90.3)	42 (87.5)	40 (85.1)	52 (83.9)	37 (77.1)	33 (70.2)
Negative, n (%)	62 (67.7)	37 (77.1)	39 (83)	10 (16.1)	7 (14.6)	7 (14.9)	6 (9.7)	6 (12.5)	7 (14.9)	10 (16.1)	11 (22.9)	14 (29.8)

Fig. 1. Serum antibody analysis during convalescence. (**A**) Comparison of IgG anti-nucleocapsid protein (OD) and correlation under anti-N concentration and days post symptoms; (**B**) Comparison of IgG anti-Spike protein (AU/mL) and correlation between anti-S concentration and days post symptoms. The cutt-off 1.280 AU/mL was highlighted to demonstrate the participants that were eligible to convalescent plasma donation in D30 (n = 30/62 [48.4%]), D60 (n = 20/48 [41.7%]) and D90 (n = 14/47 [29.8%]); (**C**) Percentage of participants with a qualitative (pos/neg) antibody production among study period, based on anti-S immunochromatographic test, and CMIA anti-S and anti-N, with absolute and relative values on table below; (**D**) Fold change comparison between D60/D30, D90/D30 and D90/60 IgG anti-S antibody concentration, using quantitative result from patients with all follow-up (n = 45). Statistical analysis was performed with One-Way ANOVA followed by Turkey's Multiple comparison test, considering significative when $p < 0.05$. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

The correlation matrix demonstrates that healthy donors have fewer interactions between cytokines and more interactions with circulating cells and subpopulations, while convalescent individuals exhibit more immune dynamics between molecules. Patients in the initial stages of convalescence, represented by D30, showed a greater number of interactions between molecules, mainly cytokines.

The number of correlations appears to decrease as convalescence progresses until the late stage, although it can be observed that anti-S antibodies have a positive correlation with AEC and CXCL10 in D30 (alongside anti-N antibodies) and a negative correlation with patrolling monocytes. In D60, the immunomodulation was related to inflammatory molecules such as IL-6 and CXCL8, and negatively correlated with B lymphocytes. In D90, the participation seems to be guided by pDC and CXCL8 (Fig. 4A,B). AEC showed a positive and significant correlation with both anti-N and anti-S antibodies in D30 and D90 (Fig. 4), which may be related to its functionality.

Inflammatory markers still may be used to characterize the initial stage of convalescence

Biomarker analysis and the Venn diagram revealed that convalescent individuals are typically characterized by higher levels of IL-15, NLR, and RDW. However, when we segregate the convalescent stage and inspect for biomarkers in each timepoint, we observe that in the beginning of convalescence, 30 days after clinical recovery, high levels of AMC, ABC, platelets, myeloid DCs and anti-S IgG were also observed. Furthermore, immunomodulation from the first (D30) to the second month (D60), can be observed through increased production of inflammatory and patrolling monocytes, B1 lymphocytes, G-CSF, IL-2, and the IFN-γ/IL4 ratio. Although B1 lymphocytes are elevated in circulation, only D30 was marked by a higher increase in anti-S antibodies.

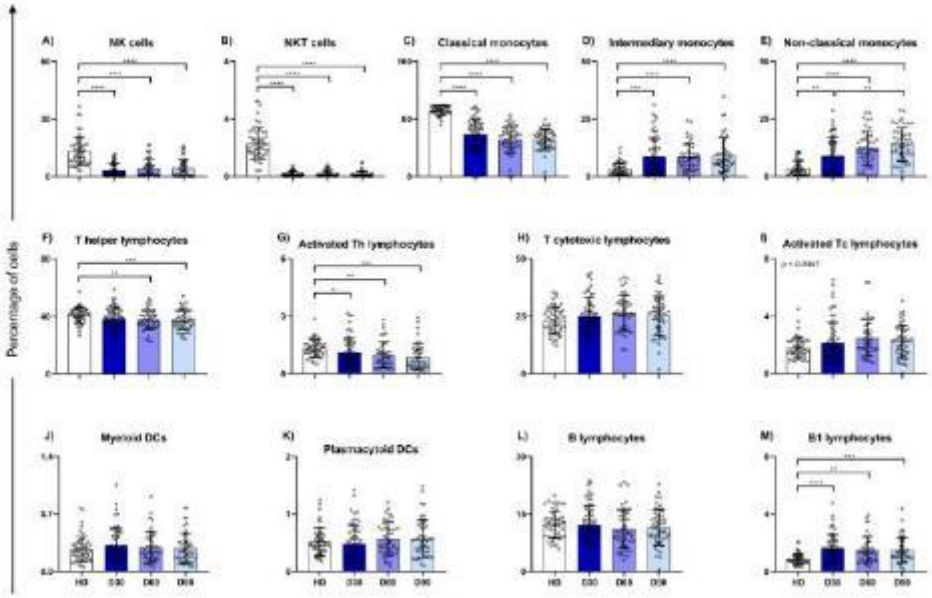


Fig. 2. Phenotypic analysis of immune cells, comparing HD, D30, D60 and D90 groups. Data is expressed as median and interquartile range in percentage of cells. Bar graphs represent analysis of: (**A**) NK cells; (**B**) NKT cells; (**C**) Classical monocytes; (**D**) Inflammatory monocytes; (**E**) Patrolling monocytes; (**F**) T helper lymphocytes; (**G**) Activated T helper lymphocytes; (**H**) T cytotoxic lymphocytes; (**I**) Activated T cytotoxic lymphocytes; (**J**) Plasmacytoid dendritic cells; (**K**) Myeloid dendritic cells; (**L**) B lymphocytes; and (**M**) B1 lymphocytes. Statistical analysis was conducted with Kruskal–Wallis and Dunn's Multiple Comparisons tests, considering significative when $p < 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Variables	HD n = 51	D30 n = 62	D60 n = 48	D90 n = 47	p value
RBC (× 10⁶/µL, median [IQR])	4.99 [4.59–5.40]	4.90 [4.56–5.20]	4.89 [4.47–5.15]	4.89 [4.71–5.29]	0.4676
Hemoglobin (g/dL, median [IQR])	14.90 [13.60–16.00]	14.40 [13.38–15.03]	14.35 [13.23–14.90]	14.40 [13.50–15.10]	0.0402
Hematocrit (%, median [IQR])	44.70 [40.6–47.4]	42.60 [40.45–45.45]	42.75 [39.13–44.50]	43.90 [41.50–46.20]	0.0660
MCV (fL, median [IQR])	87.80 [84.70–90.40]	88.15 [84.85–90.80]	87.90 [84.35–91.08]	89.10 [85.30–91.40]	0.7292
MCH (pg, median [IQR])	29.70 [28.8–30.70]	29.60 [28.38–30.60]	29.85 [28.10–30.70]	29.80 [27.90–30.80]	0.6607
MCHC (g/dL, median [IQR])	34.05 [33.10–34.60]	33.70 [32.78–34.450]	33.59 [32.75–34.48]	33.40 [32.60–34.10]	0.1667
RDW (%, median [IQR])	13.70 [13.10–14.00]	14.40 [13.75–14.95]	14.40 [13.90–15.08]	14.35 [13.68–15.03]	**< 0.0001**[a,b,c]
WBC (× 10³/µL, median [IQR])	6.33 [5.17–6.95]	6.78 [5.87–7.51]	6.34 [5.75–7.57]	6.65 [5.61–7.97]	0.2906
Neutrophil (× 10³/µL, median [IQR])	3.34 [2.8–4.17]	4.00 [3.21–4.65]	3.80 [3.14–4.61]	3.79 [3.04–4.84]	0.0757
Lymphocyte (× 10³/µL, median [IQR])	1.85 [1.59–2.18]	1.98 [1.61–2.22]	1.93 [1.6–2.3]	1.98 [1.61–2.23]	0.8081
Monocyte (× 10³/µL, median [IQR])	0.38 [0.28–0.42]	0.43 [0.37–0.48]	0.37 [0.33–0.42]	0.38 [0.28–0.43]	**0.0028**[a,c]
Basophil (× 10³/µL, median [IQR])	0.03 [0.02–0.05]	0.04 [0.02–0.06]	0.03 [0.02–0.04]	0.03 [0.02–0.05]	0.2906
Eosinophil (× 10³/µL, median [IQR])	0.19 [0.12–0.38]	0.19 [0.13–0.26]	0.18 [0.13–0.26]	0.16 [0.12–0.26]	0.6799
Platelet count (× 10³/µL, median [IQR])	243.0 [210–282]	259.5 [212–290]	245.0 [215–266]	245.00 [209–272]	0.7717

Table 2. Laboratorial parameters of healthy donors and convalescent patients at D30, D60 and D90. [a]Significant difference for HD vs D30; [b]Significant difference for HD vs D60; [c]Significant difference for HD vs D90; [d]Significant difference for D30 vs D60; [e]Significant difference for D30 vs D90; [f]Significant difference for D60 vs D90. Statistical analysis was performed with Kruskal–Wallis test, and Dunn's Multiple Comparison Test to compare hematological values from all groups. A $p < 0.05$ was considered significant and highlighted by bold type font.

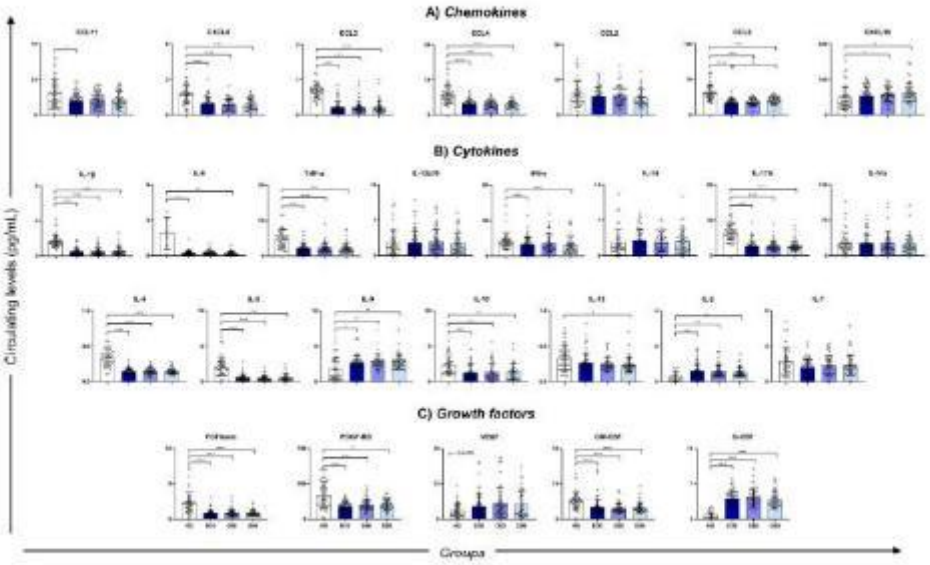


Fig. 3. Circulating level of soluble molecules comparing HD, D30, D60 and D90 groups. Data is expressed as median and interquartile range in pg/mL. Circulating levels of chemokines (**A**), cytokines (**B**) and growth factors (**C**). Statistical analysis was conducted with Kruskal–Wallis and Dunn's Multiple Comparisons tests, considering significative when $p<0.05$. *$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$.

Later stages of convalescence seen in our study was marked by both total and activated T cytotoxic lymphocytes, plasmacytoid DCs, VEGF, IL-9 and CXCL10. This profile suggests a reparative process, which can be related to the extensively injury caused by the immunity priming during acute phase. None of the convalescent groups exhibited higher production of anti-N antibodies (Fig. 5).

Discussion

COVID-19 has impacted numerous individuals worldwide, with its peak occurring between 2020 and 2021. This period was characterized by immense strain on healthcare systems, as efforts were made to find better treatments, identify effective drugs, and conduct improved clinical trials. The pandemic situation compelled researchers to enhance their understanding of how the body responds to the virus, particularly focusing on the dynamics of convalescence and the immunological factors involved in disease recurrence, antibody production, and prevention strategies. In this study, we propose perspectives on immune dynamics from 30 to 90 days after clinical recovery, aiming to characterize the immune response in terms of cells and molecules.

Our investigation into antibody dynamics revealed that anti-S IgG antibodies persisted longer than IgM antibodies, as expected. In our study, only 32% of participants had detectable IgM levels at D30, which is lower than what has been reported in the literature[28]. Conversely, more than 80% of convalescents in our study exhibited detectable IgG anti-Spike antibodies, aligning with findings from other studies[26–28]. Importantly, IgG anti-Spike antibodies demonstrated greater stability in serum compared to IgG anti-nucleocapsid antibodies, which exhibited a rapid decline during convalescence. The seroreactivity of anti-S antibodies varied by less than 5.2% over the 3-month period following viral clearance, whereas anti-N antibodies varied by 13.4% during the same timeframe. The peak period of antibody production occurred 10–20 days after the onset of symptoms and appeared to be primarily influenced by IgG1 and IgG3 subclasses[8,30]. Some studies have reported that the persistence of memory B cells and circulating Tfh cells plays a significant role in the production of anti-S antibodies, while anti-N and anti-RBD antibodies show a notable decrease[31–35].

Several concerns have been raised regarding the decrease in antibodies during convalescence. One of these concerns is the mutation that occurs in the germinal center at the onset of convalescence, which has been correlated with antibody titers[36]. It has been observed that patients who experience reinfection within a certain period do not produce antibodies, suggesting that antibody production helps prevent new cases of infection. However, this hypothesis has been questioned due to the specificity of antibodies to other SARS-CoV-2 lineages that have undergone mutations during the pandemic. Despite the reduction in antibody titers, an increase in neutralization ability has been observed[34,36].

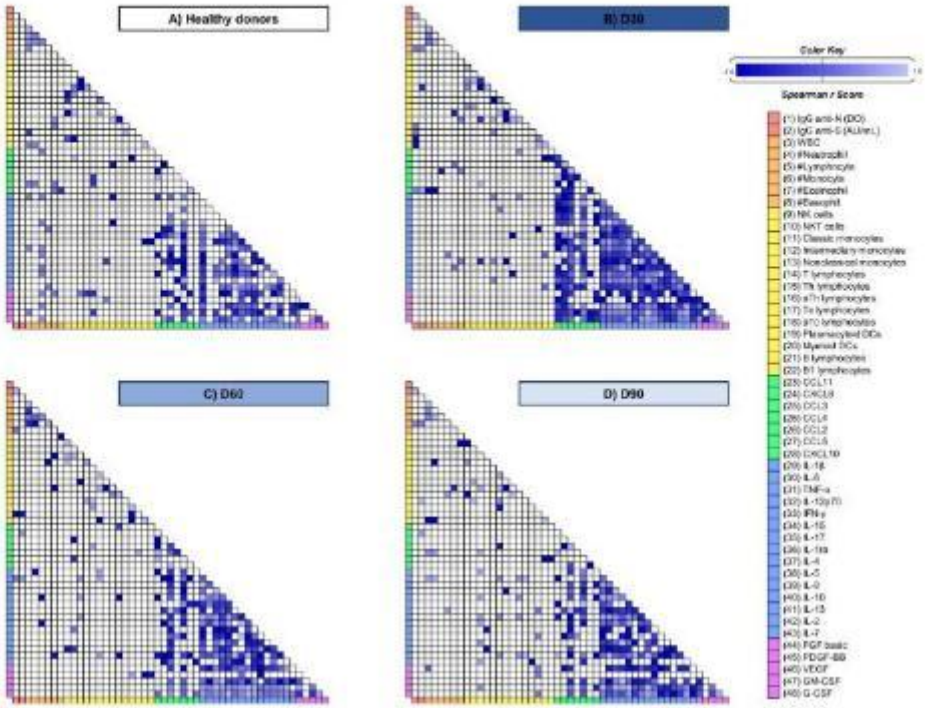


Fig. 4. Biomarker correlation matrix indicating difference in patter of healthy donors (**A**), and convalescence in D30 (**B**), D60 (**C**) and D90 (**D**). Networks were based on Spearman's correlation indices (**r**). Association was significant when $p < 0.05$ between all markers analyzed. Blue scale, ranging from −1.0 to 1.0, shows correlation strength, as represented on image. *WBC* white blood count, *ANC* absolute neutrophil count, *ALC* absolute lymphocyte count, *AMC* absolute monocyte count, *AEC* absolute eosinophil count, *ABC* absolute basophil count, *NK* natural killer, *Chemokines* CXCL8, CXCL10, CCL3, CCL4, CCL2, CCL5 and CCL11, *Cytokines* IL-1β, IL-1ra, IL-6, TNF-α, IL-12p70, IFN-γ, IL-2, IL-7, IL-9, IL-15, IL-4, IL-5, IL-13, IL-17A, IL-10, *Growth factors* VEGF, FGF basic, PDGF-BB, GM-CSF, G-CSF, *HD* healthy donors.

Some studies have shown a relationship between antibody production and the severity of the disease[37]. Patients who experience symptoms such as fever, cough, dyspnea, and pneumonia are 50 times more likely to produce higher antibody titers[34,38]. However, the severity of the disease is also related to viral load and the availability of viral antigens. It is worth noting that only anti-RBD antibodies have been found to increase over time in patients with a severe condition[39,40].

The immunological dynamics during convalescence involve a higher participation of inflammatory and patrolling monocytes. Total monocytes were found to regulate as convalescence progresses, but the activated inflammatory subpopulations appeared to increase, including an increase in patrolling monocytes. In the literature, both inflammatory and patrolling monocytes have been reported to decrease during convalescence and stabilize their levels around 5 months[12,18,41]. This difference may be related to the activation of monocytes since it has been previously shown that HLA-DR expression increases during convalescence[18,42,43]. However, there is no available data on which subpopulations express this marker during convalescence. The higher control of inflammatory status and tissue repair may be a clear reason for this increased involvement, as the increase in both subpopulations persists for up to 2 months after viral clearance.

Convalescence is characterized by a progressive decrease in antibody titers, but it can still activate local macrophages and induce inflammation through FcγRIIa receptors, thereby inducing proinflammatory molecules. M1 macrophages have been shown to be more sensitive to activated antibodies, but M2 macrophages may also polarize to M1 upon antibody recognition. M1 macrophages have been described to produce high levels of IFN-γ, CXCL8, and CCL2[44–47]. However, our patients showed a reduction in the first two markers during convalescence.

Even though our results demonstrated that convalescence can be marked by cytotoxicity, it appears to be regulated by T lymphocytes. NK and NKT cells showed a significant decrease, which has been previously

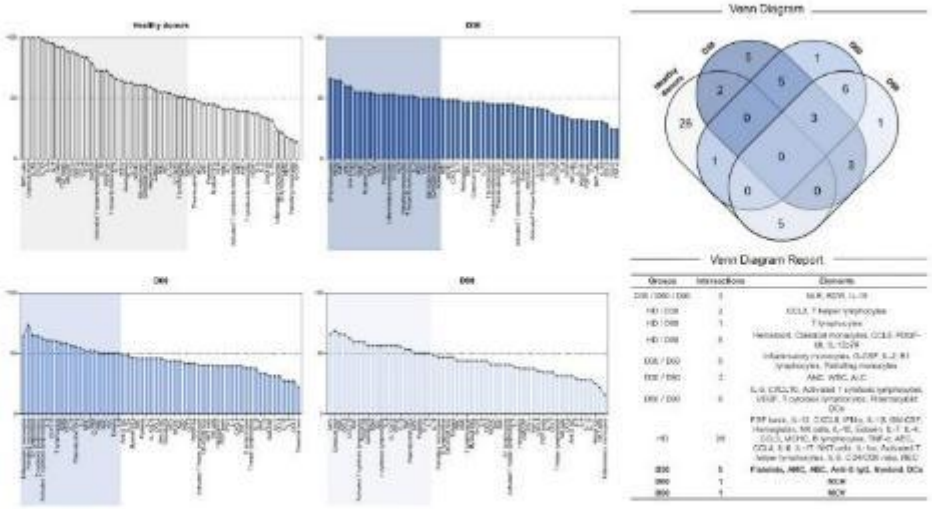


Fig. 5. Biomarker signature of groups represented in a Venn Diagram. (**A**) Frequency of subjects with biomarker level above the Cut-off; (**B**) Venn Diagram representing the groups, intersections, and elements, suggesting potential hallmarks for immunomodulation under convalescence. Global median for each parameter was measured and used to characterize participants as low (< 50%) or higher (> 50%) producers. *HD* healthy donors.

described[48–50] and may be due to the production of other cells involved in tissue repair. This same pattern was observed during the acute phase, as low NK cell counts were associated with disease severity and compromised cytotoxicity[48]. Our findings suggest that this compromise in NK cell counts persists during convalescence. Interestingly, both myeloid and plasmacytoid dendritic cells showed no difference, indicating that their role in viral clearance occurs rapidly and is stimulus-dependent.

The molecular profile showed that an inflammatory pattern remains predominant over anti-inflammatory markers. However, both pro-inflammatory and anti-inflammatory mediators were reduced compared to healthy donors. Immunosurveillance during convalescence is characterized by a lower production of inflammatory and reparative factors, along with intense regulation mediated by increased concentrations of CXCL10, IL-2, and G-CSF. Anti-N was downregulated by B lymphocytes in the beginning of convalescence, however, in D60, it seems that this cell population participates also in downregulation of anti-S antibodies as well, characteristic that is not seen in D90. Some immunological cells were still present in D60, but with a considerable reduction in antibody titers, seen in the fold change analysis, probably due to a decrease in inflammatory stimuli. Memory cells were shown to persist even with a reduction in antibody production, exhibiting reactivity to most viral antigens even after 6 months[28,51]. In later stages of convalescence, there was a loss of intensive interactions, indicating that the immune system strives to reach homeostasis a few months after viral clearance.

Throughout the entire convalescent period, there was an increase in the production of NLR. Previous studies have suggested this marker as a prognostic factor for acute patients[10,20,52–54], and our findings suggest that it remains elevated for a prolonged period during convalescence. Other studies have also reported an increase in neutrophil count with activation markers even 28 days after clinical recovery[52,55]. Furthermore, the production of neutrophil extracellular traps (NETs) has been detected even after months[17,56,57]. Our findings of increased G-CSF levels may contribute to neutrophil recruitment. The literature demonstrated that neutrophil metabolic function lowered during the acute phase and further reduced during convalescence, suggesting the production of non-reactive cells during this period. Therefore, G-CSF becomes an important factor in inducing urgent granulopoiesis, particularly in the first 2 months of convalescence[13,20].

All 3 months of convalescence were characterized by a higher production of IL-15. In the initial stage, there was also involvement of G-CSF and IL-2, while the last stage was marked by mainly producing IL-9 and CXCL10. Some studies have described the IL-15/IL-15RA axis as a crucial factor in the functional exhaustion, senescence, and apoptosis of NK and NKT γδ cells, which promotes rapid control of infection through cytotoxicity and antibody response during convalescence[39,47,58–61].

G-CSF, IL-2, and IFN-γ are molecules involved in the cytokine storm during the acute phase of COVID-19[62–65], and as observed in our study, this profile of proliferation persists in convalescence, mainly mediated by G-CSF and IL-2. This may be attributed to the functionality of repair and the migration of granulocytes from the bloodstream to the affected tissues[12]. The subsequent dynamics of IL-9 and CXCL10 at D60/D90 were also observed in chronic allergic diseases and are associated with the involvement of mastocytes, the induction of

lymphocyte adhesion to endothelium, and bone marrow inhibition[66]. CXCL10 has previously been linked to acute outcomes and the prediction of improvement in COVID-19 patients, but its role in convalescence may be associated with its anti-inflammatory properties[67].

The specific higher producers at D30 were AMC, ABC, myeloid DCs, platelets, and anti-S IgG antibodies. Myeloid DCs have been reported to be reduced or have limited involvement during the acute phase[45,57,68,69]. Antibodies were prominently produced only at D30, and we propose that this specific period of acute disease is the main source of antibody concentration.

We observed that within our convalescent groups, D30 was the optimal time for collecting convalescent plasma. Less than 50% of our patients had a sufficient antibody concentration for plasma donation, which decreased to less than 30% after 90 days. While many studies focus on evaluating the efficacy of convalescent plasma use in acute COVID-19 patients, there is limited data available to assess the factors involved in immune dynamics and antibody production, particularly in relation to obtaining convalescent plasma[32,40]. However, there is an urgent need to improve the collection of plasma samples for storage, considering future pandemics and severe cases.

The convalescent period, which is still lacking in comprehensive discussions, holds valuable data, especially regarding immunity and the factors contributing to antibody production. Understanding these mechanisms can help prevent new cases, reduce the recurrence of severe cases, inform vaccination strategies, and promote a better quality of life for the exposed population. We emphasize the dynamics of immune cells, soluble molecules, and particularly the maintenance of antibodies in COVID-19 convalescent patients.

Furthermore, we identified that inflammatory and patrolling monocytes, together with B1 lymphocytes, persist in the immune system after infection. Based on literature data, the cytokine storm triggered during SARS-CoV-2 infection lead to stimulation on immune system, mainly participation of neutrophils and cytotoxicity[70,71]. This profile was shown in our study still present in the beginning of convalescence, which immunomodulation is primarily driven by G-CSF, IL-2 and IL-15. While inflammatory markers are still present in the initial stages of convalescence, around 60 days, the profile shifts toward a proliferative pattern characterized mainly by IL-9 and CXCL10, that tend to last till 90 days of convalescence. The mechanisms and profile seem to shift towards a proliferative and reparative process, related to lymphocyte stimulation and angiogenesis (Fig. 6). Although these statements must be confirmed by future studies, we acknowledge that there is a downregulation of inflammatory process during convalescence, but still a lack on the dynamics related to improve memory on these patients.

As well, we do comprehend that understanding the dynamic of immune system is still a lack and require more approaches to better understand which mechanisms are related to antibody dynamics, but also immunoregulation. Characterize the participants based on clinical and laboratorial scores, to evaluate the acute phase and perform a follow-up till late stages of convalescence can validate the results presented here and determine more robust biomarkers for disease progression. Due to limitation of data regarding patient's medical history during acute phase of COVID-19, and due to our patients be mainly mild cases during acute, we were unable to determine clinical scores from our patients and therapies that were used, which we believe it could determine better conclusions and propose novel studies. Other factors during acute phase were shown to impact on immunity in

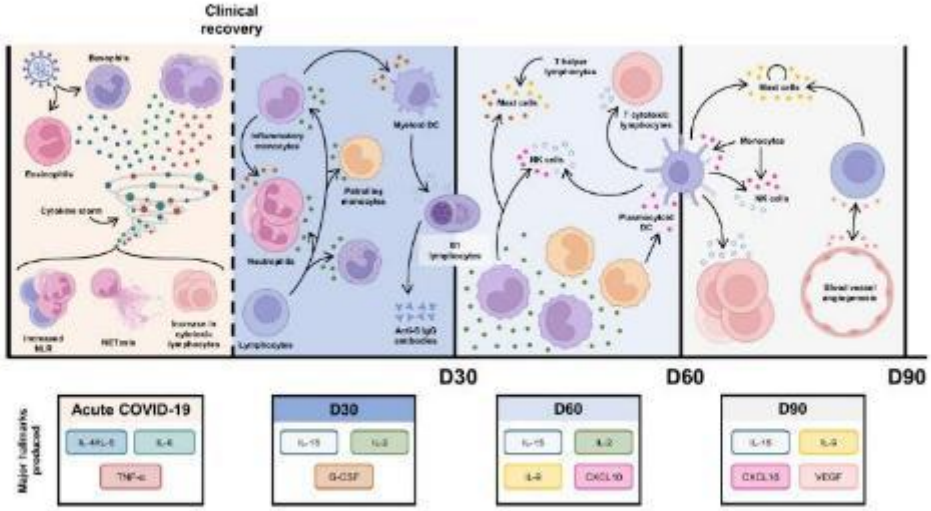


Fig. 6. Concluding remarks on immunological shifts related to acute (based on literature) and convalescence COVID-19 patients. *DC* dendritic cells, *NLR* neutrophil-to-lymphocyte ratio.

convalescent conditions, and although we considered this in our analysis, we were limited regarding the therapy used[72]. Only two had hospital admission, and mostly couldn't address the therapy used during symptomatic period. None of them received neutralizing antibodies against SARS-CoV-2 neither previously to infection, nor during acute phase. Some, however, were vaccinated during the study period, after inclusion, but the data were not included in the analysis.

We highlight the dynamics of immune cells, soluble molecules and specially, the maintenance of antibodies which are produced along COVID-19 convalescent patients. We identified that anti-N IgG reduced quickly after viral clearance, while anti-S IgG changes slowly during the first 3 months, being a good proposal for diagnostics and therapeutical strategies. We could also identify that after infection, inflammatory and patrolling monocytes are still present on immunity, together with B1 lymphocytes, and immunomodulation driven mainly by G-CSF, IL-2 and IL-15 in convalescence. Inflammation is still present on initial stages of convalescence, but around 60 days, the profile seems to start to change to a proliferative pattern, characterized by IL-9 and CXCL10. Our results contribute to hallmarks involved on antibody production and evaluate higher proportion of cells and soluble immune molecules under these patients. Our findings will support a better comprehension over the major hallmarks for immune sustainment and dynamics after SARS-CoV-2 infection.

Methods

Ethical statement

The participants enrolled in this study provided written consent by formal signature on the consent form. The study was submitted and approved by the Ethical Committee of Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas (CEP-HEMOAM) under processes of #4.126.784 and #1.982.466. The protocols followed the guidelines of the Declaration of Helsinki and Resolution 466/2012 of the Brazilian National Health Council for research involving human subjects.

Participants and samples

Our study involved 51 healthy donors (HD) who were eligible blood donors recruited before the COVID-19 pandemic, on June 13th 2017 and June 30th 2017. These donors tested negative for HIV, HBV, HCV, HTLV, Chagas disease, and syphilis. They also had no clinical symptoms prior to donation. Additionally, we included 62 COVID-19 convalescent patients who had previously tested positive for RT-PCR and experienced a symptomatic period. The age range of the convalescents was 18–60 years, and they were recruited 30 days after clinical recovery. These convalescents were followed up monthly for up to 3 months, during which blood samples were collected. The convalescent group was recruited at Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas (HEMOAM) in Manaus, Amazonas, Brazil, between July 20th 2020 and June 2nd 2021. These participants were identified based on a positive RT-PCR test and were invited to participate 30 days after clinical recovery, following the resolution of acute symptoms developed during COVID-19 phase (i.e. fever, cough fatigue or muscle aches), and when eligible for blood donation. Male gender was preferred for participation due to convalescent plasma collection, although women were also included but showed no significant differences in laboratory parameters. No other distinctions were made.

The convalescent group consisted of 62 patients at enrollment (D30), which completed all three follow-ups. As some individuals received the COVID-19 vaccine during the follow-up period, their results were not included in the analysis. Therefore, data from only 48 patients are reported at D60, and 47 at D90.

Patients who were asymptomatic for COVID-19, symptomatic at the time of recruitment, taking drugs that could inhibit ACE, had anti-erythrocyte antibodies, received any COVID-19 vaccine prior to inclusion, were pregnant, or were indigenous were not included in the study. Patients who tested positive for RT-PCR during recruitment or showed seroreactivity to other infectious diseases (HIV, HBV, HCV, syphilis, Chagas disease, or HTLV) were excluded. Samples obtained from patients who received the vaccine during the follow-up period were not included in the analysis.

Sociodemographic and clinical data collected from all participants included gender, age, ethnicity, blood type, body mass index (BMI), duration of symptoms during acute COVID-19, and the need for hospitalization and mechanical ventilation. Blood samples were collected through venipuncture, with 4 mL of whole blood collected in EDTA (BD Vacutainer® EDTA K2) tubes and 5 mL collected in separator gel (Gel BD SST™ II Advance) tubes. Serum samples were stored at − 80 °C for further procedures.

Blood count and immune cell analysis

Fresh whole blood was used to measure the parameters of red and white blood cells (RBC and WBC, respectively) and platelets. This was done using an automatic hematological blood counter (ADVIA 2120i, Siemens, USA) located at HEMOAM. Immunophenotyping of immune cells was also performed at HEMOAM using the following antibodies: anti-CD3 (PERCP), anti-CD4 (FITC), anti-CD8 (PE), and anti-CD69 (APC) to identify total T lymphocytes (CD3+), T helper lymphocytes (CD3+CD4+), activated T helper lymphocytes (CD3+CD4+CD69+), T cytotoxic lymphocytes (CD3+CD8+), and activated T cytotoxic lymphocytes (CD3+CD8+CD69+); anti-CD5 (FITC) and anti-CD19 (PE) to identify B (CD19+) and B1 (CD19+CD5+) lymphocytes; anti-CD16 (FITC), anti-CD14 (APC), and anti-HLA-DR (PE) to classical (CD14+CD16-), inflammatory (CD14+CD16+), and patrolling monocytes (CD14lowCD16+); anti-CD123 (FITC), anti-CD11c (PE), and anti-CD14 (APC) to myeloid (CD14−CD123+) and plasmacytoid (CD14−CD11c+) dendritic cells; and anti-CD3 (PERCP), anti-CD16 (FITC), and anti-CD56 (PE) to NK (CD3−CD56+CD16+) and NKT (CD3+CD56+CD16+) cells (Fig. S2). The antibodies were purchased from BD Biosciences (San Diego, CA, USA), Beckman Coulter (Brea, California, USA), and BioLegend (San Diego, CA, USA).

A sample of 100 µL of whole blood was incubated with the respective antibodies, followed by a lysis solution at room temperature. The cells were washed, resuspended in PBS, and then stored at 4 °C until they were acquired by flow cytometry within the next 24 h. A total of 30,000 events were acquired using the FACSCanto II flow cytometer at Fundação HEMOAM. The analysis was conducted using FlowJo Software v. 10.8 to define subpopulations based on morphometric characteristics and fluorescence from monoclonal antibodies. The gates and strategies used are indicated in Fig. S1. The percentage of cells was used for statistical analysis.

Chemokine, cytokine, and growth factor assay

Molecules were measured using the Luminex technique. Cytokines IL-1β, IL-1ra, IL-6, TNF-α, IL-12p70, IFN-γ, IL-2, IL-7, IL-9, IL-15, IL-4, IL-5, IL-13, IL-17, and IL-10; chemokines CXCL8, CXCL10, CCL3, CCL4, CCL2, CCL5, and CCL11; and growth factors VEGF, FGF-basic, PDGF, GM-CSF, and G-CSF were measured in serum samples at Instituto René Rachou (FIOCRUZ-MG). The procedure was conducted using the Bioplex-Pro Human Cytokine 27-Plex Kit (Bio-Rad, California, USA) following the manufacturer's instructions and protocol. Acquisition and concentration measurements were performed on a Luminex 200 System and analyzed using Bioplex Manager Software with Five Parameters Logistic Regression. The concentration is expressed in pg/mL. The detection limits for the molecules were as follows: CXCL8 = 42,150 pg/mL; CXCL10 = 31,236 pg/mL; CCL2 = 24,282 pg/mL; CCL3 = 960 pg/mL; CCL4 = 11,233 pg/mL; CCL5 = 16,533 pg/mL; CCL11 = 26,842 pg/mL; IL-1β = 8608 pg/mL; IL-1ra = 91,661 pg/mL; IL-2 = 18,297 pg/mL; IL-9 = 25,642 pg/mL; IL-15 = 22,328 pg/mL; IL-4 = 4789 pg/mL; IL-5 = 23,105 pg/mL; IL-6 = 37,680 pg/mL; IL-7 = 16,593 pg/mL; IL-10 = 35,170 pg/mL; IL-12p70 = 37,684 pg/mL; IL-13 = 8090 pg/mL; IL-17A = 28,850 pg/mL; IFN-γ = 25,411 pg/mL; TNF-α = 64,803 pg/mL; PDGF-BB = 24,721 pg/mL; FGFb = 16,046 pg/mL; G-CSF = 40,049 pg/mL; GM-CSF = 12,844 pg/mL; and VEGF = 29,464 pg/mL.

Antibody measurement

Antibodies IgG anti-Spike (anti-S) and anti-nucleocapsid (anti-N) were measured using chemiluminescence with the Chemiluminescence Microparticle Immunoassay (CMIA) by Abbott test on Architect. The procedures were conducted based on the manufacturer's instructions. A qualitative test was conducted to measure anti-N IgG, and the result was obtained in Index (S/C). It was considered positive when the Index was greater than 1.4. Quantitative data of Optical Density (OD) were also recorded and used for statistical analysis. The qualitative-quantitative test of IgG anti-S was measured, and the concentration is expressed in AU/mL. The concentration was used for statistical analysis, and according to the manufacturer's instructions, samples were considered positive when the concentration was greater than 50 AU/mL. All procedures were performed on the Architect equipment, located in the serology department of Fundação HEMOAM.

Statistical analysis

All data were stored in Microsoft Excel for further analysis in GraphPad Prism v. 8.0 (San Diego, CA, USA). The Shapiro-Wilk normality test was performed to evaluate the normality of the parameters, and the median and interquartile range (IQR) (25th and 75th percentiles) were acquired. Sociodemographic data are expressed as absolute values and percentages. Analysis of these parameters were done by Mann–Whitney test, while categorical parameters were compared with Chi-square test. Heatmap and Principal Component Analysis (PCA) were performed in R Studio v.2023.12.1 software (Project for Statistical Computing Version 3.0.1). The median of cells and molecules from all groups was compared using the Kruskal–Wallis and Dunn's Multiple Comparison tests. A confidence interval of 95% was considered, and statistical values were used when $p < 0.05$.

The correlation analysis was performed using Spearman correlation test in GraphPad Prism v. 8.0 software (San Diego, CA, USA) for all cells, cell subtypes, and molecules within each group. Significant results were used to construct biomarker correlation matrices based on the Spearman correlation coefficient (r). Significance was identified when the p-value was < 0.05.

The median value of each parameter was calculated across all groups and used as a cutoff point to categorize each participant as a "high" or "low" producer, as described previously. Median values for each parameter used are: RBC = 4.91 106/µL; hemoglobin = 14.50 g/dL; hematocrit = 43%; MCV = 88.1 fL; MCH = 29.70 pg; RDW = 14.10%; WBC = 6.53 103/µL; ANC = 3.77 103/µL; ALC = 1.95 103/µL; AMC = 0.39 103/µL; AEC = 0.18 103/µL; ABC = 0.03 103/µL; platelet = 248 103/µL; classical monocytes = 63.90%; inflammatory monocytes 5.60%; patrolling monocytes = 8.20%; T lymphocytes = 67.85%; T helper lymphocytes = 38.70%; activated T helper lymphocytes = 1.07%; cytotoxic T lymphocytes = 25.15%; activated cytotoxic T lymphocytes = 1.82%; NK cells = 5.24%; NKT cells = 0.15%; mDCs = 0.25%; pDCs = 0.47%; B lymphocytes = 11.60%; B1 lymphocytes = 1.16%; VEGF = 6.61 pg/mL; FGF basic = 1.38 pg/mL; PDGF-BB = 141.40 pg/mL; GM-CSF = 4.52 pg/mL; G-CSF = 3.94 pg/mL; IL-1β = 0.37 pg/mL; IL-1ra = 21.59 pg/mL; IL-6 = 0.20 pg/mL; TNF-α = 5.59 pg/mL; IL-12p70 = 1.97 pg/mL; IFN-γ = 13.04 pg/mL; IL-2 = 0.48 pg/mL; IL-7 = 1.33 pg/mL; IL-9 = 4.07 pg/mL; IL-15 2.36 pg/mL; IL-4 = 0.18 pg/mL; IL-5 = 0.69 pg/mL; IL-13 = 0.38 pg/mL; IL-17A = 2.87 pg/mL; IL-10 = 1.41 pg/mL; CXCL8 = 0.98 pg/mL; CXCL10 = 52.00 pg/mL; CCL3 = 0.22 pg/mL; CCL4 = 4.70 pg/mL; CCL2 = 7.56 pg/mL; CCL5 = 28.05 pg/mL; CCL11 = 15.22 pg/mL; anti-N IgG (OD) = 4.40 Index; anti-S IgG = 872.70 AU/mL. Percentage values were then obtained from these groups, and parameters that reached more than the 50th percentile were characterized as higher producers, represented in a graphic biomarker signature. Each parameter was isolated for each group and presented in a Venn Diagram, showing the respective groups, intersections, and elements. This analysis was conducted using a public website (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Linear regression was calculated to identify the relationship between the number of days from the end of symptoms and the concentration of both anti-S IgG (AU/mL) and anti-N IgG (OD), obtained by the CMIA method. A significant value was considered when $p < 0.05$. To evaluate antibody concentration, qualitative values

(positive/negative) were also calculated from immunochromatographic tests (IgM and IgG anti-S) and CMIA (IgG anti-S and anti-N). Percentage values were used to calculate the seroconversion rate for all convalescent groups. A fold change analysis was also performed using the mean value of the concentration of anti-S IgG from participants at D30/D60, D30/D90, and D60/D90, compared using the One-Way ANOVA and Tukey's Multiple Comparison test. All methodological procedures are described in Fig. S3.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Received: 13 April 2024; Accepted: 27 August 2024
Published online: 09 September 2024

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Acknowledgements

Authors would like to extend their gratitude to all staff from laboratory of Genomics, from Fundação HEMOAM, for their support to this work. Financial support was provided in the form of grants from Fundação de Amparo à Pesquisa do Estado do Amazonas (FAPEAM) (Pró-Estado Program - #002/2008, #007/2018, #005/2019, PCTI-EMERGESAÚDE - AM Program - #005/2020 and POSGRAD Program - #002/2023 and #002/2024), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (INCT-Sangue Process - #405918/2022-4) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) (PROCAD-Amazônia 2018 Program - #88881.200581/2018-01 and PDPG-CONSOLIDACAO-3-4 Program #88887.707248/2022-00). ALS-J, LSO, SD, TCCC, LAX and FAS-H have fellowships from CAPES and FAPEAM (PhD and SI students). LHF, EC and AT-C are level 1 research fellows from CNPq. OM-F is a level 1 research fellow from CNPq and a research fellow from the program supported by the Universidade do Estado do Amazonas (PROVISIT No. 005/2023-PROPESP/UEA). CAS, AGC and AM are level 2 research fellows from CNPq. The funders had no role in study design and decision to publish, or preparation of the manuscript.

Author contributions

A.L.S.-J., L.S.O., S.D. and W.L.L.N. performed laboratorial procedures of flow cytometry, A.L.S.-J., T.C.C.C. and L.A.X. recruited and collected all data and blood samples, F.S.A.-H. and C.M.M.A. measured the antibodies assays, A.L.S.-J., M.A.E.C., D.M.T. P.V.S.N., M.S.S.C., A.M.T., N.A.F., A.G.C. and A.M. conceptualized the study

and analyzed the data. A.L.S.-J. wrote the main manuscript. A.M.T., L.H.F., C.A.S., E.C.S., A.T.C., O.A.M.-F., A.G.C. and A.M. supervised and revised the final manuscript. M.A.E.C., N.A.F.,L.H.C., C.A.S., E.C.S., A.T.C., O.A.M.-F., A.G.C. and A.M. provided funding acquisition. All authors have read and agreed to the published version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-024-71419-x.

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8.3. Manuscrito 3: "Immune Contributors to Hospitalization in Patients with COVID-19 and Laboratorial Markers for Outcome Prediction" a ser submetido.

IMMUNE CONTRIBUTORS TO HOSPITALIZATION IN PATIENTS WITH COVID-19 AND LABORATORIAL MARKERS FOR OUTCOME PREDICTION

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Abstract

Background: Coronavirus Disease (COVID-19) faced a non-experienced immunity in 2020, which favored its rapidly spread, and infection in a non-prepared environment. These features led to an intense cytokine storm, tissue compromising and worse outcomes in the human population. These mechanisms were shown to be a key factor on SARS-CoV-2 management, predict hospitalization, long-COVID symptoms and sequelae. Our paper focused in unravelling cellular and molecular mechanisms with potentiality of contribution to hyperinflammatory conditions in mild, moderate and severe patients with COVID-19. **Methods:** We conducted a cross-sectional study, with 51 healthy donors, 12 mild and non-hospitalized COVID-19 patients, 6 moderates which required hospitalization but not mechanical ventilation, and 9 severe who required both hospitalization and mechanical ventilation during attendance. Whole blood and serum were obtained to measure blood count parameters, immunophenotypic and soluble proteins. Cells were identified by flow cytometry, while cytokines, chemokines and growth factors were measured by Luminex. Data was stored and analyzed by Kruskal and Dunn's tests, and Spearman correlation. A mixed model was also applied using age and gender as cofounding factors. The analysis was done with confidence interval of 95%. **Results:** Patients with COVID-19, during the acute phase, showed higher levels on inflammatory markers, mainly IFN-γ, IL-1β, GM-CSF, IL-17, and TNF-α. During mild conditions, an increase in inflammatory and proliferative markers were seen, while migration to a hospitalized condition is determined by increase mainly in NLR, NKT cells, CXCL8 and IL-6. However, eosinophil count (logFC = 1.9; $p < 0.001$) and percentage (logFC = 7.8; $p = 0.002$) were the major markers regarding those severe that

required mechanical ventilation. These markers, aligned to LUC (logFC = 2.0; p = 0.007) measurement, were shown to be increased among those who involve to death. **Conclusion:** Our findings highlight the major immune markers related to inflammation and COVID-19 worsening. As severity increases, participation on IFN-γ, IL-1β, GM-CSF, IL-17, and TNF-α are the major compounds in severe condition. Eosinophils showed the potential to act as marker for patient migration from moderate to severe hospitalized condition. There is a need to identify the main mechanisms involved in clinical worsening, and correlate to other compounds to prevent fatal outcomes in future cases.

Keywords: Hallmarks; SARS-CoV-2; Cytokine storm; Severe; Predictors.

Background

SARS-CoV-2 affected population worldwide since 2019 and was the major cause of several deaths. This virus is classified as a Betacoronavirus, with main tropism for the angiotensin-converting enzyme (ACE2), presented mainly in lung tissue, and caused the severe acute respiratory syndrome (SARS) (CEVIK et al., 2020; TURILLI; LUALDI; FASANO, 2022). Major transmission occurred rapidly by aerosol released from an infected patient to nasal the tract of a non-infected patient. For the next days, the viral replication occurs mainly in lungs to then, the transmission cycle may last from 5 (to previously immunized) to 14 (non-exposed) patients (HARRISON; LIN; WANG, 2020; KHAN et al., 2021).

Clinical symptoms reported included mild to severe fever, cough, breathing issues, intestinal compromising and in some cases, thrombosis (ARCANJO et al., 2020; MARTENS et al., 2021). Immune system showed a potentiality on hallmark research for patients' hospitalization, once mechanisms such as cytokine storm, which showed a pivotal role on disease progression (KHAN et al., 2021), but also on convalescent condition, observed after clinical recovery (ZHAO et al., 2022).

Those infected patients can progress from a mild to severe condition and death in a short amount of time. Many factors have been related to increase in severity scores, and most were related to immune components (PURWONO et al., 2024; SÁNCHEZ et al., 2023; TAO et al., 2021). Although clinical and laboratorial parameters interplay is

extremely important to comprehend the major dynamics over disease, there has been a lack over the key points between severity groups and clinical outcomes. Correlate the major parameters and comprehend how their interactions can improve future strategies and clinical management for patients with SARS.

Here we highlight the production of major immune markers in severity dynamics of patients with COVID-19 in mild condition but also hospitalized and under mechanical ventilation. Clinical outcome was also evaluated from those hospitalized, in which we highlight markers such as neutrophil-lymphocyte ratio (NLR), absolute eosinophil count (AEC) and B1 lymphocytes as possible markers of both clinical conditions, but also hospitalization outcome. To accomplish this, we recruited healthy blood donors, and COVID-19 positive and hospitalized patients. Our findings can improve clinicians' decision in future respiratory diseases that may occur in the future.

Methods

Subjects and sample inclusion

To all eligible participants, formal consent was applied after invitation and written consent was obtained, as inclusion criteria. Further, a questionnaire was applied for sociodemographic and clinical data obtainment and after, blood sample collection. This research and design were approved by the ethical committee (# 4.126.784) from both Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas (HEMOAM) and Hospital Universitário Getúlio Vargas (HUGV), located in the city of Manaus, state of Amazonas, in Brazil. All procedures were performed following Declaration of Helsinki, and Brazilian Health Ministry.

We conducted a cross-sectional study, with inclusion of 51 Healthy Donors, who were eligible for blood donation and had no previous infectious or genetic disease, and 27 patients with previous diagnosis of SARS-CoV-2 by RT-PCR. These patients were further classified in 12 Mild patients, who were symptomatic, but did not require hospital attendance; 6 Moderate patients, who required hospital attendance, but during clinical follow-up, did not required mechanical ventilation; and 9 Severe patients who were also hospitalized, but required mechanical ventilation. All participants were recruited after diagnosis and COVID-19 symptoms (for Mild group) or hospital entrance with viral symptoms (for Moderate and Severe groups), as shown in Figure 1A. Recruitment of HD

and mild groups occurred at Fundação HEMOAM, while patients from COVID-19 groups were recruited from Hospital Universitário Getúlio Vargas (HUGV), along the first SARS-CoV-2 wave (2020) and the beginning of 2021. Any participant had taken any COVID-19 vaccine before enrollment in this study (Figure 1A).

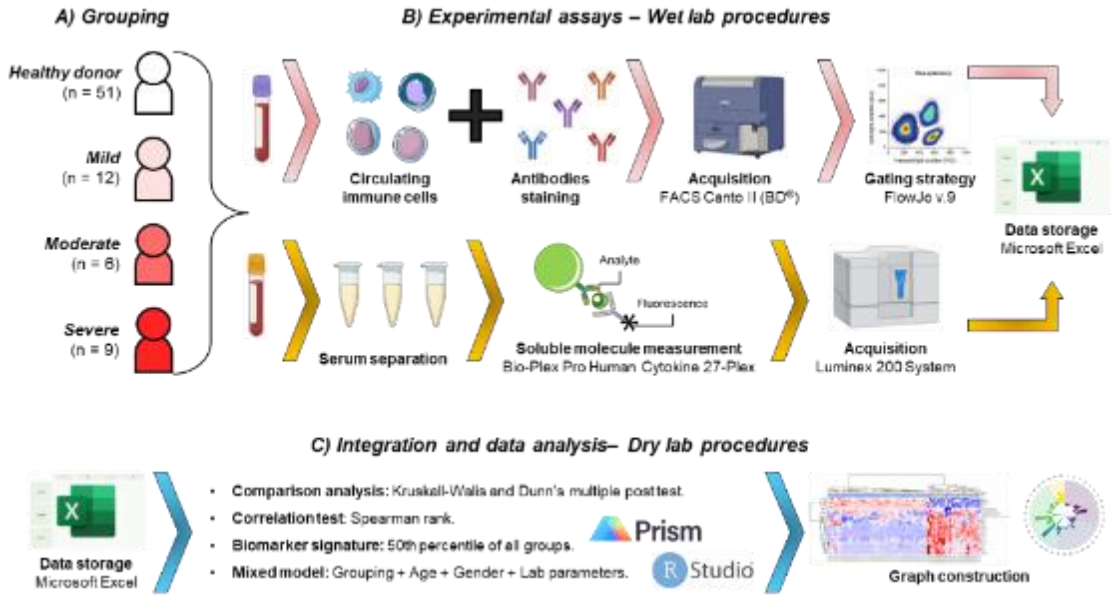


Figure 1: Methodological procedures. A) Grouping all participants included; **B)** Wet lab procedures with scheme of flow cytometry (top) and Luminex technic (bottom), following sample preparation, staining, acquisition and further data storage; **C)** Statistical analysis performed and software used to construct all graphs

From all participants, 8 ml of whole blood was obtained and separated into EDTA (BD Vacutainer® EDTA K2) tubes and Gel separator (Gel BD SST® II Advance). Whole blood was used in automatic blood count (ADVIA 2120i, Siemens, USA) at hematology lab from HEMOAM, and immunophenotyping and flow cytometry, while serum samples were collected and stored in -80 °C for further analysis.

Immunophenotyping and flow cytometry

The whole blood was marked by antibodies conjugated to fluorochromes with the objective of quantifying the immune cells subpopulation. The following markers were applied: anti-CD3 (APC), anti-CD4 (FITC), anti-CD8 (PE) and anti-CD69 (PERCP) to identify T cells (CD3+), T helper cells (CD3+CD4+, Th cells), activated T helper cells

(CD3+CD4+CD69+, aTh cells), T cytotoxic cells (CD3+CD8+, Tc cells) and activated T cytotoxic cells (CD3+CD8+CD69+, aTc cells). Anti-CD19 (PE) and anti-CD5 (FITC) for B cells (CD3-CD19+) and B1 cells (CD19+CD5+). Anti-CD14 (APC), anti-CD16 (FITC), and anti-HLA-DR (PE) for classical monocytes (CD14+CD16-, CMo), intermediary monocytes (CD14+CD16+, IMo) and non-classical monocytes (CD14lowCD16+, NcMo). Anti-CD14 (APC), anti-CD123 (FITC) and anti-CD11c (PE) for myeloid dendritic cells (CD14-CD123+, mDCs) and plasmacytoid dendritic cells (CD14-CD11c+, pDCs). Anti-CD3 (APC), anti-CD16 (FITC), anti-CD56 (PE) and anti-CD69 (PERCP) for NK cells (CD3-CD56+CD16+) and NKT cells (CD3+CD56+CD16+). All antibodies were obtained from BD Biosciences (San Diego, CA, USA), Beckman Coulter (Brea, California, USA), and BioLegend (San Diego, CA, USA). Acquisition was performed with FACSCanto II flow cytometer, also at Fundação HEMOAM, and gating strategy was conducted in FlowJo Software v.10.8, as described previously (SILVA-JUNIOR et al., 2024). The percentage of immune cells was obtained and applied to statistical analysis (Figure 1B).

Soluble mediators' measurement

Serum was used to measure chemokines (CCL11, CXCL8, CCL3, CCL4, CCL2, CCL5 and CXCL10), cytokines (IL-1β, IL-6, TNF-α, IL-12p70, IFN-γ, IL-15, IL-17A, IL1ra, IL-4, IL-5, IL-9, IL-10, IL-13, IL2 and IL-7) and growth factors (FGF basic, PDGF-BB, VEGF, GM-CSF and G-CSF) by Luminex technic at Instituto René Rachou (FIOCRUZ-MG) with the Bioplex- Pro Human Cytokine 27-Plex Kit (Bio-Rad, California, USA). Acquisition occurred in Luminex 200 System and further Bioplex Manager Software under Five Parameters Logistic Regression. The detection limits were: CXCL8 = 42,150 pg/ml; CXCL10 = 31,236 pg/ml; CCL2 = 24,282 pg/ml; CCL3 = 960 pg/ml; CCL4 = 11,233 pg/ml; CCL5 = 16,533 pg/ml; CCL11 = 26,842 pg/ml; IL-1β = 8,608 pg/ml; IL-1ra = 91,661 pg/ml; IL-2 = 18,297 pg/ml; IL-9 = 25,642 pg/ml; IL-15 = 22,328 pg/ml; IL-4 = 4,789 pg/ml; IL-5 = 23,105 pg/ml; IL-6 = 37,680 pg/ml; IL-7 = 16,593 pg/ml; IL-10 = 35,170 pg/ml; IL-12p70 = 37,684 pg/ml; IL-13 = 8,090 pg/ml; IL-17A = 28,850 pg/ml; IFN-γ = 25,411 pg/ml; TNF-α = 64,803 pg/ml; PDGF-BB = 24,721 pg/ml; FGFb = 16,046 pg/ml; G-CSF = 40,049 pg/ml; GM-CSF = 12,844 pg/ml; and

VEGF = 29,464 pg/ml. All procedures followed manufacture's protocols, and data was obtained in pg/ml (Figure 1B).

Statistical analysis

All groups were compared based on sociodemographic and laboratorial parameters using log value for continuous laboratorial values. Gender was compared using Fisher Exact Test, while age was compared with Kruskal-Wallis and Dunn's multiple comparison post-test (Figure 1C).

A mixed model was done to determine the influence of both age and gender in each laboratorial parameter. For this approach, we used all groups (and outcomes), inside package "*limma*" in R Studio R Studio v.2023.12.1 software (Project for Statistical Computing Version 3.0.1). All statistical analysis (contingency and continuous comparisons) were done also in R Studio with the proper package and considering confidence interval of 95% and significant p when $p < 0.05$.

Results

We included 51 healthy blood donors, composed by 36 (70.6%) males and 15 (29.4%) females, and mean age of 32 (± 12) years. COVID-19 mild patients were composed by 9 males (75%) and 3 females (25%), with mean age of 39 (± 8.89). The 15 patients who required hospital attendance were divided into: 6 with Moderate COVID-19, in which 2 were males (33%) and 4 females (67%) with mean age of 46 years (± 23.8) that did not require mechanical ventilation; and 9 with Severe COVID-19, in which 5 were males (56%) and 4 were females (44%), with mean age of 59 years (± 21) and required mechanical ventilation during hospital attendance. Chi-Square test showed difference among gender ($p < 0.0001$) between our groups (Figure S1A). Regarding age, Severe patients were older than Healthy Donors group ($p < 0.001$) and Mild group ($p = 0.006$) (Figure S1B).

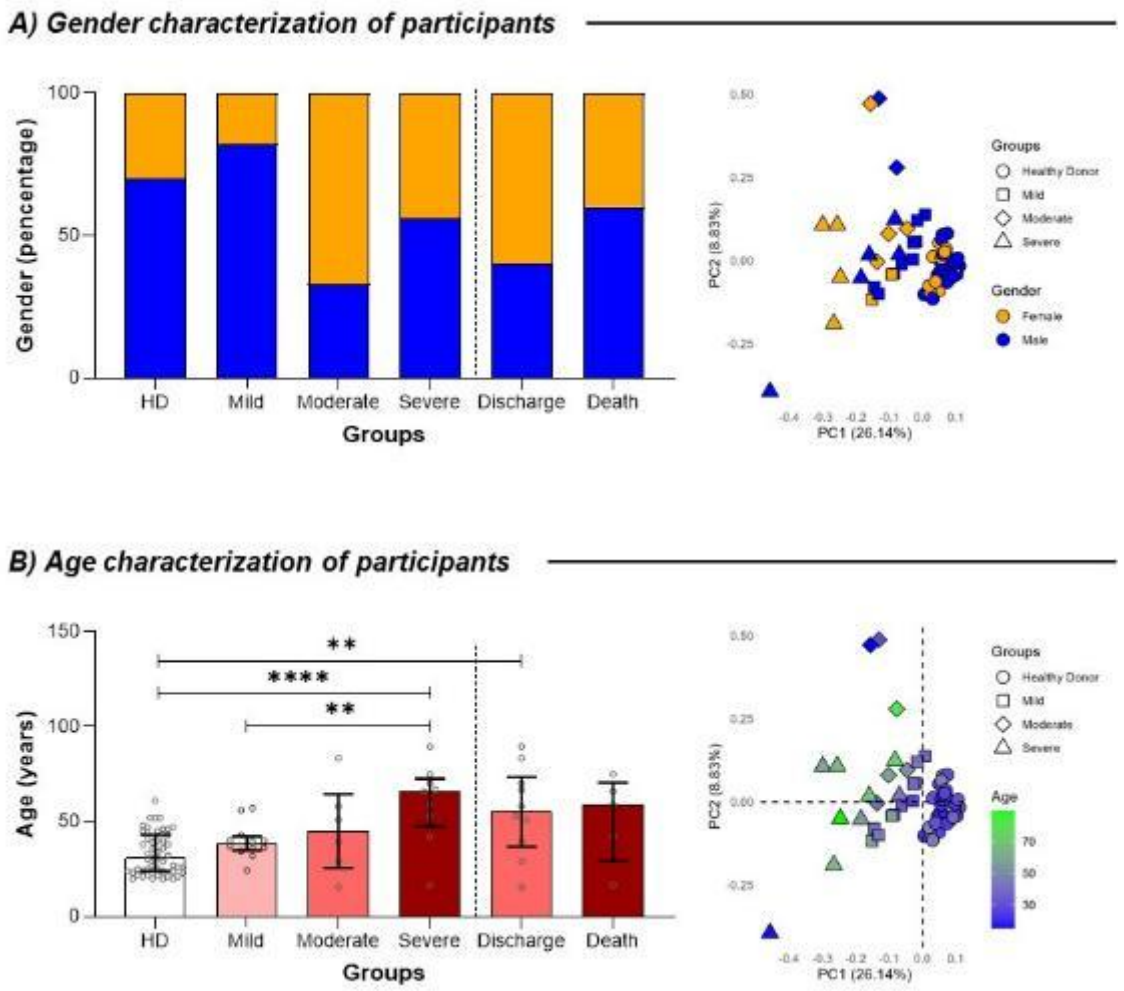


Fig S1: Participant's demographic characteristics. A) Demographic characterization of participants based on group sampling. Bar graph (left) with percentage of participants, and PCA distribution (right) based on groups and gender; **B)** Demographic characterization of participants based on age sampling. Boxplot comparison (left) with age of participants, and PCA distribution (right) based on groups and age scale.

Under 15 hospitalized patients, 2 (13.3%) had no history of previous comorbidities. 7 (46.6%) had diabetes [3/7 died], 6 (40%) had hypertension [2/6 died], 6 (40%) had neoplasm [2/6 died]. Regarding clinical outcome, these 15 patients were divided into Discharge (n = 10, 4 [40%] males and 6 [60%] females), which occurred in the mean of 33 days (± 24). Only 5 (50%) required ICU and 4 (40%) required mechanical ventilation. On the other hand, the other 5 [3 [60%] males and 2 [40%] females] patients involved to Death in the mean of 72 days (± 44) after hospital admission. Among them, all required ICU and mechanical ventilation. Statistical analysis demonstrated a

223 difference in gender analysis ($p < 0.0001$) and age (HD vs Discharge; $p = 0.002$), as shown
224 in Figure S1.
225
226 *Acute COVID-19 is marked by an inflammatory and pro-inflammatory interplay*
227 Severe patients showed lower levels of RBC, hemoglobin and hematocrit, while
228 increase in WBC, ANC and AMC (Table 1). Despite ALC falling significantly among
229 mild patients, there was no difference between moderate and severe. Neutrophil-
230 Lymphocyte ratio (NLR) was proposed to be an important marker for clinical progression,
231 and our data showed that moderate patients had a median of 36.42 $x10^3/\mu L$ [IQR 31.8-
232 48.5], higher than both Healthy Donors (HD) ($p < 0.0001$) and Mild ($p = 0.005$).
233 Regarding immune cells, only AEC decreased from HD to Mild group ($p < 0.01$) and
234 from Moderate to Severe ($p = 0.001$). We must highlight that both Mild and Moderate
235 groups had a significant reduction in AEC, even lower than 25th interquartile range from
236 HD group.
237

Hematological parameters (median [IQR])	**Healthy Donor (n = 51)**	**Mild (n = 12)**	**Moderate (n = 6)**	**Severe (n = 9)**	**p value**
RBC ($x10^6/\mu L$)	5.03 [4.6-5.4]	4.94 [4.4-5.4]	3.47 [2.4-4.4]	3.66 [3-4.4]	***< 0.001***^{b,c,d,e}
Hemoglobin (g/dL)	14.9 [13.6-16]	14.65 [13.7-15.7]	10.15 [6.5-12.2]	10.1 [8.5-12.7]	***< 0.001***^{b,c,d,e}
Hematocrit (%)	44.7 [40.6-47.4]	44.1 [40.7-48]	30.05 [19.6-37.3]	32.9 [25.7-39.5]	***< 0.001***^{b,c,d,e}
MCV (fL)	87.8 [84.7-90.4]	91.35 [87.1-94.1]	86.75 [78.8-91.8]	89.8 [84.3-92.6]	0.1773
MHC (pg)	29.7 [28.8-30.7]	30.4 [28.9-30.9]	28.7 [25.7-32]	28.4 [27.8-29.3]	0.0624
MCHC (g/dL)	34.1 [33.1-34.6]	32.55 [32.2-33.3]	33 [32.5-34.3]	32.3 [31.5-33.7]	***0.0015***^{a,c}
RDW (%)	13.7 [13.1-14]	14.25 [13.8-15]	13.85 [12.3-15.2]	13.7 [11.8-17.2]	0.0843
WBC ($x10^6/\mu L$)	6.33 [5.2-7]	5.92 [4.1-6.6]	8.9 [8.6-9]	12.26 [9.2-14.5]	***< 0.001***^{b,c,d,e}
ANC ($x10^3/\mu L$)	3.34 [2.8-4.2]	3.63 [2.6-4.5]	8.3 [7.9-8.3]	10.19 [6.4-11.7]	***< 0.001***^{b,c,d,e}
ALC ($x10^3/\mu L$)	1.85 [1.6-2.2]	1.62 [1-2.2]	0.23 [0.2-0.3]	1.35 [1.2-2]	***< 0.001***^{b,d}
AMC ($x10^3/\mu L$)	0.38 [0.3-0.4]	0.39 [0.4-0.5]	0.43 [0.4-0.5]	0.61 [0.5-1]	***0.0014***^{c}

AEC ($x10^3/\mu L$)	0.2 [0.1-0.4]	0.08 [0-0.2]	0.03 [0-0]	0.26 [0.1-0.5]	***< 0.001***^{a,b,e,f}
ABC ($x10^3/\mu L$)	0.03 [0-0.1]	0.03 [0-0]	0.04 [0-0]	0.02 [0-0.1]	0.63
LUC (%)	1.7 [1.4-2.4]	1.65 [1.2-2.3]	0.9 [0.8-1]	1.6 [1.3-2.1]	***0.0105***^{b}
NLR ($x10^3/\mu L$)	1.89 [1.5-2.3]	2.16 [1.3-3.1]	36.42 [31.8-48.5]	6.67 [4.7-9.9]	***< 0.001***^{b,c,d,e}
Platelets ($x10^3/\mu L$)	243 [210-282]	205 [168.8-278.3]	152.9 [106.2-242.4]	224.7 [147.8-360.3]	0.1284
MVP (fL)	8.1 [7.2-8.6]	8.25 [7.9-9.8]	7.45 [6.3-8.7]	7.6 [7-8.8]	0.2997

Table 1: Hematological parameters from healthy donors, and COVID-19 groups. Statistical analysis was performed with Kruskal-Wallis test and Dunn's multiple post-test. To all comparisons, a confidence interval of 95% was applied and p was considered significant when $p < 0.05$.

[a]Significant difference between HD vs Mild;

[b]Significant difference between HD vs Moderate;

[c]Significant difference between HD vs Severe;

[d]Significant difference between Mild vs Moderate;

[e]Significant difference between Mild vs Severe;

[f]Significant difference between Moderate vs Severe.

RBC: red blood cells; MCV: Mean corpuscular value; MHC: Mean corpuscular hemoglobin; RDW: Ratio Distribution Width; WBC: White blood count; ANC: Absolute Neutrophil Count; ALC: Absolute Lymphocyte Count; AMC: Absolute Monocyte Count; AEC: Absolute Eosinophil Count; ABC: Absolute Basophil Count; NLR: Neutrophil-Lymphocyte ratio; MPV: Mean platelet volume.

Acute infection demonstrated a lower on NK, NKT cells, CMo and Th cells. On the other hand, an increase in IMo, NcMo and aTc cells levels was seen in the Mild group, despite IMo having no difference among other COVID-19 groups (Figure 2).

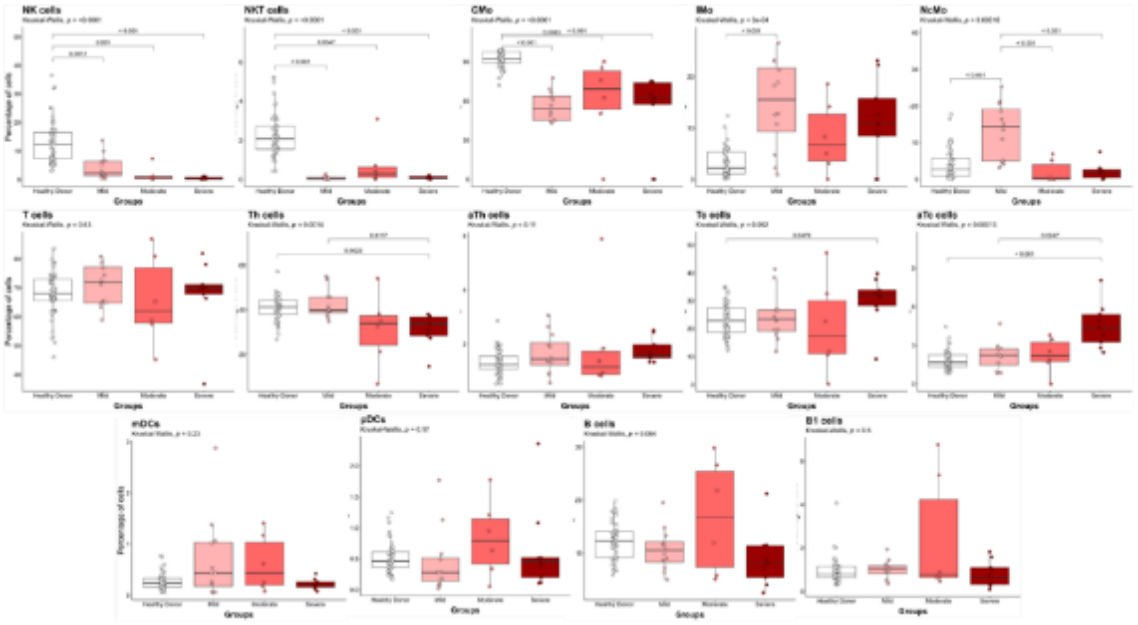


Figure 2: Acute COVID-19 cell profile. The data is expressed as percentage of cells and analyzed by Kruskal-Wallis and Dunn's Multiple Comparison posttest, with confidence interval of 95%.

Regarding soluble inflammatory and proinflammatory markers, acute COVID-19 showed an increase in CCL4, CCL11 and CXCL10, together with a reduction in CCL5 (Figure 3A). This, together with the cytokine profile with increase in IL-1β, IL-6, TNF-α, IL-12, IFN-γ, IL-17, IL-4, IL-9, IL-10, IL-13 and IL-7 mediate the inflammatory profile during acute phase (Figure 3B). Growth factor profile demonstrates an increase in VEGF, GM-CSF and G-CSF, when compared to healthy donor group (Figure 3C). Interestingly, only IL-6 was higher in Severe group, when compared to Mild ($p < 0.001$) and IL-4 was higher in Severe, compared to Moderate group ($p = 0.0292$).

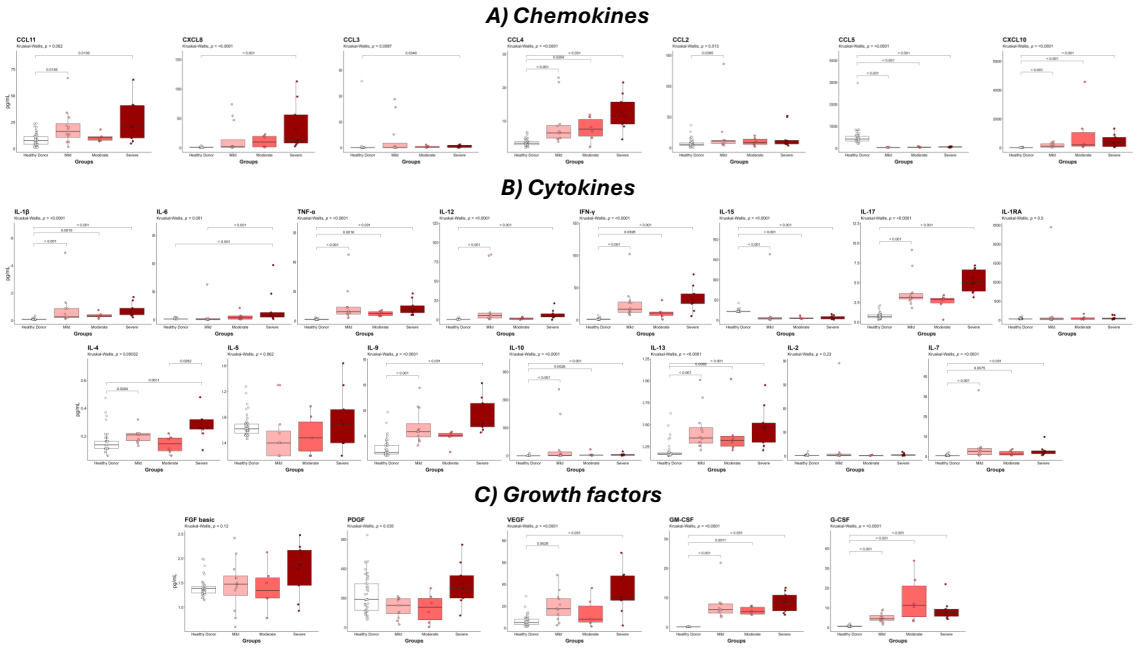


Figure 3: Acute COVID-19 soluble molecular profile. Soluble markers are distributed into chemokines (A), cytokines (B) and growth factors (C). Data is expressed as pg/mL and analyzed by Kruskal-Wallis and Dunn's Multiple Comparison posttest, with confidence interval of 95%.

Inflammatory markers are the major contributors to Severe condition, while Moderate is influenced by reparative markers

Despite general PCA showed a segregation only under Healthy Donor group and the other groups, it was seen that, based on immunological markers, all Mild, Moderate and Severe patients have a very similar profile (Figure 4A). Based on contribution, Mild, Moderate and Severe groups share the same markers, but as severity increases, inflammatory markers of GM-CSF, G-CSF, CXCL10, IFN-γ, IL-7, TNF-α, IL-1β, VEGF and IL-17 are the major markers that contribute to this severity (Figure 4B), as observed into the heatmap as well (Figure 4C).

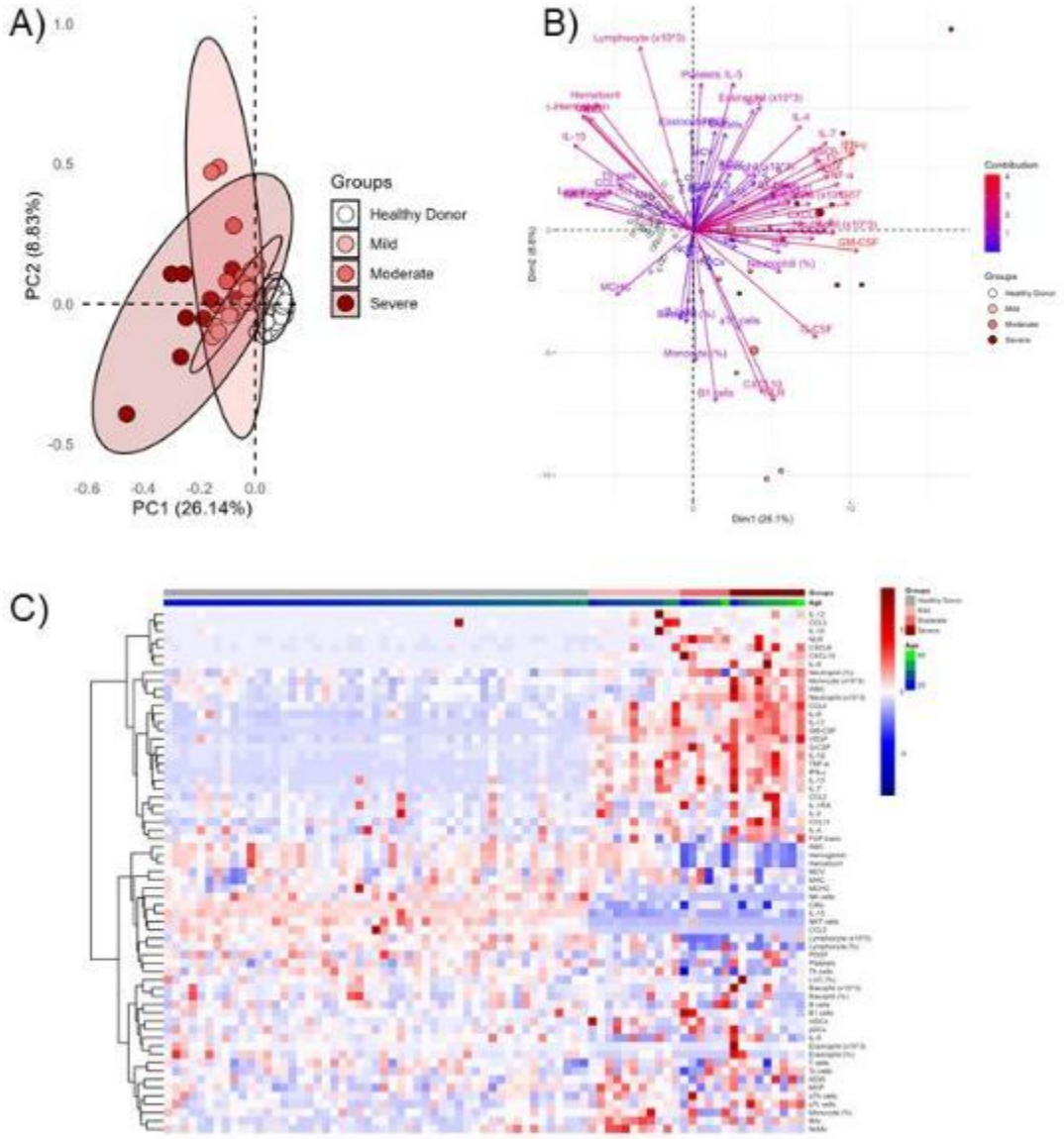


Figure 4: Inflammatory markers are the major contributors to Severe condition in acute COVID-19 patients. (A) PCA segregation based on grouping; (B) PCA with contributors to acute condition, with point colored by the groups and arrows by the contribution; (C) Heatmap with all features clustered in the rows, and groups organized by the columns.

From Healthy to Mild (symptomatic with no need of hospitalization) condition, we observed that GM-CSF, IFN-γ, IL-12, IL-10 and IMo had the biggest increase, while NKT cells, CCL5 and IL-15 had the highest decrease. From Mild to Moderate (hospitalized with no mechanical ventilation requirement), NLR, NKT cells, CXCL8, IL-6 and G-CSF were the major markers to increase, while NcMo, eosinophil (%) and ALC had the highest decrease. Thus, those patients that are under hospital attendance, and

require mechanical ventilation, transitioning from Moderate to Severe condition, eosinophil (%) and AEC and had the highest increase in blood circulation, together with NcMo, ALC and IL-12 (Figure 5). These markers suggest their capacity to act as hallmarks of disease progression.

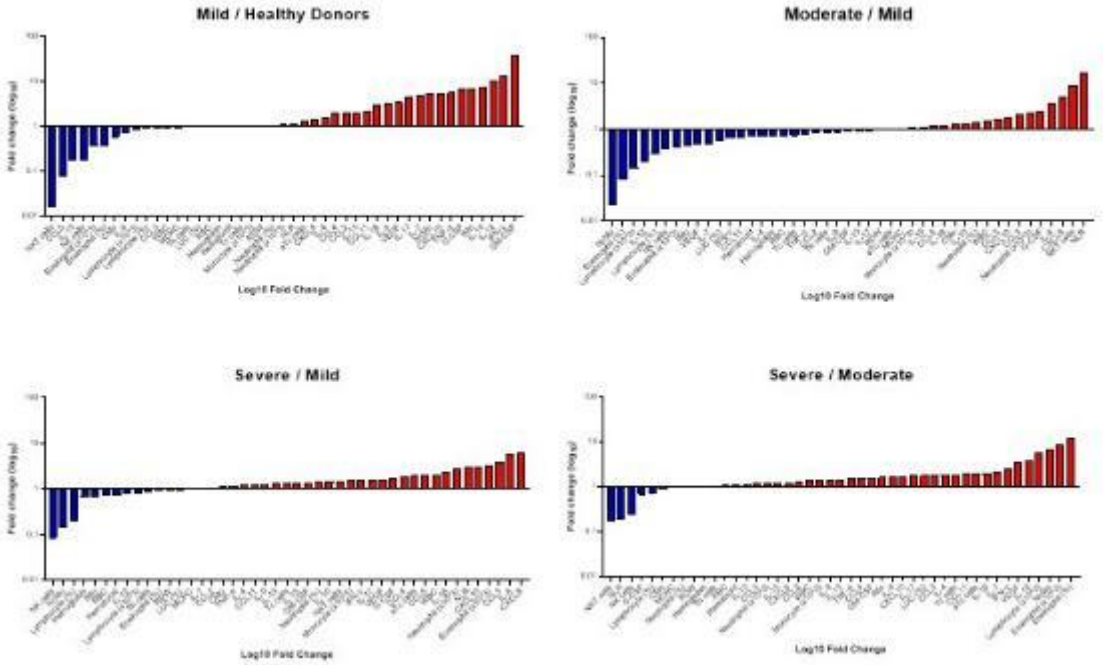
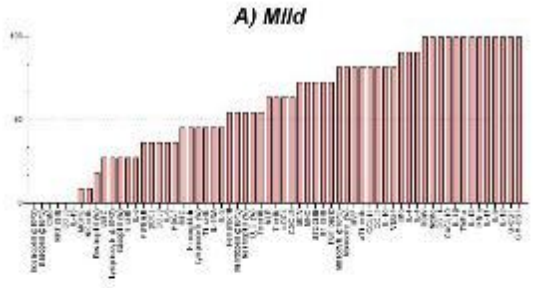
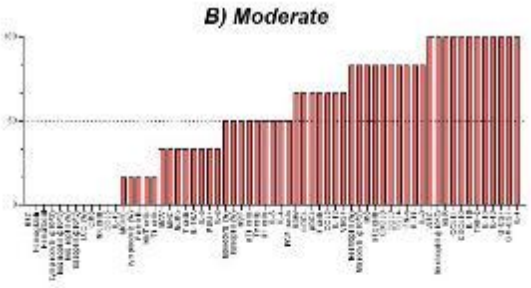


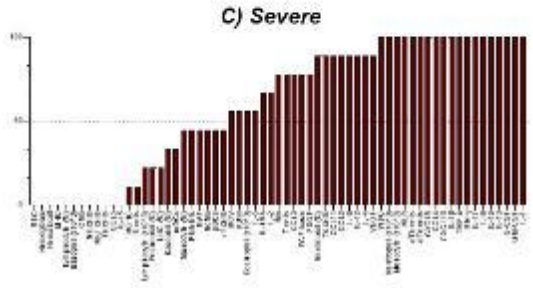
Figure 5: Fold Change (FC) analysis with the mean of each parameter from the groups, isolated by Dunn's post hoc and organized based on FC result.

Producers' analysis supports that severity is marked by high production of inflammatory markers

Furthermore, COVID-19 groups were isolated, and the global median was calculated, as described before. Our findings highlight that Mild condition was exclusively marked by higher production of 10 markers: IMo, hematocrit, hemoglobin, NcMo, B1 cells, T and Th cells, lymphocytes (%), RDW and RBC. While Moderate group was the higher producer of only basophil count, MCHC and NKT cells. Severe group was marked by higher production of 19 markers: FGF basic, AMC, IL-9, IL-13, IFN-γ, platelets, IL-1β, aTc cells, GM-CSF, aTh cells, VEGF, IL-4, TNF-α, CCL4, IL-17, IL-2, IL-1RA, PDGF and Tc cells (Figure 6).







D) Intersections report of high producers

Groups	Inters.	Elements
Mild / Moderate	7	mDCs, MHC, NK cells, MVP, Basophil (%), Monocyte (%), B cells
Mild / Severe	9	IL-7, Eosinophil (x10^2), LUC (%), MCV, IL-12, Lymphocyte (x10^3), CCL2, CCL11, Eosinophil (%)
Moderate / Severe	15	pDCs, Neutrophil (x10^3), CXCL8, cMo, CXCL10, IL-10, CCL3, Neutrophil (%), NI R, G CSF, CCL5, IL-8, WBC, IL-5, IL-15
Mild	10	IMo, Hematocrit, Hemoglobin, NcMo, B1 cells, Th cells, T cells, Lymphocyte (%), RDW, RBC
Moderate	3	Basophil (x10^3), MCHC, NKT cells
Severe	19	FGF basic, Monocyte (x10^3), IL-9, IL-13, IFN-γ, Platelets, IL-1β, aHc cells, GM-CSF, aTh cells, VEGF, IL-4, TNF-α, CCL4, IL-17, IL-2, IL-1RA, PDGF, Tc cells

Figure 6: High producers' analysis among COVID-19 groups. Higher producer calculus was used with percentile 50th in the groups of Mild (A), Moderate (B) and Severe (C). Intersection of each group and elements is described on the report (D).

Correlation matrices were performed by isolating each group and using all parameters. The Healthy Donor group showed weak correlations between the parameters, while Mild group were more strong correlations, whether positive or negative. Hospitalized groups had fewer correlations, when compared to Mild group, which could be related to hospital attendance (Figure 6).

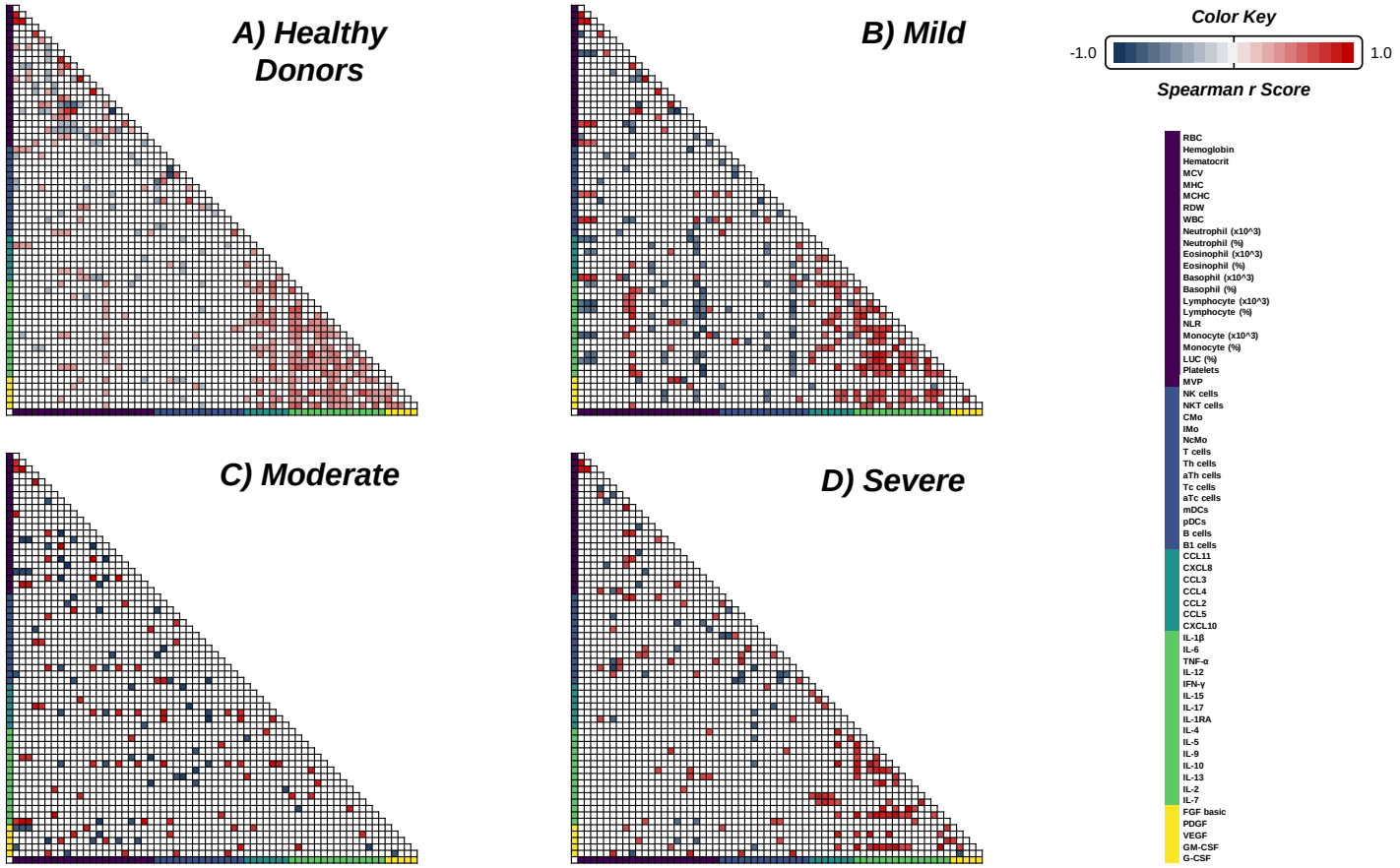


Figure 6: Correlation matrix with correlation of all parameters of Healthy Donors (A) and COVID-19 patients Mild (B), Moderate (C) and Severe (D). The network was constructed using Spearman correlation indices (r). Significant correlations ($p < 0.05$) were plotted, and r values were scaled in blue-white-scale ranging from -1.0 to 1.0. Parameters (blood Count, phenotypic profile, chemokines, cytokines and growth factors) were classified based on colors.

Eosinophil has increased on those hospitalized patients with COVID-19 with worse outcome

We applied our data into a mixed model to determine the logFC of acute COVID-19 groups, using Healthy Donors as a reference group, and including gender and age as cofounding factors. Gender did not show any significant interference among any marker, however, age demonstrated to interfere with AEC (logFC = -0.028, p = 0.0154) and eosinophil (%) (logFC = -0.147, p = 0.0163). The Spearman correlation test was made to identify the relation of both parameters to age. When grouped all patients, age had a negative correlation only to eosinophil (%) (r = -0.31, p = 0.0067), but lost significant when stratified by groups (Figure S2). Despite that, we controlled our groups based on both demographic conditions.

Mild condition had highest logFC on cytokines, mainly inflammatory and proinflammatory, IFN-γ (logFC = 18.4, p < 0.001), IL-10 (logFC = 16.7, p = 0.012), VEGF (logFC = 11.4, p = 0.002) and IL-12 (logFC = 10.8, p = 0.003), while the lowest FC were found in CCL5 (logFC = -443.3, p < 0.001), CMo (logFC = -36.6, p < 0.001) and IL-15 (logFC = -17.9, p < 0.01). A similar profile was seen in Moderate patients, with higher logFC of CXCL10 (logFC = 1,160, p < 0.01) and NLR (logFC = 40.9, p < 0.01). The Severe group also had the highest logFC on CXCL10 (logFC = 692, p = 0.002), followed by PDGF (logFC = 103, p = 0.043) and IL-1RA (logFC = 39.1, p = 0.001). All parameters are plotted into a radar graph, sorted by group in Figure 7.

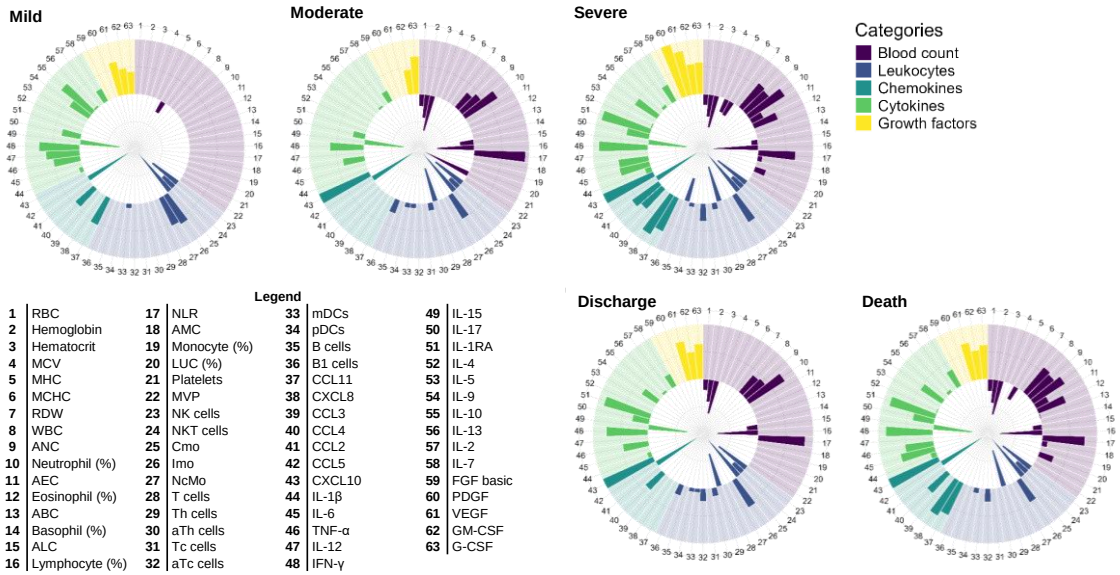
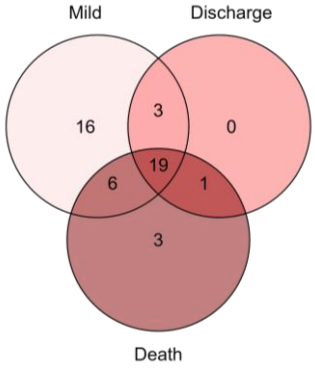
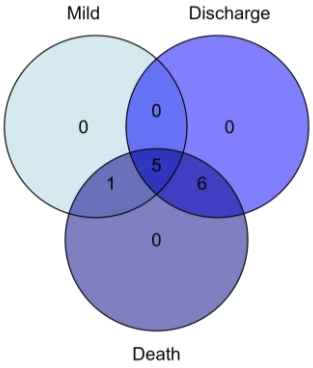


Figure 7: Radar graphs sorted based on up- and down-regulation from logFC obtained from Healthy Donor group, controlled by participant's age and gender. For plotting purposes, all data was transformed to log scale, and the size of the graphs was established in a range of -4 to 4 logFC. To negative FC, parameters are aimed to the center of the graph, while positive FC is aimed to the outside. Parameters were numbered, and categorized based into blood count markers, leukocytes (obtained by flow cytometry), chemokines, cytokines and growth factors.

Furthermore, we divided the Moderate and Severe groups based on hospital outcome, into Discharge and Death groups, from those patients that involved to discharge (n = 10) or death (n = 5) outcomes, respectfully. Even though they share a similar profile of logFC, we constructed a Venn Diagram to look for shared parameters between Mild group and both outcomes. Our findings demonstrated that the markers evaluated here were not sufficient to determine which hospitalized patients can involve to discharge. However, those who involve to death had increase in AEC, eosinophil (5) and LUC (%) (Figure 8). Hospitalization, despite the outcome, was marked by an increase in WBC, and lower on ALC, hemoglobin, lymphocyte (%), RBC, hematocrit and Th cells.



Groups	Inters.	Elements
Mild / Death	6	CCL11, CXCL8, IL-4, Monocyte (x10^3), IL-6, Basophil(x10^3)
Mild / Discharge	3	mDCs, aTh cells, B1cells
Discharge / Death	1	WBC
Mild / Discharge / Death	19	GM-CSF, IL-17, TNF-α, IFN-γ, IMo, IL-9, IL-7, CCL4, IL-1β, VEGF, IL-13, G-CSF, CXCL10, aTc cells, Neutrophil (%), IL-1RA, NLR, Neutrophil (x10^3), pDCs
Mild	**16**	**NcMo, IL-12, IL-10, RDW, MVP, Monocyte (%), CCL3, MCV, CCL2, Tcells, IL-2, FGF basic, Th cells, Tc cells, Hematocrit, MHC**
Death	**3**	**Eosinophil (x10^3), Eosinophil (%), LUC (%)**



Groups	Inters.	Elements
Mild / Death	1	MCHC
Discharge / Death	6	Lymphocyte (x10^3), Hemoglobin, Lymphocyte (%), RBC, Hematocrit, Th cells
Mild / Discharge / Death	5	IL-15, CMo, NKT cells, N Kcells, CCL5

Figure 8: Selection of major immune markers for up- and down-regulation of conditions normalized by HD group. Radar graphs (top) demonstrate the significant fold change of markers related to HD group. Venn Diagrams (bottom) shows the markers shared for the upregulated (red) and downregulated (blue) among groups.

Discussion

The entire world faced a public health issue that turned to the pandemic condition experienced in 2020. Among major contributors to the rapid spread of SARS-CoV-2 and development of extreme critical conditions, many studies highlighted the absence of immunity but also risk factors. With that in mind, we proposed to understand the immunological features related to prime infection in mild, moderate and severe patients, using a healthy donor group as reference. That, related to hospital admission and outcome allowed us to identify eosinophil laboratorial values (both absolute and percentage) as possible markers of worse outcome in COVID-19 patients.

Despite few studies determining the difference among viral characteristics based on gender, we found no interference in immune parameters observed in our study. Age by itself has been described as a factor in immune parameters, named immune senescence, and has been an important field of study (HOU et al., 2022; HU et al., 2021;

MODERBACHER et al., 2020). The distribution of patients in our study showed that the severe group was older than the reference group, which is in accordance with other studies before, relating the older ages as risk factor for severity in COVID-19 clinical management. Elderly condition follows many chronic comorbidities, that may enhance lung tissue lesion (mainly), but also other complications (SCULLY et al., 2020), which corroborate to the characteristics of our patients.

The hematological changes observed in our data are sustained by a systemic pathophysiology from the disease. Thrombotic events were previously described among patients with COVID-19, where it was shown that oxygen supplementation induces an increase in fibrinogen levels and further to RBC aggregation, RBC aggregates strength and blood viscosity. These aggregates can impact the availability of RBC into the blood vessels, but, together with RBC membrane damage and higher phosphatidylserine (PS) exposure, it contributes to removal from the spleen (KLEI et al., 2017; NADER et al., 2022). These features lead to a cascade of event where lower RBC in the blood take to a hypoxia condition (BERZUINI et al., 2021), which corroborate to the need of mechanical ventilation in Severe group.

Regarding inflammatory status seen in our patients, the neutrophil involvement was extremely increased in Severe, which was seen by other authors (BALZANELLI et al., 2021; IMRAN et al., 2020; LOURDA et al., 2021; MCELVANEY et al., 2020; MORADI et al., 2021b; ZHANG et al., 2020a; ZHAO et al., 2020). A ‘shift to the left’ effect is seen, probably due to immune dynamics related to cell production from stem cells. Many circulating neutrophils, during acute COVID-19, share immature profile (CD10+) and were previously correlated to inflammatory markers of CXCL8, CXCL10, CCL3, CCL4, IL-6, IL-1RA (CARISSIMO et al., 2020; METZEMAEKERS et al., 2021; PARACKOVA et al., 2020; WILK et al., 2020). The role of these immature neutrophils is still unclear whether regulatory or pro-inflammatory, but when associated to lymphocyte count, our data demonstrated a significant reduction based on severity. For further strategies and analysis, comprehending the mechanisms behind neutrophilia and lymphopenia, the proliferative pattern and recruitment to local inflammatory tissue can improve therapeutic approaches and acute clinical conditions in patients with a first-time infection, as SARS-CoV-2 was, in our patients.

The phenotypic profile demonstrated extremely lower counts on NK and NKT cells during severity, dynamic also found by other authors (KIM et al., 2021; TAGHILOO et al., 2021; ZHANG et al., 2020c), and to compromise acute cytotoxicity (ANTONIOLI et al., 2020; LEEM et al., 2020). Under factors related to this imbalance, issues in IL-15/IL-15RA axis were suggested as important components on NK cells functional exhaustion, senescence and a more efficient control of viral infection (FLAMENT et al., 2021; ZHANG et al., 2020c, 2020d).

As NK/NKT cells, monocytes subpopulations were also lowered on blood as severity increased. These cells participate in viral recognition and elimination, including infected cells, and although SARS-CoV-2 can infect monocytes due to their expression of the viral receptor ACE2, an increase in granularity and permeability on endothelial cells was described (KIM et al., 2021; MARTENS et al., 2021; ZHANG et al., 2020c; ZHOU et al., 2020), which may justify the reduction in Moderate and Severe of our groups. Accession of T cells showed a reduction in Th cells and increase in Tc and aTc cells in Severe group. This profile was seen before, especially on those under mechanical ventilation, guided mainly by inflammatory cytokines (CARISSIMO et al., 2020; LIU et al., 2020b; MAHMOODPOOR et al., 2021; RENNER et al., 2021).

It was suggested before that these reductions in circulation are not because there is no production or stimulus, but because they are overstimulated to migrate from circulation to the tissue (DIDANGELOS, 2020; GEBREMESKEL et al., 2021), and to determine this hypothesis, we accessed the soluble molecule profile.

Our findings highlight increased mean in CCL11, CXCL8, CCL4 and CXCL10 in severe group, which, when related to the expression of adhesion molecules in circulating leukocytes, described in the literature (GEBREMESKEL et al., 2021; MORADI et al., 2021b; ZHANG et al., 2020a), allow us to believe in the hypothesis suggested before. CXCL10 is known to induce activation and recruitment of lymphocytes, eosinophils, monocytes and NK cells (JING; VASSILIOU; GANEA, 2003; YOUNG; LEE; SONG, 2009), most cells that were reduced in our findings, and together with CCL11, CXCL8 and CCL4, enhance the inflammation and degranulation of granulocytes (BELPERIO et al., 2000; BYSTRY et al., 2001; REN et al., 2010; SMITH; HUMPHRIES, 2009).

The inflammatory profile seemed mediated by IL-1β, IL-6, TNF-α, IL-12, IFN-γ, IL-17, IL-4, IL-9, IL-10, IL-13, IL-7, VEGF, GM-CSF and G-CSF, which highlights the

dynamics on cytokine storm in our patients. These markers were shown before as key markers on disease maintenance and progression to modulate immunity, activation process, recruitment of cells, and proliferation (KNOLL; SCHULTZE; SCHULTE-SCHREPPING, 2021; KUMAR et al., 2021; QIN et al., 2021; THEOBALD et al., 2021). Our findings show that these markers are the major contributors to COVID-19 acute disease. However, moderate stage has an increased contribution of growth factors, such as GM-CSF and G-CSF, but also CXCL10, NLR and B1 lymphocytes, and stronger correlations, when compared to the severe group. Severe conditions, on the other hand, seemed to be more focused in inflammatory components, as WBC, IL-1β, IL-6, TNF-α, IFN-γ, IL-17, IL-4, IL-9, IL-13, IL-7, VEGF, GM-CSF and G-CSF. The parameters here described may be applied as markers of severe complication on hospital admission and can be proposed to prevent respiratory issues among patients with COVID-19.

The severe group had a significant increase in IL-4 concentration, when compared to Moderate group, and despite not significant, higher mean of IL-5 as well. Both cytokines were shown before to induce expression of adhesion molecules and degranulation in eosinophils, respectively (ABBAS; LICHTMAN; PILLAI, 2018). This pattern highlights the importance of eosinophil involvement in immune pathogenesis and severity of COVID-19. Tc cells (CD8+) were previously related to production and induction of IL-5, which contributes to eosinophil proliferation, even indirectly. Once disease progresses, the exhaustion in Tc cells enhance imbalance on IL-5 levels (MARTENS et al., 2021), which may be the reason behind higher concentrations of this marker in severe group.

Even though eosinophils were demonstrated to be markers of improvement in disease progression (GEORGAKOPOULOU et al., 2021; GLICKMAN et al., 2021; GONZÁLEZ et al., 2021; KWIECIEŃ et al., 2021; LIU et al., 2021; TAN et al., 2021; VITTE et al., 2020; YAN et al., 2021), our findings showed the opposite. The relation on increase in eosinophil count and a better prognosis was already suggested by other authors, however, we suggest that our patients were also degranulating, what may explain the increased severity and collaboration to worse outcomes. Determine whether the role of the axis IL-4/IL-5 regulates can contribute to comprehend the dynamics over eosinophils and other granulocytes during viral clearance and tissue damage.

During mild conditions, the eosinophil count lowered, and even more in moderate, but during severe, it increased to values like HD group. Despite we could not access if these eosinophils were activated or not, studies showed previously that activated eosinophils (CD69+) were correlated to inflammatory markers, and contributed to lung tissue infiltration, degranulation, clotting and extracellular matrix metabolization (LOURDA et al., 2021). We believe that this response can be reflection of inflammatory stimulus from the cytokine storm into the bone marrow, aiming to produce more innate cells, and clear the viral infection.

Our study brings new insights into COVID-19 immune dynamics. Our groups were obtained still during the first wave of SARS-CoV-2, when people had no immunity, and the virus had any or few mutations reported. We believe that understanding the mechanisms in a primary exposure to respiratory disease, and hospital clinical markers and predictors, can improve management on future respiratory syndromes. We acknowledge that once our recruitment period started in the very beginning of pandemics, we couldn't address a severity score to our patients, which we believe would normalize our data and results to other studies. Also, the situation that the world faces, in 2024, is an immunity better prepared, due to both previous exposure to the virus and/or immunization. Our data of a first exposure condition can be applied to future respiratory disease that may arise in the future, but also understanding the immune mechanisms in hospitalized conditions.

Conclusion

Our results highlight the immune markers, based on cells and soluble molecules, especially the ability of circulating cells and soluble molecules to participate in the dynamics related to severity in COVID-19 patients. We showed the higher contribution of molecules such as IFN-γ, IL-1β, GM-CSF, IL-17, and TNF-α, to the clinical course of the disease. Beyond that, we suggest that eosinophils, NcMo and ALC are potential markers to indicate the transition from patients hospitalized to mechanical ventilation. Eosinophils also seemed to participate in the disease progression and worse outcome, when patient is already in a severe condition. Despite not well established, unravel the role of this cell, and the mechanisms under tissue damage may propose novel insights in severity for future pulmonary diseases.

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Acknowledgments

The authors would like to extend their gratitude to all staff from the laboratory of Genomics, from Fundação HEMOAM, for their support in this work. This work was funded by Fundação de Amparo à Pesquisa do Estado do Amazonas (FAPEAM) (Pró-Estado Program [#002/ 2008, #007/2018 and #005/2019], POSGRAD Program [#005/2022] and PCTI- EMERGESAÚDE/AM - Chamada II Program [#006/2020), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). ALS-J have fellowships from CAPES (PhD student). AGC and AM are level 2 research fellows from CNPq, respectively. AGC is a research fellow from FAPEAM (PRODOC Program #003/2022). The funders had no role in study design and decision to publish, or preparation of the manuscript.

Author contributions

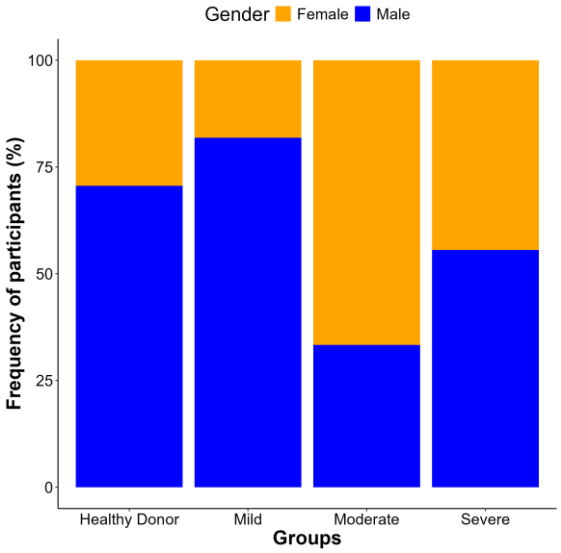
A.L.S-J., L.S.O., N.C.T.B., S.D., C.M.M.A. and W.L.L.N. performed laboratorial procedures of flow cytometry, A.T.-C. and O.A.M.-F. performed the Luminex technic, A.L.S.-J., R.K.A.A., T.C.C.C., L.A.X., F.S.A.-H., D.C.M.A., T.C.O., M.C.C.S, M.M.M.M., W.C.C.S. and G.A.S.S. recruited and collected all data and blood samples, A.L.S.-J., M.A.E.C., D.M.T., P.V.S.-N., M.P.S.S.C., A.M.T., N.A.F., A.G.C. and A.M. conceptualized the study and analyzed the data, A.L.S.-J. wrote the main manuscript, A.M.T., L.H.F., C.A.S., E.C.S., A.T.-C., O.A.M.-F., A.G.C. and A.M. supervised and revised the final manuscript, N.A.F., L.H.C., C.A.S., E.C.S., A.T.-C., O.A.M.-F., A.G.C. and A.M. provided funding acquisition. All authors have read and agreed to the published version of the manuscript.

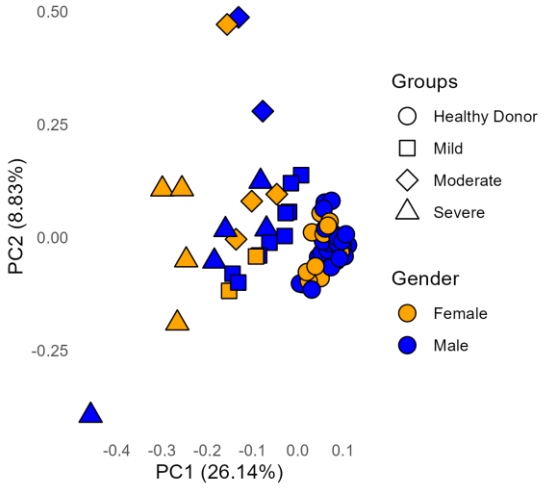
Competing of interest

The author(s) declare no competing interests. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

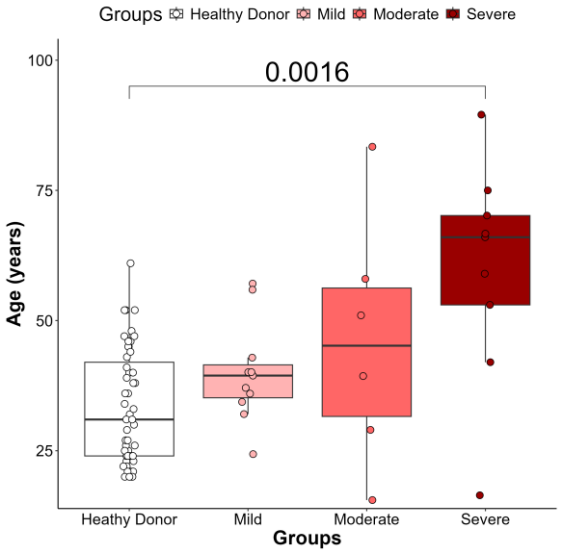
SUPPLEMENTARY MATERIAL

A) Gender characterization of participants





B) Age characterization of participants



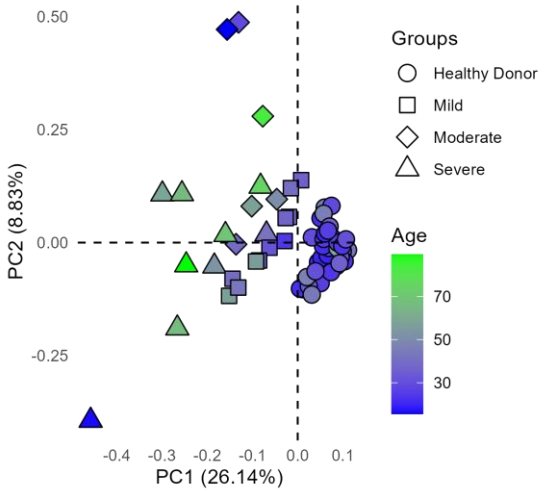
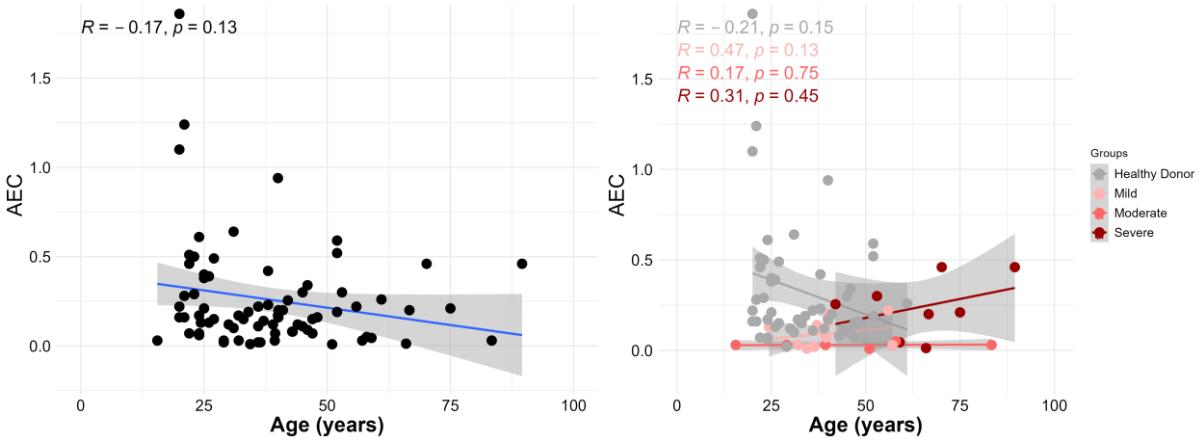


Fig S1: Participant's demographic characteristics. A) Demographic characterization of participants based on group sampling and gender. Bar graph (left) with percentage and PCA distribution based on groups and gender (right); B) Characterization based on age of all groups with barplot (left) and PCA distribution (right).

A) Age correlation to AEC using all groups (left), and separated by groups (right)



B) Age correlation to eosinophil percentage using all groups (left), and separated by groups (right)

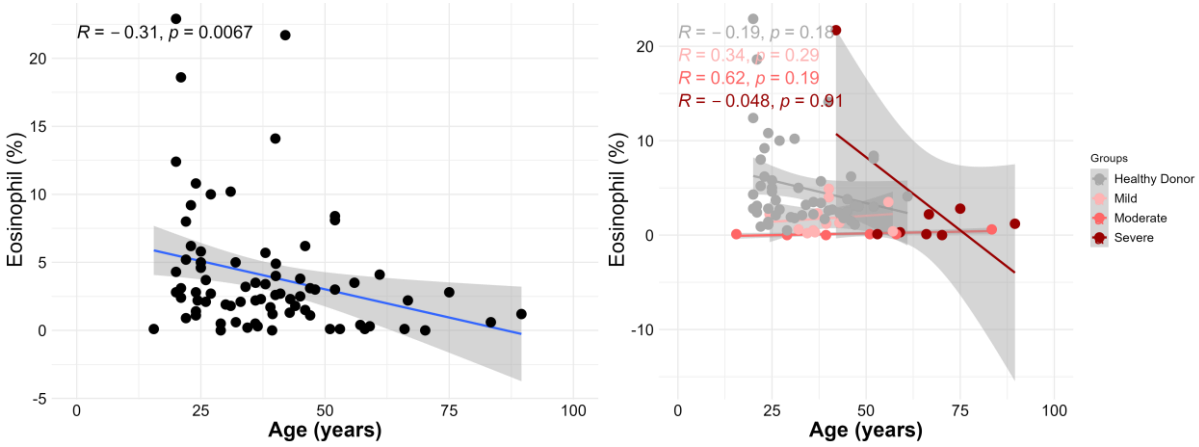


Fig S2: Correlation of age with significant parameters found in mixed model. Spearman correlation was made to age and AEC (A) and eosinophil percentage (B), group all groups together (left) and sorted by groups (right).

8.4. Resumos em congresso

ANALYSIS OF HEMATOLOGICAL PARAMETERS ON CONVALESCENT INDIVIDUALS OF COVID-19

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Introduction: SARS-CoV-2 is the agent responsible for both severe acute respiratory syndrome and COVID-19 pandemic situation. The transmission mechanism occurs by respiratory droplets, what contributes to increased incidence of disease in a curtain period of time. Hematological parameters have been described as prognostic factors to patients with COVID-19, however there is a lack over the use of hematological count to monitor COVID-19 patients into the convalescent stage. The main objective of this study was to describe hematological parameters under patients with COVID-19 diagnosis and convalescent individuals. **Methodology:** Whole blood collected in EDTA tube was obtained from 53 healthy donors (HD) as control group, 17 patients with diagnosis of COVID-19, but with no need for hospitalization (NH); and 139 convalescent individuals from COVID-19 included after 30 days of clinical recovery (CV). Hematological evaluation was performed at ADVIA2120i equipment from Fundação HEMOAM, in order to monitor parameters from red blood cells, leucocytes and platelets. Data was recorded in Microsoft Excel 2010, and statistical analysis was performed on GraphPad Prism v.5.0, with confidence interval of 95% and a significant p value when $p < 0.05$. **Results:** A lower mean value of MVC and RDW was observed in CV group, when compared to other groups, while there was an increased value on MCHC, when related to NH group. The leucocyte count was higher in CV group, when compared to HD, which seemed guided by neutrophil and monocyte count, although eosinophil count had a lower mean in NH group. The participation of neutrophils and monocytes have been reported as the main immune cells involved on COVID-19 acute response. **Conclusion:** Our data demonstrated influence of hematological parameter over COVID-19, especially leucocytes, which are directly involved in acute and chronic process of response against SARS-CoV-2. More studies are necessary to improve comprehension over the participation of blood count on patient's prognosis.

Keywords: Blood count; SARS-CoV-2; Pandemic.

LYMPHOCYTE PROFILE IN CONVALESCENT PATIENTS FROM SARS-COV-2 (COVID-19) INFECTION

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Introduction: Coronavírus is the causative agent of the pandemic of COVID-19. It is an airborne virus with a high capacity for infection and development of mild to severe symptoms. Lymphocytes are the main cells involved in the viral immune response, and although several studies describe lymphopenia as a condition of severe patients, the relationship of these cells in response of the convalescent individuals to COVID-19 is not strongly established. **Objective**: Describe the frequency of T helper (CD4+) and T cytotoxic (CD8+) lymphocytes in the immune response of convalescent individuals from COVID-19. **Methodology**: 50 convalescent individuals from COVID-19 were enrolled in this study, without symptoms for approximately 30 days prior to collection, of both genders, and without infectious diseases at the time of sample collection. 50 candidates suitable for blood donation were included as a control group. Whole blood was used for immunophenotyping of the lymphocyte population by flow cytometry with phenotype markers of CD3, CD4, CD8 and CD69. The samples were acquired in the FACS Canto II flow cytometer of the HEMOAM Foundation. The analysis was performed in FlowJo v. 10.6 software. For statistical analysis, the unpaired T-test was done, with confidence interval of 95% and significant p for $p<0.05$. **Results**: No significant difference was observed on sociodemographic data under groups. In the analysis of lymphocyte count, an increase in cytotoxic T cells and activated cytotoxic T cells was observed in the convalescent group when compared to the control group. **Conclusion**: Individuals healed from COVID-19 present increased cytotoxic T lymphocyte counts, even though 30 days after clinical recovery, suggesting activation of these cells, besides adverse effects related to tissue damage and inflammation pattern in convalescent patients. However, further studies evaluating other parameters of cellular immunity are needed to describe the relationship between clinical and immunological aspects of COVID-19.

Keywords: Inflammation; Respiratory syndrome; Brazilian Amazon.

ANTIBODY IgG PRODUCTION IN CONVALESCENT PATIENTS OF SARS-CoV-2 INFECTION IN MANAUS

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Introduction: COVID-19 is a viral disease caused by SARS-CoV-2 responsible for the pandemic stage since the beginning of 2020. Patients experience a respiratory syndrome that may evolve to death in a few weeks, marked by an inflammatory process, which includes the participation of immune cells as a key point to viral clearance, healing process and antibody production. However, there is still a lack over the antibody production among healed COVID-19 patients to maintain the protection against new infections after clinical recovery. **Objectives:** Monitor the level of IgG antibody against SARS-CoV-2 nucleocapsid protein over three months after infection. **Methods:** This is a longitudinal study, which included 138 convalescent COVID-19 donors, analyzed 30 days after clinical recovery, and with blood sample collection every month for a three-month period. Whole blood sample was collected in EDTA K2 to quantify automatic blood count, and separator gel tubes to measure anti-nucleocapside SARS CoV2 IgG levels by chemoluminescent assay. All data was recorded in Microsoft Excel 2010, to further statistical analysis in GraphPad Prism v. 5.0. **Results:** From all 138 individual enrolled, 60 completed all follow-up. Paired hematological parameters showed a significant reduction on red blood cells count, hematocrit and monocyte after one month of clinical recovery, while only monocyte had significant reduction over the three months period. Significant decrease was observed in mean hemoglobin concentration and an increase in hematocrit parameter later, on the second to the third month. Although only 49/60 (81,6%) convalescent donors were IgG+ by the time of inclusion, we could detect a rapid decline in antibody cut-off value with the 7/49 (14,3%) of the cases were classified as negative after 3 months of follow up. **Conclusion**: Anti-N IgG as measured by the Abbott test declines very rapidly and are not indicated to detect previous infected individuals. More studies are recommended to identify novel strategies related to diagnosis, and track of convalescent cases into a population.

Keyword: COVID-19; Adaptive immunity; Pandemic.

ANÁLISE DE FATORES SOCIODEMOGRÁFICOS PARA PRODUÇÃO DE ANTICORPOS IGG ANTI-SPIKE EM DOADORES DE PLASMA CONVALESCENTE NA CIDADE DE MANAUS

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Objetivos: A COVID-19 foi a doença que causou o quadro de pandemia, ocasionando diversos casos e óbitos ao longo do mundo. Uma estratégia para o tratamento de quadros graves da COVID-19 foi o uso de plasma convalescente, obtido de doadores convalescentes da doença, com alta produção de anticorpos, de forma a permitir a neutralização do vírus e melhora no quadro clínico. Embora muitos estudos destaquem o uso do plasma convalescente, poucos descrevem propostas para coletar plasma com concentração de anticorpos anti-Spike aceitáveis para o uso em indivíduos acometidos com a forma grave da COVID-19. Dessa forma, o objetivo deste estudo foi avaliar os fatores sociodemográficos ligados à produção de anticorpos IgG anti-Spike em candidatos à doação de plasma convalescente na cidade de Manaus-AM. **Material e Métodos:** Foram incluídos no estudo 123 candidatos à doação de plasma convalescente, do gênero masculino, que tiveram o diagnóstico da COVID-19 30 dias antes da doação, sem histórico de vacinação prévia. Foi realizada uma coleta de 4 mL do sangue periférico para obtenção do soro, utilizado para quantificação de anticorpos IgG anti-Spike por quimioluminescência, expressos em AU/mL, através do kit Architect SARS-CoV-2 IgG II Quant (Abbott). Os doadores foram segregados com base na concentração de anticorpos em baixa produção (< 50 AU/mL), produção intermediária (50-1,280 AU/mL) e alta produção (> 1,280 AU/mL), segundo informações do kit, e recomendação internacional. Os dados sociodemográficos de idade, gênero, cor de pele, índice de massa corporal (IMC), uso de ventilação mecânica e dias internados foram utilizados para fins de comparação entre os grupos. Foi realizado o teste de ANOVA, Qui-Quadrado e teste exato de Fisher para comparação dos dados sociodemográficos, além dos testes de correlação de Spearman. **Resultados:** Nossos dados demonstraram uma maior prevalência de doadores com alto índice de IMC na população de alto produtores de anticorpos ($p = 0.0254$). Além disso, também observamos que a concentração dos anticorpos IgG anti-Spike apresentou grau de correlação positiva com a idade ($r = 0.240$; $p = 0.008$) e IMC ($r = 0.247$; $p < 0.006$). **Discussão:** Nossos resultados demonstram uma relação entre a idade e o IMC na produção de anticorpos anti-Spike em doadores convalescentes com alta concentração de anticorpos. Essa relação pode estar atrelada à efeitos de imunomodulação, bem como ao grau de lesão tecidual e sistêmico apresentado pelos pacientes mais velhos e/ou com maior IMC. A relação desses fatores com a produção de anticorpos ainda é uma área pouco explorada, o que salienta a necessidade de compreender os mecanismos imunológicos associados à produção de anticorpos, junto com a proteção dos pacientes convalescentes. **Conclusão:** Nosso estudo demonstrou que o IMC dos acometidos pela COVID-19 pode contribuir para a alta produção de anticorpos de classe IgG anti-Spike. No entanto, estudos adicionais são necessários para compreender os aspectos imunológicos associados à maior proteção da população, bem como produção de anticorpos com maior eficácia pelos indivíduos acometidos pela COVID-19.

*AM e AGC contribuíram igualmente para este trabalho.

DINÂMICA DAS SUBPOPULAÇÕES DE MONÓCITOS NA IMUNIDADE CELULAR DE PACIENTES CONVALECENTES DA INFECÇÃO PELO VÍRUS SARS-COV-2 (COVID-19)

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Introdução: A COVID-19 tem sido uma doença de grande impacto para a saúde pública desde 2020. Sua causa se dá pela transmissão do SARS-CoV-2, um vírus altamente contagioso, que se desenvolveu rapidamente e evoluiu um quadro de pandemia. Sua veiculação pelo ar facilita a disseminação para muitos indivíduos e gera ampla variedade de sintomas, podendo ser classificados de leves a graves, além de terem alta taxa de mortalidade. Os monócitos foram descritos com uma função primordial no processo inflamatório e de retroalimentação positiva na resposta ao vírus, porém poucos estudos avaliam sua dinâmica na resposta convalescente da COVID-19. **Objetivo:** Descrever as subpopulações de monócitos circulantes na resposta imunológica de pacientes convalescentes da COVID-19. **Material e Métodos**: Foram incluídos 50 pacientes convalescentes, sem sintomas a 30 dias prévios à coleta, do sexo masculino, e sem outras doenças infecciosas. Também foram incluídos 50 candidatos aptos à doação de sangue. Foi utilizado sangue total para imunofenotipagem dos subtipos de monócitos, com base na expressão de CD14, CD16 e HLA-DR. A aquisição dos dados foi feita por citometria de fluxo, no citômetro FACS Canto II e a estratégia foi feita no software FlowJo v. 10.6. O soro foi coletado e empregado na detecção de anticorpos IgG anti-S e anti-N por quimioluminescência na Fundação HEMOAM. Para análise estatística, foi realizada a comparação dos grupos pelo teste de Kruskal-Wallis, enquanto a correlação foi realizada pelo teste de Spearman. Todas as análises foram feitas no software GraphPad Prism v. 6.0, considerando intervalo de confiança de 95% e p significativo para $p < 0.05$. **Resultados:** Não foi identificada diferença estatística quanto aos dados sociodemográficos. Já na quantificação dos monócitos totais, foi observado um aumento significativo dos monócitos ($p < 0,03$), além das subpopulações, onde foi observada uma redução dos monócitos clássicos ($p < 0,0001$), e aumento nos monócitos intermediários ($p < 0,0003$) e não clássicos ($p < 0,0001$) nos convalescente. A análise de correlação mostrou uma correlação negativa entre os monócitos não clássicos e a produção de anticorpo IgG apenas anti-S. **Discussão:** Nossos resultados demonstraram um aumento significativo de monócitos totais na fase convalescente, o que parece ser guiado pelos monócitos intermediários e não clássicos. Esses resultados corroboram com diversos outros estudos publicados, os quais podem estar atrelados à função reparadora devido ao dano causado durante a fase aguda da doença. **Conclusão:** Os dados deste estudo demonstraram uma dinâmica dos monócitos na fase convalescente, mesmo após a cura clínica, que pode estar envolvido no quadro de COVID longa. Porém, mais estudos são necessários a fim de compreender a função dos monócitos durante a fase ativa da doença, bem como no desenrolar da fase convalescente.

Palavras-chave: Resposta imune; Células mononucleares; Síndrome Respiratória Aguda Grave; Inflamação.

DENDRITIC CELLS ON ACUTE CELLULAR IMMUNITY AMONG INFECTED PATIENTS WITH SARS-COV-2: AN IMMUNE REVIEW

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Background: SARS-CoV-2 caused the 2020 pandemic in less than four months, and it is still responsible for many cases around the world. Due to higher rate of contamination, the incidence increased along time, together with death rates. Immune system plays a pivotal role on viral clearance, once is the major factor on recognition, processing, and elimination of pathogens. These events are mediated mainly by dendritic cells (DC), which are the main cells on adaptive immunity, through recognition and viral presentation to T and B lymphocytes. Yet, few studies evaluate the participation of DCs and its subtypes on COVID-19 disease activity, what highlights the need to increase comprehension of DCs on innate and adaptive immunity among those patients infected with SARS-CoV-2. **Methods:** This review was conducted on digital platforms of Pubmed and Scielo, with original papers, published between 2019 and 2022, written in English, with the following descriptors: "COVID-19", "immune response", "dendritic cells", "inflammation" and "adaptive immunity". All data was stored and used further to construct the narrative review. **Results**: On SARS-CoV-2 infection, plasmacytoid dendritic cells induce production of pro-inflammatory cytokines, such as TNF-α, IL-6 and CXCL8, what leads to cytokine storm, a key factor on severity of infected patients. During initial steps of infection, plasmacytoid DCs rapidly lose their ability to produce antiviral mediators due to intracellular mechanisms, which takes to low count, but also limits type I IFN, and contributes to viral persistence. The reduce on activation and maturation on DCs interfere on antigen presentation, culminating on a further low cellular response and worse patient's prognosis. Myeloid DCs express low HLA-DR on membrane, but there is few or any functional changes. **Conclusion:** Plasmacytoid DCs plays an important role on acute inflammation, and we strongly believe there is a pivotal participation on cytokine storm and subsequently on severity stage, however few studies support higher conclusions over DCs as leaders on inflammatory condition experienced by severe patients. Instead, we must consider that more studies are recommended to improve the knowledge over cellular immunity available, and maybe suggest a biomarker for prognosis on future cases.

Keywords: COVID-19; Immune response; Inflammation; Adaptive immunity.

Financial Support: FAPEAM, CAPES, CNPQ, HEMOAM DIAGNÓSTICOS, FUNDAÇÃO SANGUE NATIVO, FUNDAÇÃO HEMOAM.

CYTOKINE STORM RELATED TO DEATH IN COVID-19 SEVERE PATIENTS WHO REQUIRED HOSPITAL ADMISSION IN MANAUS-AM, BRAZIL

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Background: SARS-CoV-2 caused the pandemic in 2020, and worsened in 2021, with developing of new variants and many cases worldwide. The absence of effective protection against the virus, attached to the naive characteristic of public health for respiratory syndrome management culminated in a higher number of cases and deaths. Many studies determined the immune key points for disease severity, however, only a few comprehend the mechanisms related to death after hospital admission. **Methods:** In this study, we enrolled 51 healthy donors without SARS-CoV-2 infection history, 12 patients with mild disease and 15 patients who required hospital admission in Manaus-AM, Brazil. These were divided into 10 who had hospital discharge and 5 who involved to death. Whole blood was obtained and used for all methods. Cell profile was characterized by flow cytometry, while 27 soluble molecules were measured by Luminex. From data obtained, a Kruskal-Wallis and Dunn`s test was performed, using a confidence interval of 95%. **Results:** Those patients who required hospital admission were marked by low NK and NKT cells, together to a high intermediary and non-classical monocyte count. Cytotoxicity was seen and supported by inflammatory and regulatory markers, mediated mainly by IFN-γ levels, CXCL10 and CXCL8. When we analyzed the severity subgroups, a similar pattern was seen, however, B1 lymphocyte were higher on those who involved to death. **Conclusion:** Inflammation has a significant impact in severity of COVID-19, however, lower on NK, NKT and monocyte subtype cells in severe patients, together to analysis of B1 lymphocytes can have a pivotal role in disease biomarker and prediction of death under in-hospital patients. The mechanisms related to these observations must be analyzed, and a higher cohort must be performed to validate these parameters as possible biomarkers for clinical prediction.

Keywords: SARS-CoV-2; Biomarkers; Brazilian Amazon; Innate immunity.

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10. ANEXOS

10.1. Parecer consubstanciado do Comitê de Ética em Pesquisa da Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas (CEP-HEMOAM)

10.1.1. Parecer do projeto "Estudo de Biomarcadores Imunológicos em pacientes Convalescentes da Infecção pelo Vírus SARS-CoV-2 (COVID-19)"

FUNDAÇÃO DE HEMATOLOGIA E HEMOTERAPIA DO

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Estudo de Biomarcadores Imunológicos em pacientes Convalescentes da infecção pelo Vírus SARS-CoV-2 (COVID-19).

Pesquisador: Adriana Malheiro

Área Temática:

Versão: 2

CAAE: 30975020.9.0000.0009

Instituição Proponente: Fundação de Hematologia e Hemoterapia do Amazonas - HEMOAM

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 4.126.784

Apresentação do Projeto:

Este estudo será composto por três grupos. O grupo 1, pacientes convalescentes candidatos à doação de plasma, o G2 doadores de sangue saudáveis (grupo controle) e G3 pacientes internados em enfermaria ou UTI dos hospitais que atendem pacientes com Covid-19 e que aceitarem participar da pesquisa.

Os pacientes convalescentes serão incluídos por demanda espontânea à medida que forem recrutados pelo Hemoam para doação de plasma convalescente. Estima-se estudar 200 pacientes. O Hemoam, através da Secretaria de Saúde e da Fundação de Vigilância em Saúde, terá acesso à lista de pacientes convalescentes, de acordo com o projeto " aprovado pelo CEP". Estes candidatos à doação de plasma serão contactados pelo serviço social do Hemoam. Pacientes que voluntariamente procurarem a equipe do projeto ou o Hemoam também poderão ser incluídos no projeto. Para o grupo controle também serão inseridos 200 doadores de sangue saudável, que serão abordados pela equipe do projeto no momento da doação. Para o G3, serão incluídos pacientes internados em enfermarias ou UTIs de Covid-19, do HUGV que anuiu sua participação ao projeto. Estes pacientes serão contactados pela equipe de enfermagem/residentes/equipe médica (médica responsável Dra Alena Mileo), do hospital para aplicação do TCLE, para o paciente ou seus familiares. Estimamos estudar neste grupo 200 pacientes, que serão incluídos por demanda espontânea, no período de julho a dezembro de 2020.

O presente projeto visa avaliar o perfil de resposta imune na infecção pelo SARS-COVI-2,

10.1.2. Termos de Consentimento (TCLE) aplicado

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO –
DOADOR DE PLASMA CONVALESCENTE – Grupo 1
CAAE: 30975020.9.0000.0009

Centro de Pesquisa: Universidade Federal do Amazonas (UFAM)/ Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas (HEMOAM).
Projeto: Estudo de Biomarcadores Imunológicos em pacientes Convalescentes da infecção pelo Vírus SARS-CoV-2 (COVID-19).
Pesquisador Principal: Dra. Adriana Malheiro Alle Marie
Telefone de contato: (92) 99114-9478

Convidamos o Sr. a participar da pesquisa sobre o estudo de biomarcadores imunológicos (proteínas presentes no sangue) em pacientes que se recuperaram da infecção pelo Coronavírus. O objetivo desta pesquisa é descrever potenciais biomarcadores imunológicos e inflamatórios em Doadores de Plasma obtidos de pacientes que se recuperaram da infecção pelo Vírus SARS-CoV-2 (COVID-19) e associar com a cura e produção de anticorpos nestes indivíduos.

A sua participação é voluntária e se não concordarem participar ou alterar a sua decisão e retirar o consentimento a qualquer momento do estudo, o seu tratamento não será prejudicado. Será possível fazer todas as perguntas que quiser em qualquer momento do estudo. Você tem todo o tempo que quiser para decidir e discutir a situação com pessoas de sua confiança.

Para sua participação há necessidade do preenchimento do questionário de rotina para doação de amostras de sangue (equivalente a duas colheres cheias), verificação de peso e pressão arterial. Além disso, somente serão aceitos indivíduos que possuam resultado de PCR positivo para Coronavírus ou teste rápido IgM/IgG positivos, que estejam assintomáticos por pelo menos 14 dias antes da coleta. Que não tenha recebido transfusão nos últimos 12 meses. Que durante a infecção, apresentaram sintomas clínicos leves ou com necessidade de internação. Que estejam aptos nas triagens clínica, hematológica e laboratoriais de rotina para doação de sangue.

O senhor será entrevistado por um médico/enfermeiro para registro de informações sobre o tempo e sinais clínicos apresentados durante a infecção. Se o senhor estiver apto nas triagens anteriores, será coletada uma amostra de seu sangue para a verificação da presença de anticorpos que protegem contra o Coronavírus e ainda uma amostra do interior de suas narinas e garganta para verificar se o seu organismo eliminou completamente o Coronavírus. Se o senhor estiver apto nas triagens acima, serão realizados os exames de outras doenças como Hepatite B, Hepatite C, HIV, Sífilis, Chagas e tipagem sanguínea. Com a aptidão nestes exames, será agendada uma coleta de sangue para o estudo e posteriormente, será realizado a coleta de mais duas amostras de seu sangue, 60 e 90 dias após a primeira coleta de amostra para verificar a concentração de anticorpos contra o Coronavírus e das proteínas presentes no seu corpo.

Você não terá qualquer custo ou qualquer forma de pagamento/remuneração por sua participação neste estudo. Solicitamos sua autorização para armazenarmos uma amostra de seu sangue e plasma para uma nova testagem se for necessário e outros exames que não estejam previstos aqui, mas que sejam necessários para o estudo. O sangue coletado ficará armazenado no biorrepositório da Fundação HEMOAM para futuras análises e avaliações ligadas ao projeto. No caso de amostra insuficiente, quebra do tubo, hemólise, o senhor será avisado para que retorne ao HEMOAM para realizar nova coleta de sangue para a realização dos exames.

Se você sofrer algum dano ou intercorrência, deverá falar para alguém da equipe da pesquisa imediatamente ou em caso de emergência, poderá contatar diretamente Dra. Adriana Malheiro Alle Marie, pesquisadora do estudo, através do telefone (92) 99114-9478. Nós lhe daremos toda assistência integral e gratuita e que for necessária para garantir seu bem-estar. Os membros da equipe do projeto de pesquisa irão garantir que você receba assistência imediata caso tenha quaisquer tipos de danos, diretos ou indiretos, imediatos ou tardios, sofridos no decorrer de sua participação na pesquisa, previsto ou não neste documento.

Os riscos de participação neste projeto estão relacionados ao desconforto da punção da veia para coleta das amostras. Durante a coleta de sangue o senhor poderá sentir tremores e dormências nos lábios, desconforto no local da coleta e uma mancha vermelha poderá aparecer no local, mas é uma reação normal a coleta de amostras.

Os benefícios de sua participação estão direcionados ao auxílio no entendimento da doença (COVID-19) e o que ela produz no corpo dos pacientes, além de ser possível identificar proteínas que possam demonstrar os pacientes que irão produzir uma resposta eficiente do organismo ao vírus. Essa pesquisa não trará nenhum tipo de benefício direto ou remuneração de nenhuma espécie para os participantes deste projeto, mas irão ajudar a entender a doença e os pacientes que forem infectados no futuro.

Os exames e procedimentos realizados exclusivamente com fins de pesquisa não implicarão qualquer custo para você. Os acompanhamentos médicos após a doação serão realizados por telefone; se necessário, você receberá um ressarcimento pelos gastos relacionados à sua participação no estudo. Apesar de não haver compensação financeira, ao assinar este termo de consentimento você não renunciará a nenhum dos seus direitos legais, garantidos na regulamentação brasileira de pesquisa envolvendo seres humanos, sendo um de seus direitos a solicitação de indenização.

Você pode ser excluído do estudo se os pesquisadores considerarem oportuno ou se você solicitar, tanto por razões de segurança, por qualquer desconforto que ocorra ou por considerar que você não está cumprindo os procedimentos solicitados pela equipe do estudo. Em todos os casos, você receberá uma explicação adequada do motivo que ocasionou a sua retirada do estudo. Além disso, o estudo também pode ser cancelado por razões administrativas. Ao assinar este Termo de Consentimento Livre e Esclarecido, você se compromete a cumprir com os procedimentos do estudo que foram explicados.

Para obter mais informações sobre este estudo ou para qualquer informação adicional sobre os seus direitos como participante, ou se você quiser fazer uma reclamação, por favor, entre em contato com o pesquisador responsável do estudo: Dra. Adriana Malheiro Alle Marie, no endereço Av. Constantino Nery, 4397, Bloco E, 1º andar, Chapada, Manaus - AM; telefone (92) 99114-9478 ; e-mail malheiroadriana@yahoo.com.br.

Em caso de dúvidas, denúncias ou reclamações sobre a sua participação e sobre questões éticas do estudo, você pode entrar em contato com o Comitê de Ética em Pesquisas (CEP) da Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas (HEMOAM); e-mail: cephemoam@gmail.com; endereço: na Av. Constantino Nery, 4397-Chapada, Bloco A, 2° andar, Sala 13 (CEP-HEMOAM), Chapada, Manaus - AM;

telefone: (92) 3655-0114. O horário de funcionamento do CEP é de 8 às 14 horas, de segunda à sexta-feira.

Eu li este documento ou ele foi lido para mim, fico ciente e concordo em fazer a doação.

Eu (nome completo), _____

- Tive a oportunidade de tirar dúvidas sobre o estudo.
- Recebi informações suficientes sobre o estudo.
- Serei informado regularmente sobre qualquer informação que possa afetar a minha disponibilidade em continuar a minha participação no estudo.
- Autorizo o acesso às minhas informações confidenciais.
- Autorizo o processamento de minhas informações e amostras.
- Entendo que receberei uma via deste termo.
- Entendo que a minha participação é voluntária.
- Entendo que posso retirar o meu consentimento e concluir a minha participação:
    1. Em qualquer momento
    2. Sem dar qualquer explicação
    3. Sem que prejudique meu tratamento posterior

Ofereço livremente o meu acordo para participar deste estudo e dou o meu consentimento para acessar e usar os meus dados nas condições descritas neste Termo de Consentimento Livre e Esclarecido.

( ) SIM ( ) NÃO

Concordo que as amostras serão armazenadas para um biorrepositório e autorizo contato, caso necessário, para futuros estudos que possam ocorrer, aprovados pelo CEP e, quando necessário, pela CONEP.

( ) SIM ( ) NÃO

_____ _____, ____/___/_____.

Assinatura do Doador Local

_____ _____, ____/___/_____.

Assinatura da Testemunha Local

_____ _____, ____/___/_____.

Assinatura do Pesquisador / Local

Equipe da Pesquisa

10.2. Valores de sensibilidade e especificidade do teste imunocromatográfico

> 14 dias do início dos sintomas		RT-PCR		Total
		Reagente	Não reagente	
COVID-19 IgG/IgM ECO Teste	Reagente	95	0	95
	Não reagente	3	0	3
Total		98	0	98

Resultado de Sensibilidade combinada (IgG + IgM) do COVID-19 IgG/IgM ECO Teste em amostras coletadas entre 7 e 14 dias do início dos sintomas.
Sensibilidade (IgG + IgM): 96,94%.

10.3. Valores de concordância positiva do teste CMIA qualitativo

Dias após o início dos sintomas	n	Positivas	Negativas	CPP (IC 95%)
< 3	4	0	4	0.00% (0.00, 60.24)
3-7	8	2	6	25.00% (3.19, 65.09)
8-13	22	19	3	86.36% (65.09, 97.09)
≥ 14	88	88	0	100.00% (95.89, 100.00)

Concordância Positiva por dia após o início dos sintomas.

10.4. Valores de concordância positiva do teste CMIA qualitativo e quantitativo

Dias após o início dos sintomas	n	Positivas	Negativas	CPP (IC 95%)
$\leq$ 7	118	61	57	51.69% (42.77, 60.51)
8-14	163	142	21	87.12% (81.11, 91.42)
$\geq$ 15	158	157	1	99.37% (96.50, 99.97)

Concordância Percentual Positiva por Dias Após o Início dos Sintomas.

Dias após PCR positivo	n	Positivas	Negativas	CPP (IC 95%)
$\leq$ 7	220	146	74	66.36% (59.89, 72.28)
8-14	135	131	4	97.04% (92.63, 98.84)
$\geq$ 15	84	83	1	98.81% (93.56, 99.94)

Concordância Percentual Positiva por Dia Após Resultado Positivo e PCR.

10.5. Orientações de alunos de Iniciação Científica e em Trabalhos de Conclusão de Curso (TCC)

CERTIFICADO

Certificamos que **Alexander Leonardo Silva Junior** orientou o projeto de pesquisa descrito desenvolvido no âmbito do **Programa de Apoio à Iniciação Científica (PAIC)** da **Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas (HEMOAM)**, no período de Agosto de 2022 a Julho de 2023, com carga horária de 720 horas.

Bolsista: Henayara Kelly Trindade de Souza
Título do Projeto: Perfil de células dendríticas na imunidade celular de pacientes convalescentes da COVID-19

Tatiane Amabile de Lima
Coordenadora PAIC/HEMOAM

Manaus-AM, 14 de setembro de 2023

paic HEMOAM FAPEAM Secretaria de Desenvolvimento Econômico, Ciência, Tecnologia e Inovação AMAZONAS

Registro sob nº 094/2023, Livro digital, Departamento de Ensino e Pesquisa, Gerência de Pesquisa, Coordenação do PAIC/HEMOAM (+55 92 3655-0291).

UNINORTE

CERTIFICADO

Declaramos para os devidos fins, que

Prof. MSc. Alexander Leonardo Silva Junior

Atuou como orientador de Projeto de Iniciação Científica, intitulado Analise dos Fatores Envolvidos no Incentivo à Doação de Sangue em Estudantes do Ensino Superior, realizado no período de 08 de Fevereiro de 2021 a 29 de Dezembro de 2022. Tendo como orientando o aluno Jonatas Alencar Castro Campelo

Jefferson R. G. de Lemos
Coordenador do Curso de Biomedicina


UNINORTE

CERTIFICADO

Declaramos para os devidos fins, que

Prof. MSc. Alexander Leonardo Silva Junior

Atuou como orientador de Projeto de Iniciação Científica, intitulado Rastreamento e Identificação de Hotspots Transfusionais no Estado do Amazonas, realizado no período de 08 de Fevereiro de 2022 a 29 de Dezembro de 2023. Tendo como orientando o aluno Jonatas Alencar Castro Campelo

Jefferson R. G. de Lemos
Coordenador do Curso de Biomedicina

TÍTULO DO TRABALHO DE CONCLUSÃO DE CURSO DO CENTRO UNIVERSITÁRIO UNINORTE	**DISCENTE**	**CURSO**
PERFIL DA COBERTURA VACINAL CONTRA O HPV EM JOVENS E CRIANÇAS EM IDADE ESCOLAR NA AMAZÔNIA BRASILEIRA	LAÍS BRAGA DO ESPÍRITO SANTO	BIOMEDICINA
	MARCOS DÁCIO DE ARAÚJO	
ANEMIA FALCIFORME: FERRAMENTAS DE DIAGNÓSTICO E TRATAMENTO	EMILY RAYANE BENTES	BIOMEDICINA
	JESSYCA CAROLINE SOUZA LASMAR	
	VANESSA DOS SANTOS MOTA	
PROTOCOLO FOLFOX NO TRATAMENTO DE PACIENTES COM CÂNCER COLORRETAL EM ESTÁGIO III	ELLEN CAROLINE DA SILVA COSTA	FARMÁCIA
	THATIANE REBECCA SILVA GONÇALVES	
	YASMIM NASCIMENTO DE PAULA	
QUIMERISMO: MECANISMOS ENVOLVIDOS NO PÓS TRANSPLANTE DE MEDULA OSSEA – REVISÃO SISTEMÁTICA	JULIANNA CRISTINA AYRES DA SILVA	BIOMEDICINA
	JÉSSICA FERNANDA DE ALMEIDA	
	PRISCILA RETROZ MAGALHÃES	
RASTREAMENTO DA DOENÇA FALCIFORME ATRAVES DO TESTE DO PEZINHO NO BRASIL	DAIANE DA COSTA FERREIRA	BIOMEDICINA
	LEANE FERREIRA CAETANO	
	GABRIELE DOS SANTOS	
FERRAMENTAS DE DIAGNÓSTICO DA LEUCEMIA LINFOIDE AGUDA	DANIEL JACQUIMINOUTH RODRIGUES	BIOMEDICINA
	JACQUELINE PINHEIRO DA SILVA	
	LIDIANE COSTA BANDEIRA	
A EVOLUÇÃO NO TRANSPLANTE DE MEDULA ÓSSEA	NÚBIA BEATRIZ DE LIMA CLAUDIO	BIOMEDICINA

	NAYANDRA DA SILVA LOPES	
	PABLO LUCAS LOPES DE ARAÚJO	
GÊNERO E HPV: PERFIL DE SUSCETIBILIDADE AO DESENVOLVIMENTO DE CÂNCER PENIANO OU DE COLO UTERINO	AMANDA LIMA SEIXAS	BIOMEDICINA
	JUCILANE PINHEIRO DOS SANTOS	
	SILAS PRATA E SILVA	
ATUAÇÃO DOS LINFÓCITOS NA INFECÇÃO AGUDA E CRÔNICA DO VÍRUS DA IMUNODEFICIÊNCIA HUMANA (HIV)	JÓ DA SILVA VEIGA	BIOMEDICINA
	RAIANA THAÍS APARAÍ RIBEIRO	
REAÇÃO EM CADEIA DA POLIMERASE NO ÂMBITO DA GENÉTICA FORENSE: PARADÍGMA CIÊNTÍFICO AO LABORATÓRIO DE BIOLOGIA E GENÉTICA DO AMAZONAS	ANDERSON DA COSTA RODRIGUES	BIOMEDICINA
	IVANI CUNHA NOGUEIRA	
	YANARA BIANCA DA SILVA E SILVA	
RASTREIO DAS LESÕES PRECURSORAS DO CÂNCER DE COLO DE ÚTERO, VANTAGENS DOS MÉTODOS AUXILIARES	FABÍOLA RAMALHO FERREIRA DE SOUZA	FARMÁCIA
	BÁRBARA JANAÍNA PAULA DA SILVA	
LÚPUS ERITEMATOSO SISTÊMICO: CARACTERÍSTICAS CLÍNICAS EM MULHERES	AMANDA ARAUJO AMARAL	BIOMEDICINA
	ANGÉLICA ISABELLA RENDON RODRIGUEZ	
O USO TERAPÊUTICO DA ISOTRETINOÍNA ORAL EM PACIENTES COM ACNE VULGAR E SEUS EFEITOS ADVERSOS: UMA REVISÃO BIBLIOGRÁFICA	BEATRIZ VIEIRA CONDE	BIOMEDICINA
A BIOQUÍMICA CLÍNICA NO ACOMPANHAMENTO DA LESÃO HEPÁTICA EM PACIENTES COM HEPATITE A	MARJORIE PAULA MELO DA CRUZ	BIOMEDICINA
PERFIL DE LINFÓCITOS NA IMUNIDADE CELULAR DE PACIENTES CONVALESCENTES DA INFECÇÃO POR SARS-COV-2 (COVID-19)	LUCAS DA SILVA OLIVEIRA	BIOMEDICINA
SUBPOPULAÇÃO DE MONÓCITOS NA IMUNIDADE CELULAR DE PACIENTES CONVALECENTES DA INFECÇÃO PELO VÍRUS SARS-COV-2 (COVID-19)	NARA CAROLINE TOLEDO BELEZIA	BIOMEDICINA
O PAPEL DA BIOMEDICINA NO DIAGNÓSTICO E ACONSELHAMENTO GENÉTICO NOS CASOS DE ANEMIA FALCIFORME - REVISÃO LITERÁRIA	INESSA DA SILVA BENITÁH	BIOMEDICINA
	PATRICIA GISELLE ALMEIDA DE ALCANTARA	
PROTOCOLOS DE ELETROTERMOFOTOTERAPIA E EXERCÍCIOS TERAPÊUTICOS NA MELHORA DOS SINAIS E SINTOMAS EM INDIVÍDUOS COM DISFUNÇÃO TEMPOROMANDIBULAR: REVISÃO DE LITERATURA	NATHALIA CRISTINA ARRUDA CIDADE	FISIOTERAPIA
O DNA NA BUSCA DE PESSOAS DESAPARECIDAS: UMA REVISÃO DE LITERATURA	YASMIM CAROLINA DE SOUZAQUEIROZ	BIOMEDICINA
	HELIO LUCAS ASSUNCAO DA SILVA SOUZA	

PARTICIPAÇÃO DE NEUTRÓFILOS NA PATOGÊNESE DA ANEMIA FALCIFORME	HERSHILEY OLIVEIRA	BIOMEDICINA
RASTREIO DE INTERRUPÇÕES DE CONCENTRADO DE HEMÁCIAS NO AMAZONAS DE 2015 A 2019	JÔNATAS ALENCAR CASTRO CAMPELO	FARMÁCIA

10.6. Produção científica advinda da participação de outros projetos

10.6.1. Artigos em colaboração

eLife

RESEARCH ARTICLE

SARS-CoV-2 antibody dynamics in blood donors and COVID-19 epidemiology in eight Brazilian state capitals: A serial cross-sectional study

Carlos A Prete Jr[1*†], Lewis F Buss[2†], Charles Whittaker[3,4†], Tassila Salomon[5†], Marcio K Oikawa[6], Rafael HM Pereira[7], Isabel CG Moura[5], Lucas Delerino[8], Manoel Barral-Netto[8], Natalia M Tavares[8], Rafael FO Franca[8], Viviane S Boaventura[8], Fabio Miyajima[8,9], Alfredo Mendrone-Junior[10], Cesar de Almeida-Neto[10], Nanci A Salles[10], Suzete C Ferreira[10], Karine A Fladzinski[11], Luana M de Souza[11], Luciane K Schier[11], Patricia M Inoue[11], Lilyane A Xabregas[12], Myuki AE Crispim[12], Nelson Fraiji[12], Fernando LV Araujo[13], Luciana MB Carlos[14], Veridiana Pessoa[14], Maisa A Ribeiro[15], Rosenvaldo E de Souza[15], Sônia MN da Silva[15], Anna F Cavalcante[16], Maria IB Valença[16], Maria V da Silva[16], Esther Lopes[17], Luiz A Filho[17], Sheila OG Mateos[17], Gabrielle T Nunes[8,17], Alexander L Silva-Junior[12,18,19], Michael P Busch[20,21], Marcia C Castro[22], Christopher Dye[23], Oliver Ratmann[2], Nuno R Faria[3,4,23,24*], Vítor H Nascimento[1*], Ester C Sabino[24*]

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Competing interest: The authors declare that no competing interests exist.

Funding: See page 19

Preprinted: 22 February 2022
Received: 28 February 2022
Accepted: 17 September 2022
Published: 22 September 2022

Reviewing Editor: James M McCaw, The University of Melbourne, Australia

vaccines

MDPI

Article

Predicting SARS-CoV-2 Variant Spread in a Completely Seropositive Population Using Semi-Quantitative Antibody Measurements in Blood Donors

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Citation: Buss, L.; Prete, C.A., Jr.; Whittaker, C.; Salomon, T.; Oikawa, M.K.; Pereira, R.H.M.; Moura, I.C.G.; Delerino, L.; Franca, R.F.O.; Miyajima, F.; et al. Predicting SARS-CoV-2 Variant Spread in a Completely Seropositive Population Using Semi-Quantitative Antibody Measurements in Blood Donors. *Vaccines* **2022**, *10*, 1437. https://doi.org/10.3390/vaccines10091437

Academic Editors: Claudia Maria Trombetta, Emanuele Montomoli and Serena Marchi

Received: 18 July 2022
Accepted: 24 August 2022
Published: 31 August 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Abstract: SARS-CoV-2 serologic surveys estimate the proportion of the population with antibodies against historical variants, which nears 100% in many settings. New approaches are required to fully exploit serosurvey data. Using a SARS-CoV-2 anti-Spike (S) protein chemiluminescent microparticle assay, we attained a semi-quantitative measurement of population IgG titers in serial cross-sectional monthly samples of blood donations across seven Brazilian state capitals (March 2021–November 2021). Using an ecological analysis, we assessed the contributions of prior attack rate and vaccination to antibody titer. We compared anti-S titer across the seven cities during the growth phase of the Delta variant and used this to predict the resulting age-standardized incidence of severe COVID-19 cases. We tested ~780 samples per month, per location. Seroprevalence rose to >95% across all seven capitals by November 2021. Driven by vaccination, mean antibody titer increased 16-fold over the study, with the greatest increases occurring in cities with the highest prior attack rates. Mean anti-S IgG was strongly correlated (adjusted R2 = 0.89) with the number of severe cases caused by

https://www.periodicos.ufam.edu.br/index.php/revistahugv Rev HUGV (Manaus). 2023 dez-jan; v22. 12958

Revisão de Literatura

Uso terapêutico do plasma rico em plaquetas na prevenção de cicatrizes inestéticas em animais e humanos

Therapeutic use of platelet rich plasma in prevention of unaesthetic scars in animals and humans

Lima RAM[1], Belezia NCT[2], Malheiro A[3], Silva-Junior AL[4].

Lima RAM, Belezia NCT, Malheiro A, Silva-Junior AL. Uso terapêutico do plasma rico em plaquetas na prevenção de cicatrizes inestéticas em animais e humanos. *Therapeutic use of platelet rich plasma in prevention of unaesthetic scars in animals and humans*. Rev HUGV (Manaus). 2023 dez-jan; v22. 12958. DOI:10.60104/revhugv12958

RESUMO

O desenvolvimento de lesões na pele pode causar a presença de cicatrizes, às quais estão associadas a problemas estéticos, principalmente em mulheres. O uso do Plasma Rico em Plaqueta (PRP) no pós-cirurgico mostrou melhor grau de cicatrização, com menores marcas e desconfortos, devido à produção e mediadores anti-inflamatórios e melhor reparo tecidual. Desta forma, nosso objetivo é realizar um levantamento dos resultados obtidos por estudos publicados quanto a eficácia do uso terapêutico do PRP na cicatrização de feridas em animais e humanos. Com isso, foi realizada uma revisão da literatura, com artigos das plataformas digitais SciElo, PubMed, Google Schoolar, de 2000 a 2023, que utilizou seres humanos ou animais, que fizeram o uso de PRP e com uma descrição da eficácia do procedimento. Identificamos que 16/94 artigos encontrados atenderam aos critérios, onde além dos humanos, foram utilizadas pesquisas em coelhos, roedores, cães e equinos. O PRP foi preparado de formas heteróloga, homóloga e autóloga, e empregado em lesões com atraso na cicatrização, queimados, como coadjuvante em enxertia, em tecido cutâneo com processo acelerado de envelhecimento e em cicatrizes de pós-operatório de cirurgia plástica. Independente da administração, foi observado que seu efeito reparador é maior nas primeiras fases da cicatrização. Com isso, sugerimos que o PRP é um bom recurso terapêutico durante a cicatrização, e embora ainda não se tenha um protocolo estabelecido para coleta e preparação, apresenta resultados promissores no pós-operatório cirurgico. Recomendamos que mais estudos sejam realizados, e um protocolo seja padronizado para obtenção e uso do PRP de forma segura e confiável.

Palavras-chave: Lesão tecidual; reparação tecidual; fator de crescimento; tratamento reparativo.

ABSTRACT

The development of skin lesions can cause the presence of scars, which are associated to aesthetic issues, especially in women. The use of Platelet-Rich Plasma (PRP) in post-surgery showed a better degree of healing, with less marks and discomfort due to the production of anti-inflammatory mediators and better tissue repair. Our objective is to promote a survey of the results obtained by published studies regarding the effectiveness of the therapeutic use of PRP in the healing of wounds in animals and humans. To achieve our aim, we conducted a review, with articles from digital platforms, from 2000 and 2023, which used people or animals, who used PRP and who had a description of the effectiveness of the procedure. There were identified 16/94 articles that met the criteria, where in addition to humans, research also used rabbits, rodents, dogs, and horses. PRP was prepared in heterologous, homologous, and autologous ways, and used in lesions with delayed healing, burns, as an adjuvant in grafting, in cutaneous tissue with an accelerated aging process and in postoperative scars from plastic surgery. Regardless the administration, all studies agreed that its repairing effect is greater in the early stages of healing. With this, we highlight that PRP can be a good therapeutic resource in healing complications, and although there is still no established protocol for collection and prepare, it presents promising results in the surgical postoperative period. However, we suggest that more studies must be carried out, as well as a protocol be standardized to obtain and use PRP in a safe and reliable way.

Keywords: *Tissue lesion; tissue repair; growth factor; reparative treatment.*

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Acta Brasiliensis 7(2): 33-40, 2023
ISSN 2526-4338

Original Article
http://dx.doi.org/10.22571/2526-4338639

In vitro evaluation of immunomodulatory activity of the copper(I) complex and *Libidibia ferrea* in cutaneous leishmaniasis

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Received: December 14, 2022 / Accepted: November 28, 2023 / Published online: December 29, 2023

Abstract

Leishmaniasis is considered by the World Health Organization (WHO) to be one of the most neglected tropical diseases in the world. The host's immune response is crucial for parasite elimination and, although the Th1 profile is associated with control of infections, if not modulated, it can cause tissue damage. The treatment of cutaneous leishmaniasis (CL) is still a challenge because it is not adapted to the context of the patients, and is long, toxic and invasive. Thus, this study aimed to evaluate the lymphoproliferation and dosage of cytokines induced by bioactive compounds of plant and inorganic origin, with antileishmanial action, through peripheral blood mononuclear cells (PBMCs) of patients with CL. Lymphoproliferation was evaluated against the stimuli from the methanolic extract and fraction of Libidibia ferrea, copper complex and phytohemagglutinin using a BrdU cell proliferation ELISA kit after 72 hours of incubation. The dosage of IL-6, IL-8 and IL-1β was determined using a BD™ cytometric bead array (CBA) human Th1/Th2/Th17 cytokine kit. Our results indicate that the bioactive substances significantly stimulated in vitro lymphoproliferation of PBMCs (Cu(I) $p<0.000$; LFME $p<0.02$) and patients showed higher levels of IL-6 and IL-8 before treatment. It is therefore suggested that these bioactive compounds can enhance the cellular immune response.

Keywords: Cutaneous leishmaniasis; *Libidibia ferrea*; Copper complex; Therapeutic alternatives.

Avaliação in vitro da atividade imunomoduladora do complexo de cobre(I) e *Libidibia ferrea* na leishmaniose cutânea

Resumo

As leishmanioses são consideradas pela Organização Mundial de Saúde (OMS) uma das doenças tropicais mais negligenciadas do mundo. A resposta imunológica do hospedeiro é determinante para eliminação do parasito e apesar do perfil Th1 estar associado ao controle da infecção, se não modulado, pode ocasionar danos teciduais. O tratamento da leishmaniose cutânea (LC) ainda é um desafio pois não é adaptado ao contexto dos pacientes, são longos, tóxicos e invasivos. Desse modo, este estudo teve como objetivo avaliar a linfoproliferação e dosagem de citocinas induzidas por bioativos de origem vegetal e inorgânica, com ação antileishmania, através de células mononucleares do sangue periférico (PBMC) de pacientes com LC. A linfoproliferação foi avaliada frente a estímulos do extrato metanólico e Fração de Libidibia ferrea, complexo de cobre e Phytohemagglutinin utilizando BrdU Cell Proliferation ELISA Kit após 72h de incubação. A dosagem das citocinas IL-6, IL-8 e IL-1β foi determinada por BD™ Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Cytokine Kit. Nossos resultados indicam que os bioativos estimularam significativamente a linfoproliferação in vitro de PBMC (Cu(I) $p<0,000$; LFME $p<0,02$) e pacientes apresentaram maiores níveis de IL-6 e IL-8 antes do tratamento. Sugere-se então que estes bioativos podem potencializar a resposta imune celular.

Palavras-chave: Leishmaniose tegumentar; *Libidibia ferrea;* Complexo de cobre; Alternativa terapêutica.

BRAZ J INFECT DIS. 2024;28(4):103848

The Brazilian Journal of
INFECTIOUS DISEASES

www.elsevier.com/locate/bjid

Brief Communication

HIV acute infection and long-term undisclosed HIV status among blood donors from the highly endemic Amazonas state, located in the Brazilian Amazon

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ARTICLE INFO

Article history:
Received 17 January 2024
Accepted 30 June 2024
Available online 17 July 2024

Keywords:
HIV blood donors
HIV incidence
Brazilian Amazon

ABSTRACT

Background: The Amazonas state/AM and Manaus rank among the highest AIDS detection rates in Brazil. High proportion of HIV infected blood donors and transmission clusters of multidrug antiretroviral/ARV resistant viruses were described in HEMOAM blood donors, a main Amazonas public blood bank. Recent and long-term infections among previously genotyped donors are reported.

Methods/materials: The recency immunoassay Lag Avidity EIA (Maxim, USA) was employed. Clinical/CD4/viral load medical file data of the main local HIV management center (FMT-HVD) and ARV treatment/ART data were reviewed.

Results: Among 142 HIV-blood donors, chronic infection predominated (n = 87; 61.3 %), 79 based on LAg EIA and 8 undisclosed HIV identified in FMT-HVD records, mostly young adult, single males, 4 repeat donors, all ART-naive. Recent infections represented 30.3 % (n = 43), 39 identified by LAg EIA and 4 immunologic windows (antibody negative/NAT/RNA positive). The overall profile of recent and long-term infections was similar, including moderate rate of transmitted drug resistance/TDR, however with multiple resistance mutations to more than one ARV-class, suggesting ART/failure.

Discussion: Recent/acute and undisclosed/long-term HIV infections represent blood safety alerts suggesting test-seeking behavior of at-risk populations. Early ART use in Brazil, can turn HIV diagnosis more challenging representing a blood transfusion risk in the highly endemic Brazilian Amazon.

In the last decade, the Brazilian AIDS epidemic has declined especially in the Southeast and South regions, contrasting with the consistent increase observed in the North region.[1,2]

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https://doi.org/10.1016/j.bjid.2024.103848

10.6.2. Resumos em congresso em colaboração

Dias S, Silva-Junior AL, Garcia NP, Cardoso EC, Tarragô AM, Fraiji NA, Paula EV, Costa AG, Malheiro A. *Caracterização de anafilotoxinas em pacientes com anemia falciforme em crise vaso-oclusiva*. **Congresso Brasileiro de Hematologia, Hemoterapia e Terapia Celular**. 2021.

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