

UNIVERSIDADE FEDERAL DE MINAS GERAIS

Instituto de Ciências Biológicas

Programa de Pós-Graduação em Bioquímica e Imunologia

Larissa M. G. Cassiano

**CONTROLE DA HIPERINFLAMAÇÃO NA COVID-19 POR MODULAÇÃO DO
PANORAMA EPIGENÉTICO COM VITAMINA B12 EM MODELO *EX-VIVO***

Belo Horizonte

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Tese apresentada ao Programa de Pós-Graduação em Bioquímica e Imunologia, Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, como requisito parcial à obtenção do título de Doutor em Biologia Molecular.

Orientadores: Glória Regina Franco e Roney Santos Coimbra

Belo Horizonte

2025

043

Cassiano, Larissa Marcely Gomes.

Controle da hiperinflamação na COVID-19 por modulação do panorama epigenético com vitamina B12 em modelo ex-vivo [manuscrito] / Larissa Marcely Gomes Cassiano. – 2025.

194 f. : il. ; 29,5 cm.

Orientadores: Glória Regina Franco e Roney Santos Coimbra.

Tese (doutorado) – Universidade Federal de Minas Gerais, Instituto de Ciências Biológicas. Programa de Pós-Graduação em Bioquímica e Imunologia.

1. Bioquímica e imunologia. 2. COVID-19. 3. Vitamina B 12. 4. Metilação de DNA. 5. Transcriptoma. 6. Inflamação. I. Franco, Glória Regina. II. Coimbra, Roney Santos. III. Universidade Federal de Minas Gerais. Instituto de Ciências Biológicas. IV. Título.

CDU: 577.1



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ATA DA DEFESA DA TESE DE DOUTORADO DE LARISSA MARCELY GOMES CASSIANO. Aos quatorze dias do mês de novembro de 2025 às 13:00 horas, reuniu-se de forma “on line” utilizando a plataforma “Zoom”, no Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, a Comissão Examinadora da tese de Doutorado, indicada *ad referendum* do Colegiado do Curso, para julgar, em exame final, o trabalho intitulado "Controle da hiperinflamação na Covid-19 por modulação do panorama epigenético com vitamina B12 em modelo ex-vivo", requisito final para a obtenção do grau de Doutor em Ciências: Biologia Molecular. Abrindo a sessão, a Presidente da Comissão, Profa. Glória Regina Franco, da Universidade Federal de Minas Gerais, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra à candidata para apresentação de seu trabalho. Seguiu-se a arguição pelos examinadores, com a respectiva defesa da candidata. Logo após a Comissão se reuniu, sem a presença da candidata e do público, para julgamento e expedição do resultado final. Foram atribuídas as seguintes indicações: Dr. Helton da Costa Santiago (Universidade Federal de Minas Gerais), aprovada; Dra. Daniele da Glória de Souza (Universidade Federal de Minas Gerais), aprovada; Dra. Natália Satchiko Hojo de Souza (IOC-FIOCRUZ), aprovada; Dr. Marcelo Rosado Fantappiè (Universidade Federal de Rio de Janeiro), aprovada; Dr. Roney Santos Coimbra - Orientador (Fundação Oswaldo Cruz, Renê Rachou), aprovada; Dra. Glória Regina Franco - Orientadora (Universidade Federal de Minas Gerais), aprovada. Pelas indicações a candidata foi considerada:

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O resultado final foi comunicado publicamente à candidata pela Presidente da Comissão. Nada mais havendo a tratar, a Presidente da Comissão encerrou a reunião e lavrou a presente Ata que será assinada por todos os membros participantes da Comissão Examinadora. Belo Horizonte, 14 de novembro de 2025.

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*Meu corpo é igualmente memória.
Dedico esta obra aos meus ancestrais,
Cujos passos ecoam em minha alma,
Cujas vozes sussurram em meus sonhos,
Cujas lutas fortalecem minha determinação.
Avanço porque vocês persistiram.*

AGRADECIMENTOS

À medida que chego ao fim desta jornada acadêmica, gostaria de expressar minha profunda gratidão a todos aqueles que contribuíram para a realização desta tese de doutorado. Este trabalho representa não apenas um marco em minha carreira, mas também uma celebração do apoio inestimável e inspiração que recebi ao longo dos anos.

Agradeço aos meus orientadores e amigos, Roney Coimbra e Glória Franco, pela confiança, paciência e dedicação incansável que foram fundamentais para a condução desta pesquisa e para o desenvolvimento das minhas habilidades acadêmicas e profissionais;

À minha colega de equipe, Marina Oliveira, e aos membros do grupo de pesquisa Imunopatologia, da Fiocruz Minas Gerais, pelo companheirismo, suporte e por todo conhecimento e experiência compartilhados;

Aos profissionais e pesquisadores colaboradores deste trabalho, Bárbara Prado, Cristianne Cardoso, Saionara Francisco, Profa. Vânia D’Almeida, Dra. Vanessa Cavalcante, Dra. Anna Salim, Dra. Viviane Cristina, Me. Izabela Mamede, Prof. Marco Romano-Silva, Dra. Daniela Valadão, Prof. Jonas de Paula, Profa. Débora Miranda e Dr. Alex Fiorini. cujo comprometimento com a pesquisa e a busca pelo conhecimento impulsionam o avanço da ciência brasileira;

Ao Prof. David Langlais e colegas do *Inflammation Genomics Lab*, da *McGill University*, cujas discussões estimulantes, colaborações e amizades enriqueceram minha experiência acadêmica e pessoal;

Aos pacientes voluntários dos estudos que compõem esta tese, pela sua confiança na ciência. Suas contribuições são valiosas para o avanço da ciência e essenciais para o sucesso deste trabalho;

Aos meus pais e familiares, por me proporcionarem o privilégio de ser a primeira mulher da família a ter o título de doutora e pelo encorajamento, paciência e crença em mim, que foram fundamentais para me manter motivada e perseverante ao longo deste percurso desafiador;

Ao meu esposo, sogra e amigos pelo apoio incondicional e por serem meu porto seguro nos momentos mais difíceis;

Agradeço também de antemão aos membros da banca examinadora, por suas valiosas contribuições durante a defesa desta tese. Tenho certeza de que suas sugestões e questionamentos ajudarão a enriquecer o conteúdo e a qualidade deste trabalho.

Muito obrigada!

APRESENTAÇÃO

Seguindo as orientações contidas no 1º parágrafo do artigo 46º do Regulamento do Programa de Pós-Graduação em Bioquímica e Imunologia adaptado às normas gerais de Pós-graduação da UFMG, a presente tese segue um formato alternativo, onde cada capítulo contém a apresentação de um trabalho produzido pela candidata, como primeira autora, relacionado ao trabalho de tese.

“Art. 46º

§ 1º a tese poderá, ainda, ter um formato alternativo, com um mínimo de três trabalhos publicados que atendam às exigências deste regulamento, sendo o candidato o primeiro autor de pelo menos um artigo original relacionado ao trabalho de tese. Estes trabalhos não poderão ser utilizados para atender exigências de outras teses. O conjunto de trabalhos deverá ser precedido de uma introdução ou apresentação abrangente, seguida de uma discussão e conclusão globais;”

RESUMO GERAL

A doença do coronavírus 2019 (covid-19) é uma infecção respiratória aguda causada pelo coronavírus SARS-CoV-2, caracterizada por extensa desregulação imunológica, podendo levar a desfechos graves e sequelas sistêmicas a longo prazo. Estudos recentes mostraram que a vitamina B12 atua como um regulador epigenético em neuroinfecções, reduzindo a expressão de genes pró-inflamatórios como *CCL3* e *IL-1 β* . Assim, o presente estudo investigou o potencial da vitamina B12 como epifármaco para regular a hiperinflamação na covid-19. Utilizando um modelo *ex vivo* de culturas de sangue total, foram acessados o perfil transcricional e metabólico de leucócitos de pacientes durante a fase aguda da covid-19 e no déficit visuoespacial persistente pós-covid leve. Os resultados mostraram que a vitamina modulou componentes centrais da fisiopatologia da covid-19 e favoreceu o fluxo da via dos aminoácidos sulfurados, aumentando a biodisponibilidade de metil nas células. Esse fenômeno contribuiu para a hipermetilação da região promotora de genes-chave relacionados à resposta inflamatória, como *CCL3* na covid-19 aguda e *CCL11* no comprometimento visuoespacial persistente pós-covid leve. Além da normalizar a expressão de biomarcadores desregulados por meio de mecanismos epigenéticos dependentes de metil, os resultados demonstram um novo mecanismo anti-inflamatório da vitamina B12 na covid-19 aguda via regulação do processamento de mRNA, atenuando assinaturas transcricionais pró-inflamatórias em leucócitos. Essas descobertas demonstram o potencial anti-inflamatório e neuroprotetor da B12 no tratamento da covid-19 e abrem novas perspectivas para o desenvolvimento de terapias baseadas na modulação do panorama epigenético para controle da doença e suas consequências neurológicas.

Palavras-chaves: covid-19, vitamina B12; epifármaco; metilação de DNA; marcas epigenéticas; transcriptoma; hiperinflamação.

GENERAL ABSTRACT

Coronavirus disease 2019 (COVID-19) is an acute respiratory infection caused by the SARS-CoV-2 coronavirus, characterized by extensive immunologic dysregulation. This imbalance can lead to severe outcomes and long-term systemic sequelae. Recent studies have shown that vitamin B12 acts as an epigenetic regulator in neuroinfections, reducing the expression of pro-inflammatory genes such as *CCL3* and *IL-1 β* . Thus, the present study investigated the potential of vitamin B12 as an epidrug to regulate hyperinflammation in COVID-19. Using an *ex vivo* model of whole blood cultures, transcriptional and metabolic profiles of leukocytes from patients during the acute phase of COVID-19 and those with persistent visuoconstructive deficit post-mild COVID were accessed. Results showed that the vitamin modulated central components of COVID-19 pathophysiology and favoured the flux of the sulfur amino acid pathway, increasing methyl bioavailability in cells. This phenomenon contributed to the promoter hypermethylation of key inflammatory response-related genes, such as *CCL3* in acute COVID-19 and *CCL11* in post-mild COVID visuoconstructive impairment. Beyond normalizing dysregulated biomarkers through methyl-dependent epigenetic mechanisms, the results reveal a novel anti-inflammatory mechanism of vitamin B12 in acute COVID-19 via mRNA processing modulation, attenuating pro-inflammatory transcriptional signatures in leukocytes. These findings demonstrate the anti-inflammatory and neuroprotective potential of B12 in COVID-19 treatment and open new perspectives for developing therapies based on modulating the epigenetic landscape to manage the disease and its neurological consequences.

Keywords: COVID-19, vitamin B12; epidrug; DNA methylation; epigenetic marks; transcriptome; hyperinflammation.

LISTA DE ILUSTRAÇÕES

INTRODUÇÃO	27
Figura 1: Número de novos casos confirmados e mortes reportadas semanalmente em decorrência da covid-19.....	28
Figura 2: Patogênese molecular e celular do SARS-CoV-2.	33
Figura 3: Dinâmica da resposta imune na covid-19.	35
Figura 4: Patogênese, perfil imunológico e possíveis sintomas pós-covid.....	36
Figura 5: Neuroinflamação pós-covid.	38
Figura 6: Modificações epigenéticas da cromatina por metilação do DNA e modificações das histonas.	43
Figura 7: Metabolismo da vitamina B12 e o metabolismo celular.	45
Figura 8: Metilação do DNA e o <i>splicing</i> alternativo.....	47
Figura 9: Mecanismo de <i>splicing</i> e seus produtos.	49
JUSTIFICATIVA	54
CAPÍTULO I: Vitamina B12 como neuroprotetor na neuroinflamação	55
<i>Figure 1: Cobalamin and cell metabolism.</i>	<i>58</i>
<i>Figure 2: Effect of ROS in Cobalamin oxidation.</i>	<i>61</i>
<i>Figure 3: B12 regulates pivotal components of the neuroinflammation signaling pathway.</i>	<i>66</i>
CAPÍTULO II: Vitamina B12 atenua assinatura inflamatória na covid-19 via modificações dependente de metil em marcas epigenéticas	76
<i>Figure 1: Sulfur amino acid pathway</i>	<i>79</i>
<i>Figure 2: Research design.....</i>	<i>81</i>
<i>Figure 3: Vitamin B12 favorably modulated critical inflammatory mediators</i>	<i>88</i>
<i>Figure 4: Vitamin B12 increased methylation levels of CpGs in regulatory regions of CCL3.</i>	<i>91</i>
<i>Figure 5: Differently expressed genes (DEG).</i>	<i>92</i>
<i>Figure 6: Canonical Pathways affected by COVID-19 and B12.....</i>	<i>93</i>
CAPÍTULO III: Vitamina B12 como um regulador do <i>splicing</i> alternativo na covid-19 aguda.....	108

<i>Figure 1: Differentially expressed transcript isoforms and alternative splicing events analysis</i>	113
<i>Figure 2: Functional enrichment analysis of productive and unproductive transcript isoforms.....</i>	115
CAPÍTULO IV: Vitamina B12 como um epifármaco para regular biomarcadores sanguíneos na deficiência visuoesstrutiva associada à covid longa	127
<i>Figure 1: Research timeline.</i>	131
<i>Figure 2: Supplemental B12 favorably modulates persistently dysregulated inflammatory genes in the leukocytes of patients with VCD.</i>	135
<i>Figure 3: VCD patients exhibit hypomethylation of CpG in CCL11 promoter region, which are hypermethylated upon B12 supplementation.....</i>	137
<i>Figure 4: Graphical representation of the study.</i>	143
CONCLUSÃO GERAL	150
Figura 10: Resumo gráfico dos principais resultados e conclusões do trabalho de tese.....	150
REFERÊNCIAS GERAIS	152
APÊNDICES.....	169
Figura 1: Desenho experimental.	173
Figura 2: Expressão relativa de CCL11 mRNA.....	175
Figura 3: Vias KEGG alteradas.	176
Figura 5: Proporção de células imunes.	177

LISTA DE TABELAS

CAPÍTULO II

Table 1: Patient's data. 87

Table 2: Vitamin B12 increases the flow of the sulfur amino acid pathway. 90

CAPÍTULO IV

Table 1: Participant's data..... 134

ANEXO II

Tabela 1: Informações dos pacientes 174

Tabela 2: Rendimento das amostras 179

Tabela 3: Primers utilizados 180

LISTA DE ABREVIATURAS E SIGLAS

18FDG-PET	Tomografia por Emissão de Pósitrons com Fluordesoxiglicose-18	<i>Positron Emission Tomography with Fluorodeoxyglucose-18</i>
2-OGDD	Dioxigenase dependente de 2-oxoglutarato	<i>2-oxoglutarate-dependent dioxygenase</i>
5mC	5-metilcitosina	<i>5-methylcytosine</i>
ACE2	Enzima Conversora de Angiotensina 2	<i>Angiotensin-Converting Enzyme 2</i>
AD	Doença de Alzheimer	<i>Alzheimer's Disease</i>
AdoCbl	Adenosilcobalamina	<i>Adenosylcobalamin</i>
AP-1	Proteína Ativadora 1	<i>Activator Protein 1</i>
ATP	Trifosfato de Adenosina	<i>Adenosine Triphosphate</i>
ATP11A	ATPase Fosfolipídica Transportadora 11A	<i>ATPase Phospholipid Transporting 11A</i>
BCL-2	Linfoma de Células B 2	<i>B-cell lymphoma 2</i>
BCL-XL	Linfoma de Células B Extra Grande	<i>B-cell lymphoma-extra large</i>
BHE	Barreira Hematoencefálica	<i>Blood-Brain Barrier</i>
Cat	Catepsina	<i>Cathepsin</i>
Cbl	Cobalamina	<i>Cobalamin</i>
CBS	Cistationina Beta-Sintase	<i>Cystathionine Beta-Synthase</i>
CTCF	Fator de Ligação CCCTC	<i>CCCTC-Binding Factor</i>
CCL11	Quimiocina (motivo C-C) Ligante 11	<i>Chemokine (C-C motif) Ligand 11</i>
CCL2	Quimiocina (motivo C-C) Ligante 2	<i>Chemokine (C-C motif) Ligand 2</i>
CCL3	Quimiocina (motivo C-C) Ligante 3	<i>Chemokine (C-C motif) Ligand 3</i>
CCR8	Receptor de Quimiocina C-C tipo 8	<i>C-C Chemokine Receptor type 8</i>
CD4	Grupamento de Diferenciação 4	<i>Cluster of Differentiation 4</i>
CD24	Grupamento de Diferenciação 24	<i>Cluster of Differentiation 24</i>
CD74	Grupamento de Diferenciação 74	<i>Cluster of Differentiation 74</i>
CD8	Grupamento de Diferenciação 8	<i>Cluster of Differentiation 8</i>

CGL	Cistationina gamma-liase	<i>Cystathionine gamma-lyase</i>
CH3	Grupo Metil	<i>Methyl Group</i>
chr	Cromossomo	<i>Chromosome</i>
CoV	Coronavírus	<i>Coronavirus</i>
covid-19	Doença do Coronavírus 2019	<i>Coronavirus Disease 2019</i>
CpG	Citosina-fosfato-Guanina	<i>Cytosine-phosphate-Guanine</i>
CSF2	Fator Estimulador de Colônias 2	<i>Colony Stimulating Factor 2</i>
CT	Tomografia Computadorizada	<i>Computed Tomography</i>
CTF/NF-1	Fator de Transcrição CCAAT/Fator Nuclear 1	<i>CCAAT Transcription Factor/Nuclear Factor 1</i>
CTRL	Controle	<i>Control</i>
CXCL10	Quimiocina (motivo C-X-C) Ligante 10	<i>Chemokine (C-X-C motif) Ligand 10</i>
CXCL9	Quimiocina (motivo C-X-C) Ligante 9	<i>Chemokine (C-X-C motif) Ligand 9</i>
CYS	Cisteína	<i>Cysteine</i>
DAMP	Padrões Moleculares Associados a Danos	<i>Damage-Associated Molecular Patterns</i>
DEG	Genes Diferencialmente Expressos	<i>Differentially Expressed Genes</i>
DML	Locais Diferencialmente Metilados	<i>Differentially Methylated Loci</i>
DNA	Ácido Desoxirribonucleico	<i>Deoxyribonucleic Acid</i>
d-NLR	Razão Neutrófilo-Linfócito Derivada	<i>derived Neutrophil-to-Lymphocyte Ratio</i>
DNMT	DNA Metiltransferase	<i>DNA Methyltransferase</i>
DPP9	Dipeptidil Peptidase 9	<i>Dipeptidyl Peptidase 9</i>
E2F	Fator E2	<i>E2 Factor</i>
ECM	Matriz Extracelular	<i>Extracellular Matrix</i>
EGFR	Receptor do Fator de Crescimento Epidérmico	<i>Epidermal Growth Factor Receptor</i>
ERBB2	Receptor Tirosina-Quinase erb- b2	<i>Erb-b2 Receptor Tyrosine Kinase 2</i>
ESPII	Emergência de Saúde Pública de Importância Internacional	<i>Public Health Emergency of International Concern</i>

FDR	Taxa de Falsa Descoberta	<i>False Discovery Rate</i>
FGF2	Fator de Crescimento de Fibroblastos 2	<i>Fibroblast Growth Factor 2</i>
FKBP5	Proteína 5 de Ligação FK506	<i>FK506 Binding Protein 5</i>
FOS	Protooncogene FOS	<i>FOS Proto-oncogene</i>
G-CSF	Fator Estimulador de Colônias de Granulócitos	<i>Granulocyte Colony-Stimulating Factor</i>
GFAP	Proteína Fibrilar Ácida da Glia	<i>Glial Fibrillary Acidic Protein</i>
GO	Ontologia Gênica	<i>Gene Ontology</i>
GPS2	Supressor de Sinalização de Proteína G 2	<i>G Protein Pathway Suppressor 2</i>
GSH	Glutathiona	<i>Glutathione</i>
H2A	Histona 2A	<i>Histone 2A</i>
H2B	Histona 2B	<i>Histone 2B</i>
H₂S	Sulfeto de Hidrogênio	<i>Hydrogen Sulfide</i>
H3	Histona 3	<i>Histone 3</i>
H3ac	Histona 3 Acetilada	<i>Acetylated Histone 3</i>
H3k27ac	Histona 3 Lisina 27 Acetilada	<i>Histone 3 Lysine 27 Acetylation</i>
H3K27me3	Histona 3 Lisina 27 Trimetilada	<i>Histone 3 Lysine 27 Trimethylation</i>
H3K4me1	Histona 3 Lisina 4 Monometilada	<i>Histone 3 Lysine 4 Monomethylation</i>
H3k4me3	Histona 3 Lisina 4 Trimetilada	<i>Histone 3 Lysine 4 Trimethylation</i>
H3k9me2	Histona 3 Lisina 9 Dimetilada	<i>Histone 3 Lysine 9 Dimethylation</i>
H4	Histona 4	<i>Histone 4</i>
HAT	Histona Acetiltransferase	<i>Histone Acetyltransferase</i>
HAVCR2	Receptor Celular 2 de Vírus da Hepatite A	<i>Hepatitis A Virus Cellular Receptor 2</i>
HCY	Homocisteína	<i>Homocysteine</i>
HDAC	Histona Desacetilase	<i>Histone Deacetylase</i>
HDM	Histona Desmetilase	<i>Histone Demethylase</i>
HGF	Fator de Crescimento de Hepatócitos	<i>Hepatocyte Growth Factor</i>
HIV	Vírus da Imunodeficiência Humana	<i>Human Immunodeficiency Virus</i>

HLA-B	Antígeno Leucocitário Humano B	<i>Human Leukocyte Antigen B</i>
HMT	Histona Metiltransferase	<i>Histone Methyltransferase</i>
HNRNPH1	Ribonucleoproteína Nuclear Heterogênea H1	<i>Heterogeneous Nuclear Ribonucleoprotein H1</i>
HP1	Proteína Heterocromatina 1	<i>Heterochromatin Protein 1</i>
HPLC	Cromatografia Líquida de Alta Eficiência	<i>High-Performance Liquid Chromatography</i>
IFN	Interferon	<i>Interferon</i>
IFN-γ	Interferon Gama	<i>Interferon Gamma</i>
IL-10	Interleucina 10	<i>Interleukin 10</i>
IL-17A	Interleucina 17A	<i>Interleukin 17A</i>
IL-18	Interleucina 18	<i>Interleukin 18</i>
IL-1RA	Antagonista do Receptor de Interleucina 1	<i>Interleukin 1 Receptor Antagonist</i>
IL-1β	Interleucina 1 Beta	<i>Interleukin 1 Beta</i>
IL-2	Interleucina 2	<i>Interleukin 2</i>
IL31	Interleucina 31	<i>Interleukin 31</i>
IL-6	Interleucina 6	<i>Interleukin 6</i>
IL-6R	Receptor de Interleucina 6	<i>Interleukin 6 Receptor</i>
IP-10	Proteína 10 Induzida por Interferon Gama	<i>Interferon Gamma-Induced Protein 10</i>
IRF-3	Fator Regulador de Interferon 3	<i>Interferon Regulatory Factor 3</i>
IRF7	Fator Regulador de Interferon 7	<i>Interferon Regulatory Factor 7</i>
ISG	Gene Estimulado por Interferon	<i>Interferon-Stimulated Gene</i>
JMJD	Domínio Jumonji	<i>Jumonji Domain</i>
kb	Quilobase	<i>Kilobase</i>
KLF4	Fator Tipo Krüppel 4	<i>Krüppel-Like Factor 4</i>
LCR	Líquido cefaloraquidiano	<i>Cerebrospinal Fluid</i>
LGALS3	Galectina 3	<i>Galectin-3</i>
LIF	Fator Inibidor de Leucemia	<i>Leukemia Inhibitory Factor</i>
lncRNA	RNA Longo Não Codificante	<i>Long Non-Coding RNA</i>

LRRFIP1	Proteína de Interação com Repetição Rica em Leucina e Fibronectina Tipo III 1	<i>Leucine Rich Repeat Flightless Interacting Protein 1</i>
MAS1	Receptor Acoplado à Proteína G MAS1	<i>MAS1 Proto-Oncogene, G Protein-Coupled Receptor</i>
MAT	Metionina Adenosiltransferase	<i>Methionine Adenosyltransferase</i>
MHC	Complexo principal de histocompatibilidade	<i>Major histocompatibility complex</i>
MCM	Metilmalonil-CoA mutase	<i>Methylmalonyl-CoA mutase</i>
MCP-1	Proteína Químioatraente de Monócitos 1	<i>Monocyte Chemoattractant Protein 1</i>
M-CSF	Fator Estimulador de Colônias de Macrófagos	<i>Macrophage Colony-Stimulating Factor</i>
MDA-5	Proteína Associada à Diferenciação de Melanoma 5	<i>Melanoma Differentiation-Associated protein 5</i>
MeCbl	Metilcobalamina	<i>Methylcobalamin</i>
MeCP2	Proteína de Ligação a Metil-CpG 2	<i>Methyl-CpG Binding Protein 2</i>
MERS-CoV	Coronavírus da Síndrome Respiratória do Oriente Médio	<i>Middle East Respiratory Syndrome Coronavirus</i>
MIP-1α	Proteína Inflamatória de Macrófagos 1 Alfa	<i>Macrophage Inflammatory Protein 1 Alpha</i>
miRNA	MicroRNA	<i>MicroRNA</i>
MOD	covid-19 moderada	<i>Moderate COVID-19</i>
mRNA	RNA Mensageiro	<i>Messenger RNA</i>
MS	Metionina sintase	<i>Methionine synthase</i>
MT	Metiltransferase	<i>Methyltransferase</i>
MTHFR	Metilenotetrahidrofolato Redutase	<i>Methylenetetrahydrofolate Reductase</i>
mTORC1	Complexo 1 do Alvo da Rapamicina em Mamíferos	<i>Mammalian Target of Rapamycin Complex 1</i>
MUC1	Mucina 1	<i>Mucin 1</i>
NF-κB	Fator Nuclear kappa B	<i>Nuclear Factor kappa B</i>
NF-1	Fator Nuclear 1	<i>Nuclear Factor 1</i>
NFIC	Fator Nuclear I C	<i>Nuclear Factor I C</i>
NGF	Fator de Crescimento Neural	<i>Nerve Growth Factor</i>

NGS	Sequenciamento de Nova Geração	<i>Next-Generation Sequencing</i>
NK	Células Natural Killer	<i>Natural Killer Cells</i>
NLR	Receptores do Tipo NOD	<i>NOD-Like Receptors</i>
NLR	Razão Neutrófilo-Linfócito	<i>Neutrophil-to-Lymphocyte Ratio</i>
NLRP1	Proteína 1 Contendo Domínios NACHT, LRR e PYD	<i>NACHT, LRR and PYD Domains-Containing Protein 1</i>
NLRP3	Proteína 3 Contendo Domínios NACHT, LRR e PYD	<i>NACHT, LRR and PYD Domains-Containing Protein 3</i>
nM	Nanomolar	<i>Nanomolar</i>
NMD	Degradação Mediada por códon sem sentido	<i>Nonsense-Mediated Decay</i>
NOX1	NADPH Oxidase 1	<i>NADPH Oxidase 1</i>
NPNT	Nefronectina	<i>Nephronectin</i>
NSP16	Proteína Não Estrutural 16	<i>Non-Structural Protein 16</i>
OAS1	2'-5'-Oligoadenilato Sintetase 1	<i>2'-5'-Oligoadenylate Synthetase 1</i>
OAS3	2'-5'-Oligoadenilato Sintetase 3	<i>2'-5'-Oligoadenylate Synthetase 3</i>
OMS	Organização Mundial da Saúde	<i>World Health Organization (WHO)</i>
ORF	Quadro de Leitura Aberto	<i>Open Reading Frame</i>
PAMP	Padrões Moleculares Associados a Patógenos	<i>Pathogen-Associated Molecular Patterns</i>
PBS	Tampão Fosfato-Salino	<i>Phosphate-Buffered Saline</i>
pH	Potencial Hidrogeniônico	<i>Potential of Hydrogen</i>
Pi	Fosfato Inorgânico	<i>Inorganic Phosphate</i>
PI3K/AKT	Fosfatidilinositol-3-Quinase/Proteína Quinase B	<i>Phosphatidylinositol-3-Kinase/Protein Kinase B</i>
PLR	Razão de plaquetas para linfócitos	<i>Platelet-to-Lymphocyte Ratio</i>
PMF1	Fator de Maturação de Poliadenilação 1	<i>Polyamine-Modulated Factor 1</i>
Pol II	RNA Polimerase II	<i>RNA Polymerase II</i>
PPi	Pirofosfato	<i>Pyrophosphate</i>
RARA	Receptor de Ácido Retinóico Alfa	<i>Retinoic Acid Receptor Alpha</i>
RLR	Receptores do Tipo RIG-I	<i>RIG-I-Like Receptors</i>

RNA	Ácido Ribonucleico	<i>Ribonucleic Acid</i>
RNA-seq	Sequenciamento de RNA	<i>RNA Sequencing</i>
ROCF	Teste de Retenção de Figuras Complexas de Rey-Osterrieth	<i>Rey-Osterrieth Complex Figure Test</i>
RRP	Receptores de Reconhecimento de Padrões	<i>Pattern Recognition Receptors</i>
RT-qPCR	Reação em Cadeia da Polimerase Quantitativa em Tempo Real	<i>Real-Time Quantitative Polymerase Chain Reaction</i>
RXRG	Receptor X Retinóide Gama	<i>Retinoid X Receptor Gamma</i>
S	Proteína Spike	<i>Spike Protein</i>
SAHH	S-Adenosil-Homocisteína Hidrolase	<i>S-Adenosyl-Homocysteine Hydrolase</i>
SAM	S-Adenosilmetionina	<i>S-Adenosylmethionine</i>
SARS	Síndrome Respiratória Aguda Grave	<i>Severe Acute Respiratory Syndrome</i>
SARS-CoV	Coronavírus da Síndrome Respiratória Aguda Grave	<i>Severe Acute Respiratory Syndrome Coronavirus</i>
SARS-CoV-2	Coronavírus da Síndrome Respiratória Aguda Grave 2	<i>Severe Acute Respiratory Syndrome Coronavirus 2</i>
SEV	covid-19 grave	<i>Severe COVID-19</i>
SF	Fator de <i>Splicing</i>	<i>Splicing Factor</i>
SMARCA4	Regulador de Cromatina Dependente de ATP SWI/SNF Relacionado, Subfamília A, Membro 4	<i>SWI/SNF Related, Matrix Associated, Actin Dependent Regulator of Chromatin, Subfamily A, Member 4</i>
SNC	Sistema Nervoso Central	<i>Central Nervous System</i>
snRNP	Ribonucleoproteína Nuclear Pequena	<i>Small Nuclear Ribonucleoprotein</i>
SRAG	Síndrome Respiratória Aguda Grave	<i>Severe Acute Respiratory Syndrome</i>
SUMO	Pequeno Modificador Relacionado à Ubiquitina	<i>Small Ubiquitin-like Modifier</i>
SWI/SNF	Interrupor/Sacarose Não Fermentável	<i>Switch/Sucrose Non-Fermentable</i>
TC	Transcobalamina	<i>Transcobalamin</i>
TC-II	Transcobalamina II	<i>Transcobalamin II</i>

TDE	Transcritos Diferencialmente Expressos	<i>Differentially Expressed Transcripts</i>
TET	Ten-Eleven Translocation	<i>Ten-Eleven Translocation</i>
TF	Fator de Transcrição	<i>Transcription Factor</i>
TFBS	Sítio de Ligação de Fator de Transcrição	<i>Transcription Factor Binding Site</i>
TFDP1	Fator de Transcrição Dp-1	<i>Transcription Factor Dp-1</i>
TGF-beta	Fator de Crescimento Transformador Beta	<i>Transforming Growth Factor Beta</i>
Th1	Células T Auxiliares Tipo 1	<i>Type 1 T Helper Cells</i>
Th2	Células T Auxiliares Tipo 2	<i>Type 2 T Helper Cells</i>
TLR	Receptor do Tipo <i>Toll</i>	<i>Toll-Like Receptor</i>
TMPRSS2	Serina Protease Transmembrana 2	<i>Transmembrane Protease, Serine 2</i>
TNF-α	Fator de Necrose Tumoral Alfa	<i>Tumor Necrosis Factor Alpha</i>
TREM1	Receptor Desencadeador Expresso em Células Mieloides 1	<i>Triggering Receptor Expressed on Myeloid Cells 1</i>
UFMG	Universidade Federal de Minas Gerais	<i>Federal University of Minas Gerais</i>
UTR	Região Não Traduzida	<i>Untranslated Region</i>
VCD	Deficiência Vosuoconstrutiva	<i>Visuoconstructive Deficit</i>
VEGF	Fator de Crescimento Endotelial Vascular	<i>Vascular Endothelial Growth Factor</i>
VOC	Variante de Preocupação	<i>Variant of Concern</i>
WPBC	Células brancas do sangue periférico (Leucócitos)	<i>White Peripheral Blood Cells (Leukocytes)</i>

SUMÁRIO

INTRODUÇÃO	27
Epidemiologia da covid-19	27
Fisiopatologia da covid-19.....	31
Condição pós-covid	35
<i>Neuroinflamação</i>	<i>37</i>
<i>Deficiência visuoespacial como condição pós-covid.....</i>	<i>38</i>
Regulação da expressão gênica na covid-19.....	39
<i>Mecanismos epigenéticos.....</i>	<i>40</i>
<i>Vitamina B12 como regulador da expressão gênica</i>	<i>44</i>
<i>Splicing alternativo.....</i>	<i>48</i>
Modelos de infecção por SARS-CoV-2 para estudos da covid-19.....	51
JUSTIFICATIVA	54
CAPÍTULO I: Vitamina B12 como neuroprotetor na neuroinflamação	55
<i>Introduction</i>	<i>57</i>
<i>B12 regulates energy metabolism and immune response</i>	<i>59</i>
<i>B12 is antioxidant and modulates the oxidative stress response.....</i>	<i>60</i>
<i>B12 boosts the synthesis of H₂S within the CNS.....</i>	<i>62</i>
<i>B12 prevents NMDAR-mediated glutamate excitotoxicity</i>	<i>62</i>
<i>B12 rescues neurons from homocysteine-mediated cell death</i>	<i>63</i>
<i>B12 modulates the expression of inflammatory genes through cell epigenetic landscape changings</i>	<i>64</i>
<i>B12 regulates pivotal neuroinflammation signaling pathway-associated genes.....</i>	<i>65</i>
<i>Final remarks and conclusion</i>	<i>67</i>
<i>References</i>	<i>70</i>
CAPÍTULO II: Vitamina B12 atenua assinatura inflamatória na covid-19 via modificações dependente de metil em marcas epigenéticas.....	76
<i>Abstract.....</i>	<i>76</i>
<i>Introduction</i>	<i>77</i>

<i>Materials and methods</i>	79
<i>Results</i>	85
<i>Discussion</i>	94
<i>References</i>	101
CAPÍTULO III: Vitamina B12 como um regulador do <i>splicing</i> alternativo na covid-19 aguda	108
<i>Abstract</i>	108
<i>Introduction</i>	109
<i>Materials and Methods</i>	110
<i>Results</i>	112
<i>Discussion</i>	116
<i>References</i>	121
CAPÍTULO IV: Vitamina B12 como um epifármaco para regular biomarcadores sanguíneos na deficiência visuoestrutiva associada à covid longa	127
<i>Abstract</i>	128
<i>Introduction</i>	128
<i>Material and methods</i>	130
<i>Results</i>	133
<i>Discussion</i>	137
<i>References</i>	144
CONCLUSÃO GERAL	150
REFERÊNCIAS GERAIS	152
APÊNDICES	169
APÊNDICE I: Pedido de patente depositado no Instituto Nacional da Propriedade Industrial (INPI) para uso dos padrões de metilação de CpGs no promotor do gene <i>CCL11</i> como biomarcador para o diagnóstico molecular de deficiência visuoestrutiva e método diagnóstico.	169
APÊNDICE II: Projeto de doutorado sanduíche “Vitamina B12: um epifármaco para regular genes essenciais na fisiopatologia da deficiência visuoestrutiva associada à covid-19 longa e a doenças neurodegenerativas relacionadas ao envelhecimento.”.	170

APÊNDICE III: Outras produções científicas realizadas no interstício doutoral.....	186
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INTRODUÇÃO

Epidemiologia da covid-19

Os coronavírus (CoV), membros da família Coronaviridae, são vírus envelopados com genoma de RNA de fita simples e sentido positivo. Reconhecidos como os maiores vírus de RNA já descritos, os CoV possuem genomas que variam de 25 a 32 kb (1). Seu nome deriva do latim “corona” e refere-se às projeções semelhantes a uma coroa presentes na superfície do vírus (1). Grande parte dos CoV conhecidos circula em hospedeiros animais sem causar grandes problemas em humanos. No entanto, alguns coronavírus de origem zoonótica que emergiram nas últimas décadas estiveram associados a surtos de doenças respiratórias graves em humanos.

O primeiro CoV de preocupação para a saúde pública foi o da Síndrome Respiratória Aguda Grave (SARS-CoV), que surgiu na província de Guangdong, na China, em 2002 (2). O SARS-CoV disseminou-se rapidamente para diversos países na América do Norte, América do Sul, Europa e Ásia, infectando mais de 8 mil pessoas e causando cerca de 800 mortes (3). O surto de SARS foi controlado em 2003. Desde o final de 2004, nenhum caso foi relatado mundialmente (4).

Em 2012, outro coronavírus, denominado Síndrome Respiratória do Oriente Médio (MERS-CoV), surgiu na Arábia Saudita (5). Desde então, houve pouco mais de 2,6 mil casos confirmados e pelo menos 900 mortes relacionadas à doença, onde a maioria ocorreu na sua região de origem (6). O maior surto conhecido de MERS fora da Península Arábica ocorreu na República da Coreia, em 2015, associado ao retorno de um viajante procedente do Oriente Médio (7).

No final de 2019, foi identificado, na cidade de Wuhan, província de Hubei na China, o vírus da Síndrome Respiratória Aguda Grave 2 (SARS-CoV-2), responsável pela mais recente e significativa epidemia causada por um coronavírus (8). A origem do SARS-CoV-2 ainda não está totalmente elucidada, mas há evidências de que ele pode ter se originado de morcegos e então se propagado para humanos por meio de um hospedeiro intermediário, possivelmente pangolins [revisado em: (9)]. Em contraste com os coronavírus humanos anteriores, o SARS-CoV-2 demonstrou ser altamente contagioso e patogênico. Sua transmissão ocorre principalmente através de gotículas respiratórias e aerossóis, podendo se propagar antes do início dos sintomas ou mesmo por indivíduos assintomáticos. Essas características favorecem significativamente sua rápida disseminação em comunidades, tornando o SARS-CoV-2 particularmente desafiador em termos de controle epidemiológico e exigindo medidas rigorosas de prevenção para conter sua propagação [revisado em: (10)].

Embora a maioria das pessoas infectadas com SARS-CoV-2 desenvolvam uma doença leve a moderada, algumas progridem para uma pneumonia ou síndrome respiratória aguda grave (SRAG), potencialmente fatal (11). Com o aumento exponencial do número de casos e rápida propagação para diversos países ao redor do mundo, a Organização Mundial da Saúde (OMS) declarou a doença do coronavírus 2019 (covid-19) como Emergência de Saúde Pública de Importância Internacional (ESPII) em 30 de janeiro de 2020 (12) e, mais tarde, caracterizou o surto como uma pandemia em 11 de março de 2020 (13) (Figura 1). Desde seu surgimento até novembro de 2024, a covid-19 foram reportados mais de 777 milhões de casos confirmados e mais de 7,1 milhões de mortes em todo o mundo (14). No Brasil, foram registrados cerca de 39 milhões de casos e 713 mil mortes pela doença, sendo o quinto país com maior número de óbitos do mundo (14,15).

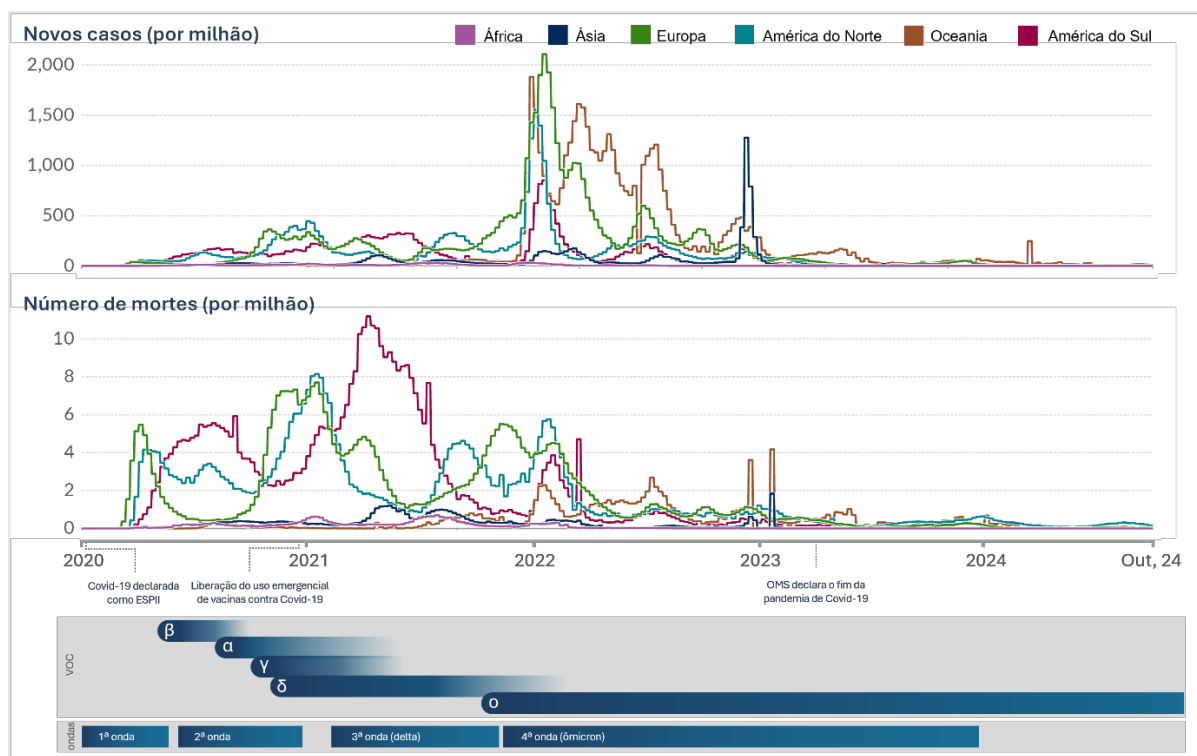


Figura 1: Número de novos casos confirmados e mortes reportadas semanalmente em decorrência da covid-19. Número de novos casos confirmados e mortes reportadas em decorrência da covid-19. Média de 7 dias consecutivos. Desde o início de 2024, vários países cessaram o acompanhamento do número de casos e mortes pela covid-19. Fonte: Adaptado de Organização Mundial de Saúde (2024) (14).

A busca por tratamentos e imunizantes eficazes contra a covid-19 tornou-se uma prioridade global desde o início da pandemia. No final de 2020, vacinas que abrangem uma variedade de tecnologias e fabricantes começaram a ser aprovadas para uso emergencial em diferentes países como um instrumento para controle da gravidade da infecção e melhora dos

desfechos clínicos da doença. As vacinas de RNA mensageiro (mRNA) produzidas pela Pzfizer/BioNtech (Comirnaty) e Moderna (Spikevax) são as mais utilizadas mundialmente devido sua maior eficácia contra variantes virais mais recentes e pela facilidade de atualização em comparação a outras plataformas vacinais (16). No Brasil, além da parceria Fiocruz - AstraZeneca para produção da vacina de vetor adenovírus recombinante desenvolvida pela Universidade de Oxford, pesquisadores do Centro Tecnológico de Vacinas (CT Vacinas) e da Universidade Federal de Minas Gerais (UFMG) desenvolveram a SpiN-Tec, uma vacina 100% brasileira. O imunizante, que atualmente está na fase de testes em humanos, utiliza uma tecnologia de proteína quimérica recombinante, e se difere de outras vacinas por estimular a imunidade celular, possibilitando maior proteção contra novas variantes virais (17). Essa iniciativa, além de ser um marco inédito para a ciência brasileira, é uma contribuição importante para o esforço global de vacinação contra a covid-19 e demonstra a capacidade do Brasil em desenvolver tecnologias inovadoras para a saúde pública. De acordo com a última atualização da OMS, estima-se que 67% da população mundial tenha completado o esquema inicial de vacinação contra covid-19 e 32% receberam pelo menos uma dose de reforço (14). Essa porcentagem diminui drasticamente em países de baixa renda, com 32,7% da população vacinada com pelo menos uma dose (14). O Brasil é o quinto país com maior número de doses administradas (16)., e, de acordo com último relatório anual de vacinação (2024), cerca de 86% da população maior de 5 anos tenha sido vacinada com o esquema inicial completo (18).

Nos últimos anos, além das vacinas, foram aprovadas opções para o tratamento de pacientes hospitalizados com covid-19 ou indivíduos com alto risco de desenvolver formas mais graves da doença. Vários países, incluindo o Brasil, liberaram o uso hospitalar ou a venda controlada de antivirais como o Remdesivir, Paxlovid (Nirmatrelvir + Ritonavir) e o Molnupiravir, que atuam inibindo a replicação viral; de anticorpos monoclonais como o Sotrovimabe e o Tocilizumabe, projetados para se ligarem à proteína *spike* do SARS-CoV-2 e impedir a entrada do vírus nas células hospedeiras; e do imunomodulador Baricitinibe, que possui propriedades anti-inflamatórias (19–21). No entanto, estes e outros medicamentos e terapias disponíveis ainda apresentam eficácia limitada ou são financeiramente inacessíveis. Com custos que variam entre 2-4 mil reais, estes medicamentos são impraticáveis para uso em larga escala, especialmente em países de baixa renda com cobertura vacinal limitada. Em resposta a essa necessidade, diversos estudos clínicos foram conduzidos em todo o mundo em busca de novas abordagens terapêuticas para a covid-19, incluindo a terapia com plasma de pacientes convalescentes, compostos imunomoduladores, e moléculas de origem natural como a vitamina D e o resveratrol [revisado em: (22)]. A estratégia de reposicionamento de fármacos

tem sido amplamente adotada como uma abordagem mais rápida, eficaz e economicamente viável no combate à covid-19. As principais vantagens desta estratégia incluem a disponibilidade de informações sobre farmacocinética, farmacodinâmica e toxicidade do medicamento de interesse, reduzindo assim o tempo e os custos necessários para ensaios clínicos, além da disponibilidade de alguns medicamentos a preços mais baixos [revisado em: (23)]. Nos primeiros meses de pandemia, a dexametasona, corticoide com propriedades anti-inflamatórias e imunossupressoras sintetizado pela primeira vez na década de 1960, emergiu como padrão ouro para tratamento de pacientes hospitalizados com covid-19. O ensaio clínico randomizado controlado *RECOVERY*, conduzido pela Universidade de Oxford, demonstrou que o medicamento reduziu a taxa de mortalidade em pacientes recebendo ventilação mecânica invasiva ou oxigênio suplementar (24). Contudo, apesar de sua eficácia no manejo da covid-19 grave, a dexametasona não é recomendada para o tratamento de casos leves ou moderados da infecção (25).

O SARS-CoV-2, bem como todos os vírus de RNA, apresenta uma tendência natural a acumular mutações em seu genoma a cada ciclo de replicação. Essa característica tem favorecido o surgimento de diferentes variantes do vírus, incluindo as Variantes de Preocupação (VOC). Essas variantes apresentam características distintas, como aumento da transmissibilidade, virulência ou redução da eficácia das vacinas e tratamentos, provocando novas ondas de surtos e necessidade de ajustes nas ações de saúde pública [revisado em: (26)] (Figura 1). A variante Alfa (B.1.1.7) foi identificada pela primeira vez no Reino Unido em setembro de 2020. Ela apresenta 17 mutações não-sinônimas, sendo a principal a N501Y na proteína *spike* (S), que favorece a ligação viral ao receptor hospedeiro ACE2 e contribui significativamente para a proliferação viral e infectividade (27,28). A variante Alfa também demonstrou ser mais eficaz em suprimir a resposta imune inata nas células epiteliais das vias aéreas do que as variantes pré-existentes (29). A variante Beta (B.1.351) foi originalmente detectada na África do Sul em maio de 2020 (30). Foi relatado que essa variante tem maior transmissibilidade, caracterizada por diversas mutações predominantemente localizadas na proteína S. Além da mutação N501Y, ela possui as mutações K417N e E484K, que demonstraram ajudar o vírus a escapar dos anticorpos e reduzir a eficácia de vacinas (31–33). A linhagem P.1 ou variante Gama foi identificada no Brasil em dezembro de 2020 e foi responsável pelo pior momento da pandemia no país nos primeiros meses de 2021 (34). A variante possui um total de 17 mutações quando comparada as VOCs anteriores, incluindo três que afetaram a proteína S (K417T, E484K e N501Y), conferindo um aumento de 1,7 a 2,4 vezes na transmissibilidade e maior proliferação viral (35). Uma das variantes mais predominantes

do SARS-CoV-2 durante a pandemia de covid-19 foi a Delta (B.1.617.2), emergente na Índia em outubro de 2020 (36). A VOC apresenta uma nova mutação (P681R) no local de clivagem da furina na proteína S (FSC). Essa mutação favoreceu a patogenicidade da linhagem, aumentando a gravidade da infecção e o surgimento de sintomas incomuns (37). A variante Ômicron (B.1.1.529), identificada mais recentemente na África do Sul no final de 2021, é a que acumula mais mutações, garantindo uma capacidade de contágio muito maior que as demais (38). Com propriedades de evasão da imunidade conferida pela vacinação ou de infecções prévias, além de apresentar baixas taxas de neutralização pela maioria das terapias com anticorpos monoclonais disponíveis, a Ômicron tem sido a VOC mais divergente vista até a data, com uma gama crescente de sub-linhagens [revisado em: (39)].

Com a redução nas mortes por covid-19 e declínio nas hospitalizações e internações em unidades de terapia intensiva relacionadas a doença, após três anos de pandemia, a OMS declarou, no dia 5 de maio de 2023, que a covid-19 já não constitui uma ESPII, marcando o fim da pandemia (40). Apesar de os casos e mortes semanais notificados terem atingido o nível mais baixo desde o início da pandemia (Figura 1), milhões de pessoas ainda são infectadas ou reinfectadas com o SARS-CoV-2 e milhares morrem todos os dias (41). Em 2025, o Brasil registrou cerca de 1.700 mortes por Covid-19, apesar de cinco anos desde o lançamento da campanha nacional de vacinação. Foram identificados ainda 10.410 casos graves decorrentes da infecção pelo vírus no período. No último ano, as autoridades sanitárias nacionais constataram uma redução da adesão vacinal, mesmo com a vacina incluída no calendário básico para grupos de risco. De 21,9 milhões de doses distribuídas aos entes federativos pelo Ministério da Saúde, apenas 8 milhões foram administradas (15,42,43). De acordo com pesquisadores, esses indicadores configuram um alerta para a vigilância contínua, uma vez que o SARS-CoV-2 permanece como um patógeno com potencial de causar morbimortalidade significativa, demandando manutenção da imunização e monitoramento epidemiológico (42).

Fisiopatologia da covid-19

A fisiopatologia da covid-19 é caracterizada por vários estágios. A primeira fase corresponde ao período de incubação, tempo definido entre a infecção pelo SARS-CoV-2 e o surgimento dos sintomas. Este tempo pode variar de pessoa para pessoa e de acordo com a variante viral responsável pela infecção, geralmente com duração de dois a 14 dias. Durante este estágio, o vírus começa a se replicar e pode ser transmitido [revisado em: (44)].

A fase aguda da covid-19 é marcada pela manifestação dos sintomas. Entre os sintomas característicos, a febre, tosse seca e fadiga emergem como os mais frequentes em casos de

manifestação leve da doença. Em casos moderados que requerem hospitalização, são comuns a dificuldade respiratória e a ocorrência de pneumonia de grau leve. Já em casos graves, a infecção pode evoluir para uma forma de pneumonia mais severa, levando à síndrome do desconforto respiratório agudo grave, falência de múltiplos órgãos e outras complicações fatais (44,45). Um dos fatores determinantes da gravidade da covid-19 e o seu desfecho é a extensão da desregulação imunológica desencadeada pela infecção (46). Esta desregulação compromete a capacidade do sistema imunológico de coordenar uma resposta eficaz contra o vírus, favorecendo o desenvolvimento de manifestações clínicas graves da doença.

Normalmente, o sistema imune inato identifica o vírus de forma rápida e ativa a expressão de *interferons* (IFN) do tipo I e moléculas antivirais relacionadas em poucas horas após a infecção [revisado em: (47)]. No entanto, ao longo de sua evolução e adaptação ao hospedeiro humano, os CoV desenvolveram alguns mecanismos de evasão da resposta imune [revisado em: (48)]. Em infecções por SARS-CoV-2, o vírus é particularmente eficaz em evitar ou retardar o desencadeamento de respostas inatas antivirais inibindo a exportação de mRNA de *IFN* para o citoplasma e/ou provocando a degradação destes transcritos no núcleo (49), além de perturbar vias de detecção viral e as funções de indução, sinalização ou efectoras de IFN (50–54). Na ausência dessas respostas, o vírus inicialmente se replica sem obstáculos e as respostas imunes adaptativas não são iniciadas até que a sinalização e ativação do sistema imune inato sejam realizadas.

Apesar das estratégias de evasão da resposta antiviral, as etapas de infecção apresentam algumas oportunidades para o sistema imunológico hospedeiro detectar componentes virais (Figura 2). As células imunes inatas, como macrófagos, monócitos, células dendríticas, neutrófilos e células *Natural Killer* (NK), possuem uma variedade de receptores de reconhecimento de padrões (RRP) à sua disposição. Esses RRP são capazes de identificar padrões moleculares relacionados a patógenos (PAMP) ou padrões moleculares associados a danos (DAMP), induzindo a ativação de vias de sinalização inflamatória e respostas imune [revisado em: (55)]. Na covid-19, a sinalização por meio desses receptores, em particular os receptores do tipo Toll (TLR), RIG-I (RLR), e NOD (NLR) e de inflamassomas, levam à ativação de diversas vias inflamatórias e, subsequentemente, fatores de transcrição, tais como o Fator Nuclear Kappa B (NF- κ B), Proteína Ativadora-1 (AP-1) e Fator Regulatório 3 e 7 de Interferon (IRF-3 e IRF-7) [revisado em: (56,57)]. Essas moléculas induzem a expressão de genes responsáveis pela codificação de citocinas, quimiocinas e fatores de crescimento, iniciando uma cascata de sinalização de morte celular para eliminar as células infectadas em resposta ao SARS-CoV-2 (56). Conforme a infecção se agrava, os níveis de citocinas,

especialmente IL-1 β , IL-6 e TNF- α (58–60), aumentam de forma desregulada levando a um ciclo pró-inflamatório. Este processo característico da covid-19 grave, denominado de hiperinflamação, resulta no influxo de várias células imunes, como macrófagos e neutrófilos, da circulação para o local da infecção, com efeitos destrutivos ao tecido pulmonar [revisado em: (61,62)] (Figura 2).

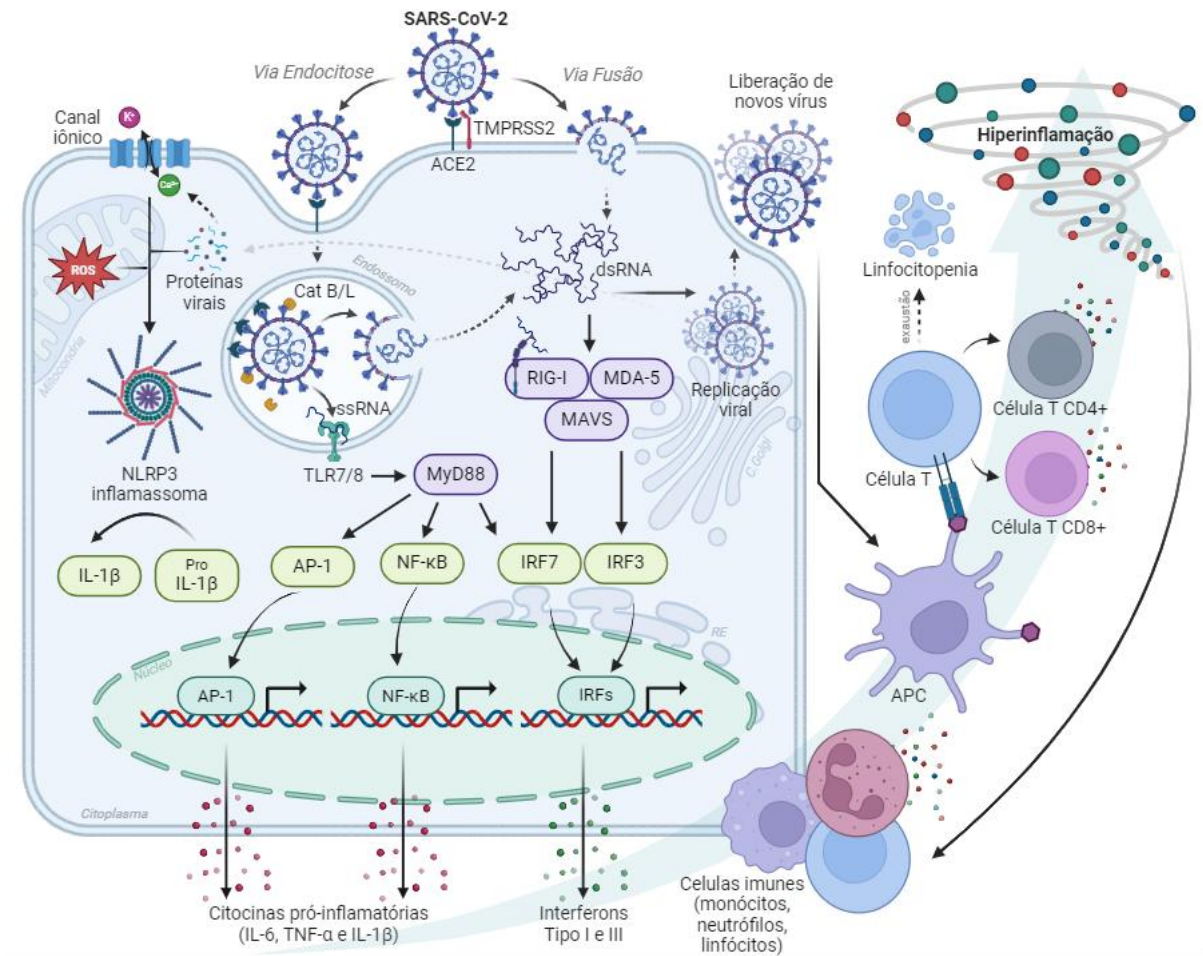


Figura 2: Patogênese molecular e celular do SARS-CoV-2. A entrada do SARS-CoV-2 na célula hospedeira acontece por meio da proteína viral estrutural *Spike* (S) que se liga ao receptor da enzima conversora de angiotensina 2 (ACE2). Se a célula alvo expressa de forma insuficiente a serina protease transmembrana tipo 2 (TMPRSS2) ou se o complexo vírus-ACE2 não encontrar a protease, o vírus é internalizado via endocitose e a proteína S é clivada pelas Catepsinas B/L (Cat B/L) no endossomo. Na presença de TMPRSS2, a clivagem da proteína S ocorre na superfície celular. Em ambas as vias de entrada, a fusão entre as membranas viral e celular permite a liberação do RNA viral no citoplasma da célula hospedeira para replicação. O vírus pode ser reconhecido pelos receptores TLR 7 e 8, sensores de RNA citosólico RIG-I/MDA-5 ou pelo receptor do tipo NOD com domínio pirina 3 (NLRP3). A sinalização destes receptores desencadeia a ativação dos fatores de transcrição AP-1, NF-κB e IRF-3 e 7 e à subsequente produção de citocinas pró-inflamatórias (por exemplo, IL-1 β , IL-6 e TNF- α) e IFN tipo I e III, respectivamente. As citocinas liberadas pelas células infectadas modulam a resposta imune adaptativa, causando recrutamento e ativação de macrófagos, células B e células T que facilitam a eliminação do vírus. No entanto, uma resposta imune desequilibrada pode causar liberação desenfreada de citocinas pró-inflamatórias, levando a uma hiperinflamação responsável pelas manifestações clínicas graves da covid-19. Fonte: Desenvolvido pelo autor.

Diversos estudos têm demonstrado que a hospitalização e o mal prognóstico da covid-19 estão correlacionados com altos níveis plasmáticos e/ou dos genes codificadores de IL-1 β , IL-1RA, IL-2, IL-6, IL-10, IL-18, IL-17A, CCL2, CCL3, CXCL9, CXCL10, G-CSF, M-CSF, IFN- γ , IP-10, MCP-1, MIP-1 α , IP-10, TNF- α , entre outros (58,60,63–65). Além disso, a hiperinflamação na covid-19 é conhecida por induzir um aumento da razão de neutrófilos para linfócitos (NLR), da razão NLR derivada (d-NLR, contagem de neutrófilos dividida pelo resultado da contagem de leucócitos menos a contagem de neutrófilos) e da razão de plaquetas para linfócitos (PLR), que contribuem para desfechos clínicos ruins (66).

Outro componente importante em infecções por SARS-CoV-2 é a resposta adaptativa celular mediada pelas células T. Sua proliferação e diferenciação contribuem para a remoção de células infectadas pelo vírus e ajudam na produção de anticorpos, principalmente nos períodos precoces da infecção (67) (Figura 3). De fato, alguns estudos já demonstraram que a presença de células T e anticorpos está associada à resolução bem-sucedida da covid-19 e à limitação da doença às suas formas mais leves (68,69). Normalmente, a circulação de células T CD4⁺ e CD8⁺ aumenta nas primeiras duas semanas a partir do surgimento dos sintomas, geralmente apresentando um fenótipo tipo 1 (Th1), que está associado a um controle viral eficaz (70). Um fenótipo tipo 2 (Th2) é frequentemente observado naqueles pacientes com a forma grave da doença (71,72). Contudo, essa subpopulação celular é particularmente afetada na fase aguda da covid-19. Pacientes com a forma grave da doença geralmente exibem uma diminuição severa de linfócitos periféricos, denominada linfopenia ou linfocitopenia (60,73–75) (Figura 3). Esse quadro é um biomarcador prognóstico da gravidade da doença e é caracterizado pela exaustão e subsequente perda de até 80% de células T periféricas (75–78). Os mecanismos subjacentes à linfocitopenia ainda não são totalmente compreendidos, mas a proliferação prejudicada de linfócitos, apoptose ou extravasamento celular para o tecido pulmonar são as hipóteses consideradas mais prováveis para explicar este fenômeno (79–81).

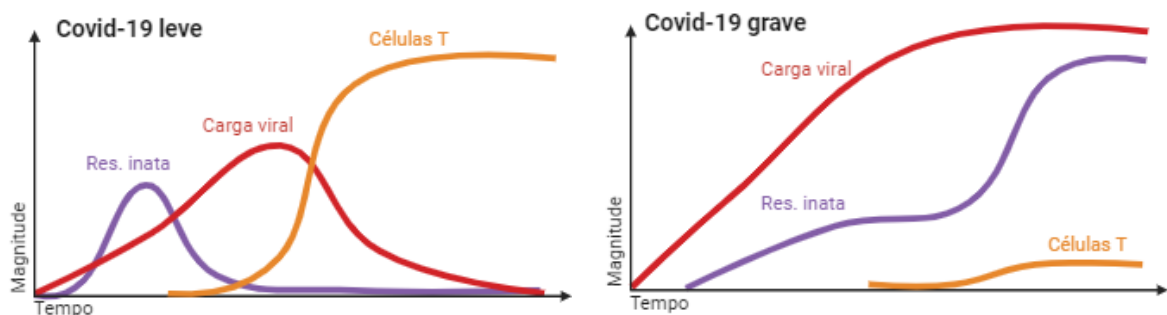


Figura 3: Dinâmica da resposta imune na covid-19. Modelização da cinética das respostas imunes ao SARS-CoV-2 em casos de covid-19 leve (que não requerem hospitalização) e grave. Fonte: Adaptado de (82).

Condição pós-covid

Além das manifestações agudas da covid-19, alguns indivíduos exibem sintomas persistentes mesmo após a eliminação do vírus (83). A gravidade destes sintomas e seu impacto na qualidade de vida da população levou a OMS a definir a “condição pós-covid” como uma nova entidade clínica que aparece em pacientes recuperados de sua fase aguda da covid-19, independentemente da gravidade da infecção inicial (84). Popularmente conhecida como covid longa, a condição é caracterizada pela continuação ou desenvolvimento de sintomas de um a três meses após a infecção inicial por SARS-CoV-2, com duração de pelo menos dois meses e sem diagnóstico alternativo (81,83).

Alguns estudos sugerem que cerca de 10-30% das pessoas que se recuperam de covid-19 leve podem desenvolver sintomas persistentes até mesmo meses ou anos após a infecção [revisado em: (85)]. O risco do desenvolvimento de síndromes pós-covid é maior em pessoas que foram hospitalizadas e em adultos mais velhos. As mulheres e pessoas em desvantagem socioeconômica também enfrentam um risco mais elevado, bem como fumantes, obesos e imunocomprometidos [revisado em: (86)]. A vacinação parece reduzir o risco, mas não previne totalmente as sequelas crônicas da infecção (87). A condição pós-covid apresenta desafios significativos para os sistemas de saúde e para o tratamento dos pacientes. A ausência de critérios de diagnóstico padronizados e a variabilidade dos sintomas dificultam a identificação e o manejo precoces da condição. Além disso, a falta de compreensão completa de seus mecanismos subjacentes dificulta o desenvolvimento de abordagens terapêuticas eficazes. O tratamento da condição pós-covid atualmente se concentra no alívio dos sintomas e no suporte paliativo aos pacientes (88).

Os mecanismos precisos por trás dos sintomas pós-covid ainda não estão completamente esclarecidos. Várias hipóteses foram propostas, incluindo a persistência viral, inflamação crônica, disfunção imunológica e desregulação autoimune (Figura 4). O desbalanço da resposta imune antiviral pelo SARS-CoV-2 pode favorecer a persistência de antígenos virais em reservatórios, provocando uma ativação inflamatória prolongada e autoimunidade. A inflamação persistente, desencadeada pela resposta imunológica inicial à infecção, pode causar danos contínuos aos órgãos e sistemas. A disfunção imunológica também pode estar ligada à produção excessiva de citocinas e à ativação prolongada das células imunes. Já a desregulação

autoimune se dá pelo aumento da produção de anticorpos autoimunes como produto da reação imune e inflamatória exacerbada. [revisado em: (85,86)].

Mais de 200 manifestações pós-covid aguda já foram reportadas, apresentando diferentes intensidades, frequências e durações, e acometendo vários órgãos do corpo humano [revisado em: (85)] (Figura 4). Os sintomas podem melhorar, agravar ou serem recorrentes ao longo prazo, com potencial evolução para eventos graves e potencialmente fatais, mesmo meses ou anos após a infecção inicial. Além dos sintomas respiratórios persistentes, como tosse e falta de ar, muitos pacientes relatam fadiga extrema, fraqueza muscular, dores de cabeça, palpitações cardíacas, distúrbios do sono, ansiedade, depressão e *brain fog* (névoa mental), caracterizado pela confusão mental, esquecimento constante e dificuldade de concentração (89). Esses sintomas podem ter um impacto substancial na qualidade de vida e na capacidade funcional dos indivíduos afetados.

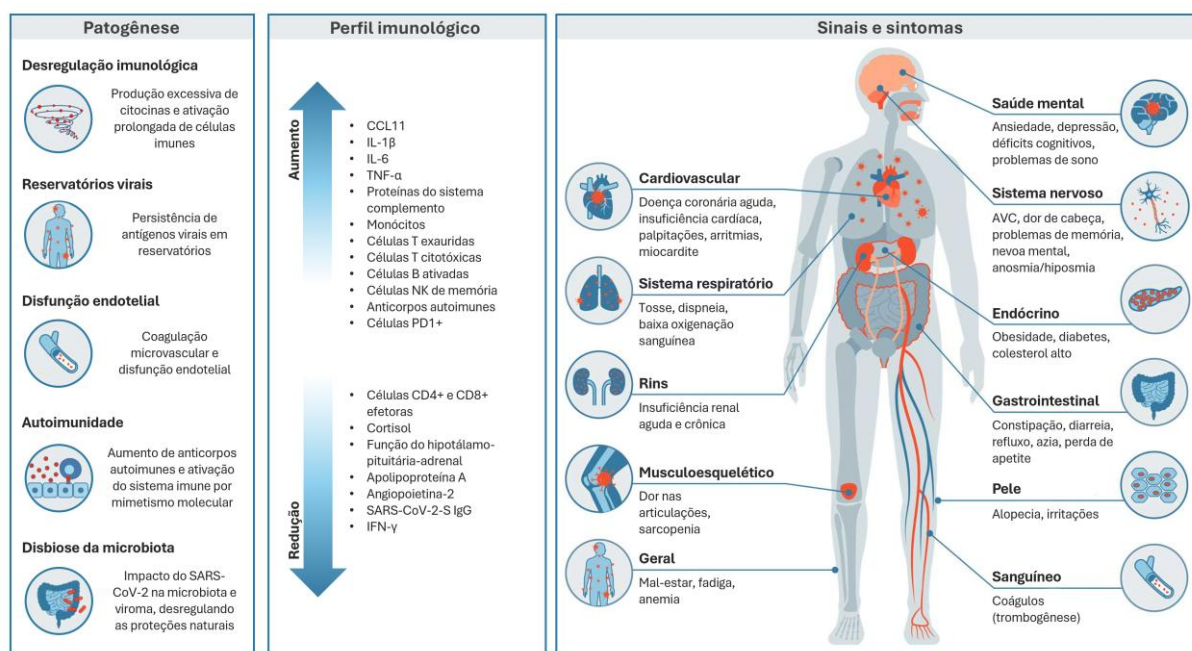


Figura 4: Patogênese, perfil imunológico e possíveis sintomas pós-covid. Postula-se que a patogênese da condição pós-covid se dá pela disfunção imunológica com ou sem reativação de patógenos subjacentes, como o herpesvírus, persistência de reservatórios de SARS-CoV-2 nos tecidos, coagulação microvascular com disfunção endotelial, impactos do vírus na microbiota, incluindo o viroma e/ou pela autoimunidade e ativação do sistema imunológico por mimetismo molecular. Pacientes com sintomas pós-covid aguda exibem um perfil imunológico caracterizado pela exaustão de células T, níveis elevados de citocinas pró-inflamatórias e anticorpos autoimunes e redução dos níveis de cortisol e IFN- γ . Os sintomas reportados variam amplamente e afetam diferentes sistemas do corpo. Além dos sintomas respiratórios persistentes, como tosse e falta de ar, muitos pacientes relatam fadiga extrema, fraqueza muscular, dores de cabeça, palpitações cardíacas, coagulopatias, distúrbios do sono, ansiedade, depressão e *brain fog*. Fonte: Elaborado pelo autor.

Os sintomas mais comuns, persistentes e debilitantes são os neurológicos, incluindo comprometimento cognitivo, parestesia, tontura e problemas de equilíbrio, sensibilidade à luz

e ao ruído, perda de olfato ou paladar e disfunção autonômica e sensório-motora (90–93). A deficiência cognitiva observada nesse contexto é comumente caracterizada por dificuldades de atenção e concentração, perda de memória, comprometimento da linguagem e déficits na função executiva, as quais são acompanhadas por alterações neuroanatômicas em indivíduos afetados (94). Um estudo realizado por pesquisadores do Reino Unido analisou imagens cerebrais de pacientes antes e após a infecção por covid-19 e indivíduos controle. Os resultados mostraram uma redução na espessura da substância cinzenta no córtex orbitofrontal e no giro parahipocampal, além de uma redução geral no tamanho do cérebro em pacientes após a covid-19 em comparação com os controles. Notavelmente, essas alterações também foram observadas em indivíduos infectados não hospitalizados (95). Os possíveis mecanismos subjacentes a essas neuropatologias envolvem processos de neuroinflamação, dano vascular induzido por coagulopatia e disfunção endotelial, além de dano neuronal (96).

Neuroinflamação

A neuroinflamação é definida como a ativação imunológica do sistema nervoso central (SNC) em resposta à estímulos inflamatórios. No contexto das alterações neurológicas pós-covid, ainda não está claro se a neuroinflamação é resultado da resposta imune persistente à infecção inicial por SARS-CoV-2 ou à neuroinvasão direta pelo vírus. Especula-se que o SARS-CoV-2 entra no SNC através do transporte retrógrado via terminações nervosas sensoriais da mucosa olfatória e regiões próximas, como o nervo olfatório, trigêmeo e o vago (97) - o que poderia explicar parcialmente a anosmia ou hiposmia observada em pacientes com covid-19-, além do tráfego sanguíneo, chegando ao cérebro através da barreira hematoencefálica (BHE) (98,99) ou pelas fenestras do plexo coroide (100). Contudo, a detecção limitada de RNA ou proteína viral no cérebro e no líquido cefalorraquidiano (LCR), além do baixo número de células infectadas pelo SARS-CoV-2 no SNC, enfraquecem a hipótese de que a neuroinflamação se deve à infecção direta pelo vírus (101–104).

Estudos que investigaram a desregulação imunológica em indivíduos afetados por sequelas neurológicas pós-covid identificaram a circulação aumentada de citocinas pró-inflamatórias como IL-1 β , IL-12, IL-6, TNF e IP-10, infiltração de macrófagos e linfócitos T CD8⁺ em regiões perivasculares, além da ativação microglial generalizada no cérebro, mesmo meses após a infecção inicial (102,103,105–107) (Figura 5). Além disso, um estudo recente apontou que o CCL11 é um biomarcador crítico de danos cerebrais de longo prazo em um modelo de camundongo com covid-19 leve (108). Também conhecido como eotaxina, CCL11 é um agente quimiotático implicado em diversas doenças neurodegenerativas associadas ao

envelhecimento, como Alzheimer e esclerose múltipla, e em distúrbios psiquiátricos como transtorno bipolar, depressão e esquizofrenia [revisado em: (109)]. Níveis elevados dessa quimiocina no SNC favorecem o estresse oxidativo e a morte neuronal por excitotoxicidade glutamatérgica, principalmente no hipocampo, comprometendo as funções de aprendizado e memória (110–112). Um dos principais mecanismos postulados para fundamentar os efeitos neurodegenerativos da CCL11 pós-covid envolve a microgliose no hipocampo, levando à diminuição da neurogênese, à deterioração dos axônios subcorticais mielinizados, e à redução da plasticidade cerebral com o aumento da poda sináptica (108) (Figura 5).

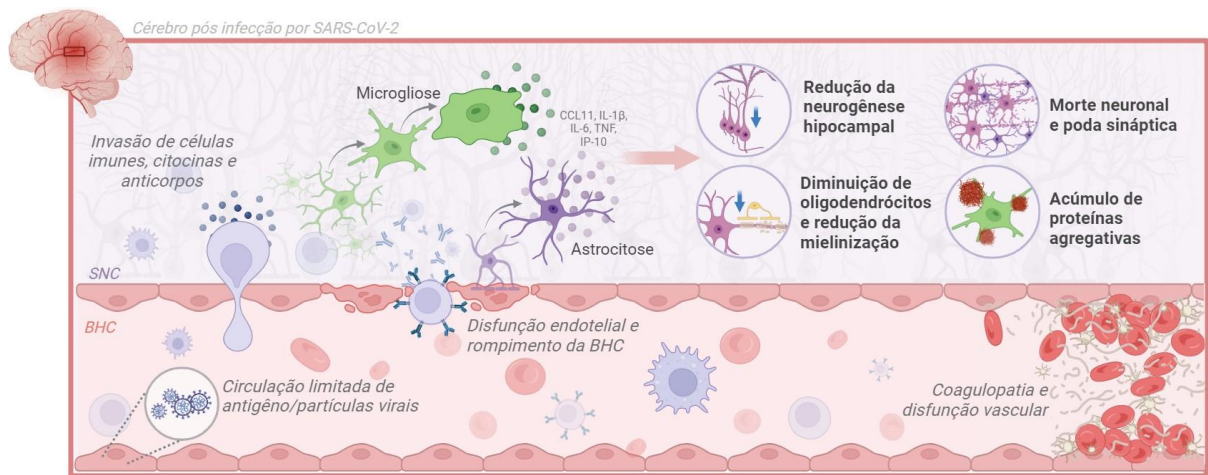


Figura 5: Neuroinflamação pós-covid. A desregulação imunológica causada durante a infecção inicial pelo SARS-CoV-2 fragiliza as barreiras do sistema nervoso central e favorece microgliose e astrocitose (reatividade e proliferação da micróglia e astrócitos). A neuroinflamação desencadeia uma série de processos que leva a neurodegeneração, diminuição da plasticidade cerebral e inibição da neurogênese. Fonte: Desenvolvido pelo autor.

Neste contexto, é crescente o número de evidências que apontam para a eficácia de intervenções voltadas para a regulação da resposta inflamatória e o suporte à neuroproteção na melhora do comprometimento cognitivo em pacientes com sequelas neurológicas pós-covid [revisado em: (113)].

Deficiência visuoespacial como condição pós-covid

As funções visuoespaciais são habilidades cognitivas que envolvem a capacidade de perceber visualmente objetos ou de desenvolver tarefas visuo-espaciais complexas. Essas habilidades são importantes para atividades cotidianas, como desenhar, montar ou organizar objetos. Os processos visuoespaciais envolvem várias áreas cerebrais de forma a integrar a percepção e interpretação da informação visual com a memória e os sistemas executivos (114). O comprometimento dessas funções é conhecido como deficiência visuoespacial e pode surgir

como uma manifestação precoce de doenças neurodegenerativas relacionadas ao envelhecimento, como a doença de Alzheimer e Parkinson [revisado em: (115,116)].

Recentemente, um estudo longitudinal conduzido por pesquisadores da Faculdade de Medicina da UFMG e da Fiocruz Minas Gerais, investigou o perfil inflamatório e o status cognitivo em indivíduos adultos que tiveram covid-19 leve (117,118). Os voluntários foram avaliados por testes neuropsicológicos, oculares e neurológicos, ensaio de marcadores imunológicos e por ressonância magnética estrutural e neuroimagem funcional 18FDG-PET com o objetivo de investigar possíveis alterações cerebrais e correlações clínicas. Aproximadamente 25% dos pacientes exibiram deficiência visuoespacial quatro meses após se recuperarem da infecção inicial (117). Os resultados revelaram uma associação entre o déficit cognitivo observado no teste de Figura Complexa de Rey-Osterrieth (ROCFT) e alterações cerebrais morfológicas e metabólicas específicas nos pacientes comprometidos. Essas alterações incluem redução do volume de substância branca em áreas cruciais, como o joelho do corpo caloso, o feixe do cíngulo, os giros fusiforme e lingual, o giro frontal inferior e os fascículos fronto-occipitais inferiores. Paralelamente, observou-se alterações no padrão do metabolismo de glicose em regiões como o giro do cíngulo anterior dorsal (hipermetabolismo), e como o giro temporal inferior esquerdo e o giro occipital inferior esquerdo (hipometabolismo) (117). As regiões afetadas desempenham papéis fundamentais na função visuoespacial, sendo essenciais para processos como integração visual-motora, planejamento espacial, memória visual e coordenação entre os hemisférios cerebrais (119,120). Os autores do estudo também constataram um aumento dos níveis de biomarcadores imunológicos periféricos como citocinas, quimiocinas e fatores de crescimento pró-inflamatórios, incluindo CCL11, em pacientes com déficits cognitivos. Curiosamente, os biomarcadores alterados se interconectam em uma rede funcional que leva a sinalização neuroinflamatória, sinalização de IL-17, além de morte celular e necrose hepática e cardíaca (117). Seis meses após a avaliação inicial, cerca de 10 meses após a recuperação da covid-19 leve, 26% dos pacientes avaliados foram diagnosticados com deficiência visuoespacial. Neste novo estudo, níveis elevados de CCL11, HGF e CXCL10 foram associados com a piora do status cognitivo dos pacientes (118).

Estes achados fornecem evidências da relação causal entre a neuroinflamação e a deficiência cognitiva persistente em pessoas que tiveram covid-19 leve, que representam uma fração grande e crescente da população mundial.

Regulação da expressão gênica na covid-19

O SARS-CoV-2 pode impactar a expressão gênica ao desestabilizar, degradar e alterar a transcrição de mRNA nas células, levando a mudanças na produção de proteínas que desempenham papéis importantes na resposta imune hospedeira e no controle viral. Essas interações entre o vírus e o genoma hospedeiro são críticas para a patogênese da covid-19 (121). Assim, a compreensão da regulação da expressão gênica durante a infecção pelo SARS-CoV-2 não só é essencial para elucidar a fisiopatologia da doença, como também fundamental para identificação de biomarcadores específicos relacionados à sua gravidade, ajudando no manejo dos pacientes, na previsão do prognóstico e sequelas pós infecção, e na identificação de potenciais alvos terapêuticos.

Pacientes com covid-19 exibem assinaturas transcricionais marcadas pela expressão elevada de genes associados a vias inflamatórias e de hipercoagulabilidade, e a inibição de genes de resposta antiviral por INF tipo I e III (121–123). Em casos mais graves da doença, são características assinaturas de ativação de neutrófilos, juntamente com as de depleção e exaustão de células T, contribuindo para piores desfechos clínicos (121,122,124). Pacientes recuperados da covid-19 apresentam mudanças persistentes nos perfis transcricionais favorecendo a disfunção da resposta imune mediada por células T, particularmente pelo aumento da expressão de PD1 em células de memória (125). Essa desregulação imune é acompanhada pela hiperexpressão dos genes codificadores da tríade pró-inflamatória IL1- β , IL-6 e TNF (126–128), e alteração da expressão de biomarcadores associados ao reparo e regeneração, como FGF2 e TGF- β [revisado em: (129)]. Na disfunção neurológica e sequelas cognitivas pós-covid, é observado o aumento da expressão de vários biomarcadores relacionados à neuroinflamação (CCL11, CXCL10, CXCL8, IL-17, IL-18, IL-6R e LGALS3), morte neuronal por apoptose (KLF4), fosforilação da proteína tau (FKBP5), e marcadores de ativação de células da glia (GFAP e EGFR) (108,130–133).

Os diversos mecanismos envolvidos na modulação da expressão genética pelo SARS-CoV-2, tanto na fase aguda da doença quanto nas condições pós infecção, ainda estão longe de ser totalmente compreendidos. Dentre eles, as modificações epigenéticas e mecanismos de *splicing* de mRNA surgem como objetos de interesse devido à sua capacidade de influenciar a expressão gênica de maneira significativa em infecções virais [revisado em: (134)].

Mecanismos epigenéticos

O termo epigenética refere-se a características que estão "acima de" ou "além de" a base genética tradicional para herança (135). Mudanças epigenéticas alteram a expressão gênica através de modificações químicas reversíveis na estrutura do DNA ou nas proteínas

constituintes da cromatina, sem a alteração do código genético e se perpetuam nas divisões celulares meióticas ou mitóticas (136). Sequências de RNA não codificantes adicionam uma camada mais complexa da regulação epigenética, controlando a transcrição através da modulação de proteínas que se ligam a regiões regulatórias no DNA. Essas mudanças permitem que as células respondam e se adaptem rapidamente a estímulos ambientais e outras exposições. Em infecções como a covid-19, a modulação da paisagem epigenética é um mecanismo fundamental para garantir a preparação, controle e memória das respostas imune do hospedeiro, sendo também uma ferramenta estratégica que o vírus utiliza para replicação e persistência nas células hospedeiras [revisado em: (137)]. A reprogramação epigenética causada na fase aguda da doença pode persistir mesmo após a resolução da infecção, contribuindo para sequelas a longo prazo (138) .

A metilação do DNA é uma das modificações epigenéticas mais estudadas e compreende a adição de um grupo metil (CH_3) à molécula de DNA. Em mamíferos, a reação é catalisada por enzimas metiltransferase (DNMT) e ocorre principalmente em dinucleotídeos de citosina sucedida por guanina (CpG), transformando-as em 5-metilcitosina (5mC) (139,140). Apesar de ocorrer amplamente na natureza em diferentes contextos de nucleotídeos, a metilação não-CpG é menos frequente em humanos e outros mamíferos, sendo observada particularmente em células-tronco embrionárias, células progenitoras hematopoiéticas e durante o desenvolvimento neural (141–144). Os sítios CpG que sofrem metilação são distribuídos por todo o genoma, formando aglomerados chamados ilhas CpG (Figura 6). A metilação destas regiões pode afetar a acessibilidade de fatores de transcrição e de RNA polimerases ao DNA. A hipermetilação de *enhancers* e promotores está associada ao silenciamento do gene correspondente, enquanto em outras regiões gênicas, como éxons e íntrons, o aumento da metilação pode favorecer ou inibir a transcrição [revisado em: (145)].

Vários genes que codificam as principais citocinas e quimiocinas com expressão sistêmica aumentada em pacientes com covid-19 (146,147) são regulados pela metilação de CpGs em seus promotores ou *enhancers* (148–154). Análises do perfil de metilação global de DNA de células mononucleares do sangue periférico de pacientes com covid-19, em particular os com a forma grave da doença, revelaram um padrão caracterizado pelo aumento da metilação de genes relacionados ao IFN e a diminuição na metilação genes inflamatórios e de citocinas (155). Esse achado corrobora os dados de expressão gênica que evidenciaram a supressão de genes da resposta antiviral mediada por IFN e aumento de genes pró-inflamatórios na covid-19 grave (124). Além disso, pesquisas têm demonstrado uma associação entre a metilação de DNA com a regulação do gene *ACE2*, codificador do receptor de entrada do SARS-CoV-2 nas células

hospedeira, sugerindo um elemento de risco para a covid-19. A hipometilação de CpGs na região promotora do gene *ACE2* nas células do pulmão, fígado e coração de pacientes com comorbidades está associada ao aumento de sua expressão nestes órgãos, tornando-os mais vulneráveis ao dano durante a covid-19 (156–158). Propõe-se que a diminuição da metilação global de DNA promovida pelo envelhecimento e condições inflamatórias associadas nestes pacientes favoreça a hipometilação de *ACE2* [revisado em: (159)].

Apenas alguns estudos publicados se concentram na observação de alterações no perfil de metilação de DNA em pacientes recuperados da covid-19, possivelmente devido ao tempo limitado de acompanhamento e ao alto custo das técnicas de análise do perfil de metilação do DNA. Resultados preliminares de Balnis *et al.* (160) sugerem que alterações nos padrões de metilação de DNA nos leucócitos circulantes induzidas pela covid-19 aguda podem persistir por pelo menos um ano. As marcas alteradas de forma persistente nessas células foram associadas a vias de respostas virais e inflamação. Análises do metiloma de amostras do sangue periférico de pacientes três meses após a recuperação da covid-19 revelaram a hipermetilação de genes relacionados a diferenciação de linfócitos e resposta imune adaptativa, além da hipometilação de genes associados a degranulação de neutrófilos, sinalização de fatores de crescimento endotelial vascular (*VEGF*) e diferenciação de leucócitos (161). Adicionalmente, quando confrontados com os dados de expressão gênica, os genes diferencialmente metilados em sua região promotora foram associados com o aumento da regulação da resposta imune humoral e da resposta imune por leucócitos (161). Estes resultados sugerem uma reação excessiva prolongada do sistema imunológico em decorrência à infecção inicial pelo SARS-CoV-2.

Uma segunda modificação epigenética de grande relevância nas infecções virais é a alteração nos aminoácidos que compõem as proteínas histonas. A cromatina é estruturada em repetições de nucleossomos, formados por um octâmero proteico composto pelas histonas H2A, H2B, H3 e H4, que se associam fisicamente a uma sequência de 147 nucleotídeos (162) (Figura 6). As histonas possuem caudas amino-terminais sujeitas a diversas reações químicas covalentes ou “marcas”, como acetilação, metilação, fosforilação e ubiquitinação. Essas modificações modulam o recrutamento de reguladores da cromatina, influenciando a condensação em eucromatina (mais acessível) ou heterocromatina (mais compacta), o que, por sua vez, controla a ativação ou repressão dos genes associados (162). O código das histonas é estabelecido e mantido por diversas enzimas modificadoras da cromatina, como histona acetiltransferases (HATs), desacetilases (HDACs), metiltransferases de histona (HMTs) e desmetilases (HDMs). Essas enzimas atuam de forma específica em locais da cromatina,

resultando em uma complexa hierarquia regulatória que controla a expressão gênica e a organização do genoma (162).

A resposta imune inata é extensivamente regulada por modificações específicas nas histonas. Já foi observado que a dimetilação na lisina 9 da histona H3 (H3k9me2) é um supressor de *IFN* e da resposta antiviral mediada por IFNs em fibroblastos e células dendríticas (163). H3k9me2 é uma marca epigenética repressiva da expressão gênica que controla processos de metilação de DNA e formação de heterocromatina (Figura 6). Além disso, vários componentes primários da resposta imune, como TLRs, possuem níveis constitutivamente elevados de trimetilação da lisina 4 na histona H3 (H3k4me3) e acetilação da histona H3 (H3ac), marcas características de promotores ativamente transcritos (Figura 6), em torno de seus sítios de iniciação da transcrição (164).

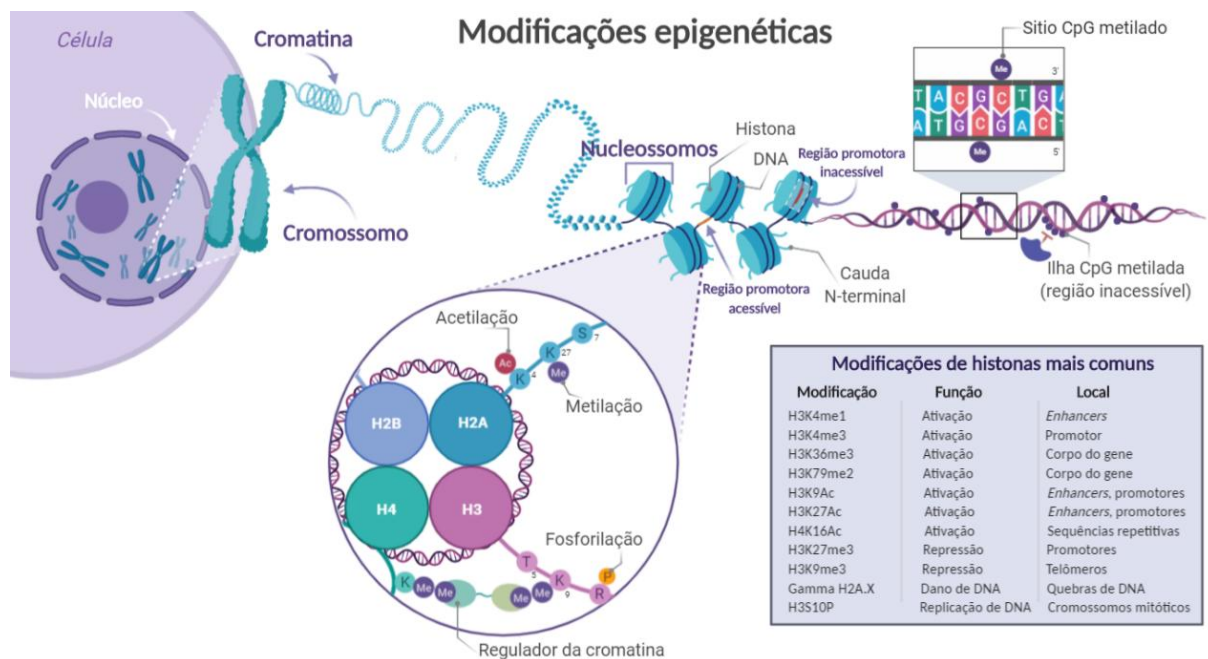


Figura 6: Modificações epigenéticas da cromatina por metilação do DNA e modificações das histonas. A expressão do gene é regulada antes da iniciação da transcrição pela modificação química do DNA ou das histonas que, juntas, formam a cromatina. A estrutura da cromatina depende da carga de suas histonas centrais, promovendo a formação de eucromatina (aberta e acessível a transcrição) ou de heterocromatina (compacta e inacessível). A transferência de um grupo metil em citosinas seguidas de guanina (CpG) forma a 5-metilcitosina, que forma pares de bases com guanina como a citosina, mas difere da base não modificada em termos de suas interações com proteínas regulatórias. Fonte: Elaborado pelo autor.

Uma análise do epigenoma de monócitos CD14⁺ de pacientes hospitalizados com covid-19 revelou alterações significativas na cromatina durante a doença (165). Pacientes com desfecho fatal apresentaram regiões cromatínicas menos acessíveis em comparação com aqueles que se recuperaram da infecção. Essas diferenças foram associadas a mudanças na

expressão gênica afetando vias biológicas específicas, como a resposta ao interferon e o metabolismo do ATP. Além disso, observou-se que as mudanças na acessibilidade da cromatina estavam relacionadas à gravidade da doença, com casos críticos apresentando menor estabilidade genômica (165).

Coronavírus como o MERS-CoV conseguem escapar da resposta imune antiviral mediada por ISGs através do aumento nos níveis de trimetilação na lisina 27 da histona H3 (H3K27me3) e redução dos níveis de H3K4me3 na região promotora destes genes (166). No contexto da covid-19, além da metilação de DNA, a expressão de *ACE2* parece ser regulada pela modificação de histonas. Em particular, HDACs aumentam a expressão de *ACE2*, favorecendo a entrada do SARS-CoV-2 *in vitro* (167). Em estudo de meta-análise incluindo dados do transcriptoma do pulmão de pacientes com comorbidades associadas a covid-19, foi observado uma correlação positiva entre o aumento da expressão de *ACE2* e a expressão de genes codificadores de enzimas epigenéticas (168). Corroborando esses achados, foi identificado um aumento da acetilação da lisina 27 na histona H3 (H3k27ac) e da mono- e trimetilação da lisina 4 na histona H3 (H3K4me1 e H3K4me3) no *locus ACE2*, sugerindo uma regulação epigenética do gene no pulmão a favor de sua superexpressão.

Essas evidências, à luz do conhecimento prévio sobre a regulação epigenética de genes implicados na tempestade inflamatória da covid-19, aponta para o potencial terapêutico de fármacos capazes de modular o panorama epigenômico, denominados epifármacos, como estratégia para o controle da inflamação exacerbada decorrente da infecção pelo SARS-CoV-2.

Vitamina B12 como regulador da expressão gênica

A vitamina B12, também conhecida como cobalamina (Cbl), é um micronutriente essencial, que, uma vez absorvido, liga-se à transcobalamina II (TC-II) e é transportado para as células através de receptores específicos (169). No citoplasma a vitamina é liberada e convertida em suas formas ativas, adenosilcobalamina, que atua no ciclo de Krebs na reação anaplerótica de conversão de metilmalonil-CoA em succinil-CoA; e metilcobalamina, cofator da metionina sintase (MS) na conversão de metionina em homocisteína (HCY) na via dos aminoácidos sulfurados (170).

A B12 desempenha papéis cruciais em diversos processos celulares, incluindo a síntese de DNA, formação de células do sangue, metabolismo energético e de lipídeos, prevenção do estresse oxidativo, mielinização de neurônios e síntese de neurotransmissores (169,170). Outra função particularmente relevante é seu papel na expressão gênica, onde a vitamina B12 influencia processos dependentes de metil. Ao participar da via de aminoácidos sulfurados, ela

promove a produção de S-adenosilmetionina, doador universal de metil para mais de 100 diferentes substratos, incluindo DNA, RNA, aminoácidos/proteínas e lipídeos (170,171) (Figura 7). O funcionamento adequado do sistema nervoso central, do metabolismo energético e dos mecanismos imunológicos, bem como da expressão gênica requer níveis adequados da vitamina, e sua deficiência pode levar a doenças neurodegenerativas e estresse oxidativo (170,171).

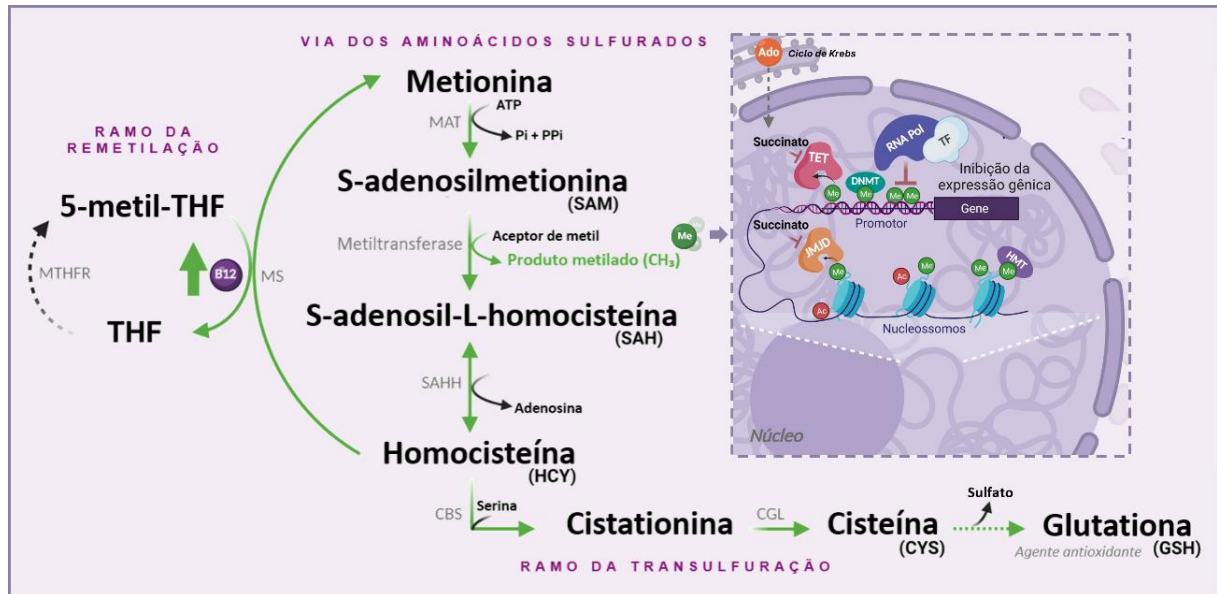


Figura 7: Metabolismo da vitamina B12 e o metabolismo celular. A metilcobalamina (MeCbl) é originária da degradação da metionina sintase (MS) na via dos aminoácidos sulfurados. Os produtos de metil gerados pela conversão de S-adenosilmetionina (SAM) para S-adenosilhomocisteína abastecem reações de metilação de DNA e de histonas, regulando a expressão gênica. O succinato produzido no ciclo de Krebs com a participação da Adocobalamina pode inibir enzimas moduladoras da metilação de DNA e histonas. Ado = Adocobalamina; ATP = Adenosina trifosfato; CBS = *Cystathionine beta-synthase*; CGL = *Cystathionine gamma-lyase*; DNMT = DNA metiltransferase; JMJD = *Jumonji C domain-containing* (desmetilase de histonas); MAT = *Methionine adenosyltransferase*; MCM = *Methylmalonyl-CoA mutase*; MS = *Synthionina synthase*; MT = Metiltransferase; MTHFR = 5,10-metilenotetrahidrofolate reductase; Pi = Fosfato (inorgânico); PPi = Pirofosfato; RNA pol = RNA Polimerase; SAHH = *Adenosylhomocysteinase* e TET = *Ten-eleven translocation methylcytosine dioxygenases*. (desmetilases de DNA); TF = *Transcription fator* (Fator de transcrição). Fonte: adaptado de: Cassiano, L.M.G et al. (2023) (171).

A capacidade da vitamina B12 de influenciar a metilação tem implicações na regulação epigenética e na expressão gênica (171). A metilação do DNA, especialmente em regiões promotoras, geralmente resulta no silenciamento de genes. Além disso, a metilação de histonas pode alterar a estrutura da cromatina, afetando a acessibilidade de fatores de transcrição ao DNA (145,172–174). Já foi demonstrado que a terapia adjuvante com B12 aumenta a metilação de DNA e de resíduos na cauda de histonas, reduzindo a expressão de mediadores inflamatórios no sistema nervoso central e favorecendo a neuroproteção em ratos infantes com meningite pneumocócica (175,176).

Alterações nos padrões de metilação do DNA também podem levar a mudanças no *splicing* alternativo [revisado em: (177)]. A relação entre estes dois mecanismos adiciona uma camada extra de complexidade à regulação gênica. Isso permite um controle fino da expressão de isoformas específicas de proteínas em diferentes contextos celulares. Estudos já demonstraram que existe uma forte associação entre a metilação do DNA e o *splicing* alternativo (178) (Figura 8). A desmetilação do DNA no corpo do gene resulta em eficiências reduzidas no alongamento ou *splicing* de transcritos. Éxons, especialmente em locais de *splicing*, têm níveis mais altos de metilação de DNA do que íntrons, e o *splicing* de cerca de 22% dos mRNAs alternativos é regulado pela metilação de DNA (177).

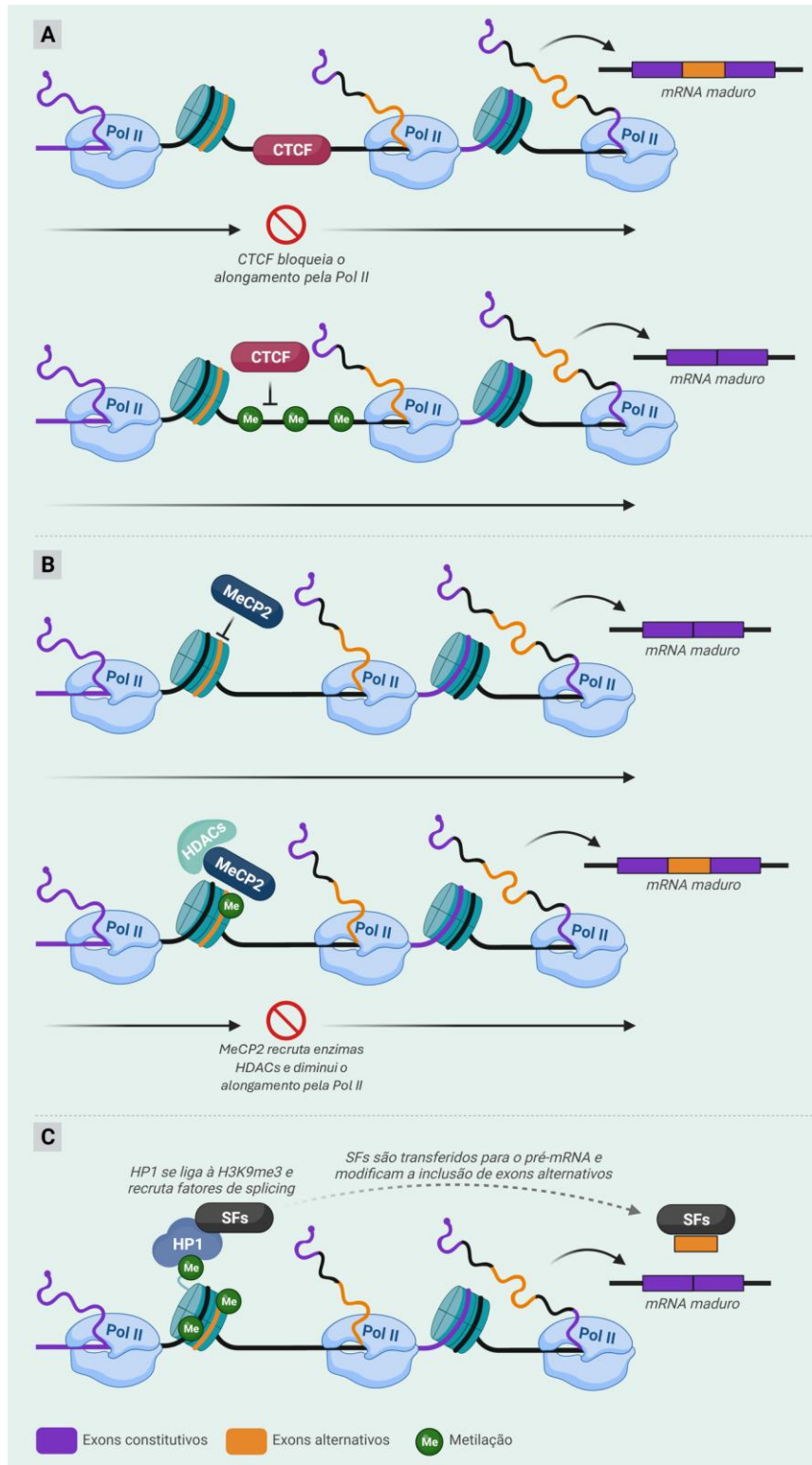


Figura 8: Metilação do DNA e o *splicing* alternativo. (A) Parte superior, quando o DNA não está metilado, o CTCF se liga a jusante do éxon alternativo e cria um bloqueio para o alongamento pela Pol II que resulta na inclusão do éxon alternativo no mRNA maduro. Parte inferior, a metilação do DNA a jusante do éxon alternativo impede a ligação do CTCF e promove o alongamento rápido da Pol II e o salto do éxon no mRNA maduro. (B) Parte superior, na ausência de metilação do DNA, MeCP2 não se liga ao éxon alternativo, o que permite o alongamento rápido de Pol II e leva ao salto de éxon no mRNA maduro. Parte inferior, a metilação do éxon alternativo faz com que o MeCP2 se ligue ao éxon alternativo e recrute enzimas com atividade HDAC, o que retarda o alongamento da Pol II e resulta na

inclusão do éxon no mRNA maduro. (C) A metilação estimula a modificação de histonas H3K9me3. HP1 liga-se a esta modificação e recruta fatores de *splicing* (SFs). Durante a transcrição, os SWI/SNF s são transferidos para o pré-mRNA e modificam a inclusão do éxon alternativo. Abreviações: H3K9me3 = Trimetilação na lisina 9 da histona H3; HDAC = histona desacetilase; Pol II = RNA polimerase II. Fonte: adaptado de Lev Maor, G., Yearim, A., & Ast, G. (2015) (177).

Mecanismos diferentes podem transmitir informações de metilação de DNA para a regulação do *splicing* alternativo. Por exemplo, a modulação da taxa de alongamento da RNA polimerase II (Pol II) pelo fator de ligação CCCTC (CTCF) (179) e pela proteína de ligação metil-CpG 2 (MeCP2) (180), e a formação de uma ponte proteica pela proteína 1 da heterocromatina (HP1), que recruta fatores de *splicing* para transcrição alternativa de éxons (181). A metilação do DNA também é frequentemente associada a modificações específicas de histonas, que podem afetar a acessibilidade da cromatina e o recrutamento de fatores de *splicing* (182).

Diante da vasta gama de propriedades protetoras da vitamina B12 e seu potencial como modulador da expressão gênica, ela é um promissor candidato para terapias no controle de doenças associadas a desregulações imunológicas inclusive em resposta a infecções.

Splicing alternativo

O *splicing* é um mecanismo fundamental para a regulação da diversidade proteômica em eucariotos visto que, através do *splicing* de um único RNA, são gerados mRNAs que codificam isoformas de proteínas com estrutura e funções distintas (183). Este processo ocorre após o capeamento do pré-mRNA e antes a poliadenilação. À medida que a RNA polimerase move-se no gene, o pré-mRNA sofre a remoção de seus íntrons pelo spliceossomo, complexo formado por ribonucleoproteínas (snRNPs) e precursores de mRNA capazes de reconhecer as sequências onde o *splicing* deve ser realizado e de juntar os éxons [revisado em:(184)]. No *splicing*, reações de transesterificações unem dois éxons, enquanto o íntron entre eles é removido como um laço. Em um mRNA típico com múltiplos éxons, o *splicing* pode ser modulado de diversas formas, resultando em uma variedade de produtos (185). A esse processo se dá o nome de *splicing* alternativo [revisado em: (186)] (Figura 9).

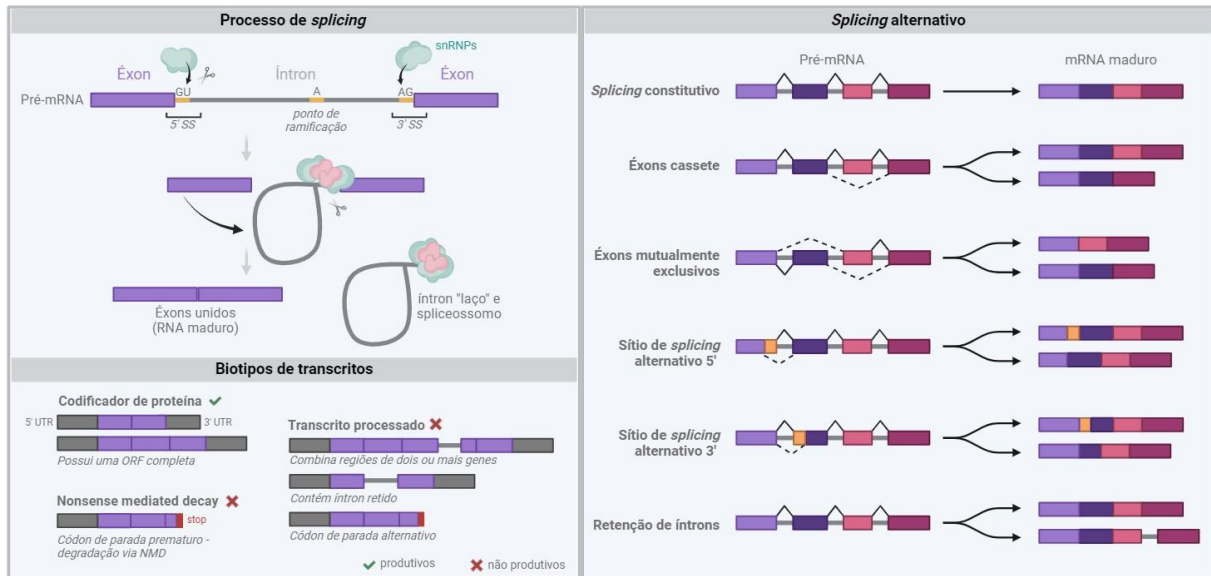


Figura 9: Mecanismo de *splicing* e seus produtos. O processo de *splicing* envolve a remoção de íntrons e a união de éxons do pré-mRNA pelo complexo do spliceossomo, resultando na formação do mRNA maduro, um passo crucial para a expressão gênica em eucariotos. Através do *splicing* alternativo, diferentes combinações de éxons podem ser selecionadas, gerando múltiplas isoformas de mRNA a partir de um único gene, o que aumenta significativamente a diversidade do transcriptoma e do proteoma. Este processo pode gerar transcritos produtivos, que resultam em proteínas funcionais completas, ou transcritos não produtivos. Estes últimos incluem transcritos com intron retido, que contêm sequências intrônicas que não foram removidas; transcritos com códon de parada prematuro (NMD), que são sujeitos à degradação pela via *nonsense mediated decay*; e transcritos sem ORF (janela de leitura aberta). Fonte: Elaborado pelo autor.

A maioria dos éxons são constitutivos, sendo sempre incluídos no mRNA maduro para manter a integridade da sequência codificante. No entanto, existem éxons regulados, conhecidos como éxons cassete, que podem ser incluídos ou excluídos do mRNA final, frequentemente na extremidade 3' (Figura 9). Em casos particulares, múltiplos éxons cassete podem ser mutuamente exclusivos, gerando RNAs que contêm apenas um dos vários éxons possíveis. O comprimento dos éxons também pode ser modificado através da alteração dos sítios de *splicing*, tanto na extremidade 5' quanto na 3'. Os éxons terminais podem ser intercambiados utilizando promotores alternativos (na extremidade 5') ou sítios de poliadenilação alternativos (na extremidade 3'), em conjunto com o *splicing* alternativo. Outro evento regulatório importante é a retenção de íntrons, onde um intron não é removido durante o processamento do mRNA. Isso pode ocorrer devido à fraqueza nos sinais de *splicing*, regulação por proteínas que se ligam ao RNA ou pela formação de estruturas secundárias do RNA que interferem no processo. A retenção pode levar à produção de proteínas com funções alteradas ou à degradação do mRNA via *Nonsense-Mediated Decay* (NMD) [revisado em: (187,188)]. Essa flexibilidade no processamento do mRNA gera uma gama de biotipos de transcritos que contribui significativamente para a diversidade proteica e a complexidade

funcional dos organismos, especialmente em eucariotos superiores (185). Os transcritos codificadores de proteína (*protein coding*) contemplam transcritos detentores de uma janela aberta de leitura (*Open reading frame* – ORF) completa, possibilitando a leitura do mRNA e tradução em uma proteína (189,190). Os transcritos que apresentam códons de parada prematuros são denominados de NMD, que surgem quando um evento de *splicing* introduz um códon de parada que está localizado mais de 50-55 nucleotídeos a montante da última junção éxon-éxon (189,190), desencadeando uma sinalização para degradação do transcrito produzido. Este mecanismo ajuda a prevenir a produção de proteínas truncadas que poderiam ser prejudiciais à célula (191). Os transcritos processados referem-se a RNAs que passaram por processamento pós-transcricional, mas não necessariamente codificam proteínas por não conterem uma ORF. Esses transcritos podem incluir RNAs longos não codificantes (lncRNAs) e aqueles com íntrons retidos (189,190). Embora esses transcritos processados tecnicamente possam não ser traduzidos em proteínas, eles podem desempenhar funções regulatórias importantes (192).

Sabe-se que vários vírus sequestram elementos do mecanismo de *splicing* para favorecer sua replicação e evitar as respostas antivirais do hospedeiro. Já foi observado que algumas proteínas do SARS-CoV-2 interagem com o complexo do spliceossomo celular, inibindo o *splicing* (193,194). Por outro lado, a infecção viral pode induzir vários *splicings* alternativos de RNAs do hospedeiro que se traduzem em proteínas alteradas, que são críticas para diversos processos, principalmente para resposta imune e ao estresse (195). Apesar de muitos estudos terem investigado os efeitos causais da expressão de RNA e proteínas circulantes na covid-19, o impacto das diferentes isoformas de transcritos resultantes do *splicing* alternativo na patogênese e desfechos da doença ainda permanece subexplorado.

Um estudo recente desenvolvido na Universidade de McGill, Canadá, demonstrou que o *splicing* alternativo de genes específicos expressos no pulmão e no sangue total estão relacionados com a susceptibilidade (*MUC1* e *PMF1*) à covid-19 e sua gravidade (*OAS1*, *ATP11A*, *DPP9* e *NPNT*) (196). Wang e colaboradores (197) observaram uma extensa desregulação do *splicing* alternativo nas células do pulmão de pacientes com desfecho fatal na covid-19, alterando a abundância relativa de diferentes isoformas de genes que são críticos para a defesa do hospedeiro. Em particular, observaram-se perdas na sequência de aminoácidos e consequente comprometimento de domínios funcionais em decorrência da exclusão de éxons durante o *splicing* em indivíduos com covid-19. Este fenômeno foi identificado em genes imunoreguladores importantes como *CD74* e *LRRFIP1*, promovendo a disfunção da imunidade antiviral. Além disso, os autores também observaram que a isoforma canônica de *IL6* não estava

aumentada nas células destes pacientes. Essa evidência sinaliza que terapias anti-IL-6 podem não ser efetivas para o tratamento de casos mais graves ou avançados da doença, como já foi observado nos resultados preliminares de ensaios clínicos com Tocilizumabe (198,199). Um estudo realizado por Dias e colaboradores demonstrou, *in vitro*, que o SARS-CoV-2 utiliza da maquinaria de *splicing* hospedeira para o escape da resposta imune antiviral. Observando o transcriptoma de células epiteliais do pulmão infectadas com o vírus constatou-se uma expressão aumentada de isoformas de transcritos não codificadoras de genes relacionados à sinalização do IFN, ISGs, MHC de classe I e genes do complexo do spliceossomo, incluindo *IRF7*, *OAS3*, *HLA-B* e *HNRNPH1*(200).

Com estas evidências, o estudo do *splicing* alternativo na covid-19 tem se mostrado importante para compreender os mecanismos moleculares subjacentes à infecção por SARS-CoV-2 e suas consequências clínicas. A investigação desses mecanismos não apenas amplia a compreensão da patogênese viral, mas também oferece perspectivas para o desenvolvimento de novos biomarcadores diagnósticos e alvos terapêuticos.

Modelos de infecção por SARS-CoV-2 para estudos da covid-19

Diferentes modelos animais e celulares têm sido empregados para mimetizar a infecção por SARS-CoV-2 e sua fisiopatologia em condições experimentais. Cada modelo apresenta vantagens e desafios únicos, contribuindo de maneira distinta para o avanço do conhecimento sobre a doença. Sua escolha depende do problema biológico específico a ser abordado e de critérios dependentes do contexto da pesquisa, como acesso, recursos e objetivo [revisado em: (201,202)].

Os modelos *in vivo*, que utilizam animais como camundongos transgênicos expressando o receptor ACE2, hamsters sírios dourados, furões e primatas não-humanos, oferecem a vantagem de reproduzir a complexidade do organismo humano e permitir o estudo da progressão da doença em tempo real. Esses modelos são particularmente úteis para avaliar respostas sistêmicas à infecção, especialmente a longo prazo e fundamentais para testar potenciais terapias. No entanto, enfrentam desafios como diferenças fisiológicas intrínsecas entre animais e humanos, questões éticas relacionadas ao uso de animais em pesquisa, além de custos elevados, necessidade de infraestrutura especializada e otimização (203).

Por outro lado, os modelos *in vitro*, que incluem culturas primárias e 3D de linhagens celulares imortalizadas como Vero E6 e Calu-3, e organoides, permitem um controle preciso das condições experimentais e facilitam a análise de mecanismos moleculares específicos. Esses modelos são essenciais para estudos moleculares detalhados e triagem de biomarcadores

e terapias. Contudo, possuem limitações na reprodução da complexidade do organismo inteiro e das interações celulares *in vivo* (204,205).

Os modelos *ex vivo*, como culturas de explantes pulmonares humanos (206,207) e de outros tecidos derivados de pacientes [revisado em: (208)], oferecem um equilíbrio entre a complexidade dos sistemas *in vivo* e o controle dos sistemas *in vitro*. Eles preservam a arquitetura, interações e programações celulares do tecido original, permitindo o estudo de respostas específicas do tecido à infecção. No entanto, são restritos pelo tempo limitado de viabilidade do tecido, dificuldade em obter amostras de tecidos humanos e variabilidade interindividual dos doadores [revisado em: (201,209)].

Dentre os modelos *ex vivo*, a cultura de sangue total de pacientes oferece vantagens significativas e contorna alguns desafios típicos dessa categoria para o estudo da covid-19. A técnica envolve a cultura de sangue total recém-coletado de pacientes ou doadores saudáveis, permitindo o estudo da resposta imune em um ambiente controlado. Em culturas de sangue total, uma amostra de sangue é diluída com meio de cultura de células e pode ser testada em exposição a diversos estímulos, como drogas ou antígenos [revisado em: (210)]. Essa abordagem preserva as proporções e interações naturais de diferentes tipos de células sanguíneas, incluindo glóbulos vermelhos, glóbulos brancos e plaquetas, bem como componentes plasmáticos como anticorpos e citocinas. Outro ponto crucial é que este método é menos invasivo e agressivo para os doadores. Enquanto outros modelos *ex vivo* podem exigir biópsias ou procedimentos mais complexos para obtenção de amostras, a cultura de sangue total requer apenas uma simples coleta de sangue. Isso não só facilita a adesão dos pacientes aos estudos, mas também permite coletas repetidas ao longo do tempo para acompanhamento da evolução da doença e seus diferentes estágios. A acessibilidade deste modelo também se estende ao aspecto econômico, pois são geralmente menos onerosas em comparação com outros modelos que podem exigir infraestruturas mais caras, tornando o modelo particularmente valioso em contextos de pesquisa com recursos limitados.

A fim de contornar as limitações da técnica de preparação e isolamento de células mononucleares do sangue periférico, a qual requer grandes volumes de amostras de sangue, desde o início da década de 1980 diversos estudos têm investigado o sistema imunológico de humanos utilizando culturas de sangue total. O modelo foi inicialmente utilizado para medição da proliferação de linfócitos, produção de citocinas e liberação de receptores de citocinas em resposta ao exercício físico e dieta (211–214), câncer (215), infecção pelo vírus da imunodeficiência humana (HIV) (216) e pelo vírus do herpes (217), além e outras condições.

É importante ressaltar, no entanto, que como qualquer modelo, as culturas de sangue total também têm limitações. Por exemplo, elas não podem replicar completamente as condições específicas de órgãos afetados pela covid-19, como os pulmões. Além disso, o tempo limitado de viabilidade da cultura (geralmente alguns dias) pode restringir estudos de respostas de longo prazo. Apesar dessas limitações, a cultura de sangue total de pacientes permanece como uma ferramenta singular no arsenal de modelos para estudo da covid-19. Sua capacidade de capturar a complexidade da resposta imunológica, combinada com sua relativa simplicidade e acessibilidade, faz dela um modelo *ex vivo* particularmente robusto e informativo. Este modelo não apenas complementa outros métodos de estudo, mas também fundamenta o avanço da compreensão da doença e o desenvolvimento de terapias mais eficazes e personalizadas.

JUSTIFICATIVA

A covid-19 é uma condição complexa e que ainda representa um grande desafio para a saúde pública. Embora tenham sido feitos avanços significativos em termos de compreensão da infecção e desenvolvimento de vacinas e tratamentos, muitas questões sobre a fisiopatologia da doença em resposta às diferentes variantes virais e seus impactos a longo prazo continuam a surgir. Neste contexto, o presente estudo busca explorar os mecanismos moleculares subjacentes a infecção aguda da covid-19, bem como os efeitos prolongados e persistentes da doença, particularmente o comprometimento cognitivo, além de investigar o potencial da vitamina B12 como um epifármaco para controlar a hiperinflamação na covid aguda e a neuroinflamação associada à deficiência visuocostrutiva pós-covid leve. A hipótese central é que a B12 pode modular a via dos aminoácidos sulfurados, aumentando a produção de SAM, metabólito importante para a regulação epigenética. O aumento da capacidade metiladora das células pela maior concentração de SAM levaria à hipermetilação dos promotores de genes de inflamação nos leucócitos, que se encontram hipometilados na covid-19 e em pacientes com deficiência visuocostrutiva após se curarem da infecção leve, atenuando a inflamação. Esta hipótese foi testada utilizando o modelo *ex vivo* de cultura de sangue total colhido de pacientes e voluntários controle.

CAPÍTULO I: Vitamina B12 como neuroprotetor na neuroinflamação

O seguinte trabalho foi publicado como capítulo no livro "*Vitamins and Minerals in Neurological Disorders*" [<https://doi.org/10.1016/B978-0-323-89835-5.00038-7>], em 2023 pela editora *Academic Press* (Elsevier). O capítulo aborda aspectos importantes sobre os mecanismos de ação da vitamina B12 na proteção do sistema nervoso contra processos inflamatórios e destaca o potencial da vitamina B12 como epifármaco em neuroinflamações e doenças neurodegenerativas. Foi discutido o papel da vitamina B12 na regulação do metabolismo energético, modulação da resposta imune, prevenção do estresse oxidativo e modulação da expressão gênica associada à neuroinflamação por meio de alterações na paisagem epigenética das células.

Vitamin B12 as a neuroprotectant in neuroinflammation

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Abbreviations

2-OGDD	2-oxoglutarate-dependent dioxygenase
3-MST	3-mercaptopyruvate sulfurtransferase
AdoCbl	adenosylcobalamin
ALS	amyotrophic lateral sclerosis
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP-1	activator protein-1
Apo E^{-/-}	apolipoprotein E deficient
APP	amyloid precursor protein
ATP	adenosine triphosphate
Aβ	β -amyloid peptide
BACE	beta-site APP cleaving enzyme
Cbl	cobalamin
CBS	cystathionine beta-synthase

CCL3	chemokine (C-C motif) ligand 3
CGL	cystathionine gamma-lyase
CNCbl	cyanocobalamin
CNS	central nervous system
DNMT	DNAmethyltransferase
FADH₂	flavin adenine dinucleotide
GSCbl	glutathionylcobalamin
GSH	glutathione
GSSG	glutathione disulfide
GTP	guanosine triphosphate
H₂O₂	hydrogen peroxide
H₂OCbl	aquacobalamin
H₂S	hydrogen sulfide
HAT	histone acetyltransferase
HCY	homocysteine
HDAC	histone deacetylase
HDM	histone demethylase
HHCY	hyperhomocysteinemia
HIF	hypoxia inducible factor
HMT	histone methyltransferase
HoCbl	hydroxycobalamin
HS⁻	hydrosulfide anion
IL	interleukin
JMJD	jumonji C domain-containing
MCM	methylmalonyl-CoA mutase
MeCbl	methylcobalamin
MET	methionine
MS	methionine synthase
MTHFR	5,10-methylenetetrahydrofolate reductase
NADH	nicotinamide adenine dinucleotide
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NMDA	N-methyl D-aspartate
NMDAR	N-methyl D-aspartate receptor

NO	nitric oxide
O₂⁻	superoxide radical
PHD	prolyl-hydroxylases
PKC	protein kinase C
PP2A	protein phosphatase 2A
<i>PSEN1</i>	presenilin 1
RNS	reactive nitrogen species
ROS	reactive oxygen species
SAM	S-adenosylmethionine
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
TC	transcobalamin
TET	ten-eleven translocation methylcytosine dioxygenase
TNF-α	tumor necrosis factor alpha

Introduction

The term vitamin B12 refers to a set of water-soluble organic compounds with a cobalamin (Cbl) in their structures. These vitamers are adenosylcobalamin (AdoCbl), cyanocobalamin (CNCbl), hydroxycobalamin (HoCbl), and methylcobalamin (MeCbl). Humans acquire B12 through diet, where it is usually found in animal products such as meat, eggs, milk, and dairy products. In plasma, vitamers form a complex with trans cobalamin (TC) protein types I, II, or III. Although it is not the most abundant plasma TC, type II (TCII) is essential for transferring Cbl into cells through interaction with specific receptors. After endocytosis of the complex, TCII is degraded in lysosomes and Cbl is released to the cytoplasm, where it can be transformed into its active forms AdoCbl and MeCbl. These vitamers act as cofactors of mitochondrial methylmalonyl CoA mutase (MCM) and cytosolic methionine synthase (MS), respectively. MCM catalyzes the conversion of L-methylmalonyl-CoA into succinyl-CoA, an intermediate in the Krebs cycle, which is pivotal for energy metabolism, and MS, in the sulfur amino acids pathway, transfers methyl between 5-methyl-tetrahydrofolate and homocysteine (HCY) forming methionine (MET), which is required for synthesis of proteins, DNA, and neurotransmitters. MET is then converted into S-adenosylmethionine (SAM), the universal methyl donor (Fig. 1). Furthermore, Cbl is also involved in processes such as myelination and neurotransmitter synthesis, combat to oxidative stress and immune response regulation (Peterson, Rodionov, Osterman, & Peterson, 2020). Therefore, maintaining adequate levels of

this vitamin is essential for proper functioning of the nervous system and its deficiency can lead to neuroinflammation-associated disorders (Hunt, Harrington, & Robinson, 2014).

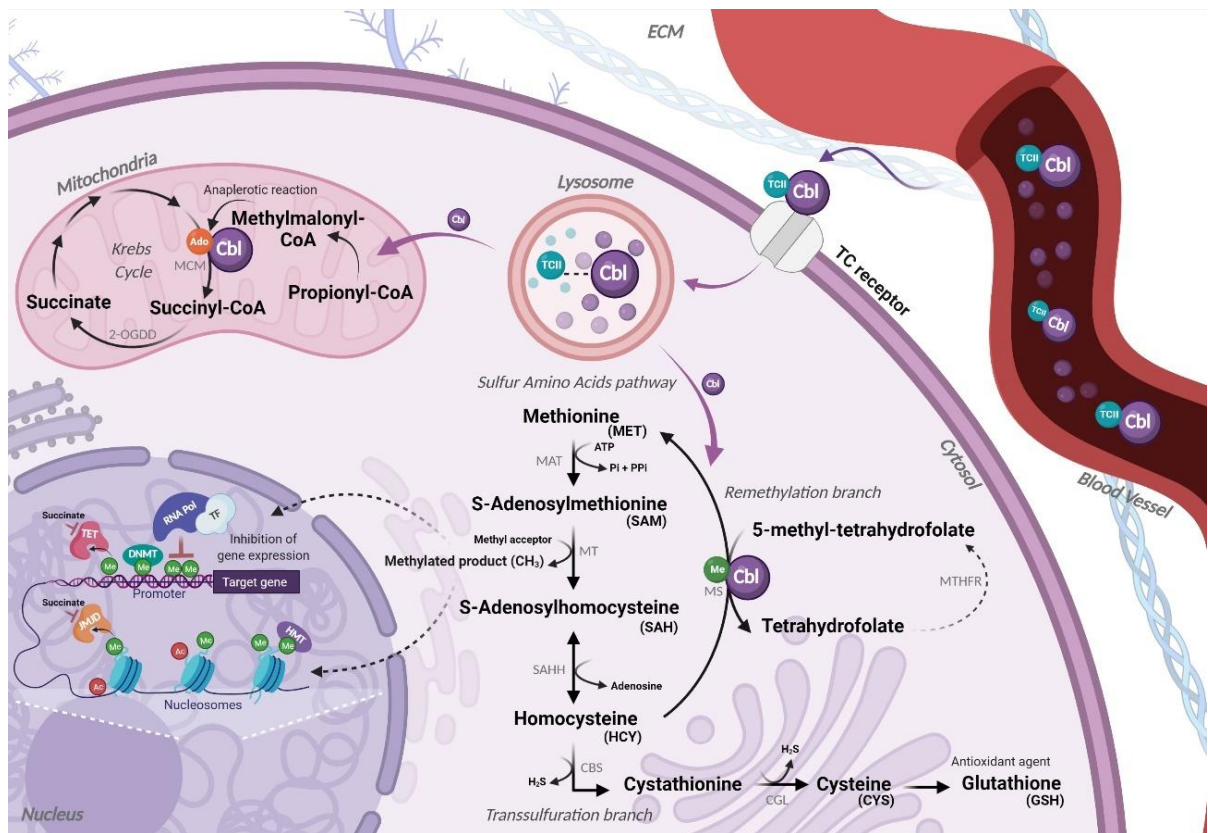


Figure 1: Cobalamin and cell metabolism. Endocytosis of cobalamin (Cbl) bound to its blood carrier transcobalamin (TCII) occurs through the transcobalamin receptor (TC receptor). In the lysosome, TCII is degraded and free Cbl is released into the cytosol for metabolism. In the mitochondria, Cbl undergoes adenosylation to form adenosylcobalamin (AdoCbl). Methylcobalamin (MeCbl) originates from the degradation of methionine synthase (MS) in the sulfur amino acids pathway. The methyl (Me) products generated by the conversion of S-adenosylmethionine (SAM) to S-adenosylhomocysteine (SAH) fuel DNA and histone methylation reactions. 2-OGDD, 2-oxoglutarate-dependent dioxygenase; Ac, acetyl; ATP, adenosine triphosphate; CBS, cystathionine beta-synthase; CGL, cystathionine gamma-lyase; DNMT, DNA methyltransferase; ECM, extracellular matrix; H₂S, hydrogen sulfide; HMT, histone methyltransferases; JMJD, Jumonji C domain containing (histone demethylase); MAT, methionine adenosyltransferase; MCM, methylmalonyl-CoA mutase; MT, methyltransferase; MTHFR, 5,10-methylenetetrahydrofolate reductase; Pi, phosphate (inorganic); PPi, pyrophosphate; RNA pol, RNA polymerase; SAHH, adenosylhomocysteinase; TET, ten-eleven translocation methylcytosine dioxygenases (DNA demethylase); and TF, transcription factor. (Created with <http://Biorender.com>.)

Inflammation in the central nervous system (CNS) is named neuroinflammation and comprises complex cellular and molecular processes triggered in response to danger signals. The main agent of this response is the microglia, whose activation involves changes in gene expression, cellular morphology, and behavior. Proinflammatory and neurotrophic factors released by activated microglia trigger a variety of biological processes, ranging from oxidative stress and excitotoxicity to profound changes in the epigenetic cell landscape. These processes involve also astrocytes, neurons, oligodendrocytes, vascular pericytes, and infiltrated peripheral

immune cells (Ransohoff & Engelhardt, 2012; Ransohoff, Kivisäkk, & Kidd, 2003). Uncontrolled neuroinflammatory response is pivotal in the pathophysiology of neurological disorders such as multiple sclerosis, Alzheimer's, Parkinson's, and Huntington's diseases, amyotrophic lateral sclerosis (ALS), meningitis and meningoencephalitis, and their role in psychiatric disorders such as depression has also been investigated (Aktas, Ullrich, Infante-Duarte, Nitsch, & Zipp, 2007).

In this chapter, the neuroprotective aspects of B12 will be discussed from the perspective of neuroinflammatory processes. Its functions in regulating energy metabolism and activating immune cells, responding to oxidative stress, glutamate-induced excitotoxicity, hyperhomocysteinemia (HHCY)-mediated cell death, and epigenetic regulation of pivotal genes in neuroinflammation signaling pathway will be emphasized.

B12 regulates energy metabolism and immune response

The Krebs cycle is a mitochondrial pathway of energy metabolism that converts the end product of glycolysis into several intermediates in a closed loop of biochemical reactions generating GTP/ATP and reduced forms of NADH and FADH₂ which provide electrons to the oxidative phosphorylation. Multiple substrates can feed the Krebs cycle, in addition to acetyl-CoA and oxaloacetate, both derived from pyruvate, the last product of glycolysis. Concomitantly, as the cycle runs, its intermediates are exported to the cytosol to become building blocks for anabolic reactions. The cycle is replenished by anaplerotic reactions, such as the conversion, within the mitochondria, of methylmalonyl-CoA into succinyl-CoA by MCM, which requires AdoCbl as cofactor (Nelson & Cox, 2021).

In response to proinflammatory stimuli, macrophages and their closely related immunocompetent cells of the CNS, the microglia, change from resting to activated state. To supply their increased energy demand, activated macrophages and microglia switch from oxidative phosphorylation to aerobic glycolysis. This metabolic shift is mediated by the inhibition of Krebs cycle and oxidative phosphorylation pathways and upregulation of glycolysis-associated genes (Ghosh, Castillo, Frias, & Swanson, 2018; Kramer, Ravi, Chacko, Johnson, & Darley-Usmar, 2014). Speculatively, Cbl, by stimulating the anaplerotic production of succinyl-CoA to replenish the Krebs cycle, may contribute to reactivate aerobic respiration, favoring the inactive phenotype of immune cells. However, it is worth noting that excess succinyl-CoA can also slow down the Krebs cycle by inhibiting citrate synthase and α -ketoglutarate dehydrogenase (Martínez-Reyes & Chandel, 2020). Other circulating immune cells, such as lymphocytes and neutrophils, can be recruited from blood to the CNS during

neuroinflammation. However, activated lymphocytes increase both glycolysis and mitochondrial respiration, and neutrophils, which have few mitochondria, use glycolysis to sustain the energy demand for their activities such as chemotaxis (Kramer et al., 2014).

In the Krebs cycle, succinyl-CoA converts to succinate, which also has nonmetabolic signaling functions, such as regulation of the innate immune response (Mills et al., 2016; Tannahill et al., 2013). For instance, the increase in succinate concentration stimulates the production of reactive oxygen species (ROS) and inhibits prolyl-hydroxylases (PHD) preventing HIF- α hydroxylation and allowing it to migrate to the nucleus where it induces the expression of glycolytic genes and IL-1 β , among other proinflammatory cytokines and chemokines (Selak et al., 2005; Tannahill et al., 2013). Succinate also plays an important role in epigenetic modulation of gene transcription. Indeed, succinate is the product of 2-oxoglutarate-dependent dioxygenase (2-OGDD) enzymes, a phylogenetically conserved family that includes histone (JMJD) and DNA (TET) demethylases. When succinate accumulates, it inhibits 2-OGDD and, consequently, histone and DNA demethylation (Letouzé et al., 2013). Thus, it is conceivable that supplemental B12, by enhancing the production of succinyl-CoA, the precursor of succinate, could also promote downregulation of neuroinflammatory genes.

B12 is antioxidant and modulates the oxidative stress response

The brain is responsible for about 20% of the body basal O₂ consumption. Furthermore, it has a reduced antioxidant capacity and a high concentration of polyunsaturated fatty acids prone to lipid peroxidation, which makes it an organ especially vulnerable to oxidative stress (Rink & Khanna, 2011). This process is characterized by the unbalance between the antioxidant response and the production of reactive species that cause oxidative damage, especially those derived from oxygen (ROS) and nitrogen (RNS). One of the most important cell sources of reactive species is the mitochondria, arising from the incomplete reduction of O₂ to H₂O. In this case, O₂ is reduced with one electron producing the superoxide radical (O₂⁻), which can be converted into peroxynitrite by reacting with nitric oxide (NO), or to hydrogen peroxide (H₂O₂) by the action of superoxide dismutase. Excessive mitochondrial ROS production can lead to functional and structural changes in the organelle and DNA damage, impairing energy production and other relevant cellular processes, such as fatty acid oxidation and amino acid metabolism. This cascade of events culminates in triggering proapoptotic signaling and cell death (Murphy, 2009). Oxidative stress is implicated in the pathophysiology of several neurological diseases, such as Alzheimer's (Butterfield & Halliwell, 2019) and Parkinson's (Guo, Zhao, Li, Li, & Liu,

2018), and is also a hallmark of the host response to CNS infections (Koedel & Pfister, 1999; Percário et al., 2012).

Oxidative stress is an inhibitor of MS activity. The Cbl present in this enzyme is an intermediate carrier of methyl groups. The cobalt that makes up Cbl can present different oxidation states in Cbl(I), Cbl(II), or Cbl(III). Cbl(I) in MS is an excellent nucleophile and acts as a methyl acceptor, forming MeCbl, which is the methyl donor to the conversion of HCY into MET by MS (Fig. 2). Alternatively, under oxidative stress conditions, Cbl(I) can act as an antioxidant by undergoing oxidation to Cbl(II). This partial oxidation temporarily inhibits MS by interrupting the conversion of HCY into MET and, consequently, favoring the production of glutathione (GSH) by the transsulfuration branch of the sulfur amino acids pathway (Banerjee & Matthews, 1990) (Fig. 1). GSH is an important cellular antioxidant due to its role as a cofactor for the enzyme glutathione peroxidase, which catalyzes the conversion of H_2O_2 into O_2 and H_2O . Moreover, GSH disulfide (GSSG) generated by oxidation of two GSH is a RNS and ROS scavenger (Aoyama, 2021; Pritsos & Pardini, 1983). Aquacobalamin (H_2OCbl) is abundant in cells and can readily combine with reduced GSH to form glutathionylcobalamin (GSCbl), an intermediary in the biosynthesis of MeCbl and AdoCbl (Pezacka, Green, & Jacobsen, 1990; Xia, Cregan, Berben, & Brasch, 2004).

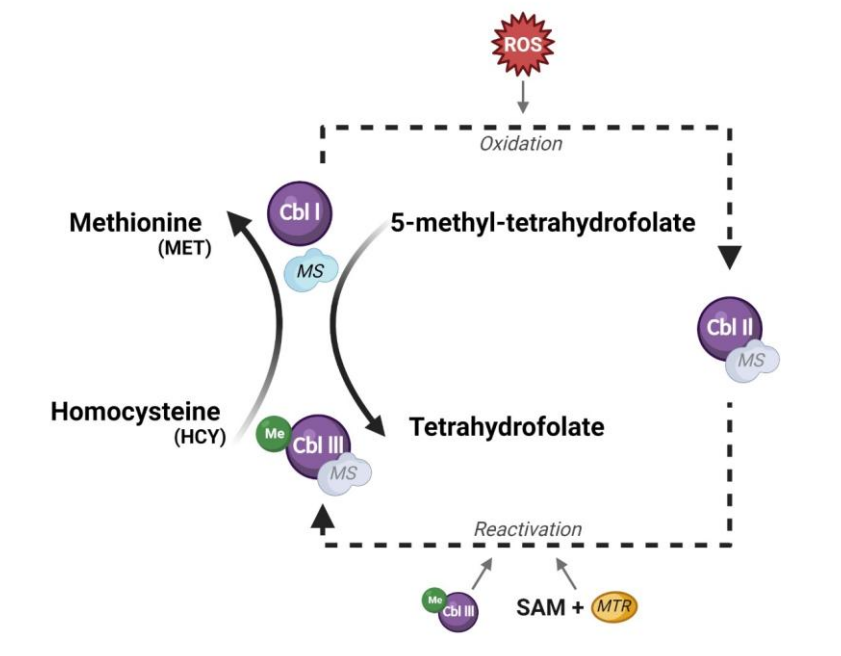


Figure 2: Effect of ROS in Cobalamin oxidation. During the primary turnover cycle of methionine synthase (MS), Cob(I)alamin (Cbl I) is occasionally oxidized by reactive oxygen species (ROS) and electrophilic xenobiotic metabolites to Cob(II)alamin (Cbl II). This reaction inhibits MS. Reactivation of the enzyme and subsequent formation of methylcobalamin (MeCbl) depends on the supply of the methyl group by SAM and methionine synthase reductase (MTR) or the replacement of oxidized Cbl(II).

Cbl(III), Cbl(III)amin; Me, methyl; MS, methionine synthase; ROS, reactive oxygen species; and SAM, S-adenosylmethionine. (Created with <http://biorender.com>.)

Thus, Cbl and MS are sensors and modulators of the CNS cell response to oxidative stress, aiding to preserve a healthy redox intracellular state and, consequently, acting antiinflammatory.

B12 boosts the synthesis of H₂S within the CNS

Hydrogensulfide (H₂S) is a gasotransmitter mainly produced from the desulfhydration of cysteine carried out by the enzymes CBS, CGL, and 3-MST (Xie, Liu, & Bian, 2016). Vitamin B6-dependent CBS and CGL participate in the transsulfuration branch of the sulfur amino acids pathway, converting HCY into cystathionine and this into cysteine and alpha-ketobutyrate, respectively (Fig. 1).

H₂S acts antiinflammatory and prevents oxidative damage through several enzymatic and nonenzymatic mechanisms, such as by enhancing cystine/cysteine transporters to increase intracellular GSH production and maintaining its reduced status. Furthermore, the hydrosulfide anion (HS⁻), resulting from the dissociation of H₂S, is a potent reducing agent against ROS (Xie et al., 2016).

It has been reported that the deficit of H₂S in patients with Alzheimer's disease correlates with low levels of SAM (McCarty, O'Keefe, & DiNicolantonio, 2019). This is probably due to the allosteric activation of CBS, the chief enzyme which synthesizes H₂S in the brain parenchyma, by SAM, whose production by MS requires MeCbl (Fig. 1).

Therefore, B12 plays a role in maintaining adequate levels of H₂S, which has a protective role in neuroinflammation. There is also an interplay between B12 and H₂S in shaping the epigenetic cell landscape that may account for their neuroprotective effects in neuroinflammation.

B12 prevents NMDAR-mediated glutamate excitotoxicity

Proinflammatory cytokines, such as IL-1 β and TNF- α , can interfere with glutamatergic transmission by increasing glutamate release, inhibiting its reuptake and modulating the expression of NMDA receptors, which are involved in excitotoxicity processes (Viviani, Boraso, Marchetti, & Marinovich, 2014). NMDAR is the main receptor of the glutamatergic system due to its fundamental role in mediating functions such as cognition, memory, neural plasticity, and neurotoxicity (Kugaya & Sanacora, 2005). Activated NMDAR opens nonselective ion channels for cations, allowing the influx of Na⁺ and Ca²⁺ and the efflux of K⁺. Hyperactivation of NMDAR and, subsequently, excessive influx of Ca²⁺ can lead to excitotoxicity. This mechanism, which induces the production of free radicals, mitochondrial

dysfunction, and cell death, is implicated in various neuropsychiatric diseases (Paoletti, Bellone, & Zhou, 2013).

CNCbl inhibited NMDAR-mediated glutamate excitotoxicity through a mechanism involving the inhibition of PKC, which, in its turn, suppresses the voltage dependent Ca^{2+} channel activity and decreases the Ca^{2+} influx into synaptosomes isolated from the prefrontal cortex of rats (Hung, Wang, Huang, & Wang, 2009). Furthermore, glutamate excitotoxicity was prevented when rat cortical neurons were cultured in a medium containing MeCbl (Akaike, Tamura, Sato, & Yokota, 1993).

Taken together, these evidences that B12 can prevent NMDAR-mediated excitotoxicity in neural cells indicate that this vitamin has a great potential as an adjuvant therapy for some neuropsychiatric diseases whose physiopathology involves imbalances in the homeostasis of excitatory amino acids.

B12 rescues neurons from homocysteine-mediated cell death

HCY is synthesized from MET as an intermediary in the sulfur amino acids pathway and this enzymatic reaction catalyzed by MS depends on MeCbl as a cofactor (Fig. 1). Indeed, reduced MS or 5,10-methylenetetrahydrofolate reductase (MTHFR) activity, due to MeCbl or folate and tetrahydrofolate deficiency, respectively, can lead to HHCY (Kim, Kim, Roh, & Kwon, 2018). HCY regulates the levels of NMDAR and AMPA (Liu, Wen, Qiao, & An, 2013) and, when in excess, causes oxidative stress (Guest et al., 2015) and impaired mitochondrial function (Zhang et al., 2020) in CNS cells, contributing to synaptic dysfunction and memory deficits (Wyse et al., 2020; Zhang, Ma, Jing, Wang, & Zhang, 2020). HHCY has been associated with increased risk for several neuropsychiatric conditions, such as stroke, epilepsy, multiple sclerosis, Parkinson's and Alzheimer's diseases, dementia, cognitive impairment, and depression (Ansari, Mahta, Mallack, & Luo, 2014).

In vitro and in vivo experimental models have been used to demonstrate the mechanisms of HCY neurotoxicity and to test whether they can be mitigated by supplementing B12. MeCbl provided neuroprotection and neurorescue in a dose-dependent manner against ROS-independent apoptosis induced by high levels of HCY in NSC-34D cells, a hybrid cell line of motor neurons. In this experimental model, MeCbl prevented the HCY activation of caspases 3 and 7 (Hemendinger, Armstrong, & Brooks, 2011). Additionally, Tripathi et al. (2016) have demonstrated the central role of endoplasmic reticulum stress, lysosomal dysfunction, and impairment of autophagy induced by excess HCY in human and mouse primary astrocyte cultures as well as in a diet-induced mouse model of HHCY. Interestingly, B12 and folate

cotreatment restored these functions. B12 and folate supplementation also reduced mitochondrial damage, autophagy, and neuronal apoptosis in apolipoprotein E-deficient (Apo E^{-/-}) mice with HHcy-enhanced brain damage (Zhang et al., 2017).

Therefore, the neuroprotectant effect of B12 in neuroinflammation involves preventing some noxious effects of excess Hcy in CNS cells, such as endoplasmic reticulum stress, lysosomal dysfunction, impaired autophagy, and ROS-independent apoptosis.

B12 modulates the expression of inflammatory genes through cell epigenetic landscape changings

DNA methylation is an epigenetic mechanism that, in mammals, occurs mainly in CpG dinucleotides. When occurring in regulatory regions, it can affect the accessibility of transcription factors and RNA polymerases to DNA, thus modulating gene expression. The transfer of a methyl group to a cytosine residue is catalyzed by a family of DNMT and requires SAM as a methyl donor. In general, DNA hypomethylation is associated with active gene expression, while its hypermethylation is related to transcriptional downregulation (Fuks, 2005) (Fig. 1).

A second level of epigenetic regulation comprises a set of posttranslational modifications in amino acids that form the amino terminal tails of histone proteins. Chromatin is composed of nucleosome repeats made up of a protein octamer containing histones H2A, H2B, H3, and H4, which is physically associated with a sequence of 147 nucleotides. The histone N-terminal tails contain amino acids that are targets of a variety of covalent modifications, such as methylation, acetylation, phosphorylation, and ubiquitination. Such posttranslational modifications of specific residues in histones alter the chromatin state at the vicinity and regulate the transcription of associated genes: the euchromatin allows transcription, while the heterochromatin silences genes. This so-called histone code is established and maintained by dozens of chromatin-modifying enzymes, including the histone methyltransferases (HMT), demethylases (HDM), acetyltransferases (HAT), and deacetylases (HDAC), which target specific histone residues and genome loci, in a complex regulatory hierarchy for the control of global gene expression (Zhang et al., 2021).

DNA and histone methylation reactions depend on the bioavailability of SAM. Accordingly, adjuvant therapy with CNCbl increased genome-wide DNA methylation and reduced expression of proinflammatory genes including *IL-1β* and *CCL3* in infant rats with pneumococcal meningitis (de Queiroz et al., 2020). In addition to this mechanism involving MS and the sulfur amino acids pathway, Cbl also regulates gene expression through MCM,

which produces succinyl-CoA in mitochondria from L-methylmalonyl-CoA (Fig. 1). In the Krebs cycle, succinyl-CoA is converted into succinate by 2-OGDD. When accumulated, succinate acts as an antagonist of histone and DNA demethylation reactions catalyzed by enzymes of the 2-OGDD family (Letouze et al., 2013).

B12 also indirectly changes the epigenetic landscape by regulating oxidative stress and inflammation. Oxidative stress inactivates the histone deacetylating enzyme HDAC2, which can contribute to the formation of euchromatin regions where DNA is more accessible to the transcription machinery (Rahman, Marwick, & Kirkham, 2004). However, oxidative stress also activates signaling pathways that increase the expression of the DNA methylating enzyme DNMT1 (Molognoni, de Melo, da Silva, & Jasiulionis, 2013), which can lead to inhibition of gene transcription. The net balance of the epigenetic changes resulting from oxidative stress alleviation by B12 remains to be determined. Inflammation also has a direct influence on DNA methylation and vice versa. Gene promoters encoding cytokines are rapidly demethylated (~6h) after activation of immune cells (Li et al., 2012), and cytokines such as TNF- α , IL-1 β , and other inflammatory molecules, such as caspases, recruited during inflammatory processes can induce histone demethylation through the activation of JMJD3 (Bayarsaihan, 2011; Greer & Shi, 2012).

B12 modulates different mechanisms of chromatin remodeling that may affect gene expression. However, the net balance of the orchestrated contributions of all these mechanisms in determining the up- or downregulation of neuroinflammatory genes is still unknown. Further research is still needed to elucidate this intriguing and complex aspect of B12 mode of action.

B12 regulates pivotal neuroinflammation signaling pathway-associated genes

Among various genes and proteins regulated by B12, APP, Tau, NF- κ B, TNF- α , and AP-1 play a central role in the regulation of neuroinflammation signaling (Fig. 3).

APP is a particularly abundant protein membrane at neuron synapses and a precursor of the β -amyloid peptide (A β), whose fibrillar form is a primary component of amyloid plaques. The accumulation of APP leads to increased activity of γ - and β -secretases, proteolytic enzymes that cleave APP generating the A β peptide. Also, extracellular deposits of A β peptide and intracellular neurofibrillary tangles, consisting mainly of hyperphosphorylated Tau protein, are observed in the brain tissue of patients affected by Alzheimer's disease or other neurodegenerative diseases. Together, these mechanisms intensify local oxidative stress and trigger a neuroinflammatory cascade (Sastre, Walter, & Gentleman, 2008).

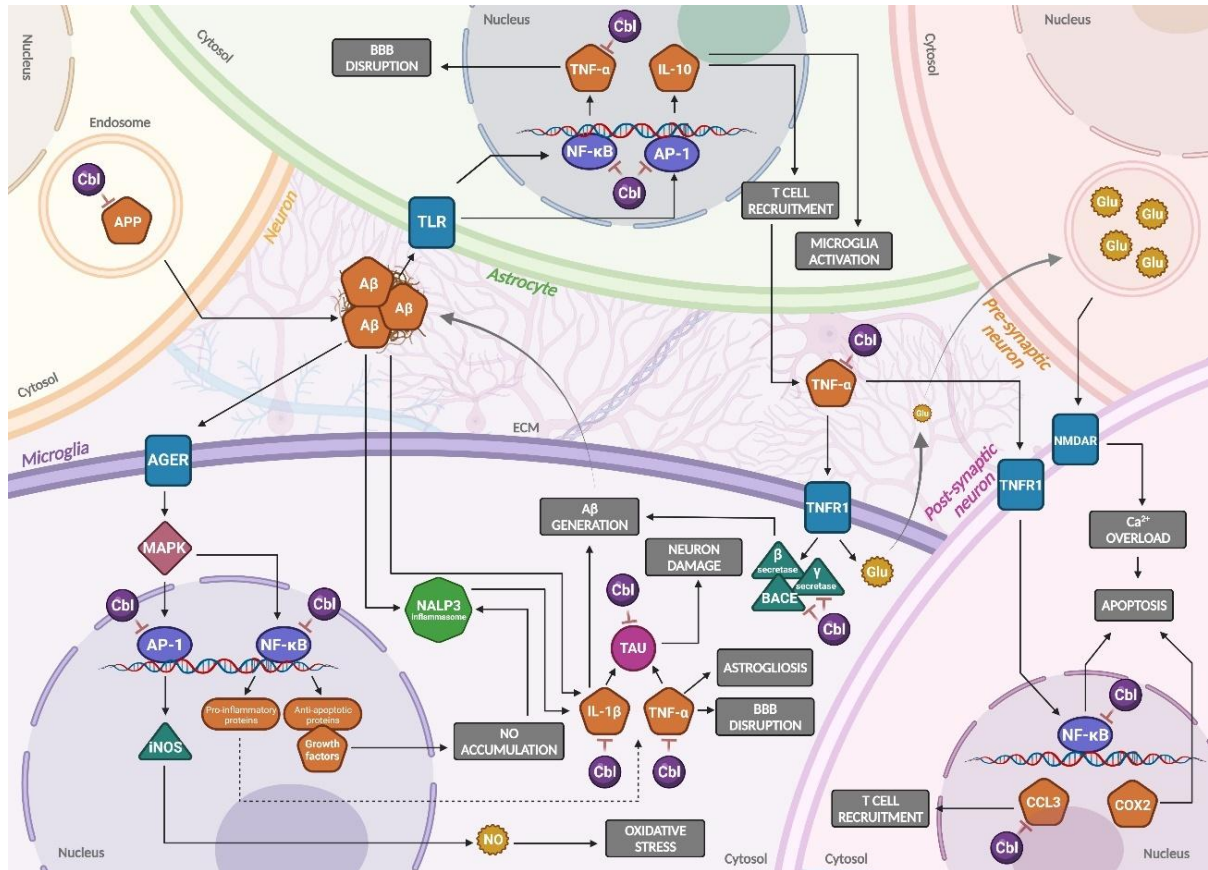


Figure 3: B12 regulates pivotal components of the neuroinflammation signaling pathway. At the early stages of neuroinflammation, accumulated APP is processed to form A β peptides that aggregate in the extracellular matrix (ECM). The deposit of A β activates membrane receptors (advanced glycosylation end-product-specific receptor—AGER and toll-like receptor—TLR) of microglia and astrocytes, starting the inflammatory response cascade. In microglia, AGER signaling triggers the activation of AP-1 and NF- κ B via MAPK. This mechanism culminates in NO accumulation, increased oxidative stress, blood-brain barrier (BBB) disruption, astroglial damage, and generation of A β in a positive feedback loop. In astrocytes, the activation of AP-1 and NF- κ B via TLR induces the production of proinflammatory mediators, contributing to BBB disruption, microglia activation, and T cell recruitment. TNF- α plays an important role in T-cell recruitment induced by neurons and in the production of glutamate (Glu). Excess glutamate hyperactivates NMDAR triggering neuronal apoptosis. Highlighted are the main molecules negatively regulated by Cbl. MAPK, mitogen-activated protein kinase; iNOS, inducible nitric oxide synthase; TNFR, tumor necrosis factor receptor 1; and COX2, cyclooxygenase 2. (Created with <http://biorender.com>.)

Cbl, through SAM production, regulates the methylation state of CpG islands of *PSEN1* promoter, a gene encoding one of the four main proteins of the γ -secretase complex. There is also evidence that *BACE*, that codes a β -secretase, is regulated by promoter methylation (Fuso, Seminara, Cavallaro, D'Anselmi, & Scarpa, 2005). HHcy induced by dietary B12 suppression increased transcriptional and protein levels of *PSEN1* and *BACE*, in addition to increasing APP phosphorylation and A β production in the rat brain (Fuso et al., 2008; Zhang et al., 2009). B12 deficiency also causes inactivation of PP2A and subsequently hyperphosphorylation and aggregation of Tau protein (Rafiee, Asadollahi, Riazi, Ahmadian, & Saboury, 2017). In line

with these findings, B12 inhibited Tau fibrillization in vitro (Rafiee et al., 2017), and combined B12 and folate supplementation prevented Tau hyperphosphorylation and HHcy-induced memory deficits in rats (Wei et al., 2011).

NF- κ B is an evolutionarily conserved transcription factor activated by several stimuli and that regulates a complex gene network not yet fully elucidated. Among the NF- κ B activators, there are neurotoxic proteins (e.g., A β) and, mainly, cytokines such as IL-1 and TNF- α that are also upregulated by the transcription factor in a feedback loop (Kaltschmidt, Widera, & Kaltschmidt, 2005). Cbl also modulates the inflammatory response and its resolution by regulating NF- κ B activity. Among the mechanisms involved, the direct or indirect removal of ROS stands out. Cbl increases the production and preservation of GSH which is a RNS and ROS scavenger. Cbl also inhibits A β production (Alam, Siddiqi, Chaturvedi, Zaman, & Khan, 2017; Andrade, Loureiro, & Pereira, 2021) and downregulates TNF- α (Peracchi, Bamonti Catena, Pomati, De Franceschi, & Scalabrino, 2001; Veber et al., 2008). B12 deficiency has actually been associated with increased CNS TNF- α expression (Scalabrino et al., 2004). Accordingly, Cbl supplementation downregulated NF- κ B and, consequently, TNF- α in the peripheral nervous system (Veber et al., 2008).

The JUN protein, associated with FOS, forms the AP-1 heterodimer. As well as NF- κ B, AP-1 is an essential transcription factor for the maintenance of the immune system, participating in the regulation of tissue homeostasis, proliferation, differentiation, apoptosis, and cell migration (Hess, Angel, & Schorpp-Kistner, 2004). AP-1 regulates transcription of genes encoding cytokines and chemokines in addition to other proteins involved in T-cell recruitment and ROS production (Weietal.,2019). The functionality of AP-1 is strongly dependent on the phosphorylation of its JUN and FOS subunits, the combinatorial patterns of these components, and the interaction with other transcription factors (Ameyar, Wisniewska, & Weitzman, 2003). It has been demonstrated that Jun mRNA levels are increased in B12-deficient mice (Liu et al., 2011), but the underlying mechanism remains to be elucidated.

Therefore, B12 regulates gene expression and the activity of central proteins in neuroinflammatory signaling, either by acting directly on these molecules or their activators or indirectly, through methyl-dependent epigenetic mechanisms.

Final remarks and conclusion

Neuroinflammation is necessary to maintain a healthy environment in the nervous system. However, an inappropriate and unbalanced immune response can cause or aggravate various neurological or psychiatric conditions. Cbl, besides its central role in MET production, which

is required for synthesis of proteins, DNA, and neurotransmitters, also acts pleiotropically preventing or attenuating several mediators of neuroinflammation, such as the imbalance in redox homeostasis, accumulation of HCY, neuronal excitotoxicity mediated by NMDAR, also ensuring adequate levels of H₂S. In addition, it acts regulating the expression and activity of pivotal transcription factors and other components of the neuroinflammation signaling cascade. This plethora of regulatory mechanisms is mostly mediated by succinyl-CoA and SAM, whose production depends directly or indirectly on reactions catalyzed by the enzymes MCM and MS that require Cbl as cofactor. Furthermore, B12 plays a key role in the control of methyl-dependent mechanisms that modify the epigenetic cell landscape regulating gene transcription. Taken together, these properties make Cbl a strong candidate medication for new adjuvant neuroprotectant therapies to neuropsychiatric diseases involving neuroinflammation.

Applications to other neurological conditions

B12 has a safe pharmacological profile and concomitantly controls several components of neuroinflammation. For these reasons, B12 supplementation has been evaluated and proposed as a neuroprotectant adjuvant therapy for various neuropsychiatric disorders with neuroinflammation involved in their pathophysiology, such as ALS, Parkinson's disease, Alzheimer's disease, stroke, epilepsy, acute bacterial meningitis, depression, among others. However, there is still room for investigating the therapeutic potential of B12 in several other diseases related to neuroinflammation, which include viral meningitis and meningoencephalitis, cerebral malaria, and infections by emerging pathogens such as dengue, the Zika virus, and, why not, SARS-CoV-2.

Other components of interest

Much of the antiinflammatory effect of Cbl is mediated by metabolites of the Krebs cycle and the sulfur amino acid pathway. Other steps in these pathways are catalyzed by enzymes that require thiamine (B1), pyridoxine (B6), and folate (B9). Therefore, it is possible that the combined supplementation of these B-complex vitamins may potentiate the neuroprotectant effect of Cbl in neuroinflammation.

Mini-dictionary of terms

- **Anaplerotic reaction**—Chemical reactions that replenish metabolic pathways, producing intermediates. One example is the conversion of methylmalonyl-CoA into succinyl-CoA by MCM to feed the Krebs cycle.

- **Autophagy**—Natural catabolic process by which cytoplasmic components are degraded and recycled in the cell lysosome. It is a protective mechanism for cleaning damaged cells. However, autophagic dysfunction is associated with neuroinflammation, neurodegeneration, and many other disease-related processes.
- **Gasotransmitter**—Gaseous signaling molecules that can chemically modify their targets and regulate various intra- and extracellular functions, such as inflammation, cellular respiration, and apoptosis. They are produced endogenously, when required, or acquired exogenously. These gaseous neurotransmitters are beneficial in physiologic concentrations but may be toxic when elevated. Well-known examples are nitric oxide, carbon monoxide, and hydrogen sulfide.
- **Epigenetic landscape**—Term coined by Conrad H. Waddington that refers to a conceptual model in which environmental factors may interact with genes determining cellular fate and modifying cell function and phenotype. According to Waddington's visual concept, epigenetic landscape consists of a series of ridges and valleys (environmental factors) that a cell can traverse on its way to a final fate (function). Later on, it has been proved that the cell epigenetic landscape is shaped with chemical modifications of specific residues in DNA, RNA, and histones, as well as by noncoding RNAs.
- **The histone code**—Proposed hypothesis in which multiple chemical modifications in histone proteins combined in different ways, primarily those of histone tails, may regulate DNA transcription, thus participating in the epigenetic code. Histones are proteins that associate with DNA to form nucleosomes, helping in the assembly of nuclear DNA to form the chromosome.

Applications to other areas

B12 supplementation has the potential as an adjuvant therapy for other clinical conditions that do not have the CNS as a primary site and whose pathophysiology involves oxidative stress, excessive cytokine, and chemokine production and imbalance in HCY and GSH production, such as influenza infections, sepsis, and others.

Summary points

- Cbl may favor the resting state of microglia and macrophages by feeding the Krebs cycle with succinyl-CoA and, consequently, contributing to reactivate the aerobic respiration to the detriment of glycolysis.
- Cbl and MS are sensors and modulators of the cell response to oxidative stress.

- B12 plays a role in maintaining adequate levels of H₂S, which has a protective role in neuroinflammation.
- There is an interplay between B12 and H₂S in shaping the epigenetic cell landscape.
- B12 can prevent NMDAR-mediated excitotoxicity in neural cells.
- B12 prevents the noxious effects of excess HCY in CNS cells that include endoplasmic reticulum stress, lysosomal dysfunction, impaired autophagy, and ROS independent apoptosis.
- B12 modulates different mechanisms of chromatin remodeling that may contribute to downregulating neuroinflammatory genes.

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CAPÍTULO II: Vitamina B12 atenua assinatura inflamatória na covid-19 via modificações dependente de metil em marcas epigenéticas

O estudo foi publicado em 2023 pela revista *Frontiers in Immunology* [<https://doi.org/10.3389/fimmu.2023.1048790>] e se concentra no uso da vitamina B12 como um tratamento adjuvante para atenuar a resposta inflamatória dos leucócitos durante a covid-19 aguda moderada e grave. Os resultados mostram que a vitamina B12 normaliza os níveis de mediadores inflamatórios críticos na covid-19 e atenua a ativação de vias de resposta imune importantes para a fisiopatologia da doença. Um dos mecanismos postulados para este fenômeno é o aumento do fluxo da via dos aminoácidos sulfurados pela B12, favorecendo a biodisponibilidade de metil e a hipermetilação do promotor de genes inflamatórios. Estes achados demonstram o potencial da vitamina B12 de atuar como um epifármaco na covid-19 e abre uma janela para a busca de novas estratégias de intervenção terapêutica baseadas na modulação epigenética para o controle da hiperinflamação causada pelo SARS-COV-2.

Os materiais suplementares que suportam os resultados do seguinte trabalho podem ser acessados através do link [<https://bit.ly/3AFM3mg>].

Vitamin B12 attenuates leukocyte inflammatory signature in COVID-19 via methyl-dependent changes in epigenetic markings

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Abstract

COVID-19 induces chromatin remodeling in host immune cells, and it had previously been shown that vitamin B12 downregulates some inflammatory genes *via* methyl-dependent epigenetic mechanisms. In this work, whole blood cultures from moderate or severe COVID-19 patients were used to assess the potential of B12 as adjuvant drug. The vitamin normalized the expression of a panel of inflammatory genes still dysregulated in the leukocytes despite glucocorticoid therapy during hospitalization. B12 also increased the flux of the sulfur amino acid pathway, that regulates the bioavailability of methyl. Accordingly, B12-induced downregulation of *CCL3* strongly and negatively correlated with the hypermethylation of CpGs in its regulatory regions. Transcriptome analysis revealed that B12 attenuates the effects of COVID-19 on most inflammation-related pathways affected by the disease. As far as we are aware, this is the first study to demonstrate that pharmacological modulation of epigenetic markings in leukocytes favorably regulates central components of COVID-19 physiopathology.

Introduction

Coronavirus disease 2019 (COVID-19), caused by SARS-CoV-2, has been reported, as of September 2022, in more than 600 million confirmed cases and has led to more than 6.4 million deaths worldwide (1), although recent studies estimate a much higher death toll (2). Since the end of 2020, large-scale population vaccination has drastically reduced the mortality rate and the burden on health systems, but factors such as inequality in access to immunizations, rapid waning neutralizing antibody titers induced either by vaccines or by exposure to SARS - CoV-2 and the emergence of new genotypic variants of the virus have contributed to successive new waves of the pandemic with more dramatic impacts on unvaccinated individuals or those with poor response to vaccines. In this context, efforts have been made in the search for treatments for COVID-19, such as the repositioning of chemicals and monoclonal antibodies that act as anti-inflammatory agents, and the development of new antiviral small molecules that target SARS-CoV-2 replication and antibodies that bind to the viral spike protein blocking virus entry into cells (3). Unfortunately, most of these drugs have limited effectiveness or are very expensive, making them unsuitable to be used on a global scale. Therefore, there is still an urgent need for new efficient treatments with safe, inexpensive, and widely available drugs (4). While most COVID-19 patients will experience mild symptoms, some will develop severe acute respiratory distress syndrome, systemic inflammation, multiple organ failure, and other more serious complications that can lead to death. Several components of the host immune system are dramatically altered during SARS-CoV-2 infection and the extent of immune

CAPÍTULO II - Vitamina B12 como epifármaco na covid-19 aguda.

dysregulation is related to the worsening of COVID-19 and progression to death (5). Patients with COVID-19 exhibit inflammatory signatures defined by low levels of type I and III interferons (IFNs) and elevated levels of some cytokines and chemokines. These two branches of the innate immune response contribute to the clinical evolution of the patients (6). As the disease worsens, increased levels of inflammatory cytokines and chemokines are observed, which can lead to depletion and exhaustion of T cell populations resulting in a significant elevation of the neutrophil/lymphocyte ratio (7–9). The increase in neutrophil population (7, 8) with transcriptional signatures related to its activated state (10) also contributes to the elevation of neutrophil/lymphocyte ratio in COVID-19.

DNA methylation is an epigenetic mechanism that, in mammals, occurs mainly in dinucleotides of a cytosine followed by a guanine (CpG) and can affect the accessibility of transcription factors (TF) and RNA polymerases to DNA, thus modulating gene expression (11). In general, DNA hypomethylation in gene promoters is associated with the activated state of their expression (12). It is already known that promoters of genes encoding cytokines are rapidly demethylated (~6 hours) after T cell activation (13) and that several genes encoding key cytokines and chemokines have increased systemic expression in patients with COVID-19 (14) are regulated by the methylation of CpGs in their promoters or enhancers (15–18). The ability of RNA viruses to hijack the epigenome of host immune cells in order to evade antiviral defense is widely acknowledged (19, 20). In early 2021, Corley and colleagues reported that the DNA methylation signature of peripheral blood mononuclear cells from critically ill COVID-19 patients is characterized by hypermethylation of genes related to the IFN-mediated antiviral response and hypomethylation of inflammatory genes (17). This finding, in light of previous knowledge about the epigenetic regulation of genes involved in the inflammatory storm of COVID-19, points to the therapeutic potential of drugs capable of modulating the epigenetic landscape as a strategy for the control of exacerbated inflammation resulting from SARS-CoV-2 infection.

The bioavailability of methyl is a determinant of the DNA methylation state and can be regulated by vitamin B12, a cofactor of the enzyme methionine synthase (MS), which transfers methyl groups from 5-methyltetrahydrofolate to homocysteine (HCY) forming methionine. This is then converted to S-adenosylmethionine (SAM), the universal methyl donor. In this same pathway of sulfur amino acids, HCY can be metabolized producing glutathione (GSH) (Figure 1) (21). It has already been demonstrated that adjuvant therapy with B12 increases DNA methylation and reduces the expression of inflammatory mediators in the central nervous system of infant rats with pneumococcal meningitis (18). Among these genes, IL1B and CCL3

also play a central role in the pathophysiology of COVID-19 (14, 22). Vitamin B12, also known as cobalamin, is an essential micronutrient, which, once absorbed, binds to transcobalamin II and is transported into the cells through specific receptors (23). Although the liver is the main source of transcobalamin II, its unsaturated form (not bound to cobalamin) is abundant in the blood (24).

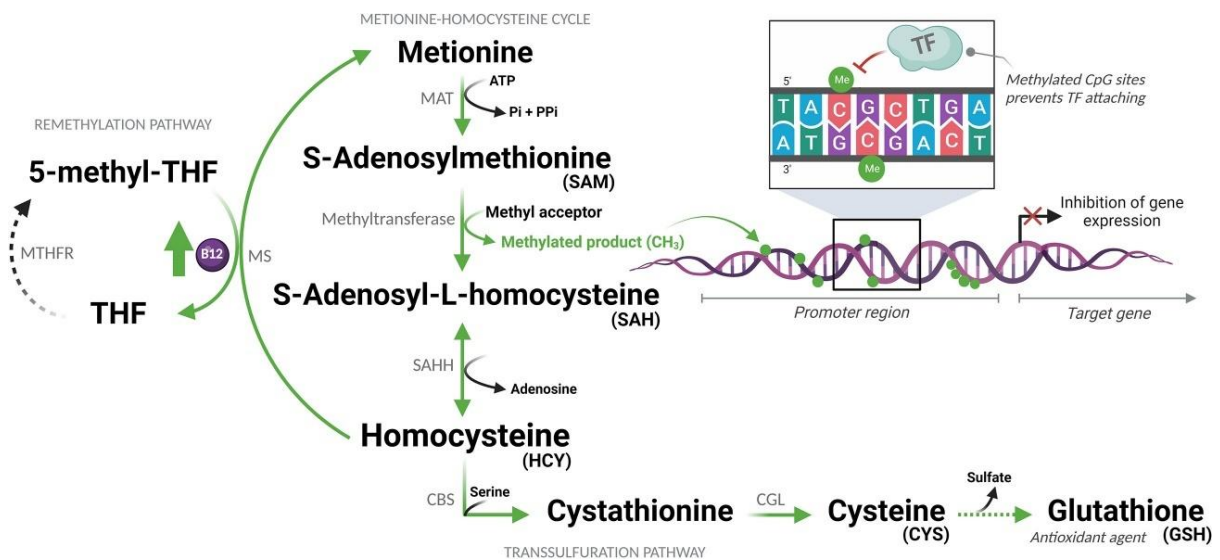


Figure 1: Sulfur amino acid pathway. Methionine is converted to S-adenosylmethionine (SAM), which is the methyl donor for numerous reactions. Upon losing its methyl group, SAM becomes S-adenosyl-L-homocysteine (SAH), which is then converted to homocysteine (HCY). This is then converted back to methionine or enters the transsulfuration pathway to form other sulfur-containing amino acids. MAT, Methionine adenosyltransferase; ATP, Adenosine triphosphate; Pi, Phosphate (inorganic); PPi, Pyrophosphate; THF, Tetrahydrofolate; MTHFR, Methylenetetrahydrofolate reductase; MS, Methionine synthase; B12, Vitamin B12; CH₃/Me, Methyl group; TF, Transcription factors; SAHH, Adenosylhomocysteinase; CBS, Cystathionine beta-synthase; CGL, Cystathionine gamma-lyase. (Created with BioRender.com).

The hypothesis of this study is that, during the advanced phase of COVID-19, characterized by hyperinflammation (cytokine storm), supplemental vitamin B12 would increase the flow of the sulfur amino acid pathway, favoring the production of SAM and the antioxidant GSH. The increased methylation capacity of cells, provided by higher concentrations of SAM, would lead to the hypermethylation of regulatory regions of pro-inflammatory genes attenuating inflammation. This hypothesis was tested using the *ex vivo* model of whole blood culture collected from patients with moderate or severe forms of COVID-19 and healthy controls.

Materials and methods

Patients

This study was approved by the National Research Ethics Committee of Brazil (CONEP: 32242620.6.0000.5091). All patients or their legal representatives were informed about the study and signed an informed consent form. Patients with moderate (MOD; n = 10) and severe (SEV; n = 16) forms of COVID-19, classified according to the World Health Organization's COVID-19 clinical severity scale (25), admitted to the Metropolitan Hospital Doctor Célio de Castro were recruited from 20th to 26th July 2020. Six healthy volunteers with similar age and biological sex distribution as the COVID-19 patients were recruited to compose the control group from 27th to 31st July 2020. At that time, no vaccine was available against COVID-19. Participants had peripheral venous blood samples (10 mL) collected in sodium heparin, at 8 a.m., and data from medical records of COVID-19 patients were analyzed. Uninfected volunteers included as controls in the study were tested for the presence of SARS-CoV-2 in the oropharynx and nasopharynx by RT-qPCR and for the presence of IgM and IgG blood antibodies against the virus by lateral flow immunochromatography (Wondfo, Guangzhou, China).

Sample processing and production of whole blood cultures

Each peripheral venous blood sample was divided into 4 aliquots of 1 mL and processed as follows: endpoint U) unprocessed blood; endpoints A and Z) added with 498 µl of RPMI 1640 culture medium (Sigma-Aldrich, Saint Louis, Missouri) and 2 µl of pH 5 citrate-phosphate buffer excipient (Merck, Darmstadt, Germany); and endpoint B) added with 500 µl of RPMI 1640 culture medium with cyanocobalamin (Merck) to a final concentration of 1 nM. Aliquots Z were immediately processed with no incubation. Aliquots A and B were incubated in 6-well plates for 24 hours at 37°C in a humidified atmosphere with 5% CO₂ (Figure 2).

Quantification of basal B12

Basal levels of vitamin B12 were quantified of plasma aliquots from endpoint U using the standard chemiluminescence method by a commercial clinical laboratory (Hermes Pardini, Belo Horizonte, Brazil).

Real time quantitative PCR

Total RNA was obtained from 1.5 mL of aliquots from endpoints Z, A and B, using the QIAamp RNA Blood kit (Qiagen, Hilden, Germany), and cDNA synthesis was performed from 2 µg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher, Waltham, Massachusetts), both according to manufacturers' protocols. All samples had their RNA

quantified by fluorometry using the Qubit RNA HS Assay Kit (#Q32852) and the Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA).

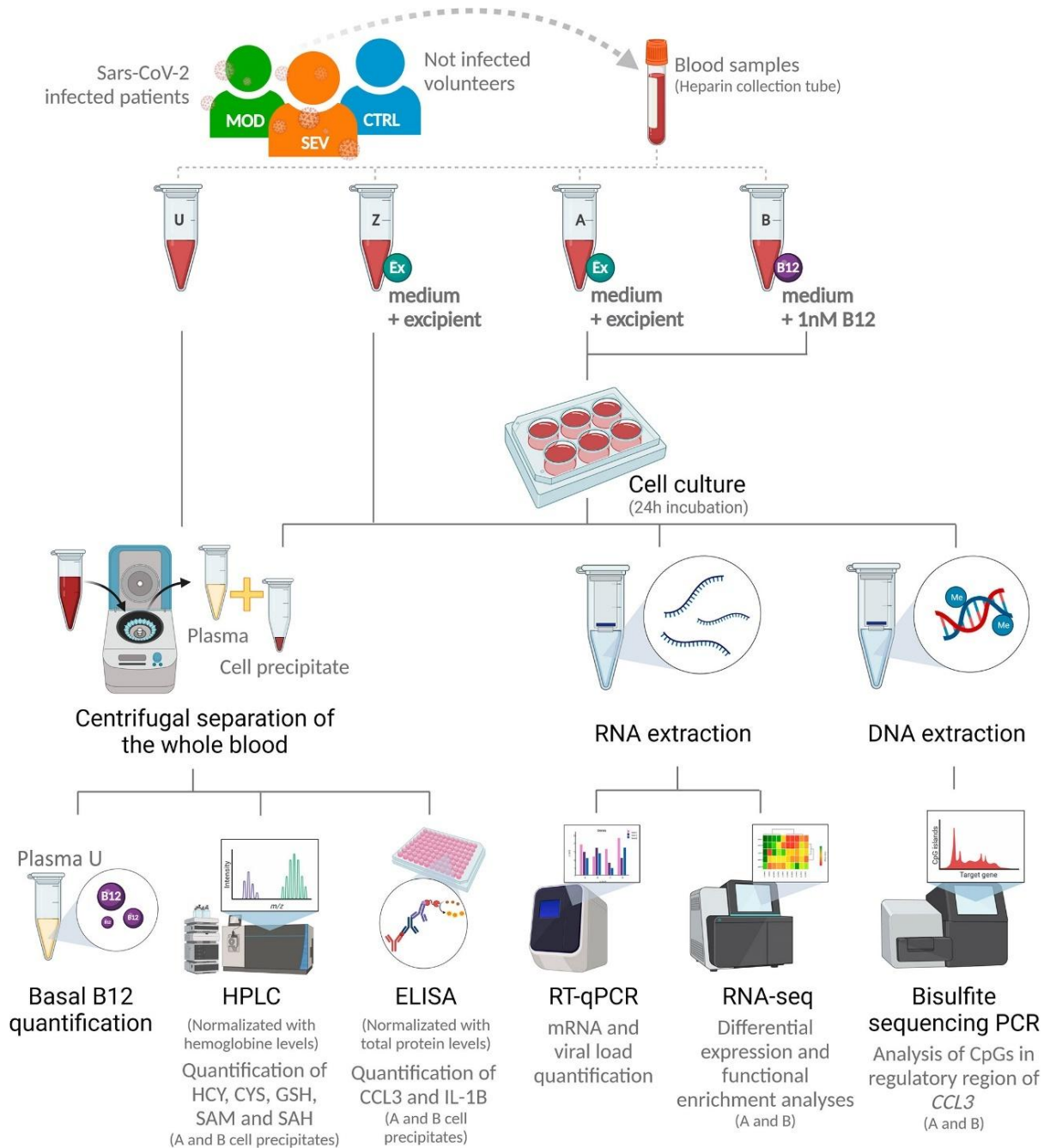


Figure 2: Research design. SEV, severe COVID-19; MOD, moderate COVID-19; CTRL, non-infected controls; U, endpoint U (unprocessed blood); Z, endpoint Z (samples immediately processed after addition of culture medium with excipient); A, endpoint A (Samples added to culture medium with excipient and incubated for 24h); B, endpoint B (Samples added to culture medium with B12 and incubated for 24h); Ex, Excipient; B12, Vitamin B12; HPLC, High Performance Liquid Chromatography; HCY, Homocysteine; CYS, Cysteine; GSH, Glutathione; SAM, S-adenosylmethionine; SAH, S-adenosyl-L-homocysteine; PCR, Polymerase Chain Reaction; RT-qPCR, Real-time quantitative PCR. (Created with BioRender.com).

Specific primers (Table S2) were used to detect the human mRNAs of *CCL1* (C-C Motif Chemokine Ligand 1) (NM_002981.2), *CCL2* (C-C Motif Chemokine Ligand 2) (NM_002982.4), *CCL3* (C-C Motif Chemokine Ligand 3) (NM_002983.3), *CXCL9* (C-X-C motif chemokine ligand 9) (NM_002416.3), *IL1B* (Interleukin 1 beta) (NM_000576.3), *IL6* (Interleukin 6) (NM_000600.5), *IL17A* (Interleukin 17A) (NM_002190.3), *TNF* (Tumor Necrosis Factor) (NM_000594.4), *HAVCR2* (Hepatitis A Virus Cellular Receptor 2) (NM_032782.5), *CD4* (CD4) (NM_000616.5) and *CD8A* (CD8a) (NM_001768.7). For all RT-qPCR assays, target gene expression levels were normalized by 18S ribosomal RNA (18S ribosomal N1) (NR_145820.1) levels. qPCR reactions were performed with FastSYBR Green Master Mix (ThermoFisher) plus 10 ng of cDNA in a final volume of 10 μ L. Thermal cycling and fluorescence detection were performed using the ViiA 7 real-time PCR system (ThermoFisher) according to the manufacturer's recommendations. The relative expression of target genes was calculated using the $2e(-\Delta Ct)$ method (26).

ELISA

To access the intracellular protein levels of CCL3 and IL-1B, cell precipitates of aliquots from endpoints A and B were homogenized in 1X PBS, diluted in Milli-Q H₂O 1:2 and the cells disrupted by mechanical lysis in the TissueLyser (Qiagen) for 2 minutes at 30 Hz and three freeze/thaw cycles (20° to -20°C).

ELISA kits (R&D Systems, Abingdon, UK) for CCL3 (#DY270) and IL-1B (#DY201) were used and assays performed as per the manufacturer's instructions. The absorbance was read at 450 nm with the Multiskan GO spectrophotometer (ThermoFisher). Standard curves were constructed from 0.1 to 100 pg/mL and cytokine concentrations were calculated with SkanIt Software 4.1 for Microplate Readers RE, version 4.1.0.43 (ThermoFisher).

High performance liquid chromatography

For measurements of HCY, cysteine (CYS), GSH, SAM and S-adenosyl-L-homocysteine (SAH), cell precipitates from aliquots A and B were diluted in Milli-q H₂O 1:2 and the cells disrupted by three freeze/thaw cycles (20° to -20°C). Metabolites were quantified by high performance liquid chromatography (HPLC) on the Shimadzu Prominence-i LC-2030C 3D Plus (GMI, Ramsey, Minnesota) according to Pfeiffer et al. (27) with some modifications. HCY, CYS and GSH were quantified using a C18 Luna column (5 mm x 150 mm x 4.6 mm) and mobile phase composed of 0.06 M sodium acetate, 0.5% acetic acid and 2% methanol (pH 4.7 adjusted with acetic acid). The flow rate was 1.1 mL $\times$ min⁻¹ and the retention time was 4.1

min for CYS, 5.9 min for HCY and 10.3 min for GSH. SAM and SAH were quantified using a method adapted from Blaise et al. (28). HClO_4 was added to the homogenized cell precipitate for protein precipitation. The supernatant was injected into a C18 LiChroCart column (5 mm x 250 mm x 4 mm) and the mobile phase applied at a flow rate of $1 \text{ mL} \times \text{min}^{-1}$, consisting of 50 mM sodium phosphate (pH 2.8), 10 mM heptane sulfonate and 10% acetonitrile. Retention time was 8.7 min for SAH and 13.6 min for SAM. All metabolites were detected by UV absorption at a wavelength of 254 nm.

The intracellular concentrations of the metabolites were normalized by hemoglobin concentrations, obtained by colorimetry (Agabe hemoglobinometer) adding 10 μL of the sample in an ampoule (Hemoglobin AP; Labtest).

RNA-Seq

Total RNA from three samples from each group (MOD, SEV and CTRL) at endpoints A and B (total 18 samples) was used to construct cDNA libraries with the TruSeq Stranded mRNA kit (Illumina, San Diego, CA) and the indexed fragments were sequenced on the NGS NextSeq 500 (Illumina) with the NextSeq 500/550 High Output 2x75 cycles kit (Illumina). All sequenced samples were quality assessed by capillary electrophoresis with the Agilent RNA 2100 Nano kit (Bioanalyzer, Santa Clara, CA).

Bisulfite Sequencing PCR

Bisulfite Sequencing PCR libraries were constructed according to Bernstein et al. (29), and the standard Illumina protocol for amplicon sequencing with modifications. Briefly, genomic DNA from five samples from each group (MOD, SEV and CTRL) at endpoints A and B (total of 30 samples) was extracted with QIAamp DNA Blood Mini kit (Qiagen) according to the manufacturer's instructions. DNA was subjected to bisulfite conversion using EpiTect Bisulfite kit (Qiagen). The region -107pb to +164pb of *CCL3* (GRCh38/hg38 Chr17: 36,090,276 - 36,090,005) that cover the promoter region and proximal portion of the first exon of the gene was PCR amplified with GoTaq DNA Polymerase (Promega, Madison, Wisconsin) from bisulfite-converted DNA using the primer listed in Table S3. DNA libraries were purified with AMPure XP beads (Beckman Coulter, Indianapolis, IN), indexed with Nextera XT DNA Library Preparation Kit (Illumina) and sequenced on the NGS MiSeq (Illumina) with MiSeq Reagent kit v2 (300 cycles) (Illumina). All sequenced samples had their quality evaluated by capillary electrophoresis with Agilent High Sensitivity DNA Kit (Bioanalyzer).

Bioinformatics analysis

Hierarchical clustering analyzes were performed using the GenePattern software (30) using Pearson correlation as a comparison method and average linkage as a linkage method.

Raw RNA-Seq reads were pre-processed with *Trimmomatic* software (31) to remove adapters, poor quality bases or very short reads (less than 36nt). The filtered reads were mapped to the *Homo sapiens* reference transcriptome (Gencode, release 36) and the total number of reads mapped per transcript and the number of transcripts per million were calculated with *Salmon* software (32). Transcript counts and abundances were summarized at the gene level using the *summarizeToGene* function of the R *Tximeta* package (33). Contrast analyses between MOD vs. CTRL at endpoint A (contrast 1), SEV vs. CTRL at endpoint A (contrast 2), MOD at endpoint B vs. CTRL at endpoint A (contrast 3), SEV at endpoint B vs. CTRL at endpoint A (contrast 4), and CTRL at endpoint A vs. CTRL at endpoint B (contrast 5) were performed with the R DESeq2 package (34). Genes with Fold Change greater than 1.5 and *P* value less than 0.05 obtained in the False Discovery Rate (FDR) analysis were considered differentially expressed (DEG) and submitted to functional enrichment analysis with *Ingenuity Pathways Analysis software* (IPA, Qiagen) using default parameters. Pathways with $-\log P$ value greater than 2 were considered to be differentially activated or inhibited. To assess the differences between moderate and severe COVID-19 (contrast 1 vs. contrast 2) and the effect of B12 (contrast 3 vs. contrast 1, and contrast 4 vs. contrast 2), only differentially regulated pathways that had more than 20% difference in Z-scores between the two contrasts were selected. Genes differentially expressed in response to B12 were submitted to gene ontology (GO) functional annotation analysis using *DAVID Bioinformatics software* (35) with default parameters. The GO terms for Biological Processes with $P < 0.05$ were summarized according to their ontology with *REVIGO* (36).

For Bisulfite Sequencing PCR analysis, raw reads were preprocessed with *Trimmomatic* (31) as described above, except that the minimum read size threshold was 50nt. Since no single nucleotide polymorphism that influence CpGs dinucleotides, which can generate or abolish a CpG site, were identified in the analyzed region, the reference sequence chr17: 36,090,276 - 36,090,005 of the genome of *Homo sapiens* (Gencode, release 38), was used to map the filtered reads. The mapping, as well as the calculation of the percentages of methylation of cytosines in CG context were performed with the *Bismark* software (37). *CCL3* functional annotations, such as transcription start site, single nucleotide polymorphisms and predicted transcription factor binding sites were identified with the *UCSC Genome Browser* (38).

Statistical analysis

Statistical analyses were performed using *GraphPad Prism* software (version 8.0.2) (GraphPad Software Inc., Irvine, CA). Data distribution was analyzed using Anderson-Darling, D'Agostino & Person, Shapiro-Wilk and Kolmogorov-Smirnov tests. For comparisons between three or more groups, one-way analysis of variance (ANOVA) or Kruskal-Wallis tests were used, followed by the multiple comparison tests of Tukey or Dunn, for parametric or non-parametric data, respectively. Two-way ANOVA followed by Tukey's multiple comparison test was used for comparisons involving the effect of two factors on a dependent variable in three or more groups. Two-tailed Student's t, paired Student's t, Mann-Whitney or Wilcoxon tests were used for comparisons between two groups, according to the experimental design and data distribution. Correlations were tested using Pearson or Spearman tests according to data distribution. Outliers identified by ROUT test ($Q = 1\%$) were excluded. Data were expressed as median $\pm$ interquartile (nonparametric) or mean $\pm$ standard deviation (parametric). Differences were considered statistically significant when $P < 0,05$.

For Bisulfite Sequencing PCR analysis, the means of methylation percentages of each CG locus of MOD, SEV and CTRL groups at endpoints A and B were compared using two-tailed Wilcoxon test and only those that presented a statistically significant difference were considered. Correlation between methylation levels of differentially methylated loci (DML) at endpoint B compared to endpoint A and *CCL3* gene expression levels from the same cultures were tested with Pearson or Spearman correlation for parametric or nonparametric distributions, respectively.

Results

Patients and healthy volunteers

The median age of patients and non-infected healthy volunteers included in the study was 64 years. 55% of recruited participants were women, with no statistically significant differences between the MOD, SEV or CTRL groups in these aspects. Infected patients had a confirmatory clinical diagnosis of COVID-19 and confirmation of SARS-CoV-2 virus infection by RT-qPCR carried out, on average, 5 days before the collection of blood samples. The time elapsed between the onset of COVID-19 symptoms and admission to the hospital was 6.3 days, and the length of hospital stay before sample collection was 11.8 days, with no statistically significant

difference between the two infected groups. Cardiovascular diseases and diabetes mellitus were the most frequent comorbidities among these patients (Table 1).

Information		All (n=26)	MOD (n=10)	SEV (n=16)	P value (MOD x SEV)
General Information					
Biological Sex	Female [n (%)]	14 (55.56)	5 (54.55)	9 (56.25)	>0.9999
	Male [n (%)]	12 (44.44)	5 (45.45)	7 (43.75)	
Age [years; median (IQ)]		64 (59.75-71)	64 (58.5-72.75)	63.5 (59.5-69.5)	0.9341
Length of stay until the date of blood collection [days; mean (SD)]		11.81 (4.205)	11.10 (3.872)	12.25 (4.465)	0.5087
Time elapsed between symptom manifestation and admission to the hospital [days; mean (SD)]		6.333 (3.460)	6.778 (4.116)	6.067 (3.127)	0.6365
Time elapsed between COVID test to blood draw [days; mean (SD)]		5.423 (4.272)	5.4 (2.547)	5.438 (5.151)	0.9832
Dexamethasone treatment [days; median (IQ)]		11 (6.5-11.5)	11 (6-11.5)	10.5 (8.5-11.75)	0.6546
Use of two or more glucocorticoids (Hydrocortisone and/or beclomethasone) [n (%)]		14 (53.85)	4 (40)	10 (62.5)	0.4216
Coexisting Medical Conditions					
Diabetes Mellitus [n (%)]		10 (38.46)	3 (30)	7 (43.75)	0.6834
Cardiovascular Diseases [n (%)]		19 (73.08)	8 (80)	11 (68.75)	0.668
Laboratory Findings - Biochemistry and Blood Count					
Glucose [mg/dL; mean (SD)]		173.3 (63.83)	131.7 (58.85)	196.7 (55.2)	0.0111 (*)
Potassium [mmol/L; median (IQ)]		4.205 (3.845-4.44)	3.82 (3.673-4.17)	4.365 (4.12-5.075)	0.0022 (**)
Total leukocytes [cells/mm ³ ; mean (SD)]		13.776 (6.119)	9.650 (2.932)	16.355 (6.230)	0.0041 (**)
% Lymphocytes [median (IQ)]		9.050 (5.75-16.65)	14.85 (10.3-22.5)	6.95 (4.7-10.5)	0.0048 (**)
% Neutrophils [median (IQ)]		81.7 (70.18-87.03)	73.85 (66.55-82.23)	86.1 (81.03-89.88)	0.0139 (*)
Neutrophil/Lymphocyte ratio [mean (SD)]		10.09 (7.097)	5.277 (3.194)	13.1 (7.26)	0.0038 (**)
Laboratory Findings - Blood Gas					
pH [median (IQ)]		7.405 (7.371-7.444)	7.435 (7.412-7.444)	7.375 (7.352-7.439)	0.0223 (*)
O ₂ Pressure [mmHg; median (IQ)]		68.7 (58.33-86.4)	58.15 (50.95-65.15)	80.1 (66.03-89.85)	0.0028 (**)
CO ₂ Tension [mmol/L; median (IQ)]		22.1 (20.4-27.28)	20.3 (19.6-24.15)	23.25 (21.83-29.6)	0.0309 (*)
O ₂ Saturation [%; mean (SD)]		93.06 (4.756)	90.25 (5.04)	94.94 (3.609)	0.0123 (*)
Antivirals					
Oseltamivir [n (%)]		19 (73.08)	10 (100)	9 (56.25)	0.0227 (*)
Antibiotics					
Clavulanate [n (%)]		11 (42.31)	7 (70)	4 (25)	0.0426 (*)
Amoxicillin [n (%)]		11 (42.31)	7 (70)	4 (25)	0.0426 (*)
Bacterial Coinfections					
Coinfections [n (%)]		14 (53.85)	2 (20)	12 (75)	0.0138 (*)
Complications					
Patients on mechanical ventilation at the time of blood collection [n (%)]		13 (50)	–	13 (81.25)	0.0001 (***)
Outcome					
Hospital discharge [n (%)]		13 (50)	9 (90)	4 (25)	0.0036(**)
Death [n (%)]		13 (50)	1 (10)	12 (75)	
Time between recruitment and patient outcome [days; median (IQ)]		8 (3.75-12)	3 (1-6.25)	10 (6.25-20.75)	0.0013 (**)
Total Length of Hospitalization [days; mean (SD)]		23.42 (12.15)	16.2 (6.07)	27.94 (12.93)	0.0132 (*)

Cardiovascular Diseases: Systemic Arterial Hypertension, Stroke, Acute Myocardial Infarction, Heart Failure, Aneurysm, Cardiopathic Patient. For parametric data, an unpaired Student's T test was used to determine statistical significance, for nonparametric data, Mann-Whitney test was used, both two-tailed. P values < 0.05 were considered statistically significant. *P < 0.05; ** P < 0.01; ***P < 0.001; ****P < 0.0001. The comparison between qualitative data was made with two-sided Fisher's exact test. One patient was excluded from glucose analysis and one from oxygen saturation analysis, as both were considered outliers (ROUT test (Q=1%)). SD, Standard Deviation; IQ, Interquartile Range; MOD, moderate COVID-19; SEV, severe COVID-19.

Table 1: Patient's data.

All infected patients were on glucocorticoid treatment (dexamethasone: 25 patients; prednisone: 1 patient) but those with severe COVID-19 most often received combinations of two or three drugs of this category, namely dexamethasone, hydrocortisone, and beclomethasone for an average of 11 days before their blood samples were collected. Among the cytochemical parameters evaluated, patients with severe COVID-19 had increased levels of blood glucose ($P = 0.0111$), potassium ($P = 0.0022$), total leukocytes ($P = 0.0041$), percentage of lymphocytes ($P = 0.0048$), percentage of neutrophils ($P = 0.0139$) and neutrophil/lymphocyte ratio ($P = 0.0038$) when compared to MOD patients. The increase in the percentage of neutrophils in SEV patients coincided with the occurrence of more frequent bacterial coinfections in this group ($P = 0.0138$). These patients also had higher pH ($P = 0.0223$), O₂ pressure ($P = 0.0028$), CO₂ tension ($P = 0.0309$) and O₂ saturation ($P = 0.0123$), compatible with the fact that SEV patients were intubated with mechanical ventilation.

The combination of Clavulanate and Amoxicillin was more frequent among MOD than SEV patients ($P = 0.0426$). Although there were no statistically significant differences between MOD and SEV groups regarding the occurrence of clinical complications, the outcomes were significantly worse for patients in the SEV group, with longer total hospitalization time ($P = 0.0132$) and more frequent deaths ($P = 0.0036$) (Table 1). All information compiled from medical records is presented in Table S1.

Control subjects had no RT-qPCR detectable SARS-CoV-2 in their oropharynx and nasopharynx and no antibodies against the virus in their blood. No statistically significant differences were found between baseline plasma B12 levels of patients in the SEV, MOD and CTRL groups (Figure S1). No SARS-CoV-2 RNA was detected by RT-qPCR in raw blood or blood cultures of any participant in this study.

Validation of the experimental model

The RT-qPCR analyses revealed distinctive transcriptional signatures for MOD and SEV forms of COVID-19 and the CTRL group in the aliquots of endpoint Z, which remained mostly preserved after 24h of incubation (endpoint A) (Figure S2 and Figure 3). Importantly, patients were already on glucocorticoid treatment before blood collection for this study, which may explain why higher levels of mRNA were not found for some genes, such as *CCL2*, *CXCL9*, *IL6*, *IL17A*, *CCL1* and *TNF* in MOD and/or SEV patients compared to CTRL at endpoints Z and A (Figure S2 and Figure 3). High levels of *CCL3* and *IL1B* mRNA were observed in the SEV and MOD groups compared to the CTRL, indicating that these

mediators of COVID-19 inflammation are not sufficiently responsive to glucocorticoid treatment. Reduced levels of mRNA of marker genes of CD4 and CD8a lymphocyte lineages were observed in MOD and SEV patients compared to CTRL at endpoints Z and A. At endpoint Z, these two genes showed a positive correlation (MOD: $r = 0.5553$, $P = 0.004$ and SEV: $r = 0.5665$, $P = 0.0032$) with the percentage of lymphocytes in the patients' blood counts (Table 1 and Table S1). On the other hand, mRNA levels of *HAVCR2* lymphocyte exhaustion marker did not differ between patients and CTRL.

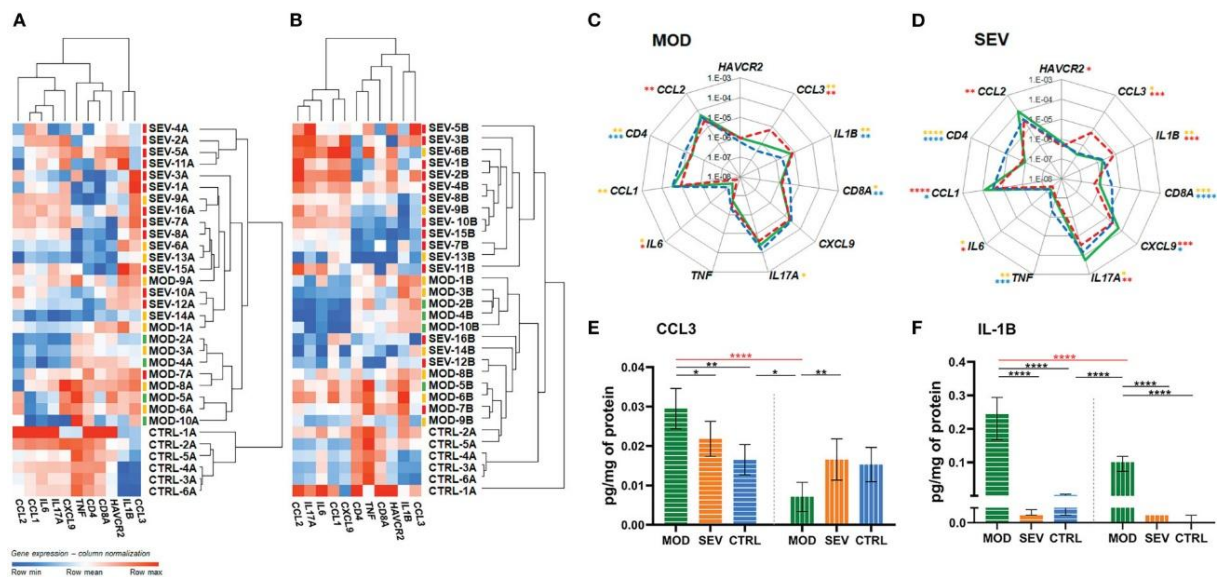


Figure 3: Vitamin B12 favorably modulated critical inflammatory mediators. (A, B): Dendrogram and heatmap depicting the hierarchical clustering and expression levels of COVID-19 hyperinflammation-related genes panel in whole blood cultures of untreated MOD and SEV patients (endpoint A) (Panel A) or treated with B12 (endpoint B) (Panel B) and untreated non-infected controls (endpoint A). Colored dashes next to the sample identification correspond to the patient's outcome. Green dashes: patients discharged from hospital one day after sample collection; Yellow lines: patients discharged from hospital two or more days after sample collection; Red lines: patients died. **(C, D):** Radar charts depicting the expression levels of COVID-19 hyperinflammation-related genes panel in whole blood cultures. The filled green lines correspond to gene expression values of infected groups (MOD, Panel C or SEV, Panel D) at endpoint (B). Dashed red lines correspond to gene expression values of infected groups at endpoint (A). Dashed blue lines correspond to gene expression values control group at endpoint (A). The gene expression values ($2e(-\Delta Ct)$) of the groups were compared pairwise using two-tailed Student's t, paired Student's t, Mann-Whitney and Wilcoxon tests according to experimental design and data distribution. Values were expressed as medians. N sample = MOD (10) and SEV (16) for each endpoint (A or B), CTRL (6). Yellow asterisks = Infected A versus controls A; Red asterisks = Infected B versus Infected A; Blue asterisks = Infected B versus controls A. **(E, F):** Bar charts depicting intracellular protein levels of CCL3 (Panel E) and IL-1B (Panel F). Filled bars with horizontal lines = endpoint (A). Filled bars with vertical lines = endpoint (B). Data were compared using two-way Analysis of Variance (ANOVA) test followed by Tukey's multiple comparison test. Values were represented as mean $\pm$ standard deviation. N sample = MOD (7), SEV (8), CTRL (4) for each endpoint (A or B). Red asterisks and red lines denote the effect of B12 on cultures of patients with moderate COVID-19. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. SEV, severe COVID-19; MOD, moderate COVID-19; CTRL, non-infected controls. Suffixes: A, endpoint A (samples added to culture medium with excipient and incubated for 24h); B, endpoint B (samples added to culture medium with B12 and incubated for 24h).

Vitamin B12 favorably modulated critical inflammatory mediators

The biomarker genes *CCL3* and *IL1B*, which were upregulated in patients despite previous glucocorticoid therapy, responded very well to treatment with B12, which reduced *CCL3* mRNA levels of MOD and SEV, and *IL1B* of SEV, matching them to the CTRL at endpoint A (Figures 3A–D). Importantly, B12 did not affect *CCL3* or *IL1B* expression in the CTRL group at endpoint B. Other genes had lower mRNA levels in MOD (*IL17A*, *IL6* and *CCL1*) and SEV (*IL17A* and *IL6*) compared to CTRL at endpoint A. B12 raised mRNA levels of *IL6* and *IL17A* in both MOD and SEV, matching them to CTRL at endpoint A. The vitamin also raised *CCL1* mRNA levels in cultures of MOD and SEV patients. In this case, *CCL1* mRNA levels of MOD group equaled those of the CTRL at endpoint A and the levels of the SEV exceeded this reference. *CXCL9* mRNA levels, which did not differ between patients and CTRL at endpoint A, increased in SEV cultures treated with B12 so that its mRNA levels exceeded those of the CTRL at endpoint A.

TNF, whose baseline mRNA levels were low only in the SEV group compared to CTRL at endpoint A, did not have their mRNA levels significantly altered by B12 in either of the two patient groups. B12 also did not affect *CD4* and *CD8A* mRNA levels in MOD or SEV, but slightly increased those of *HAVCR2* in SEV. However, this increase was not enough to achieve a statistically significant difference compared to CTRL at endpoint A.

Finally, the treatment of whole blood cultures of individuals in the CTRL group with B12 did not change the expression levels of any gene assessed. Overall, these results reinforce the security of the 1 nM dose of B12 and prove its efficiency in regulating the mRNA levels of several critical inflammatory mediators in whole blood cultures of patients with moderate and severe COVID-19. This regulation of mRNA levels of inflammatory mediators by B12 was done by down- or upregulation depending on the gene.

Vitamin B12 reduced intracellular protein levels of critical inflammatory mediators

MOD cultures treated with B12 had reduced intracellular protein levels of *CCL3* and *IL-1B* ($P < 0.0001$), both of which were elevated at endpoint A compared to CTRL ($P = 0.0011$ and $P < 0.0001$, respectively) (Figures 3E, F). Interestingly, B12 did not affect the intracellular protein levels of *IL-1B* in the cultures of the SEV group, and, regardless of the treatment, the intracellular protein levels of *IL-1B* were very low, close to or below the detection limit of the method (Figure 3F).

Vitamin B12 increased the flow of the sulfur amino acid pathway

B12 caused an increase in HCY concentrations of all groups (MOD, $P = 0.0037$; SEV, $P < 0.0001$; CTRL $P = 0.0313$) and in CYS and GSH levels of MOD and SEV (MOD: $P = 0.0029$ and $P = 0.0002$; SEV: $P = 0.0007$ and $P < 0.0001$) compared to their respective levels at endpoint A, which indicates an increased flow of the sulfur amino acid pathway (Table 2). However, the expected increase in the SAM/SAH ratio in response to B12 was not observed. In fact, at endpoint B, decreased SAM levels were observed in MOD group ($P = 0.0010$), without, however, any change in SAM/SAH ratio. In the SEV group, B12 increased SAH ($P = 0.0091$) and reduced SAM/SAH ($P = 0.0021$).

Metabolites	Group	Intracellular concentrations ($\mu\text{mol/g Hb}$) (mean [SD] or median [IQ])			P value (AxB)		
		MOD	SEV	CTRL	MOD	SEV	CTRL
HCY	A	0.04096 (0.007656)	0.04312 (0.01524)	0.054 (0.05133-0.0638)	0.0037 (**)	<0.0001 (****)	0.0313 (*)
	B	0.0561 (0.01653)	0.05762 (0.01784)	0.067 (0.06225-0.0985)			
CYS	A	0.5869 (0.2176)	0.6795 (0.2867)	0.5635 (0.04167)	0.0029 (**)	0.0007 (***)	0.1253
	B	0.7326 (0.2542)	0.9097 (0.3812)	0.8457 (0.2764)			
GSH	A	12.52 (2.123)	10.6 (4.292)	15.35 (2.735)	0.0002 (***)	<0.0001 (****)	0.0612
	B	15.1 (2.877)	14.94 (6.213)	16.24 (2.878)			
SAM	A	0.002436 (0.000673)	0.003405 (0.00225-0.00466)	0.0018 (0.001418-0.001815)	0.0010 (**)	0.4954	0.0938
	B	0.001789 (0.0003617)	0.002673 (0.002365-0.004239)	0.002081 (0.001825-0.00227)			
SAH	A	0.0002819 (0.00009389)	0.0001972 (0.00008667)	0.0001817 (0.00003608)	0.0722	0.0015 (**)	0.0087 (**)
	B	0.0002293 (0.00004081)	0.0003267 (0.0001541)	0.0002277 (0.00004052)			
SAM : SAH	A	9.093 (2.571)	16.44 (12.09)	9.632 (3.123)	0.1511	0.0021 (**)	0.7341
	B	8.041 (2.28)	8.058 (4.557)	9.243 (2.087)			

The parametric data were compared using two-tailed paired Student's T test and represented as $\pm$ standard deviation. The nonparametric data were compared using two-tailed Wilcoxon paired test and represented as $\pm$ interquartile range. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. SEV, severe COVID-19; MOD, moderate COVID-19; CTRL, non-infected controls; A, endpoint A (samples added to culture medium with excipient and incubated for 24h); B, endpoint B (samples added to culture medium with B12 and incubated for 24h).

Table 2: Vitamin B12 increases the flow of the sulfur amino acid pathway.

Vitamin B12 increased methylation levels of CpGs in regulatory regions of *CCL3*

The effects of COVID-19 and B12 on the methylation levels of the 21 CpGs located in the promoter region and proximal portion of the first exon of the *CCL3* gene (GRCh38/hg38 chr17: 36,090,276 - 36,090,005) were evaluated by Bisulfite Sequencing PCR in an NGS platform from DNA libraries produced with aliquots of endpoints A and B from individuals representing MOD, SEV and CTRL groups. At endpoint A, when compared with the CTRL group, no changes were found in the percentage of methylation of any of the evaluated positions in the cultures of MOD patients, while SEV patients had an hypomethylated CpG at chr17: 36,090,102 (Figure 4). Treatment of cultures with B12 increased methylation levels of CpGs at chr17: 36,090,097 in MOD subjects and at chr17: 36,090,097, 36,090,102 and 36,090,246 in SEV subjects compared to their respective cultures at endpoint A. Note that for methylation of

the chr17: 36,090,102 position in SEV group, COVID-19 and B12 had inverse effects. The methylation levels of all the aforementioned CpGs had negative and statistically significant correlations with the gene expression levels.

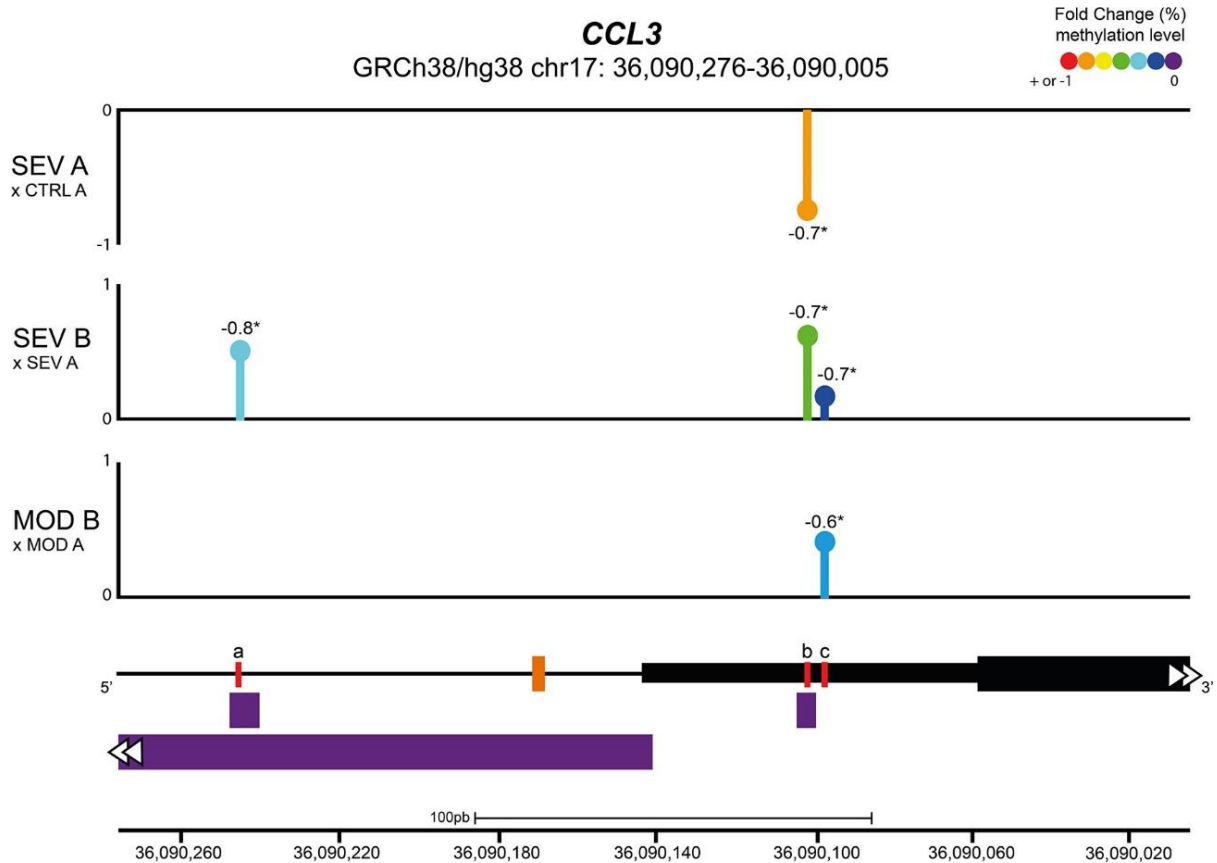


Figure 4: Vitamin B12 increased methylation levels of CpGs in regulatory regions of *CCL3*. Lollipop plot depicting the percent fold change of methylation levels of CpG sites in the promoter region and proximal portion of the first exon of the *CCL3* gene. Values next to lollipops represent correlation coefficients (Pearson or Spearman) with statistical significance ($*P < 0.05$) between methylation levels in each CpG position and gene expression values. The % fold change of methylation levels of CpG positions were compared pairwise using two-tailed Mann-Whitney. N sample = MOD (5) and SEV (5) for each endpoint (A or B), CTRL (5). In the schematic representation of the analyzed gene region, the black line represents the *CCL3* promoter region and the black blocks delimit the 5' UTR region (thin block) and proximal portion of the first exon of *CCL3* (thick block). The orange dash indicates the transcription start site of *CCL3* and the purple blocks indicate transcription factor binding sites. Red lines correspond to differentially methylated CpG sites, coordinates (GRCh38/hg38): a = chr17: 36,090,246; b = chr17: 36,090,102; c = chr17:36,090,097. Abbreviations: SEV = severe COVID-19; MOD = moderate COVID-19; CTRL = untreated non-infected controls. Suffixes: A = endpoint A (samples added to culture medium with excipient and incubated for 24h); B = endpoint B (samples added to culture medium with B12 and incubated for 24h).

Vitamin B12 attenuated the pro-inflammatory profile of leukocytes from patients with COVID-19

The effects of COVID-19 and B12 on the global gene expression in leukocytes in whole blood cultures from representative individuals of the MOD, SEV and CTRL groups were assessed by

RNA-Seq. Aiming at identifying differentially expressed genes (DEG), the following contrasts were analyzed: 1) MOD vs. CTRL at endpoint A; 2) SEV vs. CTRL at endpoint A; 3) MOD (endpoint B) vs. CTRL (endpoint A); and 4) SEV (endpoint B) vs. CTRL (endpoint A); 5) CTRL at endpoint A vs. CTRL at endpoint B.

A large number of genes had their expression affected by COVID-19 (Figure 5). In the MOD group, 3,034 DEG (2,041 upregulated and 993 downregulated) were found in contrast 1 and 3,636 DEG (2,361 upregulated and 1,275 downregulated) in contrast 3. In cultures of the SEV group, 8,565 DEG (4,464 upregulated, 4,101 downregulated) were found in contrast 2 and 8,894 DEG (4,520 upregulated and 4,374 downregulated) in contrast 4. Among the DEG identified, 2,699 had decreased (1,364) or increased (1,335) expression after treatment with B12 (Figure 5).

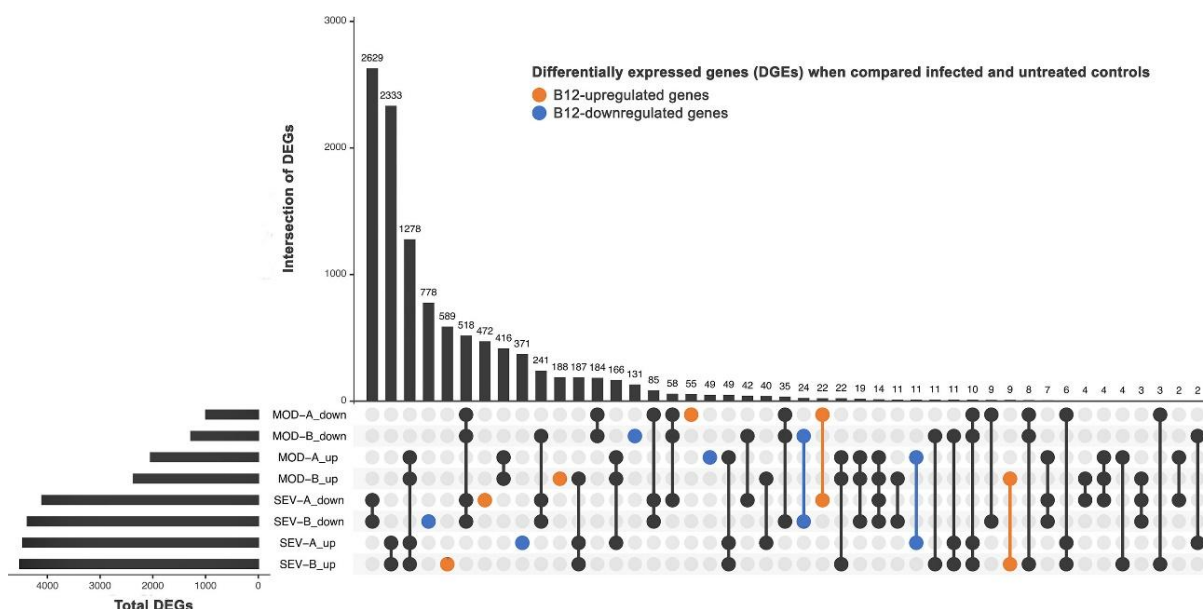


Figure 5: Differently expressed genes (DEG). DEG in infected patients (at endpoint A or B) when compared to untreated non-infected controls (endpoint A). SEV, severe COVID-19; MOD, moderate COVID-19. Suffixes: A, endpoint A (samples added to culture medium with excipient and incubated for 24h); B, endpoint B (samples added to culture medium with B12 and incubated for 24h); up, upregulated; down, downregulated.

In the leukocytes of patients in MOD group, B12-upregulated genes had GO annotations related to phagocytosis, regulation of miRNA transcription, response to virus and monocyte extravasation. In the same group, B12-downregulated genes had GO annotations related to NF-kappaB signaling, T-cell receptor signaling pathway and negative regulation of histone H3-K9 trimethylation (Figure S3). Regarding the SEV group, B12-upregulated genes had GO annotations related to cytoplasmic translation (tRNA and rRNA processing), adaptive immune response, ncRNA processing, miRNA-mediated gene silencing, proteasome-mediated

ubiquitin-dependent protein catabolic process, cobalamin transport, and negative regulation of transcription by RNA polymerase II (Figure S3). B12-downregulated genes in this group were related to positive regulation of transcription by RNA polymerase II, processing and transport of mRNA, mRNA splicing, cellular response to DNA damage stimulus, activation of innate immune response, positive regulation of IL-6 and IL-2 production, T-cell differentiation in thymus, histone modification, viral transcription and DNA conformational change. Finally, no DEG were found in contrast 5, which reinforces the safety profile of B12.

Functional enrichment analysis of DEG identified in the contrasts above mentioned revealed 90 metabolic and signaling pathways differentially regulated in the groups MOD or SEV at endpoint A directly or indirectly related to the inflammatory response in blood, although patients had received glucocorticoid treatment for approximately 11 days prior to collection of samples to the study (Figure 6A and Figure S4). In the cultures treated with B12, 45 and 74 out of these 90 pathways were still differentially regulated in MOD and SEV groups, respectively, although with a minimum 20% difference in Z-scores relatively to their untreated condition. An overall favorable effect on inflammation control was predicted for B12 treated cultures of MOD (Figure 6B and Figure S5) and SEV (Figure 6C and Figure S6) groups.

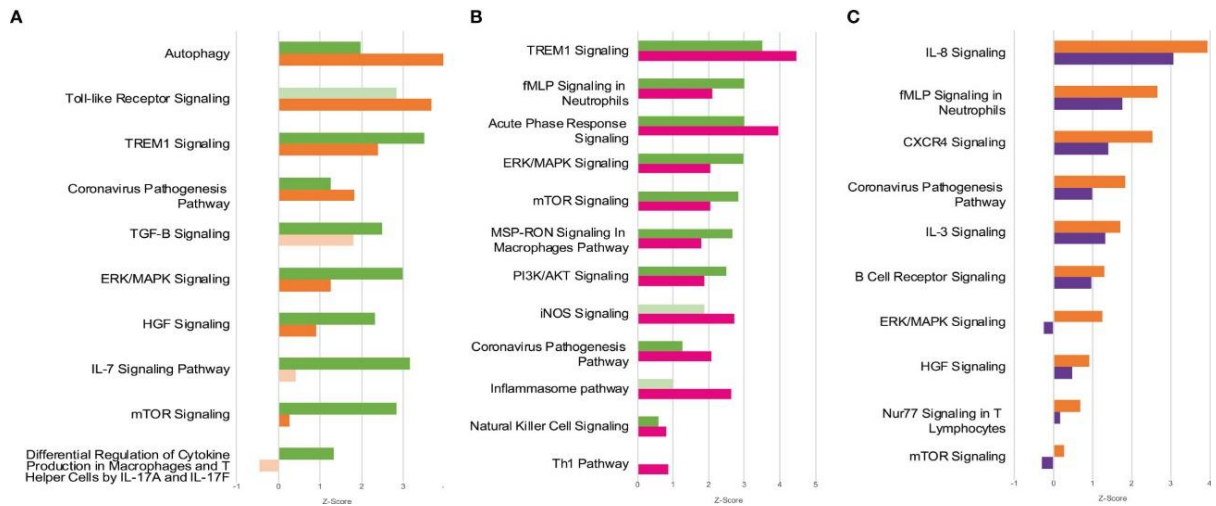


Figure 6: Canonical Pathways affected by COVID-19 and B12. Patients with moderate and severe COVID-19 previously treated with glucocorticoids had distinct global gene expression patterns (A) and vitamin B12 attenuated the pro-inflammatory profile of leukocytes from patients with moderate (B) or severe (C) forms of the disease. Green bars = contrast 1 (MOD vs. CTRL at endpoint A); Orange bars = contrast 2 (SEV vs. CTRL at endpoint A); Pink bars = contrast 3 (MOD at endpoint B vs. CTRL at endpoint A); Purple bars = contrast 4 (SEV at endpoint B vs. CTRL at endpoint A); Faded bars indicate pathways with *P* value greater than 0.05 (statistically non-significant). SEV, severe COVID-19; MOD, moderate COVID-19. Suffixes: A, endpoint A (Samples added to culture medium with excipient and incubated for 24h); B, endpoint B (Samples added to culture medium with B12 and incubated for 24h).

Discussion

Epigenetic changes in host cells during COVID-19 have already been described and are associated with the regulation of the SARS-CoV-2 cycle (39, 40), the disease severity (17, 40) or the prognosis for critically ill patients (16, 40). However, to the best of our knowledge, this is the first work to demonstrate that pharmacological modulation of the leukocyte epigenetic markings favorably regulates central components of hyperinflammation in moderate and severe forms of the disease. Initially, the whole blood culture model was validated for evaluation of anti-inflammatory drugs for COVID-19 by the identification of stable transcriptional signatures distinguishing between moderate and severe forms of the disease and between these and controls without infection, despite the fact that patients were on glucocorticoids before sample collection. Then, using this *ex vivo* model, it was demonstrated that vitamin B12 favorably modulates, by methyl-dependent epigenetic mechanisms, the expression of inflammatory genes and the activity of metabolic and signaling pathways related to the hyperinflammation associated with COVID-19 forms that require hospitalization.

Expression analysis of a panel of COVID-19 related genes at endpoint Z (blood aliquots added with culture medium and B12 excipient but not incubated) (Figure S2) in addition to revealing distinctive transcriptional signatures of moderate and severe forms of the disease, have also shed light on the effects of glucocorticoid therapy on circulating leukocytes. Exacerbated activation of inflammatory cytokines is associated with COVID-19 severity and poor prognosis (41). However, at endpoint Z, mRNA levels of *CCL2*, *CXCL9*, *IL6*, *IL17A*, *CCL1* and *TNF* were lower in the MOD and/or SEV groups compared to the controls, probably due to previous glucocorticoid treatment that patients received during hospitalization. Although these genes are components of the hyperinflammation caused by SARS-CoV-2 (41, 42), it is debatable whether their early inhibition and drastic reduction are beneficial for patients. For instance, dexamethasone is known to have a strong inhibitory effect on *IL6* and *IL17A* in patients with COVID-19 (42), but it is still unclear to what extent the beneficial effects of IL-6 blockers depend on dose, time of administration, clinical condition, among other factors (43, 44). It is also worth mentioning that IL-17A play a role in induce protective inflammatory responses, hindering viral infection (45, 46). In contrast, some biomarkers remained overexpressed in MOD and SEV cultures, despite the patients having been previously treated with glucocorticoids. Among the genes most refractory to glucocorticoid treatment, *CCL3* and *IL1B* stand out, both with a central role in the pathophysiology of COVID-19 and highly expressed in peripheral blood mononuclear cells

during the disease (14, 47). *CCL3* is a critical pyrogenic cytokine that is involved in leukocyte recruitment and activation in acute inflammation. It has been reported that *CCL3* expression is higher in COVID-19 patients with an unfavorable outcome (48). IL-1B has a broad spectrum of biological functions and participates in innate and adaptive immunity. In infections, IL-1B induces gene expression and synthesis of various cytokines and chemokines in macrophages and mast cells. SARS-CoV-2 activated IL-1B stimulates the secretion of TNF, IL-6 and other cytokines, a pro-inflammatory complex that can lead to cytokine storm and be deleterious both in the lung and systemically (49).

The transcriptional signatures observed at endpoint Z remained for the most part conserved after incubation of cultures for 24h (endpoint A), thus validating the *ex vivo* model of whole blood cultures for researching drugs with the potential to fight hyperinflammation in COVID-19.

The whole blood culture model was used to test whether vitamin B12 can regulate, *via* methyl-dependent epigenetic mechanisms, the expression of inflammatory genes in the leukocytes of patients with moderate and severe forms of the disease and who are already being treated with glucocorticoids. Indeed, in the cultures of the MOD group, B12 equaled the mRNA levels of almost all the cytokine or chemokine genes tested to those of the CTRL group that did not receive the vitamin. These genes had not been regularized by glucocorticoid therapy previously received by the patients before sample collection to this study. An exception was *IL1B*, whose mRNA levels were not significantly affected by B12. Interestingly, B12 decreased intracellular protein levels of IL-1B in the MOD group (Figure 3F). It is worth noting the magnitude of reduction of *CCL3* mRNA levels by B12 in this group, which was accompanied by the reduction of their intracellular protein levels. Likewise, the treatment of SEV group cultures with B12 brought the mRNA levels of most of the evaluated biomarkers closer to those of untreated CTRL (Figures 3A, B, D). In this group, *CCL3* and *IL1B* were the most responsive genes, at the transcriptional level, to B12, but no differences were observed in the intracellular levels of the respective proteins (Figures 3E, F). The choice to analyze the intracellular protein concentrations of IL-1B and *CCL3* was based on the premise that the inhibition of gene transcription in response to B12 added to the turnover of these proteins by the proteasome machinery present inside the cells would result in detectable reductions in their concentrations after 24 hours of culture incubation. As opposite, in the extracellular medium, a detectable decrease in the concentrations of these proteins would be less likely due to the absence of the cellular machinery for protein degradation. This strategy was suitable to detect the reduction of *CCL3* and IL-1B proteins in cultures of the MOD group. However, in cultures of the SEV

group, the reduction of CCL3 by B12 did not reach statistical significance and intracellular IL-1B levels were very low, close to the detection limit of the technique, suggesting that, in severe COVID-19, leukocytes are more prone to promptly export IL-1B. Still in the SEV group, B12 increased the mRNA levels of *CCL1* and *CXCL9* beyond those observed in the untreated CTRL group. CCL1 is produced by activated T lymphocytes and monocytes/macrophages, and is the only known chemokine capable of interacting with CCR8, a receptor expressed on Th2 cells and regulatory T cells (Treg). This chemokine mediates the inhibition of dexamethasone-induced thymocyte apoptosis (50) and its stimulation is related to autocrine anti-apoptosis (51), suggesting that CCL1-CCR8 interactions may provide survival signals for T cells at sites of inflammation. CXCL9, which is inducible by IFN- γ , is related to the activation of the Th1 antiviral immune response, as well as the trafficking of Th1, CD8 and natural killer (NK) cells (52). B12 treatment also brought *IL17A* levels of SEV closer to those of untreated CTRL. Therefore, these results indicate that B12 can stimulate T cell survival in patients with severe COVID-19. As expected, since lymphocytes expressing *CD4* and *CD8A* multiply and differentiate by clonal expansion in the thymus (53), treatment of whole blood cultures with B12 did not affect *CD4* and *CD8A* mRNA levels in either MOD or SEV groups (Figures 3A–D).

Quantification of sulfur amino acid pathway metabolites revealed an increased flux as evidenced by higher concentrations of HCY, CYS and GSH in the cultures of the MOD and SEV groups treated with B12 compared to their untreated cultures. The increased concentration of GSH favors the attenuation of exacerbated inflammation and prevents cell damage caused by COVID-19 due to its antioxidant effect (54). However, a reduction in SAM and SAM/SAH was also observed in cultures from MOD and SEV groups, respectively, in response to B12 (Table 2). When HCY and B12 levels are high, the enzyme MS converts HCY to methionine, which is then converted to SAM. Then, methyltransferases transfer the methyl group of SAM to cytosine residues in the DNA or to other acceptors, such as amino acid residues in histone tails, resulting in the formation of SAH (55). In fact, an increase in SAH was observed in cultures of the SEV group treated with vitamin B12. This set of results suggests that SAM, whose concentration was probably increased in cultures treated with B12, was readily consumed in methylation reactions of either DNA or other acceptors of methyl groups during the incubation period of 24 h. This increase in the methylation capacity of leukocytes induced by B12 was confirmed by analyzing the methylation profile of CpGs of the *CCL3* gene.

Leukocytes from cultures of patients with severe COVID-19 at endpoint A showed hypomethylation of the chr17: 36,090,102 *locus*, in the 5' UTR region of *CCL3*, when

compared to the CTRL group (Figure 4). Contrarily, no DML was identified in leukocyte cultures from patients in the MOD group at endpoint A. The hypothesis that B12 downregulates inflammatory genes of COVID-19 *via* methyl-dependent epigenetic mechanisms was proved by the hypermethylation of three CpGs located in the 5' UTR region and in the proximal portion of the first exon of *CCL3* (Chr17: 36,090,276 - 36,090,005) of leukocytes from cultures of MOD and SEV groups treated with the vitamin. The B12-induced increment in methylation levels of each of these DML negatively correlated with the *CCL3* expression level assessed by RT-qPCR (Figure 4). Two of the three DML in response to B12 are located at transcription factor binding sites. TF FOS (JASPAR ID: MA1800.1) and SMARCA4 (OREgAnno ID: OREG1238466) bind to the region encompassing the DML chr17: 36,090,246 (a), while NFIC (JASPAR ID: MA0161.2) binds to that containing the DML chr17: 36,090,102 (b). NFIC is a component of the CTF/NF-I family and a SARS-CoV-2-upregulated inflammation-associated TF (56). At endpoint A, the gene encoding NFIC is upregulated in the SEV group compared to controls (contrast 2), suggesting an important role for this TF in the transcription of inflammatory genes in severe COVID-19. AP-1, a complex formed by FOS and others TF, is crucial for transcription of *CCL3*. The AP-1 signaling pathway can be activated by cytokines, growth factors, stress, and bacterial or viral infections, including SARS-CoV proteins (57). Interestingly, the gene encoding FOS was downregulated in the SEV group at endpoint B compared to controls at endpoint A (contrast 4), indicating that B12, in addition to blocking the binding of this TF to the regulatory region of *CCL3*, also reduces its expression in severe COVID-19. SMARCA4 is a protein present in ATP-dependent chromatin remodeling complexes of the SWI/SNF type. Members of this family have helicase and ATPase activities and regulate the transcription of certain genes by altering the chromatin structure in their surroundings. Recruitment of this complex is associated with increased expression of *CCL3* (58).

Therefore, the severe form of COVID-19 induces hypomethylation of a CpG locus in the regulatory region of *CCL3*, which is coherent with its upregulation. This result corroborates the findings of Corley et al. (17), who had already demonstrated the hypomethylation of upregulated inflammatory genes in blood mononuclear cells of patients with severe COVID-19. More importantly, the results presented herein prove that vitamin B12 downregulates *CCL3* in leukocytes of patients with moderate or severe COVID-19 *via* the hypermethylation of CpGs and subsequent inhibition of TF binding to the analyzed regulatory region.

As expected, functional enrichment analysis of RNA-Seq data revealed different immune response profiles depending on the severity of the disease (Figure 6A and Figure S4). However, although it has already been shown that greater activation of hyperinflammation-related pathways is associated with disease severity (59), in the present study, cultures from patients with severe COVID-19 showed less activation or greater inhibition of pathways directly or indirectly related to inflammation compared to patients with moderate disease. It is very likely that the less inflamed profile of SEV group is due to the administration of more potent glucocorticoids to these patients during hospitalization, prior to the collection of blood samples for the study (Table 1 and Table S1). These differences in the inflammatory profile of leukocytes of patients with severe and moderate COVID-19 previously treated with glucocorticoids are evident in the activation patterns of the *Coronavirus Pathogenesis Pathway* (Figure S7).

Gene ontology (GO) functional annotation analysis of the 2,699 genes differentially expressed in response to B12 (Figure S3) indicate that, in leukocytes of patients with moderate COVID-19, the vitamin stimulates the antiviral response induced by IFNs, regulates the exacerbated immune response induced by NF-kappaB and favors the inhibition of gene expression. Regarding the SEV group, the results indicate that in addition to suppressing viral infection, B12 induces inflammatory attenuation during severe COVID-19 and promotes activation of the adaptive immune response. These processes seem to have been regulated by epigenetic mechanisms, such as changes in chromatin status, gene silencing by miRNAs and alternative *splicing* reduction, which favored the reduction of gene transcription and translation.

Functional enrichment analysis also disclosed B12-modulated pathways in the MOD (Figure 6B and Figure S5) and/or SEV (Figure 6C and Figure S6) groups. It should be noted that the effect of B12 was pleiotropic, that is, it regulates several metabolic or intracellular signaling pathways maladjusted by COVID-19 despite the glucocorticoid treatment that patients received during hospitalization. Most of the differentially regulated pathways in contrasts 1 and 2 (MOD vs. CTRL and SEV vs. CTRL, all at endpoint A) had their activation or inhibition attenuated by B12 (contrasts 3 and 4 - MOD at endpoint B vs. CTRL at endpoint A; and SEV at endpoint B vs. CTRL at endpoint A). In the cultures of patients with moderate COVID-19, B12 favored the overall reduction of inflammation and activation of the Th1-type antiviral response (Figure 6B and Figure S5). The few pathways predicted to be more activated in response to B12, such as *iNOS signaling*, regulate desirable mechanisms in the context of COVID-19. Through S-nitrosylation of cysteine residues of viral and host proteins, NO reduces the activity of viral proteases, inhibiting the fusion and replication of SARS-CoV-2 in host cells

(60). Another example is the *NK cell signaling pathway*. During infection, the coronavirus depletes NK cells and impairs their antiviral effects while activating macrophages and other immune cells leading to the cytokine storm (61). Furthermore, the activation of *TREML signaling* and *Acute phase response signaling* pathways favors the production of IFN and activation of Th1 and Th17 cells, which are fundamental components of the antiviral response (46, 62, 63). Activation of the *Th1 pathway* was also observed, which further reinforces the idea that B12 stimulates the antiviral response. Regarding the few pathways where the result seems incongruous, when analyzing their topology, it is clear that their activation by B12 is potentially beneficial to the patient. For example, in the *Inflammasome pathway*, NLRP1 was inhibited in the MOD group and B12 abrogates this inhibition (Figure S8). This inflammasome interacts with double-stranded RNA (ds), being an important detector of dsRNA viruses such as SARS-CoV-2 (64). NLRP1 activity is regulated by anti-apoptotic proteins, BCL-2 and BCL-XL, which associate with the inflammasome and inhibit its activity (65). In this study, the genes encoding these two proteins were predicted to be upregulated in the leukocytes of patients in the MOD group, however B12 did not affect their expression. Thus, the regulatory effect of B12 on NLRP1 likely depends on other mediators. In the *Coronavirus Pathogenesis Pathway*, angiotensin 1-7 was predicted to be the activated in cultures treated with B12 (Figure S7). It binds to MAS1 receptors leading to the activation of the anti-inflammatory branch of the renin-angiotensin pathway (66). Moreover, in this same pathway, *Accumulation of lungs edema fluids* and *SARS CoV Replication* are predicted to be inhibited and *Type I interferon response* and *Adaptive Immunity* are predicted to be activated by B12. The vitamin caused even more evident attenuation of most of the inflammatory pathways activated by the disease in the cultures of patients with severe COVID-19 (Figure 6C and Figure S6). In summary, the pro-inflammatory transcriptional profile observed in leukocytes from patients with moderate and severe COVID-19 was clearly attenuated by vitamin B12, with no adverse effects predicted from these results.

Besides regulating DNA methylation acting as a cofactor of the enzyme MS in the sulfur amino acid pathway, B12 also modulates different chromatin remodeling mechanisms that can affect gene expression. In the Krebs cycle, adenosylcobalamin can induce the accumulation of succinate, an antagonist of histone and DNA demethylation reactions catalyzed by enzymes of the 2-oxoglutarate-dependent dioxygenase family, which may favor the activation or inhibition of gene expression (67). The antioxidant activity of B12 (68) can also indirectly influence the epigenetic landscape by regulating oxidative stress, which, depending on the context, can determine an accessible or non-accessible state of chromatin. Oxidative stress inactivates the

histone-deacetylating enzyme HDAC2, contributing to the greater accessibility of transcriptional machinery to DNA. However, oxidative stress also activates signaling pathways that increase the expression of the enzyme DNMT1 that methylates DNA (67, 69). All these mechanisms may contribute to the down- (1,364) or upregulation (1,335) of the 2,699 genes in the leukocytes of patients in the MOD and/or SEV groups in response to B12 (Figure 5).

It is noteworthy that when patients were recruited to this study, in July 2020, vaccines were not yet available and dexamethasone and other glucocorticoids had recently been integrated to the COVID-19 therapeutic protocol. At that time, there was no record of SARS-CoV-2 variants of interest/concern circulating in Brazil. Therefore, caution is needed when extrapolating the findings of this work to COVID-19 patients previously vaccinated or infected with SARS-CoV-2 variants.

In conclusion, vitamin B12 has a great potential as an adjuvant drug for alleviating inflammation in patients with moderate or severe COVID-19 in addition with the other established treatments. Beyond favorably regulating the expression of several inflammatory genes *via* methyl-dependent epigenetic mechanisms, B12 also acts as an antioxidant, has an excellent safety profile and is widely available at low cost. Therefore, phase II/III clinical trials are enthusiastically recommended. Furthermore, the results presented herein neither endorse the prophylactic use of B12 to prevent SARS-CoV-2 infection, nor its therapeutic use in mild COVID-19.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA862565> and <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA887739>.

Ethics statement

The studies involving human participants were reviewed and approved by National Research Ethics Committee of Brazil (CONEP). The patients/participants provided their written informed consent to participate in this study.

Author contributions

LC performed the experiments, contributed to experimental design, analyzed data and wrote the manuscript; VC-S and VD'A performed the HPLC assays, analyzed data and reviewed the

manuscript; BP, CC and SF recruited patients, collected blood samples and clinical and sociodemographic data from patients records; MO contributed to data analysis and reviewed the manuscript; AS made substantial contributions to the NGS experiments; GF contributed to the experimental design and data analysis and reviewed the manuscript; RC designed and oversaw the study, analyzed data and wrote the manuscript. All authors read and approved the final manuscript. All authors have approved the final article and agree with its publication.

Acknowledgments

This work received financial support from FIOCRUZ, CAPES and INCT-Vacinas. The authors declare that this study received funding from Cláudia Garcia Martins. The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article, or the decision to submit it for publication. Technical support was provided by FIOCRUZ Network of Technological Platforms: Real Time PCR and Digital PCR (P04-006), NGS Sequencing (P01-007), and Bioinformatics (P08-002). We thank Dr. Alex Fiorini de Carvalho for testing the presence of SARS-CoV-2 in blood samples by RTqPCR, Dr. Ludmila R.P. Ferreira for valuable discussions about the functional enrichment analysis, and Dr. Diana Bahia and Prof. Dulciene M.M. Queiroz for critically reviewing this manuscript.

Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2023.1048790/full#supplementary-material>

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Keywords: COVID-19, vitamin B12, epidrugs, chromatin remodeling, DNA methylation, epigenetic markings, inflammation, antiviral response

Received: 21 September 2022; **Accepted:** 27 February 2023;

Published: 13 March 2023.

CAPÍTULO III: Vitamina B12 como um regulador do *splicing* alternativo na covid-19 aguda

O seguinte trabalho surge como uma extensão do estudo anterior, no qual foram aprofundados os efeitos da covid-19 e do tratamento com vitamina B12 na expressão diferencial de isoformas de transcritos. Observou-se que a proporção de isoformas não codificadoras é afetada de acordo com a gravidade da infecção. Além disso, foi demonstrado que a vitamina B12 influencia o *splicing* alternativo dos transcritos em leucócitos, atenuando a ativação das vias relacionadas à resposta pró-inflamatória. Em conjunto, os dados revelam um novo mecanismo de ação da vitamina B12 na modulação da resposta inflamatória exacerbada associada à infecção por SARS-CoV-2.

O manuscrito está em fase de submissão para publicação. Os materiais suplementares que subsidiam as conclusões deste trabalho são apresentados neste documento [[Capítulo3-Materiais suplementares](#)].

Vitamin B12-mediated regulation of alternative splicing in acute COVID-19

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Running title: Regulation of splicing in COVID-19

Keywords: Alternative splicing, COVID-19, vitamin B12, transcript isoforms, inflammation

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Abstract

This study investigates the impact of SARS-CoV-2 infection and vitamin B12 treatment on protein-coding and non-protein-coding transcript isoform expression in leukocytes from patients with moderate and severe COVID-19. Using an *ex vivo* model of whole blood cultures, significant alterations in alternative splicing patterns associated with disease severity were identified. Leukocytes from patients with moderate COVID-19 showed a favorable trend towards protein coding and expression, maintaining antiviral and adaptive response capacity through functional isoform preservation. Conversely, patients with severe COVID-19 exhibited greater immune response dysregulation and increased inhibition of coding transcript isoforms. Moreover, the study reveals a novel mechanism by which vitamin B12 exerts anti-inflammatory effects in COVID-19 by modulating mRNA processing and isoform usage. Vitamin B12 attenuated pro-inflammatory gene expression in leukocytes of both moderate and severe COVID-19 patients, promoting a balance between antiviral and adaptive immune responses. These findings highlight the importance of alternative splicing in host response to viral infections and position vitamin B12 as a promising regulator of gene expression during COVID-19, offering insights into potential therapeutic strategies for disease management.

Introduction

Alternative splicing is a fundamental process in the regulation of gene expression, enabling a single messenger RNA (mRNA) to be processed in multiple ways, thereby producing diverse protein isoforms from a single gene (183). This mechanism is particularly significant in the context of viral infections, where pathogens can manipulate the host's splicing machinery to favor their replication and persistence within cells (218–220). Such interference can have profound implications for the host's antiviral response, notably through the generation of non-protein-coding splicing isoforms (221). These isoforms, which fail to encode functional proteins due to alterations in the splicing process, may result from intron retention, the presence of premature stop codons, or alternative selection of splicing sites that disrupt the coding sequence (reviewed in: (192)).

The coronavirus disease 2019 (COVID-19) pandemic has illuminated novel aspects of the interaction between the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) and the host immune system. Recent studies have revealed that certain viral proteins, such as NSP16, sequester components of the cellular spliceosome complex, thereby altering the splicing process (193,194,222). This strategy particularly favors the suppression of interferon (IFN) expression and antiviral response genes, consequently modulating B and T lymphocyte populations and promoting increased circulation of macrophages and neutrophils

(194,222,223). Furthermore, virus-induced dysregulation of alternative splicing contributes to enhanced expression of coding isoforms of genes encoding pro-inflammatory cytokines and chemokines, including *IL6*, *CXCL8*, and *TNF*, potentially exacerbating disease severity (200).

DNA methylation is one of the epigenetic mechanisms known to regulate alternative splicing (178). It is estimated that this process regulates the splicing of approximately 22% of alternative mRNAs (224). Typically, exons containing splice donor sites exhibit hypermethylation relative to adjacent intronic regions (178,224). Moreover, reduced methylation levels within gene bodies can result in diminished efficiency of RNA polymerase II (Pol II) elongation during transcription, potentially leading to the generation of incomplete transcripts (181).

Vitamin B12, known for its crucial role in cellular metabolism and DNA synthesis, is also fundamental for methyl bioavailability and regulation of the cellular epigenetic landscape (171). The vitamin acts as an essential cofactor in the sulfur amino acid pathway, catalyzing the conversion of homocysteine to methionine. The product of this reaction, S-adenosylmethionine (SAM), serves as the primary methyl donor in cells, fueling methylation reactions of epigenetic marks such as DNA and histone methylation (225). Vitamin B12 has been demonstrated to modulate the expression of key genes involved in inflammatory processes during infections, particularly in COVID-19, by promoting hypermethylation of their promoter regions and consequently attenuating their expression (175,176,226).

Considering these findings, the present study aims to elucidate the consequences of SARS-CoV-2-induced splicing perturbations, with a particular emphasis on the expression of noncoding transcript isoforms. Additionally, this research evaluates the potential of vitamin B12 as a regulator of this process, utilizing an *ex vivo* whole blood culture model derived from patients with moderate and severe COVID-19.

Materials and Methods

Dataset

RNA sequences were obtained from a previously bulk transcriptome analysis of leukocytes from patients with moderate COVID-19 (MOD), severe COVID-19 (SEV), and uninfected controls (CTRL) (226). The raw sequencing data are publicly available through the Sequence Read Archive (SRA) under accession number PRJNA862565. Comprehensive demographic, clinical and laboratory data for the study cohort have been published and are available for reference in the original article (226). Patient categorization was performed according to disease

severity using the World Health Organization (WHO)-established clinical progression scale (11). The study groups were age-matched, with a average of 64 years, and exhibited a balanced sex distribution (55.5% female, 44.5% male). All COVID-19 patients were undergoing glucocorticoid therapy at the time of blood sample collection, with severe cases more frequently receiving combination therapy involving two or three glucocorticoid agents. A total of 18 whole blood culture samples were analyzed. These samples were equally distributed among the three study groups (MOD, SEV, and CTRL), with each group comprising six samples. Within each group, three samples were treated with an excipient (endpoint A) and three with 1 nM vitamin B12 (endpoint B).

Reads alignment and transcripts differential expression analysis

Raw RNA-seq sequences were pre-processed using Trimmomatic software (227) to remove reads shorter than 36 nucleotides and low-quality bases, consistent with the previous gene-level differential expression analysis. Transcript abundance estimation was performed using the Salmon pseudo-alignment software (228) with an alignment index created using 31-mers and the *Homo sapiens* GENCODE v44 reference transcriptome (189). Transcript quantification was executed using the default settings along with additional parameters “*—validateMappings*”, “*—numGibbsSamples 100*”, “*—gcBias*”, and “*—seqBias*”. Transcripts were grouped based on their inferential uncertainty using the *group* and *collapse* functions of the Terminus software (229), refining the transcript set for analysis. Transcript abundances were then imported into the R environment using the tximport package (230) and summarized to transcript and gene counts.

Differential expression analysis was conducted on transcript counts using the *Swish* function from the Fishpond package (231). Expression levels of infected patients were compared to those found in the excipient-treated control group (CTRL-A), resulting in four contrasts (MOD-A vs. CTRL-A, SEV-A vs. CTRL-A, MOD-B vs. CTRL-A, SEV-B vs. CTRL-A). Transcripts with an adjusted *P* value less than 0.05 and an absolute log2 fold change greater than 0.58 were considered differentially expressed (DET). Splicing event analyses were conducted using the IsoformSwitchAnalyzeR package (232,233).

Isoform annotation and functional enrichment analysis

Transcript per million (TPM), log2 fold change, and adjusted *P* values were used as input for isoform annotation and functional enrichment analysis with the Isoformic package (234). A gene dictionary was constructed based on the reference transcriptome, containing identifiers,

official symbols, biotypes, and gene names for each transcript. This information was used to annotate tables containing DETs from the study dataset and classify them as coding (those derived from protein-coding genes annotated as "*protein_coding*") or noncoding (derived from protein coding genes and annotated as "*retained_intron*" – isoforms containing introns in the final transcript due to alternative splicing; "*processed_transcript*" – transcripts without open reading frames; or "*nonsense_mediated_decay*" – transcripts subject to degradation via the nonsense-mediated decay pathway due to premature stop codons). Other transcript isoforms were disregarded due to their lower abundance. The final tables are available in Table S1. Canonical pathway gene sets from the Reactome database (235) were used for functional enrichment analysis of coding and noncoding isoforms using *fgsea* (236). Enriched pathways with an adjusted *P* value less than 0.05 in at least one of the analyzed experiments (coding or noncoding) were considered significantly affected. The complete set of analyzed pathways is available in Table S2.

Differential expression and functional enrichment results were visualized using dot plots created with *ggplot2* (237) in the R environment.

Results

Vitamin B12 favors alternative splicing of noncoding transcripts during acute COVID-19

The effects of moderate and severe acute COVID-19, as well as vitamin B12 treatment, on the differential expression of coding and noncoding transcript isoforms were investigated. A total of 1,723 DETs were identified in the MOD-A group, 2,124 in MOD-B, 4,993 in SEV-A, and 5,991 in SEV-B when compared to the excipient-treated control group (CTRL-A) (Figure 1, panel A). Coding transcript isoforms (*protein_coding*) were found to be more abundant in all groups (MOD-A 77%, MOD-B 76.6%, SEV-A 77.3%, and SEV-B 73.5%). However, vitamin B12 treatment decreased the proportion of coding isoforms more notably in leukocytes from SEV patients (Δ MOD B-A -0.4%, Δ SEV B-A -3.8%). Among noncoding isoforms, those containing retained introns (*retained_intron*) were the most prevalent (MOD-A 17.1%, MOD-B 17.8%, SEV-A 16%, and SEV-B 19.4%), followed by those with premature stop codons (*nonsense_mediated_decay*) (MOD-A 5.6%, MOD-B 5.4%, SEV-A 6.4%, and SEV-B 6.9%). The proportion of noncoding isoforms lacking an open reading frame (*processed_transcript*) averaged 0.1% in both MOD and SEV groups.

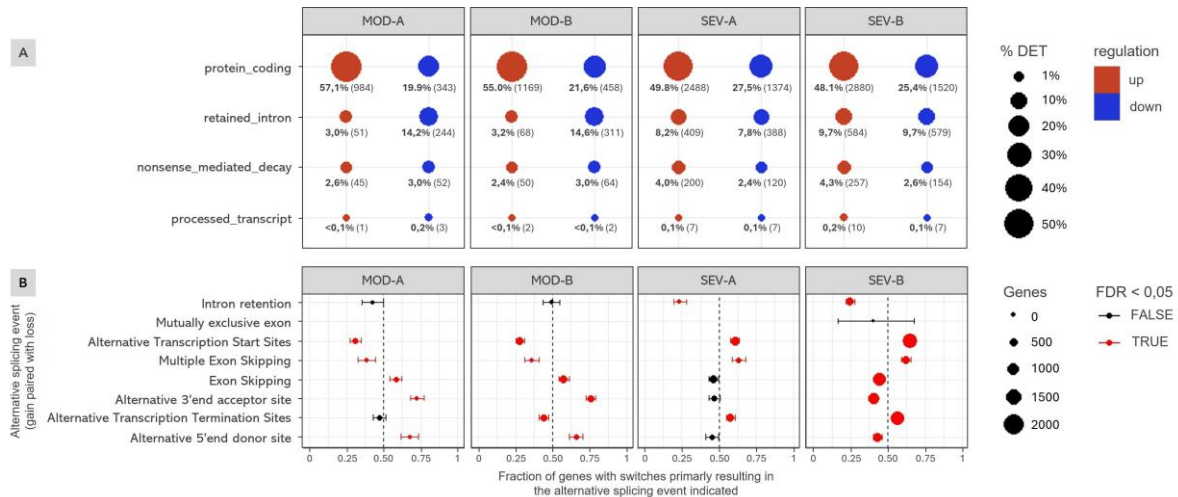


Figure 1: Differentially expressed transcript isoforms and alternative splicing events analysis. (A) Fraction (and number) of differentially expressed transcripts with an adjusted P value threshold less than 0.05 and an absolute $\log_2$ fold change greater than 0.58. Transcripts categorized as "retained_intron", "processed_transcript", or "nonsense_mediated_decay" were considered noncoding". **(B)** Alternative splicing events. The x-axis indicates the fraction (and 95% confidence interval) of isoform switches resulting in gain of a specific alternative splice event (indicated by y-axis) in the switch from CTRL (endpoint A) to COVID-19. The dashed line indicates no enrichment/depletion. Abbreviations: DET = differentially expressed transcripts; FDR = False Discovery Rate; up = upregulated; down = downregulated; MOD = moderate COVID-19; SEV = severe COVID-19. Suffixes: A = whole blood cultures incubated for 24h with excipient; B = whole blood cultures incubated for 24h with vitamin B12.

When evaluating the up- and downregulation of coding *versus* noncoding transcript isoforms in each contrast of endpoint A, a higher fraction of upregulated coding isoforms and downregulated noncoding isoforms was observed in patients with moderate COVID-19, suggesting a favorable trend towards protein coding and expression (Figure 1, panel A). This phenomenon was particularly evident in the greater inhibition of transcript isoforms with intron retention in leukocytes of these patients. Compared to the proportions found in the MOD-A, patients with severe disease exhibited a higher percentage of downregulated coding isoforms (27.5% SEV-A vs. 19.9% MOD-A) and an increased fraction of upregulated noncoding isoforms (8.2% SEV vs. 3.0% MOD for *retained_intron*; 4.0% SEV vs. 2.6% MOD-A for *nonsense_mediated_decay*). Furthermore, the distribution of transcripts with intron retention showing increased or decreased expression was more balanced in SEV patients (8.2% up vs. 7.8% down) compared to MOD patients (3.0% up vs. 14.2% down).

The vitamin B12 treatment resulted in an overall increase in the number of DETs, consistent with the previous gene-level transcriptome analysis (226). This effect was observed across all functional categories (Figure 1, panel A). However, the treatment led to a reduction in the

proportion of upregulated coding isoforms in leukocytes from both moderate and severe COVID-19 patients. In addition, vitamin B12 increased the proportion of downregulated coding isoforms in MOD samples, while in SEV, it decreased the fraction of downregulated coding isoforms. Regarding noncoding isoforms, the vitamin maintained a higher number of downregulated isoforms in MOD, whereas in SEV, it increased the proportion of both upregulated and downregulated isoforms.

Alternative splicing events resulting in differentially expressed isoforms were also analyzed (Figure 1, panel B). The results revealed various mechanisms activated according to the severity of the infection. Significantly affected events in leukocytes of patients with moderate COVID-19 included exon skipping and gain of alternative splicing sites. In the SEV group, intron retention was exclusively affected. COVID-19 also altered mRNA processing through the gain of transcription start sites and multiple exon skipping, with these events being more predominant in patients with severe disease. Interestingly, vitamin B12 treatment modulated distinct splicing mechanisms. In the MOD group, the treatment reduced genes with gain of alternative transcription termination sites. In the SEV group, B12 reduced genes with exon skipping and gain of alternative donor and acceptor splicing sites.

Vitamin B12 attenuates inflammatory response in COVID-19 through the activation of noncoding transcript isoforms

To characterize the functional consequences of alternative splicing induced by COVID-19 and vitamin B12 in patient leukocytes, a biological pathway enrichment analysis was performed using coding (*protein_coding*) and noncoding (*retained_intron*, *nonsense_mediated_decay*, and *processed_transcript*) transcript isoforms (Figure 2). The results revealed distinct patterns of immune response and cellular signaling in patients with moderate and severe COVID-19 at endpoint A. Overall, the MOD group exhibited a more prominent activation of immune response pathways compared to the SEV group, particularly through increased production of coding transcript isoforms from genes in these pathways. In contrast, gene expression regulation mechanisms were more active in SEV, as inferred by the higher quantity of coding isoforms and lower quantity of noncoding isoforms of genes in these pathways. Interestingly, the mRNA splicing mechanism itself was affected by increased expression of noncoding isoforms in the MOD group, while in the SEV group, the nonsense-mediated decay signaling pathway was affected by an increase in inhibited noncoding isoforms.

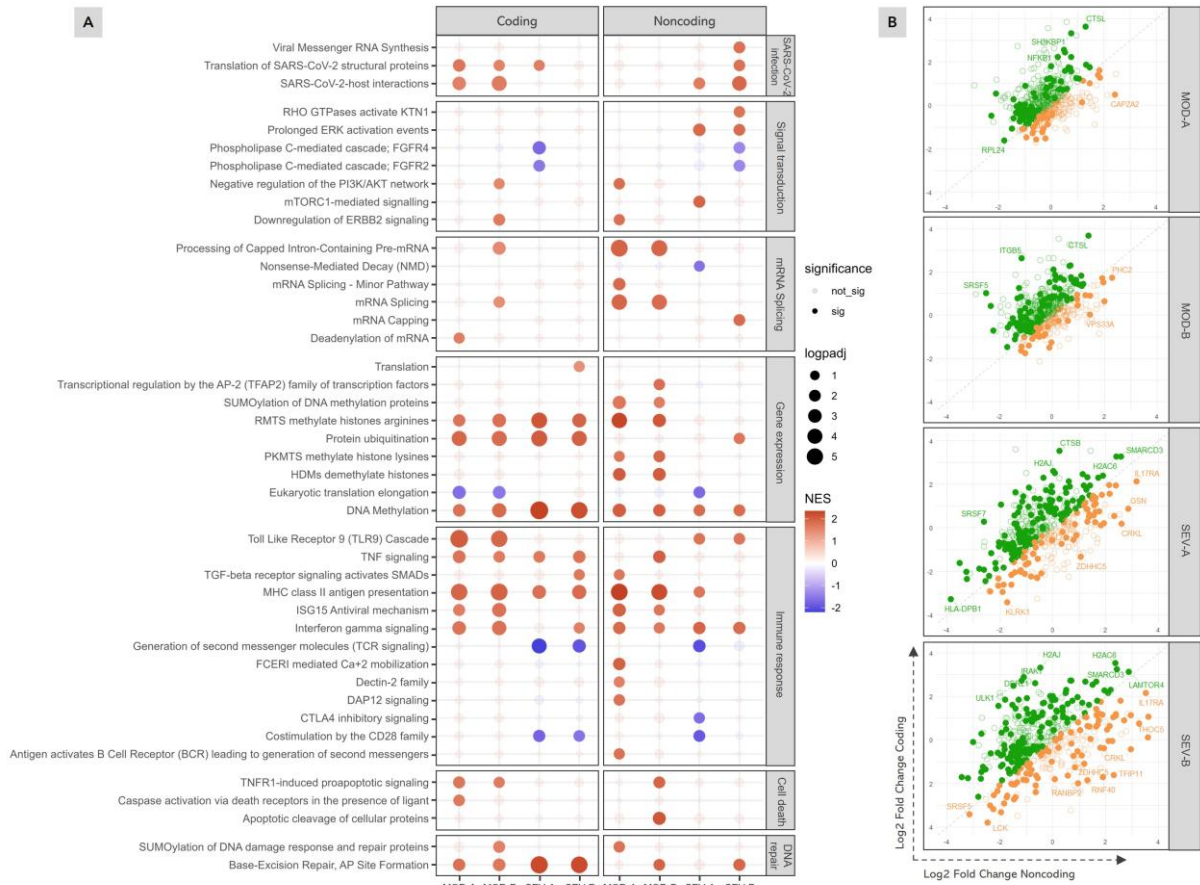


Figure 2: Functional enrichment analysis of coding and noncoding transcript isoforms. (A) REACTOME pathways affected by coding and noncoding transcript isoforms during infection and vitamin B12 treatment. Only pathways relevant in the context of COVID-19 and with a NES variation of at least 10% between endpoints A and B are displayed. Other altered pathways can be found in Table S2. **(B)** Average expression levels (log2 fold change) of coding *versus* noncoding transcript isoforms of genes involved in the altered pathways represented in panel A. For each gene, the average log2 fold change value of all coding or noncoding isoforms was calculated separately. Green points above the diagonal dotted line indicate higher expression of coding isoforms, while orange points below indicate higher expression of noncoding isoforms. Empty points represent genes without statistically significant differences in expression levels in the analyzed contrasts. Abbreviations: sig = significant (adjusted P value < 0.05); not_sig = not significant; logpadj = log of adjusted P value; NES = Normalized enrichment score; MOD = Moderate COVID-19; SEV = Severe COVID-19. Suffixes: A = whole blood cultures incubated for 24h with excipient; B = whole blood cultures incubated for 24h with vitamin B12.

In whole blood cultures treated with vitamin B12 (endpoint B), was observed a normalization of the inflammatory response and attenuation of deleterious pathways during SARS-CoV-2 infection (Figure 2). This effect was mediated both by reducing pathway activation through a decrease in coding transcript isoforms and by increasing noncoding isoforms related to these pathways (Figure 2, panel B). In the MOD group, vitamin B12 favored the reduction of translation of SARS-CoV-2 structural proteins, of crucial inflammatory pathways such as TNF signaling and Toll-like receptor 9 cascade, and of cell death signaling pathways (TNFR1-

induced proapoptotic signaling, Caspase activation via death receptors in the presence of ligand, and Apoptotic cleavage of cellular proteins). The treatment activated pathways inhibiting cellular signaling (Negative regulation of the PI3K/AKT network and Downregulation of ERBB2 signaling), while increasing genes related to MHC class II antigen presentation and ISG15-mediated antiviral response. Additionally, in the MOD group, vitamin B12 also modulated mRNA processing pathways (activation of Processing of capped intron-containing pre-mRNA and mRNA splicing, and inhibition of Deadenylation of mRNA) and regulated DNA repair pathways (activation of SUMOylation of DNA damage response and repair proteins and inhibition of Base-excision repair, AP site formation). In the SEV group, the treatment contributed to the inhibition of SARS-CoV-2 replication mechanisms (Viral messenger RNA synthesis, Translation of SARS-CoV-2 structural proteins, and SARS-CoV-2-host interactions), RHO GTPase-mediated signaling, nonsense-mediated decay RNA degradation signaling, attenuation of epigenetic mechanisms regulating gene expression (RMTS methylate histones arginines and DNA methylation), and DNA repair pathways (Base-excision repair, AP site formation). Vitamin B12 also increased protein translation, TGF-beta signaling (TGF-beta receptor signaling activates SMADs), enhanced MHC class II antigen presentation and IFN- γ signaling, and favored T cell signaling (Generation of second messenger molecules (TCR signaling)) and costimulation by the CD28 family.

Discussion

The potential of vitamin B12 as an epidrug for attenuating SARS-CoV-2-induced hyperinflammation had been previously demonstrated (226). However, the present study was pioneering in evaluating the role of B12 in modulating alternative splicing and the production of noncoding transcript isoforms in immune cells during COVID-19.

Transcriptomic analysis provides a more comprehensive view of the intricate molecular landscape underlying cellular responses to infection (238). This level of investigation elucidates how pathogens can distinctly modulate the expression of specific transcript biotypes, offering important insights into host-pathogen interactions. The importance of analyzing alternative RNA processing is exemplified in the inflammatory response. Studies have shown that the abundance of functional isoforms of inflammatory genes in response to tumor necrosis factor (TNF) is predominantly regulated by alternative splicing mechanisms rather than changes in gene transcription rates (239). The substantial number of DETs observed in leukocytes from moderate (MOD) and severe (SEV) patient groups underscores the impact of SARS-CoV-2 infection on gene expression. The increase in DETs from moderate to severe cases (1,723 in

CAPÍTULO III - Vitamina B12 como regulador do *splicing* na covid-19 aguda.

MOD-A to 4,993 in SEV-A) suggests an association between disease severity and the extent of transcriptional dysregulation (Figure 1, panel A). This escalation in transcriptional changes may reflect the intensification of immune response and cellular stress associated with more severe COVID-19 cases (63).

Alternative splicing can have diverse functional implications and significantly influence protein expression. Depending on the stimulus, mRNA processing can produce modified transcripts exhibiting distinct molecular characteristics, such as alterations in the proportion of coding isoforms and changes in mRNA stability. The proteins resulting from these altered transcripts may undergo important modifications, including the addition or removal of protein domains, an increase in intrinsically disordered regions, or alterations in signaling peptides. These changes in splicing patterns have the potential to affect the function and regulation of the resulting proteins, impacting several cellular processes (reviewed in: (240,241). During infection, SARS-CoV-2 sequesters splicing elements to favor its replication and evade host antiviral responses (193,194). This mechanism could explain the larger fraction of coding isoforms with reduced expression in leukocytes of severe COVID-19 patients treated with excipient compared to those with the moderate form of the disease at the same endpoint (Figure 1, panel A). Additionally, the SEV group received a more potent combination of glucocorticoids during hospitalization than the MOD group (226), which may have helped suppress gene expression. Previous studies have reported extensive dysregulation of immune and stress responses caused by the production of proteins with altered functions through alternative splicing in COVID-19, especially in severe cases (196,197). This evidence corroborates the observation of a larger fraction of upregulated noncoding isoforms in the SEV group compared to MOD at endpoint-A, suggesting an association between the extent of splicing mechanism impairment and disease severity.

To identify the mechanisms responsible for producing differentially expressed isoforms during COVID-19, the frequencies of alternative splicing events associated with transcripts identified in patient leukocytes were analyzed (Figure 1, panel B). The events significantly altered in both moderate and severe forms of COVID-19 were the gain of alternative transcription start sites and multiple exon skipping, with individuals in the SEV group showing a larger fraction of altered genes. Other studies have already reported a higher frequency of these events in blood and lung cells of infected patients (242,243). In addition to the aforementioned events, an increase in exon skipping and gain of alternative splice sites was observed in the MOD group, while intron retention was less frequent only in the SEV group. Decreased intron retention has

been implicated in the pathogenesis of autoimmune diseases and contribute to the abnormal expression of genes encoding regulators of macrophage transcription, phagocytosis and inflammatory signaling (244–246). These findings suggest that changes in alternative splicing may be implicated in affecting various biological pathways that lead to the aggravation of COVID-19.

The treatment of whole blood cultures from infected patients with vitamin B12 resulted in a decrease in the proportion of coding isoforms, particularly in the SEV group, and an increase in noncoding isoforms (Figure 1, panel A). This modulation may represent a novel and important mechanism by which B12 exerts its anti-inflammatory effects. Based on the results of previous analysis of sulfur amino acid pathway activity and DNA methylation using the samples included in the present study (226), it can be speculated that increased methyl bioavailability by B12 favored exon hypermethylation, leading to distinct splicing outcomes in leukocytes from patients with moderate and severe COVID-19 (Figure 1, panel B). In the MOD group, B12 reduced the fraction of genes containing alternative transcription termination sites. This finding aligns with previous studies demonstrating that DNA methylation can influence the recognition of polyadenylation signals and alter transcription termination (247,248). The B12-mediated hypermethylation may enhance the fidelity of canonical termination site usage, potentially preserving the integrity of key immune response transcripts. In the SEV group, B12-treatment decreased exon skipping events and reduced gain of alternative donor and acceptor splicing sites. This observation is particularly intriguing, as it suggests that the impact of B12-induced methylation on splicing may be context-dependent. The hypermethylation could be altering the binding affinity of splicing factors or modifying the secondary structure of pre-mRNA, thereby influencing spliceosome assembly and function (178,181,224).

The functional analyses of coding and noncoding isoforms at endpoint A indicates that the mRNA processing imbalance caused by infection impacts important regulatory processes and signaling pathways related to the immune system, promoting a dysfunctional immune response associated with disease severity (Figure 2). Despite the dysregulation of innate immune response pathways, evidenced by enrichment with noncoding isoforms, leukocytes from MOD-A patients seem to maintain antiviral and adaptive response capacity through the preservation of functional isoforms. The processing of coding isoforms in this group preserved the activation of fundamental pathways for antiviral response, such as mechanisms mediated by the ISG15 protein, which plays an essential role in defense against viral infections (249), and interferon gamma signaling, crucial for controlling viral replication and coordinating cellular immune

response (250,251). Additionally, the maintenance of these coding isoforms sustains critical components of the adaptive immune response, such as antigen presentation by MHC class II, fundamental for developing specific and lasting immunity against SARS-CoV-2 (252). In MOD-A group, suppression of splicing and less activation of DNA and histone methylation mechanisms was also observed through modulation by noncoding isoforms. These processes may have contributed to the regulation of gene expression and greater functional control in these patients compared to those with the severe form of the disease.

The imbalance in immune response and cellular signaling processes due to differential mRNA processing is more evident in leukocytes from SEV-A patients (Figure 2). Besides suppressing important antiviral response pathways, this group showed inhibition of secondary messenger generation responsible for T cell signaling and costimulation by the CD28 family. T cell exhaustion is one of the central components of severe COVID-19 pathophysiology (73,78,79,81,253). The signaling pathways modulated by T cells are essential for virus elimination and development of a robust adaptive immune response (254). The CD28 family consists of a group of immunoglobulin-like molecules that regulate immune response by modulating T cell activity and are essential for controlling the course of adaptive response (255). Another important aspect modulated by noncoding isoforms in leukocytes of SEV-A patients is the favoring of the signaling pathway known as nonsense-mediated decay (NMD). NMD is a molecular mechanism for controlling gene expression, responsible for surveying newly synthesized mRNAs and signaling the degradation of those containing premature stop codons. This process prevents the production of truncated or aberrant proteins that could result in deleterious functions (256). Alterations in the NMD signaling can have far-reaching implications for global gene expression potentially contributing to the dysregulated immune response observed in severe COVID-19 patients.

The biological pathway enrichment analysis revealed that vitamin B12 attenuates inflammation in COVID-19 through alternative mRNA processing. Previous studies had demonstrated that B12 can act as an epidrug in modulating the exacerbated inflammatory response during acute COVID-19 and neuroinfections (175,176,226). In this study, it was observed that vitamin treatment mitigates the activation of immunity pathways, favoring the balance between innate and adaptive immune responses, while also regulating important pathways for viral infection, mRNA splicing, cell signaling, cell death, and DNA repair (Figure 2). The few pathways that showed greater activation exclusively in response to B12 regulate desirable mechanisms in the context of COVID-19. For example, the negative regulation of PI3K/AKT, mTORC1, and

ERBB2 (negative regulation of PI3K/AKT network, mTORC1-mediated signaling, and downregulation of ERBB2 signaling) (Figure 2). These are components of highly conserved eukaryotic signal transduction networks that regulate metabolism, cell proliferation, and survival, and are directly involved in initiating the inflammatory response (257,258). During infection, SARS-CoV-2 utilizes mechanisms from these and other metabolic pathways for the synthesis of proteins necessary for its replication, initiating an aberrant signaling cascade (259). It is postulated that mTOR pathway inhibitors may prevent damage caused by SARS-CoV-2 infection through the re-establishment of antiviral CD8⁺ T lymphocyte populations and reduction of cytokine production (260). In a study using a murine model, inhibition of ErbB family receptors was shown to have antiviral and protective effects during infection (258). Another example is the activation of SUMOylation of DNA damage response and repair proteins (Figure 2). SUMOylation is the result of the covalent attachment of a small ubiquitin-like modifier (SUMO) to lysine residues (261). This post-translational process is important for regulating cellular response to genetic material damage and maintaining genomic integrity (262). The activation of SUMOylation by vitamin B12 may favor the repair of DNA damage caused by inflammation and cellular stress. The activation by B12 of pathways related to the processing of capped pre-mRNA containing introns, mRNA splicing, and protein translation evidences the modulation of coding transcript isoform by the treatment. Furthermore, the modulation of antigen presentation via MHC class II and IFN- γ signaling, in addition to less inhibition of costimulation by CD28 family by B12, suggests a role in enhancing adaptive immune response and regulating T cell signaling, which are compromised by infection (73,78,79,81,253).

This study elucidates a novel mechanism through which vitamin B12 exerts its anti-inflammatory effects in the context of COVID-19. By modulating RNA processing and isoform usage, vitamin B12 attenuates the expression of pro-inflammatory genes in leukocytes of patients with both moderate and severe COVID-19. These findings provide crucial insights into the molecular mechanisms underlying SARS-CoV-2 infection and open new avenues for therapeutic interventions based on splicing modulation. However, further research is needed to determine whether these alterations in transcript isoform patterns have clinical consequences for COVID-19 pathogenesis.

Acknowledgments

This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES (Doctoral scholarship received by LC: 88887.518397/2020-00). We thank MSc. CAPÍTULO III - Vitamina B12 como regulador do *splicing* na covid-19 aguda.

Izabela Mamede Conceição for valuable discussions about transcript isoform analyses and technical support using the Isoformic R package.

Conflict of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicial to the impartiality of the reported research.

Authors Contributions:

LC, RC and GF conceived the study, LC conducted the experiments and analyzed the data, LC, RC and GF wrote and reviewed the manuscript. All authors read and approved the final manuscript. All authors have approved the final article and agree with its publication.

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Supplementary materials

The following online material is available for this article:

Table S1 – Transcripts expression levels

Table S2 – Functional enrichment analysis of biological pathways

This material is available as part of the online article from <http://www.scielo.br/gmb>

The dataset presented in this study can be found in the SRA online repository www.ncbi.nlm.nih.gov/bioproject/PRJNA862565

The patient's data are available as part of the online article from Cassiano et al. 2023a (doi: 10.3389/fimmu.2023.1048790)

CAPÍTULO IV: Vitamina B12 como um epifármaco para regular biomarcadores sanguíneos na deficiência visuoconstrutiva associada à covid longa

O seguinte trabalho foi publicado em 2025 na revista *Scientific Reports* [<https://doi.org/10.1038/s41598-025-86637-0>] e investiga as alterações epigenéticas e transcricionais de leucócitos em mulheres que apresentam déficit visuoconstrutivo persistente (DVC) após infecção leve por covid-19. O estudo faz parte de uma coorte longitudinal composta por indivíduos infectados antes da vacinação completa e do surgimento das variantes Ômicron, acompanhados por até doze meses após o diagnóstico. Utilizando análises de expressão gênica, metilação do DNA e avaliação de biomarcadores inflamatórios, foi explorado a relação entre alterações epigenéticas, resposta inflamatória e sintomas cognitivos persistentes, com destaque para o papel potencial da vitamina B12 como agente modulador desses processos. Os resultados sugerem que a persistência do DVC está associada a um declínio sistêmico de respostas neuroprotetoras e anti-inflamatórias, e apontam para a possibilidade de a vitamina B12 atuar como uma intervenção terapêutica promissora para mitigar sequelas neurocognitivas da covid-19 leve.

Vitamin B12 as an epidrug for regulating peripheral blood biomarkers in long COVID-associated visuocognitive deficit

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Abstract

Approximately four months after recovering from a mild COVID-19 infection, around 25% of individuals developed visuoconstructive deficit (VCD), which was found to be correlated with an increase in peripheral immune markers and alterations in structural and metabolic brain imaging. Recently, it has been demonstrated that supplemental vitamin B12 regulates hyperinflammation during moderate and severe COVID-19 through methyl-dependent epigenetic mechanisms. Herein, whole peripheral blood cultures were produced using samples obtained from patients with confirmed persistent VCD, and controls without impairment, between 10 and 16 months after mild COVID-19. This experimental model was used to assess the leukocyte expression patterns of 11 biomarkers previously associated with VCD in long COVID and explore the potential of pharmacological B12 in regulating these genes. The results showed that patients with persistent VCD displayed continued upregulation of *CCL11* and *LIF* compared to controls. It is worth noting that elevated serum levels of CCL11 have been previously linked to age-related neurodegenerative diseases. Notably, the addition of 1 nM of vitamin B12 to blood cultures from individuals with VCD normalized the mRNA levels of *CCL11*, upregulated the neuroprotective *HGF*, and, to a lesser extent, downregulated *CSF2* and *CXCL10*. There was an inverse correlation observed between *CCL11* mRNA levels and methylation levels of specific cytosines in its promoter region. These findings underscore the significance of systemic inflammation in persistent VCD associated with long COVID. Moreover, the study provides evidence suggesting that B12, acting as an epidrug, shows promise as a therapeutic approach for addressing this cognitive impairment.

Keywords: Long COVID; Visuoconstructive deficit; *CCL11*; DNA methylation; Epigenetics.

Introduction

Long COVID is a term used to describe the prolonged symptoms of COVID-19, including cognitive dysfunction, also known as "brain fog," which can harm memory, attention, and problem-solving abilities. These symptoms may persist for weeks or even months after the infection has resolved (1,2). While the exact cause of long COVID is not fully understood, it is

thought to be related to the immune system's response to the virus and the systemic inflammation that it causes (reviewed in: (3)).

In a previous prospective cohort study of individuals who had mild COVID-19, it was found that approximately 25% of young adults (with an average age of 38 years) exhibited significant cognitive impairment in the copy stage of the Rey-Osterrieth Complex Figure (ROCF) Test four months after their recovery from the infection (4). The ROCF is a neuropsychological test that involves drawing a complex figure to assess visuospatial abilities, executive functions, and memory retention (reviewed in: (5)). Performance on visuoconstruction and memory tests is mainly associated with learning, problem-solving skills, and several activities of daily living, such as orientation and navigation (6). These processes integrate the perception and interpretation of visual information with memory and executive systems by involving various brain areas, including the occipito-parietal regions, the dorsal and ventral streams, and connections with the cingulate, medial temporal, and frontal cortices (7). Notably, negative correlations were found between performance on the ROCF test and white matter volume in both the left and right genu of the corpus callosum, extending to the cingulum bundle, as well as glucose metabolism in the right dorsal anterior cingulate gyrus of individuals diagnosed with visuoconstructive deficit (VCD). Moreover, a functional interaction network was observed among ten out of 11 plasma biomarkers that were upregulated in VCD patients. Four of these biomarkers are components of the *Neuroinflammation Signaling pathway*, and four are components of the *IL-17 Signaling pathway*. Indeed, evidence suggests that peripheral innate immune system molecules can trigger a secondary neuroinflammatory response in the central nervous system (CNS) (8–10).

Epigenetic mechanisms regulate the expression of genes crucial for immune response, neuronal function, and synaptic plasticity in neuroinflammatory processes. In this regard, it is possible that epigenetic changes may contribute to cognitive dysfunction in long COVID. For example, Lee and colleagues reported that individuals with long COVID had higher levels of DNA methylation in certain genes involved in immune response and inflammation (11). DNA methylation is a process that involves the addition of a methyl group (-CH₃) to a cytosine base in DNA. This modification can regulate gene expression by altering the interaction between the DNA and the proteins that bind to it (12,13). It is worth noting that dysregulation of DNA methylation patterns has been linked to various brain disorders, including depression, anxiety, and cognitive decline (reviewed in: (14,15)).

Vitamin B12 is an essential cofactor for the enzyme methionine synthase, which catalyzes the conversion of homocysteine to methionine in the sulfur amino acid pathway. S-

adenosylmethionine, the methyl donor utilized in all cellular methylation processes, is derived from methionine (16). Accordingly, adjuvant vitamin B12 has been shown to increase methylation of key genes in inflammatory disorders, resulting in their downregulation. This effect has been previously demonstrated in an infant rat model of pneumococcal meningitis (17) and in whole blood cultures from human patients with acute COVID-19 (18). These findings highlight the potential therapeutic role of vitamin B12 as an epidrug in attenuating hyperinflammation, providing promising directions for further research in neuroinflammatory conditions associated with long COVID.

The primary objective of this study was to identify peripheral blood biomarkers that are linked to persistent VCD in long COVID patients who have previously experienced a mild acute COVID-19 episode. Additionally, the study aimed to explore the methyl-dependent epigenetic mechanisms that play a role in regulating these biomarkers as well as investigate the potential of vitamin B12 in modulating the gene expression of these genes in leukocytes.

Material and methods

Patients

This study is part of a longitudinal research project involving a cohort of individuals who experienced mild COVID-19 before completing their vaccination schedule and prior to the emergence of the Omicron variants. This prospective observational cohort study received approval from the Institutional Review Board (IRB) of the Federal University of Minas Gerais (UFMG) (CAAE3768820.1.0000.5149). All participants provided written informed consent. The study has been performed in accordance with the Declaration of Helsinki. Volunteers were recruited approximately four months after having mild COVID-19 confirmed through RT-qPCR testing. Disease severity was categorized as 1 or 2 according to the WHO clinical ordinal scale (19). During the recruitment appointment and subsequent follow-up appointments at six and twelve months, the clinical status and mental health of all volunteers were evaluated, peripheral blood samples were collected, 45 plasma biomarkers (cytokines, chemokines, and growth factors) were assessed, and a comprehensive investigation was conducted using neuropsychological and ophthalmological tests, PET-CT, and magnetic resonance neuroimaging. These procedures are thoroughly detailed in (4). Exclusion criteria were self-reported history of autoimmune diseases, chronic mental or neurological disorders, recurrent infections, substance abuse, previous brain surgery, and endotracheal or orotracheal intubation during COVID-19 treatment. Detailed information on neuropsychological assessment, and

exclusion criteria have been thoroughly documented in previous publications based on this cohort study (4,20). The research timeline can be found in Figure 1. For the present study, seven individuals diagnosed with VCD during the recruitment appointment were enrolled. Additionally, five individuals who had mild COVID-19 but did not exhibit any signs of VCD or other neuropsychological sequelae that could be related to the infection at the recruitment and at six- and 12-month follow-ups were included as controls. Approximately 30% of participants discontinued their involvement between recruitment and the 6-month follow-up (20). Women were more consistent in attending follow-ups, while men participation significantly declined after six months. Consequently, men were excluded from the present study. Due to the unique characteristics of the studied population - individuals infected before completing vaccination and prior to the Omicron variants - it is not feasible to increase the sample size.

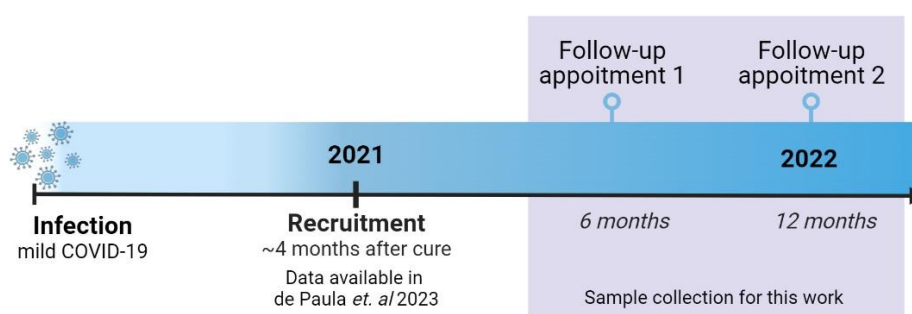


Figure 1: Research timeline. (Created with BioRender.com).

Assessment of Folate, Homocysteine and Vitamin B12 basal levels

The standard chemiluminescence method was used by a commercial clinical laboratory (Geraldo Lustosa, Belo Horizonte, Brazil) to quantify the basal levels of folate (vitamin B9), homocysteine (HCY), and vitamin B12 in plasma samples from all volunteers.

Whole Blood Cultures

Whole peripheral blood cultures were generated using samples collected in sodium heparin at 8 a.m., following the protocol outlined by Cassiano et al. (18). In brief, the blood samples were mixed with 50% (v/v) RPMI 1640 medium (Sigma-Aldrich, Saint Louis, Missouri). Two different conditions were employed: endpoint A, where the medium was supplemented with an excipient (citrate-phosphate buffer, pH 5, Merck, Darmstadt, Germany), and endpoint B, where the medium was supplemented with cyanocobalamin (Merck) at a final concentration of 1 nM.

The cultures were then incubated for 24 hours at 37°C in a humidified atmosphere with 5% CO₂.

Real-time quantitative PCR (RT-qPCR)

The total RNA was extracted from leukocytes using the QIAamp RNA Blood Kit (Qiagen, Hilden, Germany), and cDNA was produced with the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher, Waltham, MA) as previously described (18). The mRNA levels of *CCL2* (C-C Motif Chemokine Ligand 2), *CCL11* (C-C Motif Chemokine Ligand 11), *CSF2* (Colony Stimulating Factor 2), *CXCL10* (C-X-C Motif Chemokine Ligand 10), *HGF* (Hepatocyte Growth Factor), *IL1RA* (Interleukin 1 Receptor Antagonist), *IL6* (Interleukin 6), *IL10* (Interleukin 10), *IL31* (Interleukin 31), *LIF* (LIF Interleukin 6 Family Cytokine), and *NGF* (Nerve Growth Factor) were quantified using specific primers in SYBR Green (Thermo Fisher) or TaqMan (Thermo Fisher) platforms following the manufacturer's instructions. These genes encode for the plasma inflammatory and growth factors previously associated with VCD in long COVID in this cohort (4). Target gene expression levels were normalized by 18S ribosomal RNA (18S ribosomal N1), and relative gene expression was calculated using the 2e(-ΔCt) method (21). The primer sequences and TaqMan assay IDs are listed in Additional file 1. Each sample was tested in two technical replicates.

Bisulfite Sequencing PCR

To assess the impact of VCD and supplemental vitamin B12 on the methylation levels of 17 CpG sites located in the promoter region and proximal portion of the first exon of *CCL11*, DNA libraries obtained from cultures of endpoints A and B were analyzed using Bisulfite Sequencing PCR (BSP). The individuals included in the analysis represented both the impaired and non-impaired groups.

The production of BSP libraries, sequencing, and bioinformatics analysis were performed as described in (18) with minor modifications. Following DNA isolation, unmethylated cytosine nucleotides were converted into uracil with the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, California). The DNA region spanning from -2 569 pb to +310 pb of *CCL11* (GRCh38/hg38 chr17: 34283173 - 34286052) was amplified by PCR from bisulfite-converted DNA using a primer set designed to account for cytosine conversion to uracil (Bisulfite Primer Design Tool, Zymo Research) (Additional file 2) and GoTaq DNA Polymerase (Promega, Madison, Wisconsin). The mapping of filtered reads, as well as the calculation of methylation percentages at CG loci, were performed using the Bismark software (22). Only reads that

uniquely mapped to the reference sequence chr17: 34283173 - 34286052 of the *Homo sapiens* genome (Gencode, release 38) were considered for the methylation analysis. The functional annotations for *CCL11* were obtained from the UCSC Genome Browser (23).

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (version 8.0.2) from GraphPad Software Inc., Irvine, CA. The data distribution was assessed using Anderson-Darling, D'Agostino & Person, Shapiro-Wilk, and Kolmogorov-Smirnov tests. Data were considered parametric if they passed ($\alpha = 0.05$) at least one of the normality tests. Based on the distribution, a two-tailed Student's t-test, paired Student's t-test, Mann-Whitney test, or Wilcoxon test were employed to compare the groups. Statistical significance was defined as a P-value less than 0.05. The data were presented as median $\pm$ interquartile range.

The fold change methylation levels of each CG locus were compared between groups using an unpaired t-test. Only the loci that exhibited statistically significant differences between the compared groups were chosen for further analysis. To evaluate the correlation between the fold change methylation levels of differentially methylated loci (DML) and *CCL11* gene expression levels quantified by RT-qPCR in the same cultures, Pearson or Spearman tests were employed based on data distributions.

Search for transcription factor binding sites at the DML

Transcription factor binding sites (TFBS) at the DML were searched in the JASPAR CORE 2022 database (<https://jaspar.genereg.net>), an open-access and regularly updated resource featuring manually curated transcription factor (TF) binding profiles (24,25).

Results

Sample characterization

The study included female participants with an average age of 40.7 ± 8.5 years. There were no statistically significant differences in age between the groups initially diagnosed with or without VCD, as indicated in Table 1. It is worth mentioning that all participants had received at least two doses of SARS-CoV-2 vaccine (Sinovac-CoronaVac, Pfizer or AstraZeneca) at the time of the follow-up appointments.

Among the seven individuals initially diagnosed with VCD, four (57%) still exhibited the condition during the follow-up appointments, while three showed no further signs of

impairment. The five non-impaired control individuals maintained their initial diagnosis, as shown in Table 1. No significant statistical differences were observed in the basal levels of serum folate, HCY, and B12 among individuals with VCD, those who had recovered from the impairment, and non-impaired individuals, as detailed in Additional file 3.

Participant ID	Age (years)	Baseline diagnosis	Follow-up appointment (months)	Diagnosis at follow-up appointment
BRA-106	34	Impaired	12	Impaired
BRA-108	51	Impaired	12	Normal
BRA-111	32	Normal	12	Normal
BRA-112	56	Normal	12	Normal
BRA-126	31	Impaired	6	Normal
BRA-138	35	Normal	6	Normal
BRA-140	33	Normal	6	Normal
BRA-143	36	Impaired	6	Impaired
BRA-187	49	Impaired	6	Impaired
BRA-196	44	Impaired	6	Impaired
BRA-200	51	Normal	6	Normal
BRA-202	37	Impaired	6	Normal

Table 1. Participant's data

Supplemental B12 favorably modulates persistently dysregulated inflammatory genes in the leukocytes of patients with VCD

After 6 to 12 months from the initial diagnosis, *CCL11* ($P = 0.0127$) and *LIF* ($P = 0.0485$) continued to show upregulation in leukocytes from individuals with persistent VCD compared to those from non-impaired subjects (Figure 2). Importantly, when the whole peripheral blood cultures were incubated with 1 nM of B12 for 24 hours, the expression of *CCL11* in the impaired group was normalized to the levels observed in the cultures from the non-impaired group incubated with the excipient ($P > 0.05$).

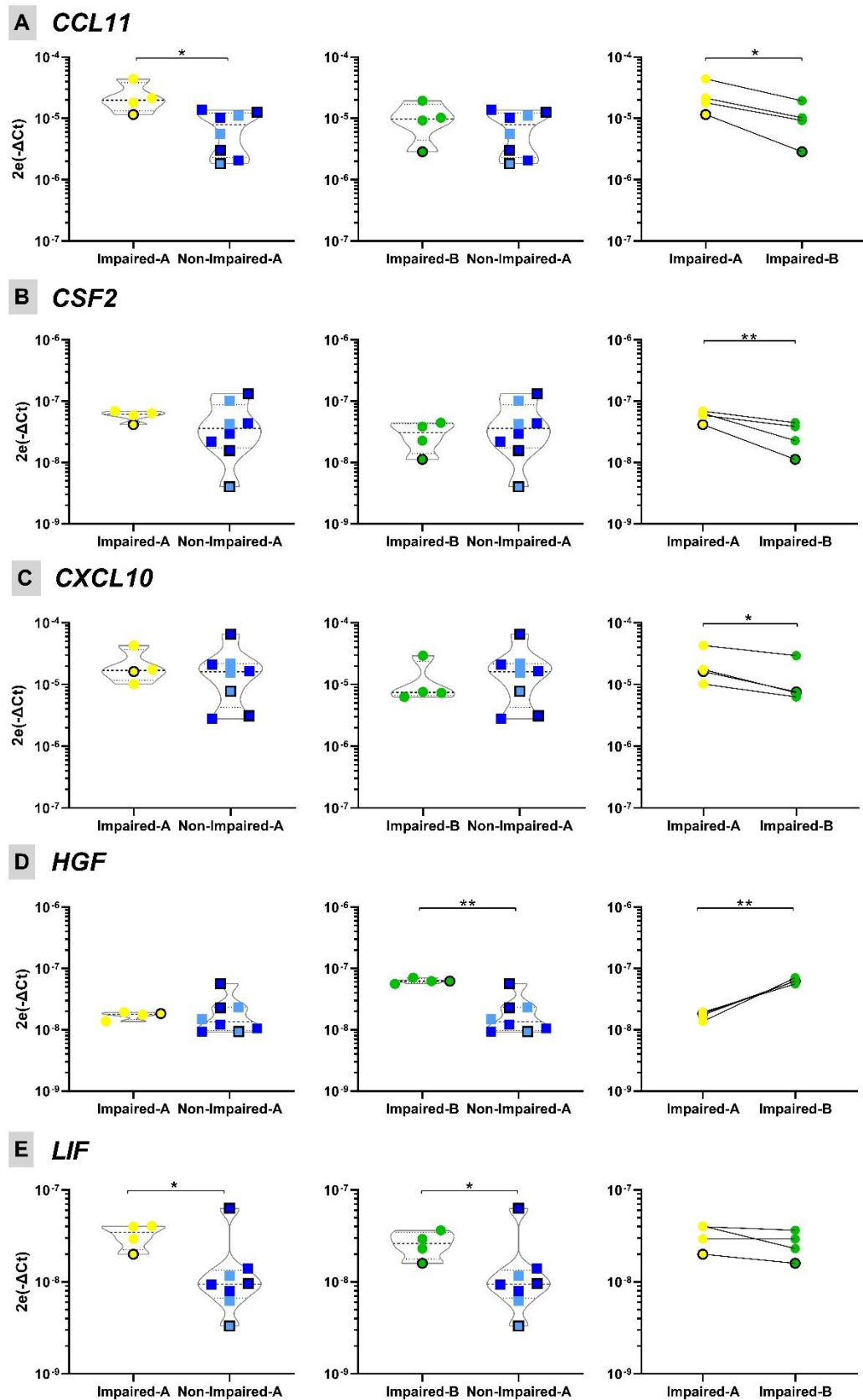


Figure 2: Supplemental B12 favorably modulates persistently dysregulated inflammatory genes in the leukocytes of patients with VCD. Violin charts depicting the expression levels of a panel of

inflammatory and growth factor genes in leukocytes from whole peripheral blood cultures (WPBC). Endpoint A: WPBC incubated with excipient. Endpoint B: WPBC incubated with 1 nM vitamin B12. Light blue squares represent individuals diagnosed with VCD at the time of recruitment who subsequently recovered before the follow-up appointment. Black-outlined circles or squares denote individuals who attended the 12-month follow-up appointment. In all other cases, both circles and squares represent individuals who attended the 6-month follow-up appointment. The pairwise comparison of the gene expression values ($2e(-\Delta Ct)$) between groups was conducted using two-tailed Student's t, paired Student's t, Mann-Whitney, or Wilcoxon tests according to the experimental design and data distribution. Values were expressed as medians. * $P < 0.05$; ** $P < 0.01$.

B12 also downregulated *CSF2* ($P = 0.0071$) and *CXCL10* ($P = 0.0205$) in leukocytes from impaired individuals, although these genes were not differentially regulated when compared to the non-impaired group. Additionally, B12 increased the mRNA levels of *HGF* in the impaired group compared to the non-impaired group incubated with the excipient ($P = 0.0081$). *CCL2*, *IL10*, *IL1RA*, and *IL6* were not differentially expressed when impaired and non-impaired individuals were compared, and these genes did not respond to incubation with B12 (Additional file 4). *IL31* and *NGF* mRNA were not detected in leukocytes from any of the volunteers.

Impaired individuals exhibit hypomethylation of CpG sites in the *CCL11* promoter region, which are then hypermethylated upon supplementation with B12

In impaired individuals, three CpG sites exhibited hypomethylation when compared to the non-impaired group. Notably, treatment of cultures with B12 significantly increased the methylation level of two of these CpG sites.

Furthermore, a strong and negative correlation was observed between the methylation status of these DML and the mRNA levels of *CCL11*. These DML are located at predicted transcription factor binding sites (TFBS) according to the JASPAR CORE 2022 database (Figure 3).

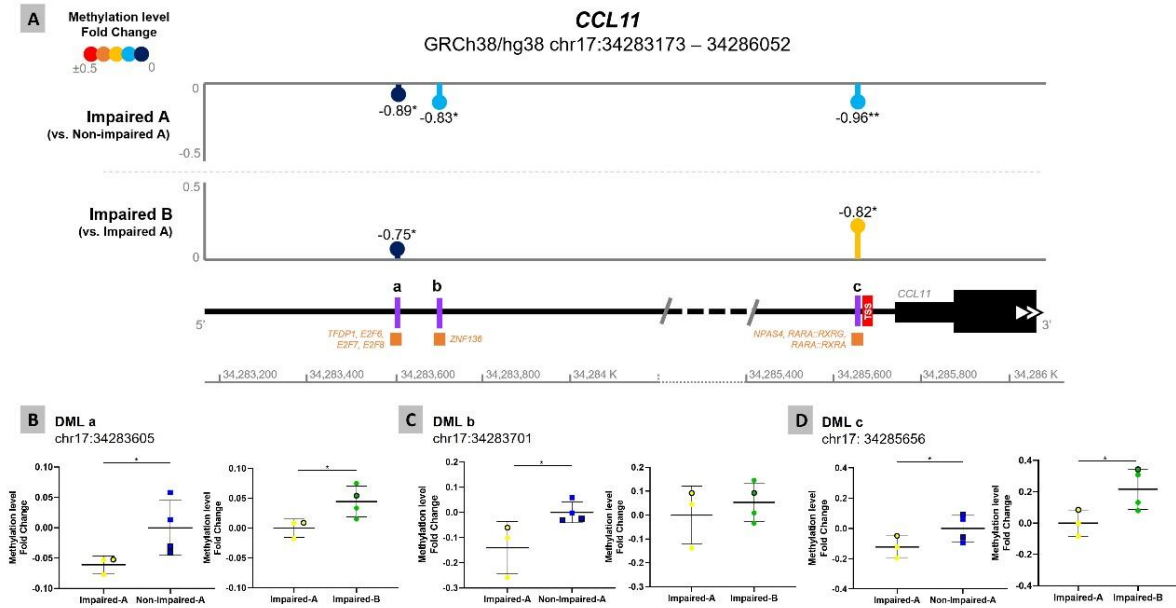


Figure 3: VCD patients exhibit hypomethylation of CpG in *CCL11* promoter region, which are hypermethylated upon B12 supplementation. (A) = a lollipop plot depicting the methylation level fold change of DML in the promoter region and proximal portion of the first exon of *CCL11*. (B), (C), and (D) = The fold change methylation levels of CpG positions were compared between groups using an unpaired t-test. Values next to lollipops represent correlation coefficients (Pearson or Spearman) with statistical significance (* $P < 0.05$ or ** $P < 0.01$) between methylation level fold change in each DML and gene expression values. In the schematic representation of the analyzed gene region, the black line represents the *CCL11* promoter region, and the black blocks delimit the 5' UTR region (thin block) and proximal portion of the first exon of the gene (thick block). The red bold dash indicates the transcription start site of *CCL11*, and the orange boxes indicate predicted transcription factor binding sites. Purple lines correspond to the DML at coordinates (GRCh38/hg38): a = chr17: 34283605; b = chr17: 34283701; c = chr17: 34285656. In the graphs, black-outlined circles or squares denote individuals who attended the 12-month follow-up appointment. In all other cases, both circles and squares represent individuals who attended the 6-month follow-up appointment. Line at mean $\pm$ standard deviation. * $P < 0.05$. Abbreviations: DML = differentially methylated locus; TSS = transcription start site. Suffixes: A = endpoint A (whole peripheral blood cultures (WPBC) incubated with excipient); B = endpoint B (WPBC incubated with 1 nM B12).

Discussion

VCD, characterized by an impaired ability to visually perceive and construct complex visual-spatial designs or tasks, is a well-recognized feature of aging-related neurodegenerative diseases, including Alzheimer's disease (26). The occurrence of this symptom in the young adults enrolled in this study is noteworthy. It suggests the possibility that individuals who experience mild COVID-19 may be at a higher risk of developing severe conditions associated with neurodegeneration. Furthermore, the high prevalence (57%) of persistent VCD six to 12 months after the initial diagnosis is a cause for concern.

Of the 11 plasma inflammatory and growth factors previously associated with VCD in long COVID at the recruitment, four months after mild COVID-19 (4), only *CCL11* and *LIF* displayed a transcriptional upregulation in leukocytes from individuals with persistent impairment. Accordingly, elevated CCL11 protein levels were identified in the raw plasma from individuals who developed VCD, compared with individuals who recovered from the impairment between the recruitment and the six-month appointment (20). CCL11, also known as eotaxin-1, is a chemotactic agent for eosinophils into inflammatory sites. In the CNS, CCL11 has been implicated in age-associated cognitive decline, psychiatric disorders, multiple sclerosis (MS), and Alzheimer's disease (AD) (reviewed in: (27)). Under stimuli, CCL11 can be transported from blood to the brain across the blood-brain barrier (BBB) or be primarily secreted by activated astrocytes. CCL11 predominantly acts via the CCR3 receptor, which is expressed by microglia, astrocytes, and neural progenitor cells (reviewed in: (28)). High levels of this chemokine can upregulate nicotinamide adenine dinucleotide phosphate-oxidase 1 (NOX1) in glial cells, triggering the production of reactive oxygen species (ROS) and potentiating glutamate-induced neuronal death (29). CCL11 also inhibits neural progenitor cell proliferation, impairing neurogenesis and hippocampal-related learning and memory (30–32). Recent findings have revealed CCL11 to be a critical biomarker of long-term brain damage in a mouse model of mild COVID-19 (33). One of the primary mechanisms postulated to underlie the neurodegenerative effects of CCL11 in long COVID involves the induction of hippocampal microgliosis, leading to diminished neurogenesis and the deterioration of myelinated subcortical axons (27). Altogether, it is reasonable to conclude that CCL11 is a strong biomarker of persistent cognitive sequelae following mild COVID-19 and could be a key target for VCD treatment. LIF is a multifunctional neurotrophic cytokine from the interleukin-6 family. Despite its intricate and context-specific function within the CNS, exhibiting both protective and detrimental impacts on neuronal and glial survival and function, LIF signaling is crucial for the maintenance of the nervous system (reviewed in: (34)). LIF has been identified as a regulator of neuronal phenotype and a coordinator of glial and inflammatory cell responses in the CNS (reviewed in: (34,35)). Moreover, the LIF protein has been shown to increase the transcription of *HGF*, which encodes an important neuroprotective molecule (36). Nonetheless, the specific role of LIF in the CNS during long COVID remains to be further elucidated.

No significant alteration in the mRNA levels of *CCL2*, *CSF2*, *CXCL10*, *HGF*, *IL10*, *IL1RA*, or *IL6* was observed in leukocytes from individuals with persistent VCD during the follow-up. However, it is unclear whether the reduction of some of these molecules before the resolution

of the impairment is beneficial. HGF is a potent neurotrophic factor that promotes neurogenesis and has been shown to have neuroprotective effects in neurodegenerative diseases (reviewed in: (37)). IL-10 is an anti-inflammatory cytokine that negatively regulates the immune response, reduces inflammation, and protects against neuroinflammatory processes associated with neurodegenerative diseases (reviewed in: (38)). IL-1RA dampens the pro-inflammatory effects of IL-1, preventing excessive inflammation and mitigating neuroinflammatory processes associated with neurological disorders (reviewed in: (39)). These findings indicate an orchestrated decline in the neuroprotective and anti-inflammatory response in individuals with persistent VCD. It is important to mention that elevated CXCL10 and HGF levels, along with decreased IL-10 protein levels, have been associated with worsening cognitive function from recruitment to the six-month follow-up in the previous study by Souza-Silva *et al.* 2024 with this prospective cohort (20). These authors analyzed protein levels in raw plasma from patients whose cognitive status changed from VCD to healed, and vice versa, between recruitment and the six-month follow-up, while the present study evaluated the mRNA levels of these biomarkers in whole blood cultures from volunteers who maintained the same diagnosis between recruitment and the six or 12-month appointment. However, a direct comparison between both experiments is not recommended and further research is needed to elucidate the transcription-translation dynamics of the investigated biomarkers in long COVID-associated persistent VCD.

The absence of *IL31* and *NGF* mRNA in leukocytes from the volunteers suggests that the proteins they encode, which were previously found to be elevated in the plasma samples of patients with VCD during the recruitment appointment four months after mild acute COVID-19 (4), might be primarily produced by other cell types (40–42).

Methylation is a fundamental biochemical mechanism that plays a critical role in modulating gene expression during neuroinflammation and neurodegenerative disorders (43–45). Folate and vitamin B12 are important cofactors of this mechanism, acting in the one-carbon metabolism. Insufficient levels of these vitamins hinder the efficient conversion of HCY to methionine, thereby disrupting the methylation cycle and diminishing the cells' methylation capacity. At the same time, HCY accumulates in the blood, leading to hyperhomocysteinemia. The association between the severity of acute COVID-19 and low concentrations of folate and B12 has been reported (46,47). Conversely, a few studies reported high levels of vitamin B12 in the blood of patients with a worse COVID-19 outcome (48,49) potentially due to cytolysis leading to the release of intracellular B12 into the plasma and disrupted B12 clearance by the

CAPÍTULO IV - Vitamina B12 como epifármaco na covid-19 longa.

liver, which is often affected in these patients (50). Nevertheless, the impact of B12 levels on cognitive sequelae following mild COVID-19 still needs to be investigated. In the present study, no change was observed in the basal serum levels of folate, vitamin B12 and HCY among patients with VCD, those who recovered from the impairment, and those without impairment. Notably, incubating peripheral whole blood cultures with vitamin B12 resulted in the normalization of *CCL11* expression, the increase of the neuroprotective *HGF* and the decrease *CSF2* and *CXCL10* mRNA levels in leukocytes from individuals with persistent VCD (Figure 2). It is important to highlight that *CSF2* and *CXCL10* encode pro-inflammatory proteins linked to neurodegenerative diseases (51–53), and, as mentioned above, elevated levels of CXCL10 protein are associated with persistent VCD in individuals from the same cohort of the present study (20). In addition to its role in regulating gene expression through methyl-dependent epigenetic mechanisms, vitamin B12 is recognized for its neuroprotective, antioxidant, and anti-inflammatory properties (54). In the acute COVID-19 context, previous studies have demonstrated that vitamin B12 increases the flow of the sulfur amino acid pathway, favoring production of glutathione, an important antioxidant agent, in leukocytes of patients with moderate and severe COVID-19 (18). Moreover, daily B12 supplementation has improved the clinical outcome of hospitalized patients with COVID-19 (55) and was also effective in alleviating long COVID symptoms from a patient recovered from mild COVID-19 (56). Altogether, these findings suggest a potential beneficial effect of vitamin B12 in reducing neuroinflammation and promoting neuroprotection in patients with persistent VCD long after experiencing mild acute COVID-19.

The observed differently methylated CpG sites in the promoter region of *CCL11* are located at predicted TFBS (25). The transcription factors (TF) E2F7 (JASPAR ID: MA0758.1) and E2F8 (JASPAR ID: MA0865.2) can bind to the region encompassing the DML a (chr17: 34283605), while E2F6 (JASPAR ID: MA0471.2) and TFDP1 (JASPAR ID: MA1122.1) can bind to the complementary region of this DML in the minus strand. E2F6, E2F7, and E2F8 are members of the E2F family of TF, which play important roles in cell cycle regulation, cell proliferation and survival, and gene expression (57). TFDP1 heterodimerizes with E2F proteins to enhance their DNA-binding activity and promote transcription from E2F target genes (57). Unfortunately, there is limited research specifically investigating the relationship between E2F6-8 and *CCL11* transcription in the CNS context. Regarding DML b (chr17: 34283701), ZNF136 (JASPAR ID: MA1588.1) can bind to the complementary region in the minus strand, but there is no evidence of its action on *CCL11* expression. NPAS4 (JASPAR ID: MA2042.1)

can bind to the region containing the DML c (chr17: 34285656), and RARA::RXRA (JASPAR ID: MA0159.1) and RARA::RXRG (JASPAR ID: MA1149.1) to the complementary region of this locus. NPAS4 is a neuroprotective protein and acts as a TF, regulating the transcription of a diverse set of genes involved in synaptic plasticity, neurotransmission, and neuronal survival (58). However, there is limited research on its specific interaction with *CCL11*. The RARA::RXRA and RARA::RXRG complexes are heterodimeric protein complexes formed by the retinoic acid receptor alpha (RARA) in conjunction with either retinoid X receptor alpha (RXRA) or retinoid X receptor gamma (RXRG) (59). These complexes are involved in mediating the effects of retinoic acid signaling, which plays crucial roles in development, cell differentiation, and immune response (60). Previous studies have demonstrated that the use of RXR partial agonists can reduce eosinophilic airway inflammation, a condition mediated, in part, by the chemoattractant activity of CCL11 (60). RXR agonists are also effective in the treatment of some neuropathies, such as Alzheimer's disease (61) and Parkinson's disease in animal models (62). While direct evidence linking the RARA::RXRA and RARA::RXRG complexes to *CCL11* expression in the CNS is currently not well established, it is plausible to speculate that retinoic acid signaling through these complexes could inhibit *CCL11* expression in specific brain conditions. Furthermore, the DML c is situated within regulatory regions that contain binding sites for EZH2, CTCF, GPS2, and TCF21 across various cell types (63). These TF act as epigenetic regulators of chromatin structure through interactions with histone modifiers, chromatin remodeling complexes, transcriptional regulators, and/or DNA methylation machinery that modulate gene expression. It is reasonable to assume that changes in CpG methylation levels in this region may reflect on the epigenetic landscape, possibly favoring the expression of *CCL11*. Overall, this significant discovery highlights the potential of using CpG methylation patterns as diagnostic biomarkers for neurodegenerative conditions characterized by *CCL11* upregulation.

The hypothesis that B12 could modulate *CCL11* expression during persistent VCD via methyl-dependent epigenetic mechanisms was confirmed by the hypermethylation of DML a and c in leukocytes from cultures of impaired individuals incubated with the vitamin. The B12-induced increment in methylation levels of each of these DML negatively correlated with the *CCL11* expression level (Figure 3). These findings provide compelling evidence for the role of epigenetic regulation of *CCL11* and suggest that supplemental B12 has potential as an epidrug to treat neurodegenerative conditions associated with aberrant *CCL11* expression.

This study offers valuable insights into the mechanisms underlying persistent VCD associated with long COVID and highlights the therapeutic potential of vitamin B12 as an epidrug to prevent or mitigate this impairment. However, several limitations should be considered when interpreting the findings. The small sample size, restricted to women, limits the generalizability of the results.

It is noteworthy that the volunteers included in this study had mild COVID-19 in 2021, prior to the emergence of the SARS-CoV-2 Omicron variant and when the Brazilian population had not completed the full COVID-19 vaccination schedule. COVID-19 vaccination has been associated with a lower risk of several, but not all, post-COVID-19 symptoms (64). The present study was not designed to assess the protective effect of vaccination against VCD as a post COVID-19 sequelae. Therefore, caution should be taken when extrapolating findings from this work to individuals who were infected after completing their vaccination or were infected with more recent SARS-CoV-2 variants.

The whole blood culture model serves as a valuable tool for studying immune responses, inflammation, and molecular processes in a physiologically relevant context, offering a more realistic representation of blood tissue complexity compared to in vitro studies with single type cells. However, further research using animal models is needed to evaluate the potential of B12 as an epidrug for in vivo modulation of peripheral biomarkers associated with persistent VCD in long COVID and to determine whether this contributes to neuroprotection.

Conclusion

This study provides compelling evidence highlighting the importance of systemic inflammation in persistent VCD associated with long COVID (Figure 4). Notably, the study reports a persistent upregulation of *CCL11* in peripheral leukocytes in patients with VCD. This upregulation has been found to be strongly and negatively correlated with the methylation state of three cytosines in the promoter region of the *CCL11* gene. Collectively, these findings support the potential use of *CCL11* mRNA levels or promoter methylation patterns as biomarkers for VCD in long COVID. Moreover, the results suggest that supplementation with vitamin B12 holds promise as a therapeutic approach for patients with VCD in the context of long COVID.

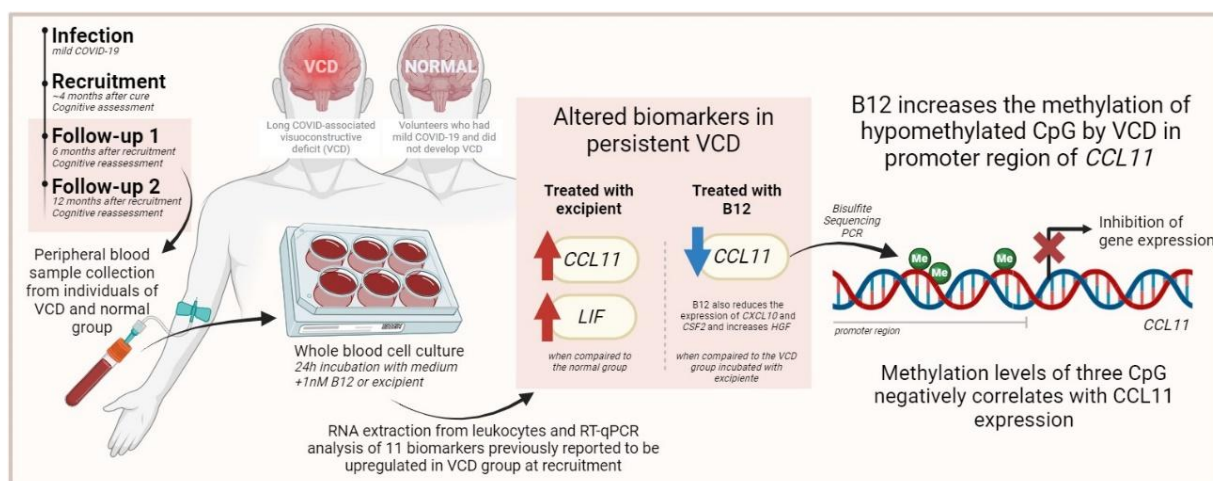


Figure 4: Graphical representation of the study. (Created with BioRender.com).

Data availability

The dataset supporting the conclusions of this article is available in the SRA repository (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA992778>).

Supplementary information is available in the Additional files 1 to 4.

Abbreviations

BBB: Blood brain barrier

BSP: Bisulfite sequencing PCR

CNS: Central nervous system

COVID-19: Coronavirus disease 2019

CpG: Cytosine-phosphate-guanine

DML: Differentially methylated locus

HCY: Homocysteine

MS: Multiple sclerosis

PET-CT: Positron emission tomography - computed tomography

ROCF: Rey-Osterrieth complex figure

ROS: Reactive oxygen species

CAPÍTULO IV - Vitamina B12 como epifármaco na covid-19 longa.

RT-qPCR: Real time quantitative polymerase chain reaction

SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2

TF: Transcription factor

TFBS: Transcription factor binding site

VCD: Visuoconstructive deficiency

WHO: World Health Organization

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Author contributions

Study conception and/or design: JJP, DMM, MAR-S, and RSC. Clinical assessment: JJP. Data acquisition: LMGC, JJP, DVR. Data analysis: LMGC, JJP, DVR, DMM, MAR-S, and RSC. Manuscript writing and/or revision: LMGC, JJP, DVR, DMM, MAR-S, and RSC.

Funding

This work was supported by INOVA FIOCRUZ (VPPCB-005-FIO-20-2-115), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—CAPES (88881.504749/2020-01. 9951), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (408817/2022-4), Instituto Nacional de Ciência e Tecnologia em Vacinas (INCT- Vacinas), and Instituto Nacional de Ciência e Tecnologia em Neurotecnologias Responsáveis (INCT-NeuroTec-R; 406935/2022). LMGC is the recipient of a CAPES doctoral scholarship.

Ethics approval and consent to participate

This study received approval from the Institutional Review Board (IRB) of the Federal University of Minas Gerais (UFMG) (CAAE3768820.1.0000.5149).

Consent for publication

All participants included in this study provided written informed consent.

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/s41598-025-86637-0>

CONCLUSÃO GERAL

O presente trabalho fornece evidências substanciais sobre o potencial terapêutico da vitamina B12 no tratamento da covid-19 e suas complicações a longo prazo, bem como elucidar mecanismos moleculares cruciais para patogênese da doença. Os resultados obtidos demonstram que a vitamina B12 é um epifármaco com propriedades anti-inflamatórias e mecanismos de ação singulares, capazes de modular a resposta inflamatória sistêmica observada em pacientes com covid-19 moderada a grave, e regular a neuroinflamação na deficiência visuoconstrutiva (DVC) pós infecção. Apesar de ter um excelente perfil de segurança, são necessários estudos *in vivo* adicionais para confirmar a eficácia do tratamento com vitamina B12 em diferentes contextos clínicos da covid-19 e suas complicações a longo prazo. Contudo, os trabalhos apresentados fornecem uma base sólida para futuros estudos clínicos que visam validar o uso da vitamina B12 como terapia adjuvante para covid-19 e oferecem novas perspectivas para estratégias terapêuticas inovadoras baseadas na regulação epigenética da expressão gênica.

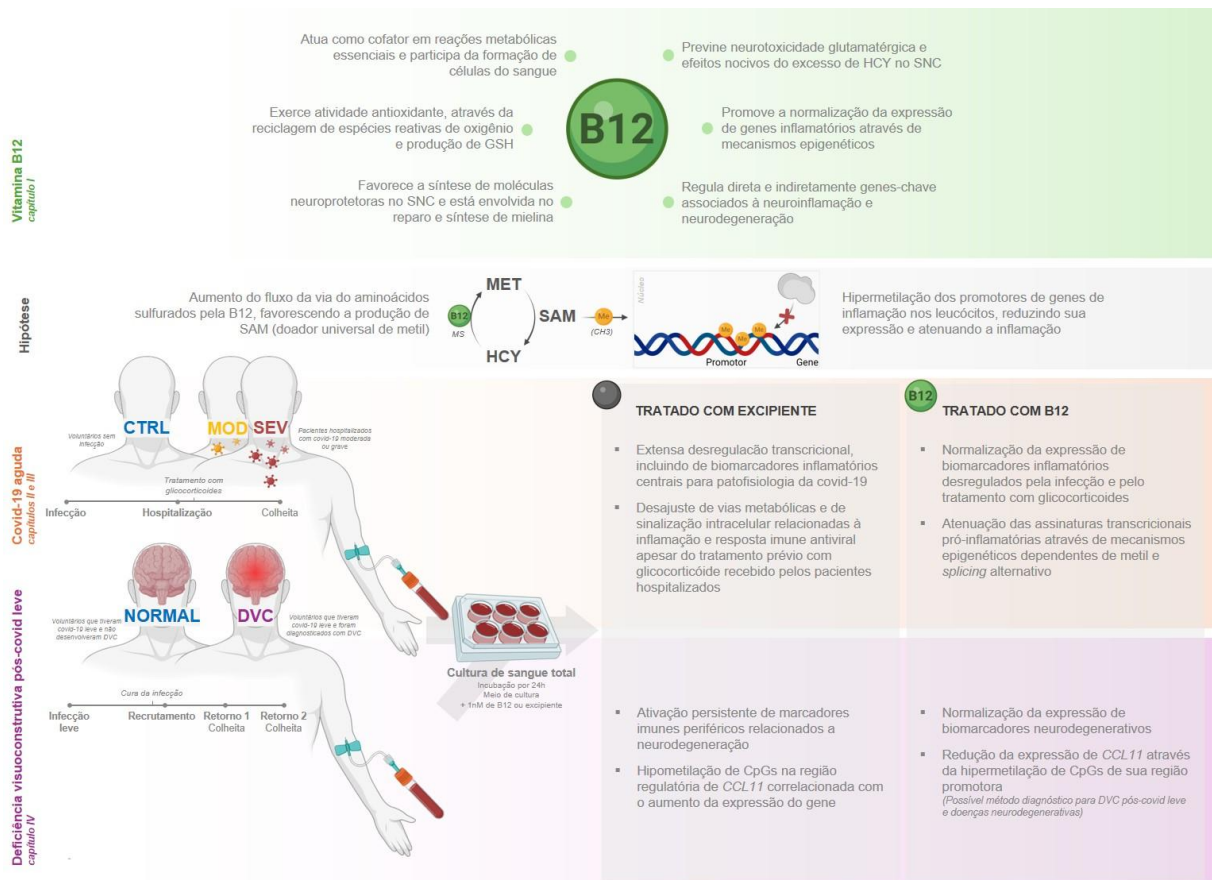


Figura 10: Resumo gráfico dos principais resultados e conclusões do trabalho de tese. GSH = Glutathiona; SNC = Sistema Nervoso Central; MET = Metionina; HCY = Homocisteína; MS = Metionina sintase; CH₃/Me = metil; SAM = S-Adenosil metionina; CTRL = controles; MOD = covid-19 moderada;

SEV = covid-19 grave; DVC = Deficiência visuoespacial; CpG = dinucleotídeos de citosina e guanina.

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APÊNDICES

APÊNDICE I: Pedido de patente depositado no Instituto Nacional da Propriedade Industrial (INPI) para uso dos padrões de metilação de CpGs no promotor do gene *CCL11* como biomarcador para o diagnóstico molecular de deficiência visuoestrutiva e método diagnóstico.

A invenção é um produto do projeto principal de doutorado (Capítulo IV), e propõe um método inovador para o diagnóstico da deficiência visuoesrutiva relacionada à neurodegeneração causada pela covid longa e por doenças neurodegenerativas. A tecnologia baseia-se na análise dos padrões de metilação de sítios CpGs específicos no promotor do gene *CCL11*. O pedido de patente de invenção foi depositado no Instituto Nacional da Propriedade Industrial (INPI) no dia 10 de outubro de 2024 (Número do Processo: BR 10 2024 021139 1).

Método de diagnóstico molecular de deficiência visuoesrutiva a partir do uso dos padrões de metilação de CpGs no promotor do gene *CCL11* como biomarcador

[01] A tecnologia baseia-se no desenvolvimento de um método inovador para diagnosticar deficiência visuoesrutiva (DVC) relacionada à neurodegeneração causada pela Covid longa e por doenças neurodegenerativas. A tecnologia utiliza-se da análise dos padrões de metilação de citosinas seguidas de guaninas (CpGs) e, portanto, metiláveis, em pelo menos dois dos três locus diferencialmente metilados, DMLa (chr17: 34283605; genoma do *Homo sapiens*, Gencode, versão 38), DMLb (chr17: 34283701; genoma do *Homo sapiens*, Gencode, versão 38) e DMLc (chr17:34285656; genoma do *Homo sapiens*, Gencode, versão 38), que se encontram hipometilados no promotor do gene *CCL11* dos pacientes com DVC. As CpGs diferencialmente metiladas atuam como biomarcadores no diagnóstico da deficiência.

Trecho da petição 870250093184, de 10/10/2025, página. 20/49.

O documento completo da patente de inovação pode ser consultado em: [BR102025022110-1_870250093184-Adendo.pdf](https://patents.google.com/patent/BR102025022110-1_870250093184-Adendo.pdf).

APÊNDICE II: Projeto de doutorado sanduíche “Vitamina B12: um epifármaco para regular genes essenciais na fisiopatologia da deficiência visuoconstrutiva associada à covid-19 longa e a doenças neurodegenerativas relacionadas ao envelhecimento.”.

O seguinte trabalho é um desdobramento do projeto principal de doutorado, desenvolvido em parceria com a *McGill University (McGill University Genome Centre)* sob supervisão do professor Dr. David Langlais. O projeto foi executado de maio a dezembro de 2023 e financiado pelo Programa Institucional de Internacionalização – CAPES – PrInt. Seu principal foco foi identificar os mecanismos epigenéticos subjacentes ao efeito da B12 na modulação da expressão, em leucócitos periféricos, de genes associados à deficiência visuoconstrutiva (DVC) persistente pós-covid-19 leve.

O centro de Genoma da Universidade McGill (MGC) é destaque internacional em iniciativas em genética e genômica além de ser um polo para o desenvolvimento de novas abordagens ômicas e de bioinformática. O MGC conta com plataformas de sequenciamento de nova geração (NGS) e de bioinformática com equipamentos de última geração para realização pesquisas na fronteira do conhecimento. Dr. Langlais, é o diretor do laboratório COVID-19 BCL2+, onde são processadas amostras clínicas de pacientes com covid-19, principal pesquisador do MGC e professor assistente nos departamentos de Genética Humana e Microbiologia & Imunologia da Universidade McGill. Dr. Langlais tem expertise em genômica funcional, bioinformática e biologia molecular e sua atual linha de pesquisa é baseada na identificação e validação de novos alvos terapêuticos para doenças inflamatórias, inclusive para covid-19.

Vitamina B12: um epifármaco para o tratamento da deficiência visuoconstrutiva persistente após a covid-19 leve

INTRODUÇÃO

Alguns estudos pioneiros demonstraram que a covid-19 induz o remodelamento da cromatina nas células imunes do hospedeiro (1, 2). Anteriormente, nós já havíamos relatado que a vitamina B12 regula negativamente alguns genes inflamatórios por meio de mecanismos epigenéticos dependentes de metil (3). Estas duas premissas motivaram um trabalho recente de nosso grupo no qual, utilizando um modelo *ex vivo* de culturas de sangue total, nós demonstramos que a vitamina B12 normaliza a expressão de um painel de genes inflamatórios

ainda desregulados nos leucócitos de pacientes com covid-19 moderada e grave, apesar da terapia com glicocorticoides durante a hospitalização. Além disso, a análise do transcriptoma destes pacientes revelou que a B12 atenua os efeitos da covid-19 na maioria das vias relacionadas à inflamação afetadas pela doença. Como esperado, a B12 aumentou o fluxo da via de aminoácidos sulfurados, elevando a biodisponibilidade de metil. Consequentemente, a regulação negativa do gene *CCL3* induzida pela B12 se correlacionou forte e negativamente com a hipermetilação de CpGs em seu promotor. No entanto, nossos resultados sugerem que, além de aumentar a metilação do DNA, o efeito anti-inflamatório da B12 no contexto da covid-19 moderada e grave também pode envolver modificações de histonas. Até onde sabemos, este foi o primeiro estudo a demonstrar que a modulação farmacológica de marcas epigenéticas em leucócitos regula favoravelmente os componentes centrais da fisiopatologia da covid-19 (4).

Em outro estudo, recrutamos uma coorte de adultos pelo menos quatro meses após se recuperarem da covid-19 leve. Esses voluntários foram avaliados por testes neuropsicológicos, oculares e neurológicos, ensaio de marcadores imunológicos e por ressonância magnética estrutural e neuroimagem funcional 18FDG-PET com o objetivo de investigar possíveis alterações cerebrais e correlações clínicas. Em aproximadamente um quarto dos indivíduos que tiveram covid-19 leve, detectamos uma deficiência visuoespacial específica, que foi associada a alterações nas imagens moleculares e estruturais do cérebro e correlacionada com a regulação positiva de marcadores imunológicos no sangue periférico. O dano visuoespacial tem sido reconhecido como uma manifestação precoce da doença de Alzheimer (5, 6) e é caracterizado pelo comprometimento da capacidade de coordenar habilidades motoras finas, dificuldade em entender as relações visuoespaciais, e pelo prejuízo em tarefas de planejamento e nas funções executivas. Nossos achados fornecem evidências da relação causal entre a neuroinflamação e a deficiência cognitiva persistente em pessoas que tiveram covid-19 leve, uma fração já grande e crescente da população mundial (7-9). Portanto, há que se investir na pesquisa de tratamento para esta manifestação da covid longa.

Alinhados com esta demanda urgente de saúde pública, nossos resultados mais recentes indicam que a B12 normaliza a expressão de *CCL11*, um biomarcador inflamatório crítico regulado positivamente no sangue periférico de pacientes com deficiência visuoespacial persistente por pelo menos 10 meses após a covid-19 leve. Curiosamente, *CCL11* foi recentemente apontado como um importante biomarcador de danos cerebrais a longo prazo em um modelo de camundongos de covid-19 leve (10). Assim como demonstrado em nosso estudo sobre covid-19 aguda moderada e grave (4), nossos resultados mais recentes indicam que a paisagem epigenética também é um importante espaço de busca por novos tratamentos para a

deficiência cognitiva na covid longa. E estudos mais aprofundados são necessários para elucidar os mecanismos pelos quais a B12, um epifármaco seguro, barato e amplamente acessível modula o epigenoma das células imunes controlando os mediadores inflamatórios associados à deficiência visuocognitiva após a covid-19 leve.

OBJETIVO

Nosso objetivo é investigar de forma ampla e aprofundada os efeitos da covid longa e do tratamento com vitamina B12 na paisagem epigenética de leucócitos de pacientes com ou sem deficiência visuocognitiva persistente após se curarem da covid-19 leve.

METODOLOGIA

Utilizamos um modelo de culturas de sangue total, já estabelecido pelo nosso grupo (4), com amostras de participantes da coorte de um estudo longitudinal coordenado por nosso colaborador Prof. Marco Aurélio Romano-Silva, da Faculdade de Medicina de Universidade Federal de Minas Gerais (UFMG) (estudo aprovado pelo Comitê Nacional de Ética em Pesquisa - CAAE3768820.1.0000.5149). Voluntários que tiveram covid-19 leve desde janeiro de 2022 foram recrutados e avaliados por testes neuropsicológicos, oculares e neurológicos, ensaio de marcadores imunológicos de sangue periférico e por ressonância magnética estrutural e neuroimagem funcional 18FDG-PET. Nas consultas de acompanhamento 24 a 36 meses após o recrutamento, os pacientes foram reavaliados e tanto aqueles com diagnóstico de deficiência visuocognitiva persistente quanto aqueles que não desenvolverem sequelas neurológicas tiveram amostras de sangue venoso periférico coletadas (Figura 1). O projeto inclui análise dos níveis basais de vitamina B12, homocisteína (HCY) e ácido fólico e produção de culturas de sangue total, incubadas com 1 nM de cianocobalamina ou com excipiente (tampão citrato-fosfato) por 24h. Também são propostas a quantificação dos níveis de expressão de mRNA do biomarcador *CCL11*, além de análises de modificações de histonas dependentes de metil, metilação global de DNA e transcriptoma da população de leucócitos com as técnicas de ChIP-Seq, “Reduced Representation Bisulfite Sequencing” (RRBS) e RNA-Seq, respectivamente, e integração dos dados dessas diferentes abordagens com técnicas de bioinformática (Figura 1).

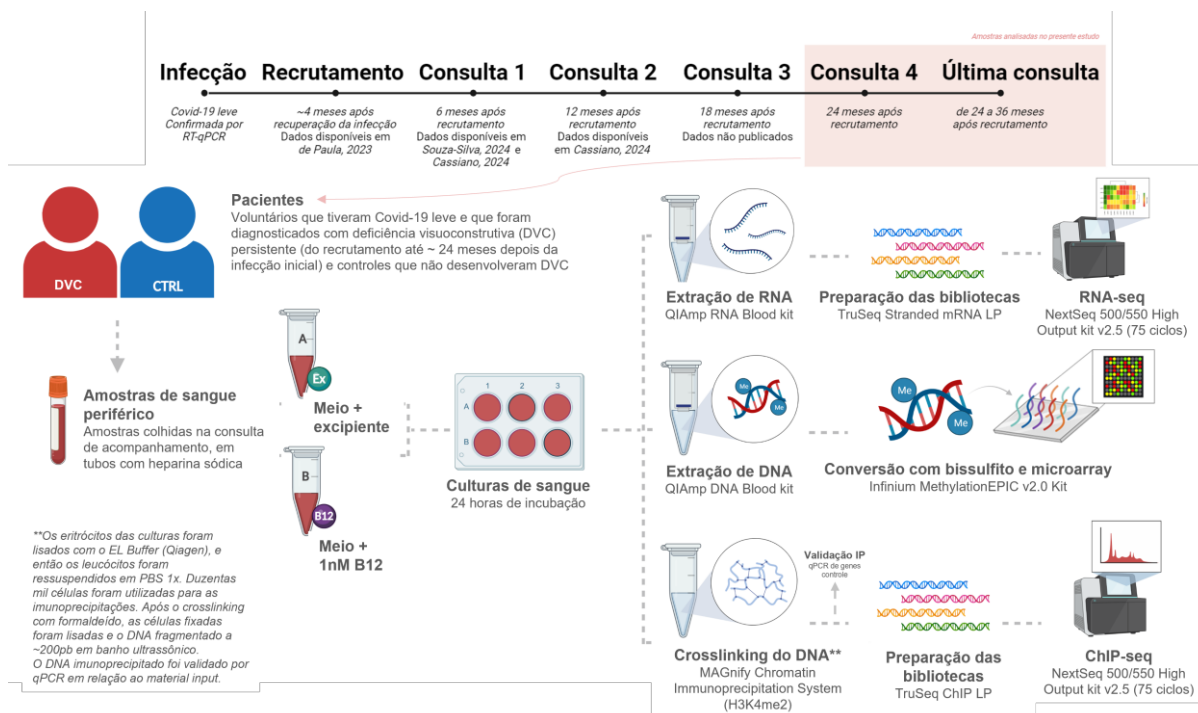


Figura 1: Desenho experimental. DVC = Deficiência visuoconstrutiva; CTRL = Controles; IP = Imunoprecipitação

RESULTADOS

Durante o intercâmbio no centro de Genoma da Universidade McGill, foram realizados treinamentos em bioinformática para análise de dados de RNA-Seq, ChIP-Seq e metilação global de DNA com *microarrays*. Os protocolos produzidos podem ser encontrados nos Materiais Suplementares 1 a 3.

Foram recrutadas para este estudo 14 voluntárias com deficiência visuoconstrutiva persistente (n= 5) ou que não apresentaram esse sintoma (N = 9) até 28 ou 34 meses depois de terem tido covid-19 leve (Tabela 1).

A expressão do gene *CCL11*, medida por RT-qPCR, continua elevada nos leucócitos dos pacientes com deficiência visuoconstrutiva persistente (DVC) confirmada nas consultas de acompanhamento aos 28-34 meses após a covid-19 aguda leve (24 ou 30 meses após o recrutamento para o estudo longitudinal). A incubação das culturas de sangue total desses pacientes com 1 nM de B12 durante 24h anulou esse efeito da doença (Figura 2). Este resultado confirma que a expressão aumentada de *CCL11* pode servir como um biomarcador da deficiência visuoconstrutiva persistente em pacientes que tiveram covid leve (1, 2).

Paciente	ID	Consultas de acompanhamento						Data consulta 24 meses	Colheita da amostra	Idade	Sexo biológico
		Recrutamento	6 meses	12 meses	18 meses	24 meses	Última consulta				
BRA-002.5	1	normal	normal	normal	normal	normal*	-	28/10/2022	28/10/2022	40	Feminino
BRA-005.5	2	DVC	DVC	normal	-	DVC*	-	28/10/2022	28/10/2022	26	Feminino
BRA-008.6	3	normal	normal	normal	normal	normal	normal	22/10/2022	10/10/2023	31	Feminino
BRA-014.6	5	normal	normal	normal	normal	normal	normal	01/12/2022	14/11/2023	50	Feminino
BRA-028.5	9	normal	normal	normal	normal	normal*	-	27/01/2023	27/01/2023	39	Feminino
BRA-040.5	10	normal	normal	normal	normal	normal*	-	27/01/2023	27/01/2023	46	Feminino
BRA-056.5	11	normal	normal	normal	normal	normal*	-	27/01/2023	27/01/2023	59	Feminino
BRA-061.6	12	DVC	DVC	DVC	-	DVC	DVC	27/01/2023	14/11/2023	37	Feminino
BRA-068.5	17	normal	normal	normal	-	normal*	-	24/02/2023	24/02/2023	44	Feminino
BRA-041.6	18	DVC	normal	DVC	-	DVC*	-	10/12/2022	06/10/2023	33	Feminino
BRA-131.5	21	normal	normal	normal	-	normal	normal	16/09/2023	06/10/2023	45	Feminino
BRA-119.5	22	normal	normal	-	normal	normal	normal	10/10/2023	20/10/2023	30	Feminino
BRA-201.5	23	DVC	-	-	-	DVC	DVC	10/10/2023	10/10/2023	47	Feminino
BRA-196.5	24	DVC	DVC	DVC		DVC	DVC	27/10/2023	27/10/2023	44	Feminino

Tabela 1: Informações dos pacientes. DVC = Deficiência Visuoconstrutiva. - Paciente não compareceu à consulta. * Última consulta.

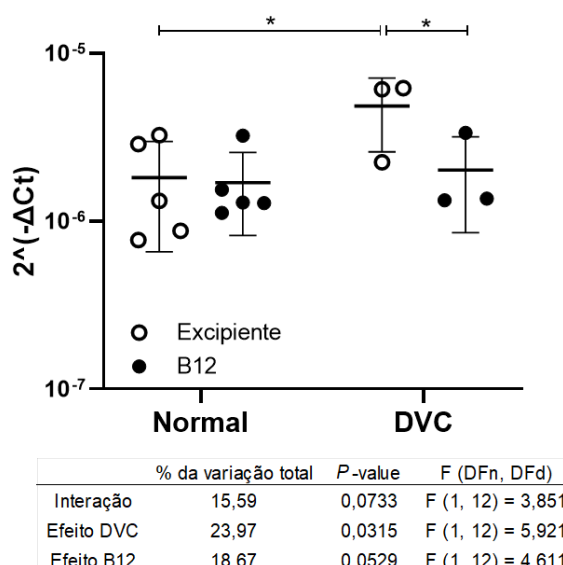


Figura 2: Expressão relativa de *CCL11* mRNA. O gene *18s* foi utilizado como calibrador para os cálculos de $2^{(-\Delta Ct)}$. Amostras fora do intervalo interquartil foram excluída da análise. O efeito da DVC, do tratamento com B12 e da interação dos dois fatores foi avaliado através do teste ANOVA *two-way*, seguido de múltiplas comparações corrigidas por *False Discovery Rate* (FDR) através dos métodos de Benjamini, Krieger e Yekutieli. * $q < 0.05$. As linhas horizontais representam a média e o desvio padrão. DVC = Deficiência Visuoespacial

Ocorreu um atraso na execução das etapas de análises do transcriptoma e do epigenoma de leucócitos humanos. Os RNAs das amostras já disponíveis quando este projeto foi iniciado estavam parcialmente degradados, comprometendo a qualidade das bibliotecas para sequenciamento. Por isto, foi necessário colher novas amostras dos voluntários quando estes retornaram para as consultas de acompanhamento 34 meses após a covid-19 leve (30 meses após o recrutamento para o estudo) e adaptar o planejamento da análise inicialmente proposta. Por se tratar de um estudo longitudinal com voluntários infectados antes do surgimento da variante ômicron do SARS-CoV-2 e quando a vacina ainda só estava disponível para grupos prioritários, como profissionais de saúde, não foi possível aumentar o número de voluntários e de amostras até o número que seria ideal para a análise do transcriptoma leucocitário. O pequeno número de amostras disponíveis para o RNA-Seq limitou a robustez dos resultados obtidos. Apesar dessa limitação, a análise de enriquecimento funcional do transcriptoma das amostras disponíveis com o software *Gene Set Enrichment Analysis* (GSEA) (3) revelou que, nos leucócitos dos pacientes com DVC, a B12 inibe a ativação das vias KEGG "*Oxidative phosphorylation*", "*Parkinson's disease*", "*Notch signaling pathway*", "*VEGF signaling pathway*" e "*P53 signaling pathway*". Estudos prévios têm relatado alterações relacionadas a algumas dessas vias em pacientes com covid longa. A disfunção mitocondrial e o estresse

oxidativo podem contribuir para a fadiga e os sintomas neurológicos persistentes (4-6). A via da doença de Parkinson compartilha algumas semelhanças com os mecanismos patológicos observados na covid longa. Ambas as condições envolvem disfunção mitocondrial, estresse oxidativo e neuroinflamação, podendo acelerar processos neurodegenerativos (7,8). A ativação da via Notch pode promover a entrada viral e a inflamação (9), enquanto a sinalização VEGF aumentada leva a complicações vasculares e edema (10,11). A modulação da via p53 pelo vírus pode perturbar a homeostase celular e contribuir para a inflamação crônica (12). Ademais, algumas das vias ativadas pela vitamina B12, como as vias "*B Cell receptor signaling pathway*", "*Toll-like receptor signaling pathway*" e "*Regulation of Autophagy*" desempenham um papel ambivalente na covid-19, atuando tanto na defesa antiviral quanto na patogênese da doença. Um estudo mais aprofundado sobre os biomarcadores alterados nessas vias está em andamento, a fim de compreender as consequências imunológicas neste cenário.

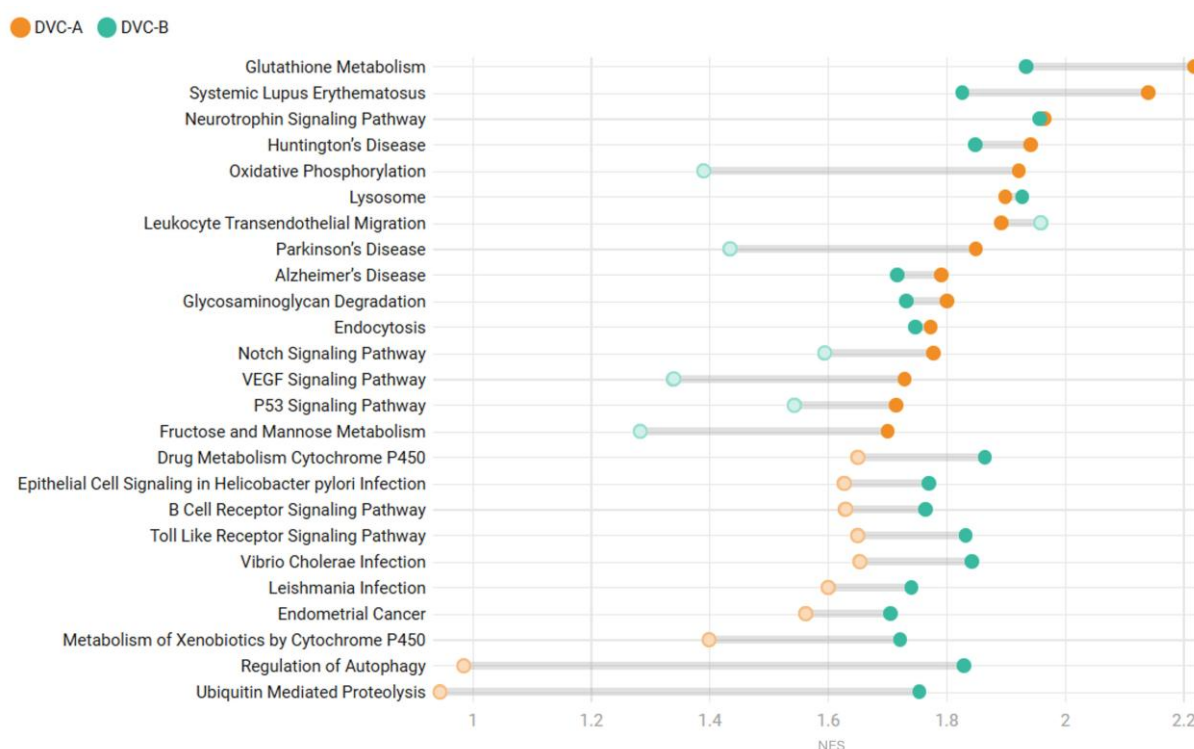


Figura 3: Vias KEGG alteradas. Análise de enriquecimento funcional dos genes ranqueados nos contrastes 1 (DVC-A vs. normal-A) e 2 (DVC-B vs. normal-A). Círculos desbotados denotam enriquecimento estatisticamente não significativo (Valor de FDR > 0.05). NES = *Normalized enrichment score*; DVC = Deficiência Visuoconstrutiva; A = endpoint A (culturas incubadas com excipiente); B = endpoint B (culturas incubadas com B12).

A análise de deconvolução dos dados do RNA-Seq revelou uma diminuição significativa na proporção das subpopulações de leucócitos T CD8+ devida à DVC (Figura 5).

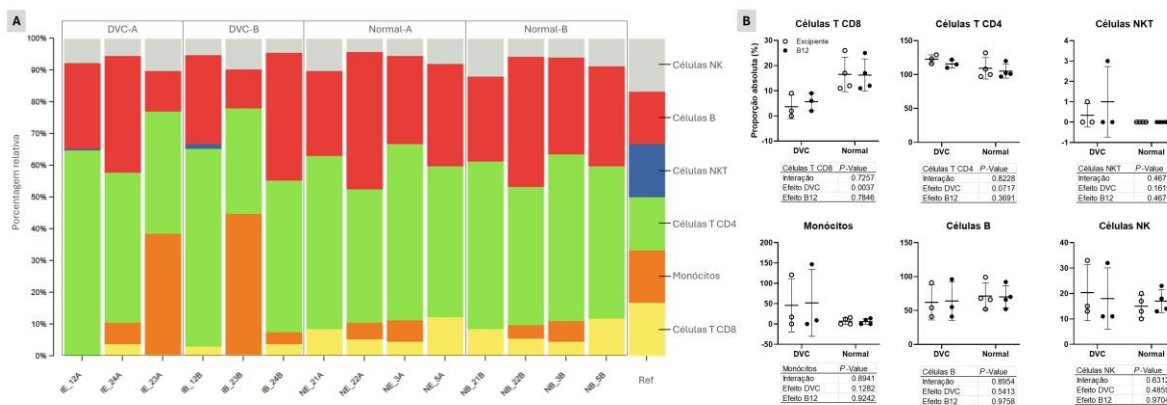


Figura 5: Proporção de células imunes. (A) A análise de deconvolução dos dados de RNA-Seq para avaliar o efeito da DVC e B12 nas proporções dos principais tipos de leucócitos foi realizada usando o software CIBERSORTx, especificamente utilizando o módulo "*Impute Cell Fractions*", e ajustando parâmetros para correção de lote (B-mode) e número de permutações (1000). As assinaturas específicas do tipo de célula de referência foram obtidas a partir de dados validados de RNA-Seq de célula única de células imunes derivadas de humanos oferecidos pelo software (NSCLC PBMCs Single Cell RNA-Seq - Fig. 2a,b). (B) Frações celulares em cada grupo experimental. Para todos os gráficos, as barras horizontais representam médias com desvio padrão. Os efeitos da DVC e do tratamento com vitamina B12 nas frações celulares foram comparados com *ANOVA two-way* seguido do pós-teste de Tukey. DVC = Deficiência Visuoconstrutiva; A = endpoint A (culturas incubadas com excipiente); B = endpoint B (culturas incubadas com B12).

A depleção de células T na covid longa é pilares da desregulação imunológica prolongada característica dessa condição (13-15). Estudos demonstram que pacientes desenvolvem alterações persistentes nos subtipos linfocitários, com redução significativa de células T de memória efectoras CD4+ e CD8+, além de expansão anômala de populações citotóxicas associadas a sintomas específicos. A exaustão celular mediada pela superexpressão de PD-1 em células T centrais de memória cria um ambiente propício à persistência viral residual e à reativação de herpesvírus latentes, contribuindo para desequilíbrios na tolerância imunológica e elevando a produção de autoanticorpos contra receptores neurológicos. Dentre os mecanismos principais sustentam essa disfunção estão o dano mitocondrial direto pelo SARS-CoV-2, que reduz a capacidade oxidativa necessária para a proliferação e diferenciação linfocitária; e a ativação crônica de vias inflamatórias via interferons tipo I/III, levando à diferenciação preferencial de células T efectoras de curta duração em detrimento das de memória (12-15).

As amostras para o metiloma de DNA foram extraídas com sucesso (Tabela 2). Novas amostras destinadas às análises do epigenoma produzidas a partir do sangue colhido nas consultas de 28 e 34 meses após a covid-19 aguda leve já foram enviadas para a *McGill University* e as análises de bioinformática estão em andamento.

As amostras de todos os indivíduos recrutados, colhidas nas consultas de 28 ou de 34 meses após a covid-19 leve, foram processadas para as análises do epigenoma. A imunoprecipitação de cromatina para H3k2me2 foi bem-sucedida, como comprovado pela análise de enriquecimento de regiões associadas de forma estável a essa marca epigenética (a maioria das amostras tiveram valores acima de 1) (Tabela 2). A quantidade dessas regiões nas amostras imunoprecipitadas foi estimada por qPCR utilizando os iniciadores apresentados na Tabela 3. Não foi possível obter enriquecimento por imunoprecipitação das regiões genômicas associadas à H3k9me3, o que pode ser devido, em parte, à pouca quantidade de células utilizadas e, sobretudo, ao fato dessa marca epigenética estar associada a regiões de heterocromatina, o que reduz a exposição dos epítomos reconhecidos pelo anticorpo utilizado na imunoprecipitação. Contudo, devido à limitação orçamentária para o projeto, não foi possível dar continuidade ao sequenciamento (ChIP-Seq) das amostras imunoprecipitadas.

Os resultados parciais confirmam que a expressão aumentada de *CCL11* pode servir como biomarcador da deficiência visuocognitiva persistente e que a vitamina B12 regula a expressão deste e de vários outros genes que participam de vias relacionadas à fisiopatologia da covid longa.

Paciente	ID	Grupo	Contagem de leucócitos (células /mL)		Viabilidade celular (%)		[] RNA				[] DNA (ng/uL)		Fold Enrichment (H3K4me2)	
			Excipiente (A)	B12 (B)	Excipiente (A)	B12 (B)	Excipiente (A)		B12 (B)		Excipiente (A)	B12 (B)	Excipiente (A)	B12 (B)
							[] (ng/uL)	RIN	[] (ng/uL)	RIN				
BRA-002.5	1	normal	6x10 ⁵	4x10 ⁵	81%	75%	9,5	NA	9,5	NA	16,9	13,6	0,47	0,82
BRA-005.5	2	DVC	7x10 ⁵	6x10 ⁵	76%	78%	6,4	NA	6,8	NA	14,7	17,4	1,07	1,25
BRA-008.6	3	normal	4,7x10 ⁶	3,8x10 ⁶	86%	83%	37,7	6,3	46,3	6,7	26,2	22,0	1,99	1,04
BRA-014.6	5	normal	2x10 ⁶	1,6x10 ⁶	91%	85%	35,9	8,9	44,1	8,2	13,2	14,4	1,61	2,22
BRA-028.5	9	normal	2x10 ⁶	2x10 ⁶	60%	60%	6,9	2,4	4,4	2,4	20,8	31,9	1,28	0,88
BRA-040.5	10	normal	3x10 ⁶	3x10 ⁶	62%	70%	4,2	2,9	10,7	3	26,8	31,5	1,74	0,58
BRA-056.5	11	normal	5x10 ⁶	4x10 ⁶	82%	60%	8,1	1,9	8,8	2,3	33,4	35,4	0,64	1,25
BRA-061.6	12	DVC	2,9x10 ⁶	2x10 ⁶	90%	88%	82,8	8,9	78	8,5	23,2	21,6	1,07	1,33
BRA-068.5	17	normal	3x10 ⁶	2x10 ⁶	82%	86%	2,5	2,5	3,0	2,7	9,7	16,7	1,33	0,91
BRA-041.5	18	DVC	2x10 ⁶	3x10 ⁶	89%	88%	4,7	NA	5,0	NA	17,3	14,4	2,08	1,20
BRA-131.5	21	normal	6x10 ⁵	5,2x10 ⁵	96%	90%	30,4	8,5	30	7,7	7,29	7,26	1,46	0,85
BRA-119.5	22	normal	2,2x10 ⁶	2,5x10 ⁶	91%	84%	52,5	8,2	52,1	9,6	13,8	13,5	1,38	1,03
BRA-201.5	23	DVC	6,1x10 ⁶	6,4x10 ⁶	95%	92%	29,3	5,9	37,1	8,8	36,4	37,0	1,11	2,33
BRA-196.5	24	DVC	4,9x10 ⁶	5,7x10 ⁶	88%	84%	84,2	7,7	68,1	7	23,4	25,5	2,51	3,26

Tabela 2: Rendimento das amostras. DVC = Deficiência Visuoconstrutiva; Excipiente (A) = culturas incubadas com excipiente; B12 (B) = culturas incubadas com B12; ([] = concentração; RIN = *RNA integrity number* (qualidade do RNA); *Fold enrichment* = Fold change de enriquecimento da amostra imunoprecipitada em relação ao *input*. Calculado a partir do dCT dos controles positivos dividido pelo dCT dos controles negativo.

Gene	Número de acesso	Região	Orientação	Sequência (5'→3')	Tm (°C)	Produto (pb)
<i>RNA18SN1</i>	NR_145820.1	-	Forward	CTCAACACGGGAAACCTCAC	58.7	110
			Reverse	CGCTCCACCAACTAAGAACG	58.9	
<i>CCL11</i>	NM_002986.3	Exon 2/3	Forward	CAGGAGAATCACCAGTGGCA	59.6	74
			Reverse	CACAGATATCCTTGCCAGTTTG	59.6	
<i>GAPDH</i>	NC_000012.12	Promotor	Foward	CCCCGGTTTCTATAAATTGAGC	61.1	82
			Reverse	AAAAGAAGATGCGGCTGACT	62.3	
<i>BIRC3</i>	NC_000011.10	Promotor	Foward	TGGTTATTACCGCTGGAGTTC	61.9	130
			Reverse	CTGTCCTTTTAAATGCGTCACCC	61.9	
<i>POMC</i>	NC_000002.12	Promotor	Foward	TTCGCATTTGGCGGTAAATATC	61.8	118
			Reverse	CCCAAAGTGGAACAGAGAGAATA	62.0	
<i>TBX19</i>	NC_000001.11	Intron 1	Foward	GACTCTTTGTGTCCTTCAAATC	61.5	121
			Reverse	GGCTAGGACTGGGTAATCATAC	61.4	

Tabela 3: Primers utilizados. Primers desenhados com o software Primer-BLAST (NCBI). *GAPDH* e *BIRC3* - eucromatina em células imunes (controle positivo do enriquecimento das amostras em regiões genômicas associadas à marca H3K4me2). *POMC* e *TBX19* – não são expressos em leucócitos (controle negativo do enriquecimento das amostras em regiões genômicas associadas à marca H3K4me2). pb = pares de base.

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MATERIAIS SUPLEMENTARES

Material suplementar 1: Análise de dados de transcriptoma

Protocolo de processamento de leituras de RNA-Seq em massa (*bulk RNA-Seq*) Illumina, compatível para análise de sequências pareadas (*paired-end*) ou não pareadas (*single-end*). Os parâmetros descritos são sugeridos para análise de leituras curtas de 75 pares de base e bibliotecas de RNA construídas com método de captura de cauda poli-A.

```

1 ## Required softwares ##
2 # Terminal (protocol executed in Ubuntu >= 22.0.0)
3 # Java Runtime Environment (for Ubuntu users - https://ubuntu.com/tutorials/install-jre#2-installing-openjdk-jre)
4 # R(>=4.0.0) and Rstudio (Optional)
5 # FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/)
6 # Trimmomatic (http://www.usadellab.org/cms/?page=trimmomatic)
7 # Salmon (https://salmon.readthedocs.io/en/latest/salmon.html)
8
9 ## R dependencies ##
10 # RSeQC (https://rseqc.sourceforge.net)
11 # ReadR (https://cran.r-project.org/web/packages/readr/index.html)
12 # tximeta (https://bioconductor.org/packages/release/bioc/html/tximeta.html)
13 # SummarizedExperiment (https://bioconductor.org/packages/release/bioc/html/SummarizedExperiment.html)
14 # DESeq2 (https://bioconductor.org/packages/release/bioc/html/DESeq2.html)
15 # apeglm (https://bioconductor.org/packages/release/bioc/html/apeglm.html)
16 # pheatmap (https://www.rdocumentation.org/packages/pheatmap/versions/1.0.12/topics/pheatmap)
17 # data.table (https://cran.r-project.org/web/packages/data.table/vignettes/datatable-intro.html)
18 # tidyverse (https://www.tidyverse.org)
19 # ggrepel (https://cran.r-project.org/web/packages/ggrepel/vignettes/ggrepel.html)
20 # cluster (https://cran.r-project.org/web/packages/cluster/index.html)
21 # factoextra (https://cran.r-project.org/web/packages/factoextra/index.html)
22 # dendextend (https://cran.r-project.org/web/packages/dendextend/vignettes/dendextend.html)
23 # org.Hs.eg.db (https://bioconductor.org/packages/release/data/annotation/html/org.Hs.eg.db.html)
24 # fgsea (https://bioconductor.org/packages/release/bioc/html/fgsea.html)
25
26 ## Files ##
27 # Reference transcriptome (trans_ref.fa or trans_ref.fa.gz)
28 # Reference genome (genome_ref.fa or genome_ref.fa.gz)
29 # Reference annotation BED file (genome.bed)
30 # Fasta files (.fq or fq.gz)
31 # Illumina Adapter file (TruSeq3-SE.fa or TruSeq3-PE.fa - for Illumina reads)
32 # Samples information table (samples_sheet.txt - samples names (column 1) x samples condition (column 2))
33
34 # Step 1: Quality Check and reads filtering
35 fastqc reads.fq.gz
36 java -jar /path/trimmomatic-0.39.jar PE -phred33 reads.fq.gz reads_trim.fq.gz ILLUMINACLIP:/path/TruSeq3-SE.fa:2:30:10
   LEADING:5 TRAILING:5 SLIDINGWINDOW:4:15 MINLEN:36 CROP:75 # Suggested parameters
37 fastqc reads_trim.fq.gz
38
39 # Step 2: Transcript quantification
40 grep "^>" <(gunzip -c genome_ref.fa.gz) | cut -d " " -f 1 > decoys.txt
41 sed -i.bak -e 's/>/g' decoys.txt
42 cat trans_ref.fa.gz genome_ref.fa.gz > gentrome.fa.gz
43 salmon index -t /path/gentrome.fa.gz -d /path/decoys.txt -p 12 -i ./index --gencode -k 31
44 salmon quant -i /index -l A -r /path/reads_trim.fq.gz --validateMappings -o /path_output/sample_quant --threads 12 --
   numGibbsSamples 100 -d --gcBias --seqBias --writeMappings=/path_output/sample.sam
45 samtools sort sample.sam -o sample.bam
46 samtools index -b sample.bam
47
48 # Step 3: RNA-seq reads coverage (Quality check)
49 geneBody_coverage.py -r genome.bed -i sample1.bam,sample2.bam,sample3.bam -o samples_coverage
50
51 # Step 4: Differential expression (DE) analysis (R)
52 # Load the data
53 library("readr")
54 dir <- "path/salmon_results"
55 samples <- read.table(file.path(dir,"samples_sheet.txt"), header=TRUE)
56 rownames(samples) <- samples$sample
57 samples$sample
58 files <- file.path(dir, samples$sample, "quant.sf")
59 names(files) <- samples$sample
60 all(file.exists(files))
61 coldata <- data.frame(files, names = samples$sample, condition = samples$condition, stringsAsFactors=FALSE)
62
63 # Run DE analysis
64 library("tximeta")
65 library("SummarizedExperiment")
66 se <- tximeta(coldata)
67 colData(se)
68 gse <- summarizeToGene(se)

```

```

69
70 library("DESeq2")
71 library("apeglm")
72 ddsData <- DESeqDataSet(gse, ~condition)
73 ddsData$condition <- releve(ddsData$condition, ref = "Normal")
74 dds <- DESeq(ddsData)
75 res <- results(dds)
76 resultsNames(dds_con)
77 res.lfs <- lfcShrink(dds, coef = "condition_Treated_vs_Normal")
78 select <- which(res.lfs$padj < 0.05 & abs(res.lfs$log2FoldChange) >= log2(1.5)) # number of differentially expressed genes
79 length(select)
80 resOrder <- res.lfs[order(res.lfs$padj),]
81 res.data <- data.frame(resOrder)
82
83 # Quality Check - PCA and Hierarchical Clustering
84 library("ggplot2")
85 library("ggrepel")
86 transform.dds <- rlog(dds, blind = TRUE)
87 dds.pca <- plotPCA(transform.dds, intgroup = c("condition"), returnData = TRUE, ntop = 1000) # select genes with most
# variance across rows to inspect data behaviour
88 percentVar <- round(100 * attr(dds.pca, "percentVar"))
89 ggplot(dds.pca, aes(PC1, PC2, color = condition)) +
90   geom_point(size = 5) +
91   geom_label_repel(aes(label=name)) +
92   xlab(paste0("PC1: ", percentVar[1], "% variance")) +
93   ylab(paste0("PC2: ", percentVar[2], "% variance")) +
94   coord_fixed() +
95   scale_color_manual(values = c("orange", "blue"))
96
97 library("cluster")
98 library("factoextra")
99 library("dendextend")
100 tmp_top_var <- 1000
101 tmp_rowVars <- matrixStats::rowVars(as.matrix(assay(transform.dds)))
102 select <- order(tmp_rowVars, decreasing=TRUE)[seq_len(min(tmp_top_var, length(tmp_rowVars)))]
103 select <- row.names(transform.dds)[select]
104 dist_mat <- dist(t(assay(transform.dds[select, ])), method = 'euclidean')
105 hclust_avg <- hclust(dist_mat, method = 'complete')
106 dend <- as.dendrogram(hclust_avg)
107 plot(dend, main = "Hierarchical Clustering - Euclidean/complete")
108
109 # Step 5: Functional enrichment analysis (R)
110 library("data.table")
111 setDT(res.data, keep.rownames = TRUE)
112
113 library("tidyverse")
114 res.data$rn <- sub("^[0-9]*", "", res.data$rn)
115
116 library("org.Hs.eg.db")
117 ens2symbol <- AnnotationDbi::select(org.Hs.eg.db,
118                                   key=res.data$rn,
119                                   columns="SYMBOL",
120                                   keytype="ENSEMBL")
121 ens2symbol <- as_tibble(ens2symbol)
122 res.gene <- inner_join(res.data, ens2symbol, by=c("rn"="ENSEMBL"))
123 res.gene <- na.omit(res.gene)
124
125 library("fgsea")
126 KEGG_paths <- gmtPathways("c2.cp.kegg.v2023.1.Hs.symbols.gmt") # can choose different databases
127 log <- res.gene.f$log2FoldChange
128 names(log) <- res.gene.f$SYMBOL
129 all_path_fgsea_res <- fgsea(stats = log, pathways = KEGG_paths)
130 pathOrder <- all_path_fgsea_res[order(all_path_fgsea_res$padj),]
131 path.tab <- data.frame(pathOrder)
132 path.padj <- path.tab[path.tab$padj<0.05, ]
133
134 library("ggplot2")
135 path.padj %>%
136   ggplot(aes(pathway, NES)) +
137   geom_point(aes(size = size, color = NES > 2)) +
138   coord_flip() +
139   scale_color_manual(values = c("#00BFC4", "#F8766D"))
140 ggplot(path.padj, aes(reorder(pathway, NES), NES)) +
141   geom_col(aes(fill=NES > 2)) +
142   coord_flip() +
143   labs(x="Pathway", y="Normalized Enrichment Score",
144        title="KEGG Pathways") +
145   theme_minimal()

```

Material suplementar 2: Análise de dados de ChIP-Seq

Protocolo de processamento de leituras de ChIP-Seq, compatível para análise de sequências pareadas (*paired-end*) ou não pareadas (*single-end*). Os parâmetros descritos são

sugeridos para análise de marcações epigenéticas de metilação de histonas (H3K4me2 e H3K9me3).

```

1 ## Required softwares ##
2 # Python >=3.6
3 # R(>=4.0.0) and Rstudio (Optional)
4 # FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/)
5 # Bowtie2 (https://bowtie-bio.sourceforge.net/bowtie2/index.shtml)
6 # Samtools (http://www.htslib.org/)
7 # MACS2 (https://pypi.org/project/MACS2/)
8 # Deeptools (https://deeptools.readthedocs.io/en/develop/#)
9 # Bedtools (https://bedtools.readthedocs.io/en/latest/)
10
11 ## R dependencies ##
12 # ChIPQC (https://bioconductor.org/packages/release/bioc/html/ChIPQC.html)
13 # rGREAT(https://bioconductor.org/packages/release/bioc/html/rGREAT.html)
14
15 ## Files ##
16 # Reference genome (genome_ref)
17 # Fasta files (.fq or .fq.gz - Treated and control samples)
18 # Bed file containing regions of interest (region.bed)
19
20 # Step 1: Reads Quality analysis (Check the results before proceed)
21 fastqc reads_R1.fq.gz reads_R2.fq.gz
22
23 # Step 2: Alignment
24 bowtie2-build -f ./path/reference/genome_ref genome_index #create genome index
25 bowtie2 --very-sensitive-local -p 3 --trim5 1 -x ./path/genome_index -1 ./path/reads_R1.fq.gz -2 ./path/reads_R2.fq.gz -S
  ./path/results/sample_map.sam #or -U ./path/reads.fq.gz for unpaired reads
26 samtools view -c ./path/results/sample_map.sam #count the number of reads that are in the SAM file
27
28 # Step 3: Sort and index alignments
29 samtools view -h -F 1804 -q 10 -b sample_map.sam | samtools sort -o sample_map.sorted.bam #the -F flag exclude alignments
  with a given FLAG field. The 1804 FLAG excludes the following reads unmapped, mate unmapped, not primary alignment, read
  quality low, PCR / optical duplicate.
30 samtools index sample_map.sorted.bam
31 rm sample_map.sam #check the bam files before remove it
32
33 # Step 4: Calling peaks
34 macs2 callpeak -f BAMPE -g hs --mfold 10 50 -q 0.05 --treatment ./path/treated_sample_map.sorted.bam --control
  ./path/control_sample_map.sorted.bam --name ./MACS2_peaks --outdir ./results/macs2 #Use -f BAM for unpaired reads
35
36 # Step 5: Peaks Quality Control (R) (Check the results before proceed)
37 library(ChIPQC)
38 library(TxDb.Hsapiens.UCSC.hg38.knownGene)
39 df <- read.table("../path/peaks.tsv", header=T)
40 chip <- ChIPQC(df, annotation="hg38") #Use the same genome version as reference
41 plotCC(chip) #plot the cross-correlation profile
42 plotRegI(chip) #heatmap plot for relative enrichment of reads around annotated genomic features
43 plotPeakProfile(chip) #plot peak profiles
44 plotCorHeatmap(chip) #plot correlation heatmap between samples
45 plotPrincomp(chip) #compute a PCA based on all the peak scores for each sample
46
47 # Step 6: Visualization
48 bamCoverage --bam ./path/sample_map.sorted.bam -o sample.bigwig --normalizeUsing BPM --extendReads
49 computeMatrix reference-point -S ./path/sample.bigwig \
50   --referencePoint center \
51   -R ./path/region.bed \
52   --beforeRegionStartLength 3000 \
53   --afterRegionStartLength 3000 \
54   --skipZeros \
55   -o ../path/deeptools/matrix.mat.gz
56 #-R: File name or names, in BED or GTF format, containing the regions to plot. If multiple bed files are given, each one is
  considered a group that can be plotted separately
57 plotHeatmap -m ../path/deeptools/matrix.mat.gz \
58   --heatmapHeight 5 \
59   --refPointLabel 'Peak summit' \
60   --regionsLabel 'Peaks' \
61   --plotTitle 'Peak summits' \
62   -out ../results/deeptools/heatmap.png
63
64 # Step 7: Gene set enrichment analysis (R)
65 library(rGREAT)
66 inp_bed <- read.table("../path/MACS2/sample.bed")
67 colnames(inp_bed) <- c("chr", "start", "end", "name", "score", "strand")
68 head(inp_bed)
69 gr <- makeGRangesFromDataFrame(inp_bed)
70 job = submitGreatJob(gr, species="hg38") #Use the same genome version as reference
71 tbl = getEnrichmentTables(job)
72 head(tbl[["GO Biological Process"]]) #options include "GO Molecular Function" "GO Biological Process" "GO Cellular
  Component"
73 plotVolcano(job, ontology = "GO Biological Process") #visualize relations between log2 fold change and (adjusted) p-values.
74 plotRegionGeneAssociations(job) #Get region-gene associations
75 getRegionGeneAssociations(job) #export associations as a table

```

Material suplementar 3: Análise de dados de metiloma global de DNA

Protocolo de processamento de dados de metilação de DNA obtidos com *microarrays* Illumina EPIC. Os parâmetros descritos são sugeridos para análise de níveis de metilação em células do sangue.

```

1 ## Adapted from Morris, T. J (2014).
  (https://bioconductor.org/packages/release/bioc/vignettes/ChAMP/inst/doc/ChAMP.html#section-champ-pipeline)
2
3 ## Required softwares ##
4   # R(>=3.3) and Rstudio (Optional)
5
6 ## R packages ##
7   # ChAMP (https://bioconductor.org/packages/release/bioc/html/ChAMP.html)
8
9 ## Files ##
10  # EPIC files (.idat or beta-valued matrix)
11  # Sample Sheet (.csv - included in the results folder - check the file for important information as 'sample_group')
12
13 # Full pipeline at once (Optional)
14 library(ChAMP)
15 dataDirectory <- system.file("../path/files")
16 champ.process(directory = dataDirectory)
17
18 # Step-by-step
19 library(ChAMP)
20 dataDirectory <- system.file("../path/files")
21 myLoad <- champ.load(dataDirectory) # load the data
22 myLoadF <- champ.filter() # filtering data - default
23 myLoadF <- CpG.GUI(CpG=rownames(myLoadF$beta), arraytype="EPIC") # check distribution of CpGs
24 myLoadF <- CpG.GUI(beta=myLoadF$beta, arraytype="EPIC") # quality control
25 myNorm <- champ.norm(beta=myLoadF$beta, arraytype="EPIC", cores=5, PDFplot=TRUE) # BMIQ method recommended for EPIC arrays
26 champ.SVD(beta=myNorm, pd=myLoadF$pd) # variation analysis
27 # If Batch detected, run myCombat <- champ.runCombat(beta=myNorm, pd=myLoadF$pd, batchname=c("Slide")) here.
28 DMP.GUI()
29 myDMP <- champ.DMP(beta = myNorm, pheno=myLoadF$pd$Sample_Group, arraytype="EPIC") # Differential methylation probes - set
  the desired contrasts
30 DMR.GUI()
31 myDMR <- champ.DMR(beta=myNorm, pheno=myLoadF$pd$Sample_Group, method="Bumphunter", arraytype="EPIC") # Differential
  methylation regions - check for different DMR algorithms
32 myBlock <- champ.Block(beta=myNorm, pheno=myLoadF$pd$Sample_Group, arraytype="EPIC") # Differential methylation blocks
  (region clusters)
33 Block.GUI(Block=myBlock, beta=myNorm, pheno=myLoadF$pd$Sample_Group, runDMP=TRUE, compare.group=NULL, arraytype="EPIC") #
  Set the desired comparison in compare.group.
34 myGSEA <- champ.GSEA(beta=myNorm, DMP=myDMP[[1]], DMR=myDMR, adjPval=0.05, method="fisher", arraytype="EPIC")
35 myRefbase <- champ.refbase(beta=myNorm, arraytype="450K") # For blood samples - detect cell proportion and remove cell
  type bias

```

APÊNDICE III: Outras produções científicas realizadas no interstício doutoral

Durante o período de doutoramento, foram produzidos outros seis trabalhos científicos de primeira autoria e uma patente de inovação não relacionados ao tema principal da tese.

ARTIGOS CIENTÍFICOS

- *Antibiotic-induced dysbiosis of the gut microbiota shifts host tryptophan metabolism and increases the susceptibility of mice to pulmonary infection with *Pseudomonas aeruginosa*.*

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Immunology

DOI: 10.1111/imm.13932

Publicado em: 19 de maio de 2025

Resumo: *Pseudomonas aeruginosa* is an opportunistic bacterium that mainly infects those who have previously been treated with antibiotics. We hypothesised that antibiotic treatment disrupts tryptophan metabolism, leading to increased susceptibility to *P. aeruginosa* infection. Our results showed that mice receiving antibiotics exhibited intestinal dysbiosis with alterations in host tryptophan metabolism, a higher mortality rate and a higher bacterial load compared to eubiotic mice. In the lungs of the dysbiotic mice, there was an increase in IDO1 (Indoleamine 2,3-dioxygenase 1) activity and an accumulation of kynurenine after infection, and IDO1-/- mice were resistant to infection

after induction of dysbiosis. Importantly, dysbiosis led to increased expression and activation of AHR (Aryl Hydrocarbon Receptor) in an IDO1-dependent manner. Blocking AHR activation in dysbiotic mice resulted in a lower bacterial load. Our data showed that increased AHR activation by kynurenine was associated with decreased phagocytosis of P. aeruginosa by macrophages and neutrophils. In conclusion, our results indicate that dysbiosis resulting from prolonged antimicrobial treatment alters tryptophan metabolism, leading to activation of the IDO1-AHR axis and increasing susceptibility to P. aeruginosa infection. Furthermore, these data suggest that IDO1 or AHR are potential host targets for the prevention of opportunistic infections in patients undergoing antimicrobial therapy.

- ***Impact of Zika virus non-structural protein mutations on hippocampal damage***

Larissa Marceley Gomes Cassiano; Roney Santos Coimbra

Neural Regeneration Research – volume 20:8

DOI: 10.4103/NRR.NRR-D-24-00493

Publicado em: 29 de julho de 2024

Resumo: The Zika virus (ZIKV), a member of the Flaviviridae family, attracted worldwide attention for its connection to severe neurological effects, notably microcephaly in newborns, first reported during the 2015 epidemic in Brazil. Yet, its impact goes beyond fetal and neonatal abnormalities, also affecting the central nervous system (CNS) in both children and adults, leading to enduring cognitive and behavioral impairments. The neuropathogenesis of Zika fever in the CNS involves a complex interplay between various brain cells, host factors, and viral elements. ZIKV crosses the blood-brain barrier by infecting brain endothelial cells and altering tight junction proteins. ZIKV-infected monocytes can also cross the blood–brain barrier, acting as a “Trojan horse” for the virus. The hippocampus, a primary site of postnatal neurogenesis and a center for memory and learning, is particularly vulnerable to infection due to its abundant vascularization and proximity to the lateral ventricles. This

proximity increases the risk of virus exposure circulating in the cerebrospinal fluid. Upon entry into the CNS, ZIKV primarily targets neuronal precursor cells, immature neurons, and mature neurons, thereby inducing cell cycle dysregulation and neuronal loss. ZIKV can also infect glial cells (e.g., microglia, astrocytes, and oligodendrocytes), resulting in microglial hyperplasia, reactive gliosis, and myelination impairment. Microgliosis and astrocytosis lead to increased production of cytokines, chemokines, and reactive oxygen/nitrogen species, thereby amplifying neuroinflammation and contributing to neuronal death and dysfunction (reviewed in Bhagat et al., 2021). These processes are shaped by variations within the ZIKV genome, leading to unique patterns of viral spread and host response. Recent research is uncovering the significance of mutations in viral non-structural proteins (NSP) concerning host immune evasion and epigenetic alterations in surviving neurons, especially regarding the severity of hippocampal damage caused by the infection. Comprehending these molecular intricacies is crucial for developing therapeutic approaches to mitigate ZIKV-related neurological complications.

- ***Neonatal overfeeding attenuates microgliosis and hippocampal damage in an infant rat model of pneumococcal meningitis***

Larissa Marcely Gomes Cassiano; Karina Barbosa de Queiroz; Thais Veronez de Andrade Martins; Ana Luiza Azevedo Maia; Milene Alvarenga Rachid; Sarah J. Spencer; Roney Santos Coimbra

Frontiers in Immunology – volume 15:1429157

DOI: 10.3389/fimmu.2024.1429157

Publicado em: 14 de outubro de 2024

Resumo: Pneumococcal meningitis (PM) triggers apoptotic neuronal and progenitor cell death in the hippocampal dentate gyrus (DG), resulting in subsequent cognitive impairment. Microglia play a crucial role in PM-induced hippocampal damage. While the lasting effects of neonatal nutrition on health are well documented, the influence of early-life overfeeding on the host response to neuroinfections remains uncertain. This study aimed to examine whether neonatal overfeeding affects the outcome of PM in the hippocampus (HC). Overfeeding was induced by adjusting litter size immediately after

birth. On the eleventh day of life, rats were intracisternally injected with *Streptococcus pneumoniae* or saline, followed by euthanasia after 24 hours for brain dissection. Histological analysis evaluated apoptosis in the DG and the extent of inflammatory infiltrate in the hippocampal fissure, while microgliosis was assessed by immunohistochemistry. The hippocampal transcriptome was analyzed using RNAseq, and the mRNA levels of specific inflammatory biomarkers were evaluated via RT-qPCR. Overfed rats exhibited 40.5% greater body mass compared to their normal-fed counterparts. Intriguingly, PM-induced apoptosis in the DG was 50% lower in overfed rats. This effect was accompanied by significant alterations in the hippocampal transcriptional profile, particularly the lack of activation of the Programmed cell death pathway in overfed infected animals. RT-qPCR analysis of *Aif1* and examination of *Iba1*-immunostained cells revealed mild microgliosis in the HC of infected-overfed animals. This reduced microglial reaction may be attributed to the diminished activation of interferon signaling pathways, as disclosed by the transcriptome analysis, potentially preventing microglial priming. Additionally, evidence of reduced neuroinflammation in overfed rats with PM was observed through the milder activation of pathways associated with Toll-like receptors, interleukins, and chemokine signaling. Furthermore, overfed animals exhibited increased transcription of proinflammatory *Il6* and anti-inflammatory *Il10* genes, with the latter showing higher expression even in the absence of PM, suggesting a priming effect of overfeeding on hippocampal immune cells. This study sheds light on the complex interplay between early-life overfeeding, immune response, and neuroprotection in infant rats with PM. The findings demonstrate the neuroprotective impact of early-life overfeeding in the context of PM, linked to the modulation of microglial function.

- ***Uncovering the neuroprotective effect of vitamin B12 in pneumococcal meningitis: insights into its pleiotropic mode of action at the transcriptional level***

Larissa Marceley Gomes Cassiano; Marina da Silva Oliveira; Karina Barbosa de Queiroz; Alice Muglia Thomaz da Silva Amancio; Anna Christina de Matos Salim; Gabriel da Rocha Fernandes; Cláudia Martins Carneiro; Roney Santos Coimbra

Frontiers in Immunology – volume 14:1250055

DOI: 10.3389/fimmu.2023.1250055

Publicado em: 03 de outubro de 2023

Resumo: The interplay between bacterial virulence factors and the host innate immune response in pneumococcal meningitis (PM) can result in uncontrolled neuroinflammation, which is known to induce apoptotic death of progenitor cells and post-mitotic neurons in the hippocampal dentate gyrus, resulting in cognitive impairment. Vitamin B12 attenuates hippocampal damage and reduces the expression of some key inflammatory genes in PM, by acting as an epidrug that promotes DNA methylation, with increased production of S-adenosyl-methionine, the universal donor of methyl. Eleven-day-old rats were infected with S. pneumoniae via intracisternal injection and then administered either vitamin B12 or a placebo. After 24 hours of infection, the animals were euthanized, and apoptosis in the hippocampal dentate gyrus, microglia activation, and the inflammatory infiltrate were quantified in one brain hemisphere. The other hemisphere was used for RNA-Seq and RT-qPCR analysis. In this study, adjuvant therapy with B12 was found to modulate the hippocampal transcriptional signature induced by PM in infant rats, mitigating the effects of the disease in canonical pathways related to the recognition of pathogens by immune cells, signaling via NF- κ B, production of pro-inflammatory cytokines, migration of peripheral leukocytes into the central nervous system, and production of reactive species. Phenotypic analysis revealed that B12 effectively inhibited microglia activation in the hippocampus and reduced the inflammatory infiltrate in the central nervous system of the infected animals. These pleiotropic transcriptional effects of B12 that lead to neuroprotection are partly regulated by alterations in histone methylation markings. No adverse effects of B12 were predicted or observed, reinforcing the well-established safety profile of this epidrug. B12 effectively mitigates the impact of PM on pivotal neuroinflammatory pathways. This leads to reduced microglia activation and inflammatory infiltrate within the central nervous system, resulting in the attenuation of hippocampal damage. The anti-inflammatory and neuroprotective effects of B12 involve the modulation of histone markings in hippocampal neural cells.

- ***The neurotoxic branch of the kynurenine pathway is highly activated in the central nervous system of patients with pneumococcal meningitis***

Larissa Marcelly Gomes Cassiano; Danilo Bretas de Oliveira; Talitah Michel Sanchez Candiani; Ana Carolina Campi-Azevedo; Olindo Assis Martins-Filho; Erna Geessien Kroon; Markus Kohlhoff; Roney Santos Coimbra

Cytokine – volume 168:156237

DOI: 10.1016/j.cyto.2023.156237

Publicado em: 29 de maio de 2023

Resumo: *Acute bacterial meningitis (ABM) causes excessive activation of N-methyl-D-aspartate receptors (NMDAr), leading to cortical and hippocampal neuron death. As opposite, enteroviral meningitis is more frequently benign. The kynurenine (KYN) pathway is the major catabolic route of tryptophan (TRP) and some of its metabolites are agonists or antagonists of NMDAr. In order to investigate the pathogen-specific patterns of KYN pathway modulation in the central nervous system of children with acute meningococcal (MM), pneumococcal (PM) or enteroviral (VM) meningitis, the cerebrospinal fluid (CSF) concentrations of TRP, KYN, kynurenic acid (KYNA) and quinolinic acid (QUINA) were evaluated by ultra-high performance liquid chromatography (uHPLC) coupled to mass spectrometry. In addition, CSF levels of IL-6, IL-10 and TNF- α were quantified by multi-analyte flow assay. The data was mined and integrated using statistical and machine learning methods. The three forms of meningitis investigated herein up-regulated the neurotoxic branch of the KYN pathway within the intrathecal space. However, this response, represented by the concentration of QUINA, was six and nine times higher in PM patients compared to MM or VM, respectively. CSF levels of IL-6, TNF- α , and IL-10 were increased in MM and PM patients when compared to controls. In VM, CSF IL-6 and IL-10, but not TNF- α were increased compared to controls, although not reaching the high levels found in bacterial meningitis. No correlation was found between the concentrations or the ratios of any pair of KYN metabolites and any cytokine or standard cytochemical parameter tested. CNS infection with meningococci, pneumococci, and enteroviruses intrathecally activate the KYN pathway, favoring its neurotoxic branch. However, in PM, higher CSF levels of QUINA, compared to MM and VM, may contribute to its poorer neurologic outcome.*

- ***Neurotoxic effects of hallucinogenic drugs 25H-NBOMe and 25H-NBOH in organotypic hippocampal cultures***

Larissa Marcelly Gomes Cassiano; Marina da Silva Oliveira; Wellington Alves de Barros; Ângelo de Fátima; Roney Santos Coimbra

Heliyon – volume 9: e17720

DOI: 10.1016/j.heliyon.2023.e17720

Publicado em: 29 de junho de 2023

Resumo: NBOMes and NBOHs are psychoactive drugs derived from phenethylamines and have hallucinogenic effects due to their strong agonism to serotonin 5-HT_{2A} receptors. Although cases of toxicity associated with the recreational use of substituted phenethylamines are frequently reported, there is a lack of information on the possible neurotoxic effects of NBOMe and NBOH in the brain hippocampus, a major neurogenesis region. This study aimed at assessing the phenotypic and molecular effects of prolonged exposure of the hippocampus to the drugs 25H-NBOMe and 25H-NBOH. The ex vivo organotypic culture model of hippocampal slices (OHC) was used to investigate, by immunofluorescence and confocal microscopy, and transcriptome analyses, the mechanisms associated with the neurotoxicity of 25H-NBOMe and 25H-NBOH. Reduction in the density of mature neurons in the OHCs occurred after two and seven days of exposure to 25H-NBOMe and 25H-NBOH, respectively. After the withdrawal of 25H-NBOMe, the density of mature neurons in the OHCs stabilized. In contrast, up to seven days after 25H-NBOH removal from the culture medium, progressive neuron loss was still observed in the OHCs. Interestingly, the exposure to 25H-NBOH induced progenitor cell differentiation, increasing the density of post-mitotic neurons in the OHCs. Corroborating these findings, the functional enrichment analysis of differentially expressed genes in the OHCs exposed to 25H-NBOH revealed the activation of WNT/Beta-catenin pathway components associated with neurogenesis. During and after the exposure to 25H-NBOMe or 25H-NBOH, gene expression patterns related to the activation of synaptic transmission and excitability of neurons were identified. Furthermore, activation of signaling pathways and biological processes related to addiction and oxidative stress and inhibition of the inflammatory response were observed after the period of drug exposure. 25H-NBOMe and 25H-NBOH disrupt the balance between neurogenesis and neuronal death in the hippocampus and, although chemically similar, have distinct neurotoxicity mechanisms.

- ***Organotypic hippocampal culture model reveals differential responses to highly similar Zika virus isolates***

Marina da Silva Oliveira*; Larissa Marcelly Gomes Cassiano*; Jeanne Pioline; Ketyllen Reis Andrade Carvalho; Anna Christina Matos Salim; Pedro Augusto Alves; Gabriel Rocha Fernandes; Alexandre de Magalhães Vieira Machado; Roney Santos Coimbra

*Compartilham primeira autoria do trabalho

Journal of Neuroinflammation – volume 20:140

DOI: 10.1186/s12974-023-02826-6

Publicado em: 10 de junho de 2023

Resumo: *Zika virus (ZIKV) caused an outbreak in Brazil, in 2015, being associated to microcephaly. ZIKV has a strong neurotropism leading to death of infected cells in different brain regions, including the hippocampus, a major site for neurogenesis. The neuronal populations of the brain are affected differently by ZIKV from Asian and African ancestral lineages. However, it remains to be investigated whether subtle variations in the ZIKV genome can impact hippocampus infection dynamics and host response. This study evaluated how two Brazilian ZIKV isolates, PE243 and SPH2015, that differ in two specific missense amino acid substitutions, one in the NS1 protein and the other in the NS4A protein, affect the hippocampal phenotype and transcriptome. Organotypic hippocampal cultures (OHC) from infant Wistar rats were infected with PE243 or SPH2015 and analyzed in time series using immunofluorescence, confocal microscopy, RNA-Seq and RT-qPCR. Unique patterns of infection and changes in neuronal density in the OHC were observed for PE243 and SPH2015 between 8 and 48 h post infection (p.i.). Phenotypic analysis of microglia indicated that SPH2015 has a greater capacity for immune evasion. Transcriptome analysis of OHC at 16 h p.i. disclosed 32 and 113 differentially expressed genes (DEGs) in response to infection with PE243 and SPH2015, respectively. Functional enrichment analysis suggested that infection with SPH2015 activates mostly astrocytes rather than microglia. PE243 downregulated biological process of proliferation of brain cells and upregulated those associated with neuron death, while SPH2015 downregulated processes related to neuronal development. Both isolates downregulated cognitive and behavioral development processes. Ten genes were similarly regulated by both isolates. They are*

putative biomarkers of early hippocampus response to ZIKV infection. At 5, 7, and 10 days p.i., neuronal density of infected OHC remained below controls, and mature neurons of infected OHC showed an increase in the epigenetic mark H3K4me3, which is associated to a transcriptionally active state. This feature is more prominent in response to SPH2015. Subtle genetic diversity of the ZIKV affects the dynamics of viral dissemination in the hippocampus and host response in the early stages of infection, which may lead to different long-term effects in neuronal population.

PATENTES

- **Método para triagem de fármacos ou substâncias com potencial neuroprotetor, e kit para triagem de fármacos ou substâncias.**

Roney Santos Coimbra; Marina da Silva Oliveira; Larissa Marcelly Gomes Cassiano; Gabriel Rocha Fernandes; Pedro Augusto Alves; Alexandre de Magalhães Vieira Machado; Anna Christina Matos Salim

Depósito: 18/05/2022

Número do registro: BR 10 2022 009685 6

A invenção se refere a um método para triagem de fármacos ou substâncias com potencial neuroprotetor. A invenção se refere ainda a um kit para triagem de fármacos ou substâncias com potencial neuroprotetor. A tecnologia baseia-se no uso de um painel de genes biomarcadores associados à neurodegeneração e resposta inflamatória induzida pela infecção por vírus Zika em um modelo de cultura organotípica de hipocampo (COHs) como processo para avaliar o potencial neuroprotetor de fármacos e compostos químicos.