UNIVERSIDADE FEDERAL DO AMAZONAS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM IMUNOLOGIA BÁSICA E APLICADA

BÁRBARA BATISTA SALGADO

CARACTERIZAÇÃO DAS RESPOSTAS HUMORAIS EM INFECÇÕES POR FLAVIVÍRUS E POR SARS-COV-2

Orientador: Prof. Dr. Pritesh Jaychand Lalwani
Co-orientadora: Profa. Dra. Jaila Dias Borges Lalwani

MANAUS
JULHO – 2021

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Tese apresentada ao Programa de Pós-graduação em Imunologia Básica e Aplicada do Instituo de Ciências Biológicas da Universidade Federal do Amazonas, como requisito final para a obtenção do título de Doutora em Imunologia Básica e Aplicada.

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Esta tese foi julgada para a obtenção do título de Doutora em Imunologia Básica e Aplicada e aprovada em sua versão final pelo Programa de Pós-Graduação em Imunologia Básica e Aplicada da Universidade Federal do Amazonas.

Prof. Dra. Jerusa Araújo Quintão Arantes Faria
Coordenador PPGIBA

Apresentada perante a Banca Examinadora composta pelos professores:

Dr. Pritesh Jaychand Lalwani, Presidente
Instituto Leônidas e Maria Deane – ILMD/FIOCRUZ-AM

Dra. Silvia Beatriz Boscardin
Universidade de São Paulo – USP

Dra. Viviane Sampaio Boaventura de Oliveira
Instituo Gonçalo Moniz – IGM/FIOCRUZ-BA

Dr. André Machado de Siqueira
Instituto Nacional de Infectologia – INI/FIOCRUZ-RJ

Dr. Mauricio Lacerda Nogueira
Faculdade de Medicina de São José do Rio Preto – FAMERP

Manaus, 30 de julho de 2021

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RESUMO

As doenças infecciosas são capazes de afetar a saúde humana com seu potencial de gerar grandes pandemias e epidemias locais. A região amazônica é endêmica para flavivírus emergentes de relevância em saúde pública e agora está enfrentando uma pandemia sanitária causada por coronavírus que causa uma síndrome respiratória aguda grave. Por isso, nosso objetivo foi avaliar as respostas de anticorpos em infecções por flavivírus e SARS-CoV-2 em residentes na cidade de Manaus. As amostras foram submetidas a análise sorológica por meio de testes de inibição da hemaglutinação (IH), ensaios imunoenzimáticos (ELISA) usando antígenos recombinantes e testes de neutralização, investigando fatores de risco associados. Flavivírus: em militares saudáveis, as taxas de soropositividade por IH foram de 60,4%, 30,9%, 46,6%, 54,7%, 51,3% e 48,7% para o vírus da dengue (DENV), vírus zika (ZIKV), vírus do Nilo Ocidental (WNV), vírus da encefalite de Saint Louis, vírus Ilhéus e vírus Rocio, respectivamente. Em indivíduos da área urbana de Manaus, observamos uma alta soropositividade do DENV (93,7%) e da cepa vacinal de febre amarela (92,8%) por IH, com maior prevalência no sexo masculino e elevadas reatividades a partir dos vinte anos. Anticorpos anti-ZIKV foram os menos prevalentes (52,6%). Demonstramos o mesmo cenário da elevada reatividade para DENV e menor para ZIKV por ELISA contra a proteína do envelope. Adicionalmente, soros com altos títulos de DENV neutralizaram o ZIKV de forma mais eficiente. Em ambas as populações, observamos uma baixa reatividade para DENV-3 e DENV-4 em comparação com DENV-2 e DENV-1 por IH; e altos títulos de DENV reagiram mais frequentemente com ZIKV ou WNV. SARS-CoV-2: analisamos de forma multidimensional e observamos um padrão temporal de aumento progressivo da resposta de anticorpos para diferentes regiões imunogênicas. A resposta robusta foi diretamente proporcional à gravidade da doença; no entanto, a resposta robusta do anticorpo não foi suficiente para proteger os indivíduos com infecção grave e o controle da inflamação. Em conclusão, militares servindo na Amazônia podem servir como sentinelas para fortalecer a vigilância global de doenças infecciosas, especialmente em áreas remotas. A pandemia da COVID-19 afetou negativamente as medidas de controle de mosquitos. Atualmente, o Brasil e a Região Amazônica enfrentam um cenário epidemiológico complexo caracterizado pela circulação simultânea de vários arbovírus, portanto, um programa funcional de controle de vetores e vigilância da síndrome febril é essencial para identificar ameaças epidemiológicas imprevistas. Além disso, estudos de coorte adicionais para avaliar a persistência de anticorpos contra SARS-CoV-2 pós-infecção natural ou vacinação na resposta imune protetora podem ser necessários.

Palavras-chave: arbovírus, covid-19, anticorpos, saúde pública, Amazônia

ABSTRACT

Infectious diseases are capable of affecting human health with their potential to generate large pandemics and local epidemics. Amazon Region is endemic for emerging flaviviruses of public health relevance and is now facing a health pandemic caused by coronaviruses that cause severe acute respiratory syndrome. Hence, our aim was evaluating antibody responses in flavivirus and SARS-CoV-2 infections in Manaus city residents. The samples were submitted to serological analysis by hemagglutination inhibition assay (HIA), enzyme-linked immunosorbent assay (ELISA) using recombinant antigens and neutralization tests, investigating correlating risk factors. Flavivirus: for healthy military personnel, HIA-seropositivity rates were 60.4%, 30.9%, 46.6%, 54.7%, 51.3% and 48.7% for the dengue virus (DENV), Zika virus (ZIKV), West Nile virus (WNV), Saint Louis encephalitis virus, Ilhéus virus and Rocio virus, respectively. Regarding individuals from Manaus urban area, we observed a high DENV seropositivity (93.7%) and also yellow fever vaccine strain (92.8%) by HIA, showing greater prevalence in males and elevated reactivity since age twenty years. Anti-ZIKV antibodies were the least prevalent (52.6%). We demonstrate the same scenario of increased DENV reactivity and lower for ZIKV by ELISA against the envelope protein. Additionally, high DENV serum titers neutralized ZIKV more efficiently. We observed decreased reactivity for DENV-3 and DENV-4 compared to DENV-2 and DENV-1 by IH; and great DENV titers reacted more frequently with ZIKV or WNV in both populations. SARS-CoV-2: a multidimensional analysis revealed a temporal pattern of progressive increase of antibody response toward different immunogenic regions. The robust response was directly proportional to the disease severity; however, the robust antibody response was not sufficient to protect individuals with severe infection and control of inflammation. In summary, military personnel serving in Amazon could serve as sentinels to strengthen global infectious disease surveillance, particularly in remote areas. COVID-19 pandemic has negatively affected mosquito control measures. Presently, Brazil and Amazon region faces a complex epidemiological scenario characterized by simultaneous circulation of several arboviruses, hence a functional vector control program and febrile-syndrome surveillance is essential to identify unforeseen epidemiological threats. Furthermore, additional cohort studies assessing post-infection or vaccination persistent antibodies against SARS-CoV-2 in protective immune response may be needed.

Keywords: arbovirus, covid-19, antibodies, public health, Amazon.

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LISTA DE ABREVIATURAS E SIGLAS

ACE2	enzima conversora de angiotensina 2
ADE	*antibody-dependent enhancement* (aumento dependente de anticorpos)
C	proteína capsídeo
CoV	coronavírus
COVID-19	doença causada pelo coronavírus de 2019
DENV	vírus dengue
DENV-1/2/3/4	vírus dengue sorotipos 1 / 2 / 3 / 4
DI/DII/DIII	domínio I / domínio II / domínio III
E	proteína do envelope
IFN	interferon
IgA	imunoglobulina A
IgG	imunoglobulina G
IgG1/2/3/4	imunoglobulina G subclasses 1 / 2 / 3 / 4
IgM	imunoglobulina M
IL-	interleucina
ILHV	vírus Ilheus
JEV	vírus da encefalite japonesa
LTC	linfócito T citotóxico
M	proteína de membrana
MERS-CoV	coronavírus associado a Síndrome Respiratória do Oriente Médio
N	nucleocapsídeo ou nucleoproteína
nAc(s)	anticorpo(s) neutralizante(s)
NFκB	*nuclear factor kappa-light-chain-enhancer of activated B cells*
NS(s)	proteína não-estrutural(is)
NSP	proteína não-estrutural
OMS	Organização Mundial da Saúde
ORF	*open reading frame* (região de leitura aberta)
POWV	vírus Powassan
prM	precursor da proteína de membrana
PRR	receptores de reconhecimento padrão
RBD	região de ligação do receptor
RNA	ácido ribonucleico
ROCV	vírus Rocio
S	proteína *spike*
S1	subunidade 1 da proteína *spike*
S2	subunidade 2 da proteína *spike*
SARS	síndrome respiratória aguda grave
SARS-CoV	coronavírus associado a síndrome respiratória aguda grave
SARS-CoV-2	coronavírus associado a síndrome respiratória aguda grave tipo 2
SLEV	vírus da encefalite de Saint Louis
ssRNA	ácido ribonucleico de fita simples
TBEV	vírus da encefalite proveniente de carrapato
TLR	receptores do tipo Toll
TNF-α	fator de necrose tumoral – alpha
UTI	unidade de terapia intensiva
VOC	variantes de preocupação
WNV	vírus do Oeste do Nilo ou Nilo Ocidental
YFV	vírus da febre amarela
ZIKV	vírus zika

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Capítulo 1

1. INTRODUÇÃO

As doenças infecciosas afetam profundamente a saúde humana com seu potencial de impactar globalmente de forma imprevisível e intensa. Grandes pandemias e epidemias locais podem determinar o destino de nações e atingir o progresso da sociedade, especialmente naquelas mais vulneráveis. Uma das características marcantes das doenças infecciosas é a estreita dependência da natureza e da complexidade do comportamento humano em conjunto com a evolução dinâmica dos agentes infecciosos (Fauci & Morens 2012).

Arboviroses emergentes são problemas importantes e constantes em saúde pública em regiões endêmicas como o Brasil, especialmente espécies de flavivírus (Donalisio, Freitas, & Von Zuben, 2017; Marchi, Trombetta, Trombetta, & Marchi, 2018).

FLAVIVÍRUS: Dentre os principais arbovírus endêmicos e emergentes estão a família *Flaviviridae*, gênero *Flavivirus*. Os flavivírus são vírus de ARN (ácido ribonucleico) transmitidos por vetores artrópodes hematófagos, cujos hospedeiros vertebrados são os primatas não humanos e o homem. Alguns patógenos importantes que causam doença em humanos pertencem a esse gênero, como os vírus do dengue, zika, febre amarela, Oeste do Nilo, encefalite japonesa, além de outros com potenciais emergentes como vírus rocio e ilheus (Frenk & Moon, 2013; Kuno et al., 1998; Moreli & da Costa, 2013).

Os flavivírus podem causar diversas manifestações clínicas, dependendo da espécie e do indivíduo acometido. Mais de 50% das infecções por flavivírus são assintomáticas ou causam síndromes febris não-específicas, manifestando febre, mialgia, artralgia, cefaleia, conjuntivite e erupçãp cutânea de rápida resolução (Donalisio et al., 2017; Vasconcelos & Calisher, 2016). As doenças mais graves causadas por flavivírus podem ser categorizadas em hemorrágicas ou viscerais e neurotrópicas, alguns deles podendo exibir os dois fenótipos. Estes vírus representam uma preocupação contínua na saúde brasileira devido ao seu potencial epidêmico e por serem responsáveis por taxas consideráveis de morbidade e mortalidade (Pierson & Diamond, 2020).

Flavivirus é um gênero com espécies altamente relacionadas. No Brasil, circula-se endemicamente os vírus dengue, enquanto outra espécie, como a febre amarela,

tem sua vacinação como obrigatória e eficiente na prevenção de infecções (Fares, Souza, Añez, & Rios, 2015; Monath & Vasconcelos, 2015). A emergência de zika causou uma epidemia devastadora com complicações autoimunes e neurológicas, como a Síndrome de Guillain-Barré em adultos e microcefalia em crianças (Beaver, Lelutiu, Habib, & Skountzou, 2018). No entanto, existe a hipótese de que a endemicidade para dengue e a vacinação da febre amarela podem ter desempenhado um papel parcial na proteção dos indivíduos contra as patologias do ZIKV, ao invés de agravar a infecção pelo vírus zika devido à presença de anticorpos antigenicamente relacionada (Dussupt et al., 2020; Hattakam, Elong Ngono, McCauley, Shresta, & Yamabhai, 2021; Rodriguez-Barraquer et al., 2019). Considerando a circulação simultânea de flavivírus na cidade de Manaus-AM, é importante compreender a dinâmica da resposta imunológica na população.

Concomitantemente à problemática das arboviroses, o país enfrenta no atual momento uma das maiores crises sanitárias de sua história com a doença por coronavírus de 2019 (COVID-19) que causa uma síndrome respiratória aguda grave (Araujo et al., 2020; Hallal et al., 2020).

SARS-CoV-2: o mundo e a nação brasileira estão encarando a maior pandemia da história recente da nossa humanidade. No final de 2019, surgiu o coronavírus SARS-CoV-2 em Wuhan, na China, causando uma onda de ocorrências com problemas respiratórios em humanos (Gralinski & Menachery, 2020; Zhu et al., 2020). A sua alta transmissibilidade pelo contato pessoa-pessoa por meio de gotículas respiratórias ou por superfícies contaminadas levou a uma rápida disseminação global (Harrison, Lin, & Wang, 2020), sendo caracterizada como pandemia em 11 de março de 2020 pela Organização Mundial da Saúde (WHO, 2020a).

O vírus SARS-CoV-2 se liga a enzima conversora de angiotensina 2 (ACE2), receptor celular humano, e estabelece a infecção (Q. Wang et al., 2020). A COVID-19 comumente causa febre, cansaço e tosse seca em humanos, contudo possui um grande potencial de desencadear quadros respiratórios graves, especialmente em grupos de risco (C. Huang et al., 2020). É caracterizada, em sua maioria, por um desequilíbrio imunológico, levando a uma exarcebada resposta inflamatória sistêmica, que, por sua vez, afeta os outros sistemas extra-pulmonares (Olbei et al., 2021).

Uma das formas de proteção contra a COVID-19 é a resposta imune humoral por meio da indução de anticorpos neutralizantes. Estudos vêm mostrando que

maioria dos indivíduos infectados assintomáticos ou pouco sintomáticos apresentam respostas de anticorpos mais fracas, tardias ou até inexistentes comparadas a pacientes sintomáticos graves (Long, Liu, et al., 2020; Long, Tang, et al., 2020; N. Okba et al., 2020; Röltgen et al., 2020; W. Tan et al., 2020). Uma das hipóteses levantadas é que os indivíduos com sintomas leves da doença podem ter estimulado a secreção de anticorpos IgA anti-SARS-CoV-2 nas mucosas durante uma resposta inata, sem necessitar da resposta imune adaptativa (Cervia et al., 2021; Yongchen et al., 2020).

Uma das características particulares em infecções por SARS-CoV-2 é produção precoce de anticorpos IgG anti-SARS-CoV-2 nos primeiros dias após a infecção, especialmente em casos mais graves de COVID-19 (W. Tan et al., 2020). Adicionalmente, a resposta humoral de IgA tem sido mais intensa e persistente comparado aos anticorpos IgM, surgindo precocemente e estabelecendo maiores reatividades ((Lee, Lin, Renia, & Ng, 2020; N. Okba et al., 2020; Padoan et al., 2020). Além disso, anticorpos IgA mostraram maior capacidade de neutralização comparado a anticorpos IgG, e permaneceram por mais tempo com a função neutralizante em espécimes de saliva (Sterlin et al., 2021).

As glicoproteínas do capsídeo viral e nucleoproteínas são os principais focos na indução da resposta humoral de IgG (e subclasses), IgM e IgA em infecções por SARS-CoV-2 (Poland, Ovsyannikova, & Kennedy, 2020). Dentre as subclasses de IgG, as maiores reatividades específicas para SARS-CoV-2 foram dos anticorpos IgG1 e IgG3, seguida por IgG2, e a subclasse IgG4 com os menores títulos (Amanat et al., 2020; Dogan et al., 2021; Hunt et al., 2021; Iles et al., 2021; Yates et al., 2021).

No entanto, quase um ano e meio depois do início da pandemia, o número de infecções por SARS-COV-2 passou de 180 milhões (WHO 2021). E a pandemia ainda está em andamento, embora as taxas globais de letalidade e o as formas clínicas graves da doença tenham reduzido consideravelmente. Isso está diretamente relacionado com a eficácia das vacinas. Contudo, muitos estudos vêm mostrando a queda dos anticorpos neutralizantes em tempos curtos após a convalescência, o que permite que ocorram reinfecções (Cromer et al., 2021; Khoury et al., 2021).

Além dessa durabilidade e manutenção de anticorpos neutralizantes contra SARS-CoV-2, a capacidade de neutralizar as variantes de preocupação (VOC)

emergentes é também um dos maiores desafios a serem enfrentados durante a trajetória dessa pandemia em curso.

Existem ainda muitos assuntos não compreendidos em relação a resposta imunológica humoral a flavivírus e ao SARS-CoV-2.

Considerando a co-circulação de flavivírus de importância clínica no bioma amazônico em conjunto com a vacinação contra a febre amarela, procuramos responder sobre as reatividades aos diferentes flavivírus, endêmicos e potenciais emergentes em populações saudáveis, e o papel de anticorpos neutralizantes de reação cruzada entre dengue e zika na imunidade protetora.

Quanto ao SARS-CoV-2, buscamos analisar o perfil dos anticorpos em grupos de pacientes com sintomas leves e graves durante a infecção por COVID-19. Avaliamos também as respostas de anticorpos IgA, IgG total e subclasses de IgG contra o nucleocapsídeo e a proteína spike, de comprimento total ou truncadas, com o objetivo de avaliar a resposta humoral em relação ao desfecho da doença.

2. REFERENCIAL TEÓRICO

2.1 FLAVIVÍRUS

2.1.1 Histórico e Epidemiologia dos flavivírus

Os arbovírus, vírus provenientes de artrópodes, de maior interesse em saúde pública pertencem à família *Flaviviridae*, gênero *Flavivirus*. A extensa disseminação global e transmissão epidêmica de flavivírus nas últimas décadas tem sido notável (figura 1). O potencial epidêmico dos arbovírus está diretamente relacionado a fatores como desmatamento, urbanização inapropriada, carência de saneamento básico, mudanças climáticas, habitat ideal para reprodução dos artrópodes, distribuição geográfica dos vetores, além da introdução de novos vírus como os vírus Zika e Chikungunya (*Togaviridae*) (Guzmán et al., 2020).

Dengue é considerada uma das doenças virais transmitidas por artrópodes mais prevalente no mundo. Existem quatro sorotipos de vírus da dengue (DENV) (DENV-1 a DENV-4) distintos, mas antigenicamente relacionados. Os DENV infectam cerca de 400 milhões de humanos a cada ano. Atualmente, mais de um quarto da população mundial, em mais de 100 países, vive em áreas endêmicas para dengue. O DENV causa aproximadamente 500.000 apresentações da forma grave da doença por ano no mundo, com pelo menos 40% da população mundial sob risco de dengue de acordo com a estimativa da Organização Mundial da Saúde (OMS) (Bhatt et al., 2013; Fares et al., 2015). Nos últimos quatro anos, nas Américas, o Brasil tem apresentado altos números de casos de dengue reportados, com cerca de 4,5 milhões (PAHO/WHO, 2021c).

O vírus zika (ZIKV) foi identificado primeiramente em primatas não-humanos em 1947 em Uganda, mas passou a ser considerado uma vírus emergente quando ressurgiu provocando epidemias febris na Micronésia em 2007 e Polinésia Francesa em 2013-14 (Cao-Lormeau et al., 2014; Lanciotti et al., 2008). O ZIKV atraiu a atenção internacional devido a sua associação sem precedentes com complicações autoimunes e neurológicas, como a Síndrome de Guillain-Barré em adultos e microcefalia em crianças, no surto nas Américas que começou no Brasil em abril de 2015. Durante a evolução do ZIKV, foi identificado duas grandes linhagens, a africana isolada em 1947, e a asiática estabelecida no surto em 2007 e predominante nas

epidemias atuais (Beaver et al., 2018; WHO, 2016). De acordo com a OMS existem atualmente cerca de 90 países e territórios com evidência de transmissão vetorial do ZIKV, e mais de 60% concentrado no continente americano (WHO, 2018). No Brasil, desde os primeiros relatos de 2015 até os dias de hoje, estima-se que foram notificados entre 350.000-400.000 casos suspeitos de infecção por ZIKV, com incidência média de 40,0 casos/100 mil habitantes (PAHO/WHO, 2021a).

O vírus do Oesto do Nilo (WNV) é outro flavivírus de grande ameaça com potencial epidêmico no Brasil. É a arbovirose de maior disseminação mundial. Desde a sua introdução nos Estados Unidos em 1999, foi notório sua rápida dispersão espacial nas Américas e emergência na Europa, causando inúmeros casos de encefalite. A infecção por WNV teve uma taxa de letalidade de 50% nos últimos 20 anos (CDC, 2021), causando doenças neuroinvasivas nos Estados Unidos, e continua se expandindo e causando epidemias em área urbanas (Donalisio et al. 2017; Vasconcelos & Calisher 2016). No Brasil, o primeiro caso de encefalite aguda causada pelo WNV foi confirmado sorologicamente em uma área rural em 2014 (Vieira et al., 2015). Considerando o clima tropical brasileiro e o intenso desmatamento, é possível a ocorrência de surtos de WNV no Brasil e causar sérios problemas para a saúde pública.

Outro flavivírus clinicamente significativo é o vírus da febre amarela (YFV). Acredita-se que surtos gerados nos séculos XVIII e XI causados por YFV provocaram graves epidemias que tornaram necessária a produção de uma vacina. Apesar da eficácia da vacina, o YFV permanece endêmico na África e América do Sul. Surtos periódicos ocorrem frequentemente na África, representando cerca de 90% dos casos de YFV no mundo. Por outro lado, a taxa de letalidade por YFV na África (~20%) é menor do que na América do Sul (40-60%). No Brasil, casos humanos por YFV são esporádicos, pois o vírus habita predominantemente em ambiente silvático, causando surtos associados com epizootias. Contudo, considerando que a epidemiologia do YFV é determinada pela distribuição de seu vetor, a adaptação desses mosquitos em ambientes peri-urbanos pode disseminar a circulação desse vírus e provocar desafios para a saúde pública (Monath & Vasconcelos, 2015). No Amazonas, a vacinação de YFV é obrigatória e alguns outros flavivírus são endêmicos.

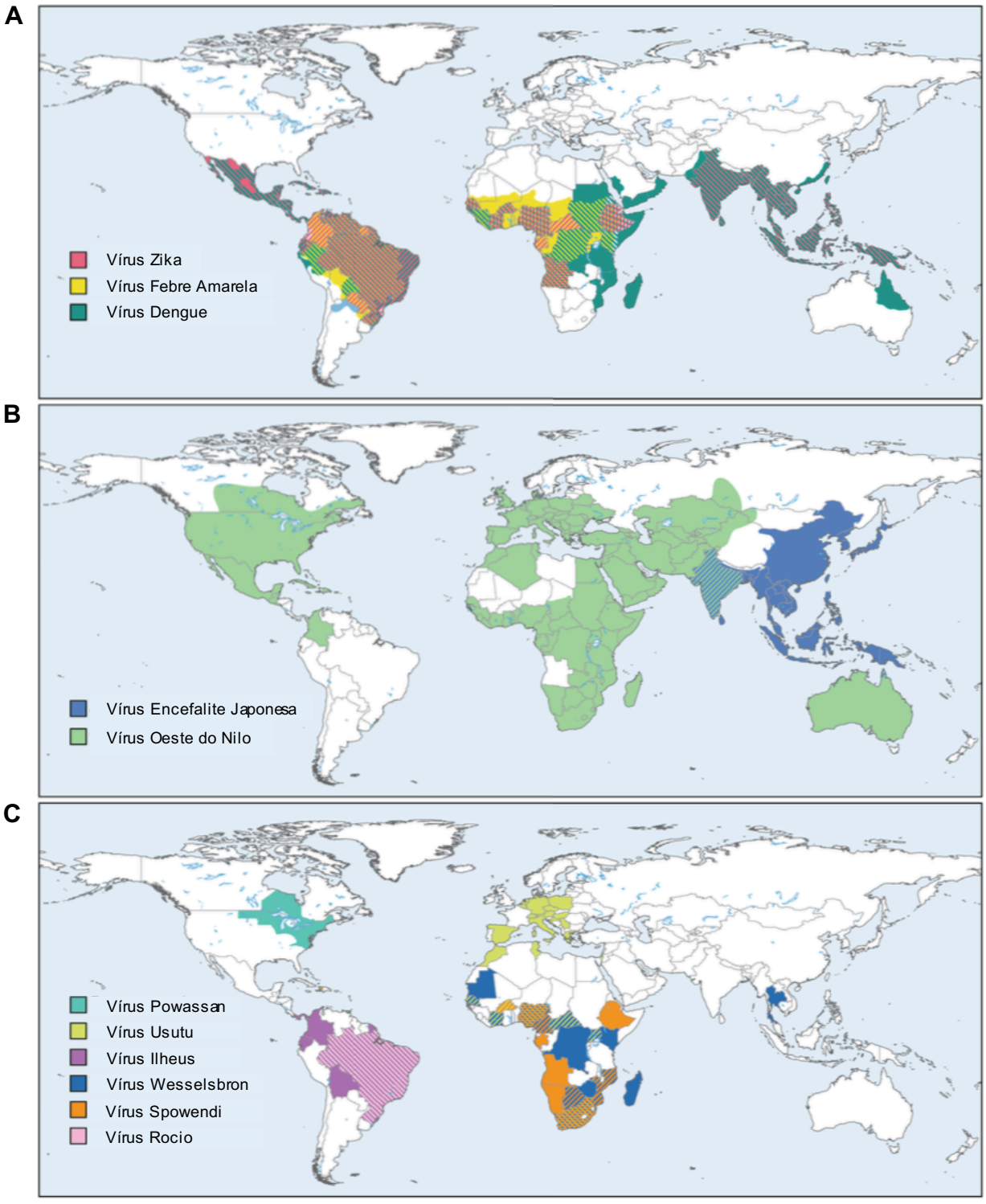


Figura 1. Distribuição dos Flavivírus no mundo. Estimativa da distribuição geográfica dos (A) principais flavivírus transmitidos por mosquitos *Aedes* spp., (B) flavivírus com ameaças estabelecidas, e (C) flavivírus com potencial para emergência em populações humanas (adaptado de Pierson & Diamond 2020).

O vírus da encefalite Japonesa (JEV) causa doenças neurológicas graves e é prevalente em países da Ásia, sendo responsável por aproximadamente 35.000-50.000 casos anualmente, com uma taxa de letalidade variando entre 20-40%. Apesar da implantação de vacinas, o JEV continua sendo uma importante preocupação global para encefalite viral. Existe também um alerta quanto à emergência de JEV nas Américas, semelhante ao processo de dispersão do WNV, uma vez que os vetores *Culex* são suscetíveis à infecção pelo JEV, e uma variedade de espécies de aves são suscetíveis ao JEV e podem servir como hospedeiros (Y. J. S. Huang et al., 2015; Tsai, 2000). A tropicalização do Brasil favorece o trânsito de culicídeos e animais silvestres.

Outros flavivírus apresentam riscos contínuos à saúde ou estão emergindo em diferentes partes do mundo, incluindo os vírus neutrotrópicos: da Encefalite de Saint Louis (SLEV), Ilheus (ILHV), Rocio (ROCV), da Encefalite transmitida por carrapatos (TBEV) e o vírus Usutu. Já reportaram casos humanos no Brasil de SLEV (Dos Santos, Sallum, Franco, Oshiro, & Rocco, 2006) , ILHV e ROCV (Lopes, Nozawa & Linhares 2014)

Das arboviroses de maior circulação no país (dengue, chikungunya e zika), a infecção por ZIKV apresenta as menores taxas de mortalidade. O impacto da co-circulação desses vírus ainda é pouco conhecido e muito discutido. Existem dificuldades no manejo clínico e diagnóstico laboratorial, e também implicações na imunidade, patogenicidade e capacidade infecciosa em virtude do trânsito simultâneo desses vírus (Brasil et al. 2016; Cardoso et al. 2015; Ioos et al. 2014; Roth et al. 2014).

Entre 2012 e 2016, ocorreram diferentes cenários nos estados brasileiros comparando as incidências de DENV e ZIKV (figura 2). Bahia, Rio de Janeiro e Mato Grosso apresentaram baixos índices de incidência de DENV, mas exibiram os maiores índices de ZIKV, quando o vírus emergiu no Brasil em 2015 e no ano epidêmico em 2016. Contrariamente, Acre, Minais Gerais e Mato grosso do Sul mostraram alta incidência para DENV e baixa para ZIKV. O Amazonas apresenta um cenário similar a baixas incidências para DENV e consideravelmente altas pra ZIKV (SVS/MS, 2012, 2015, 2017).

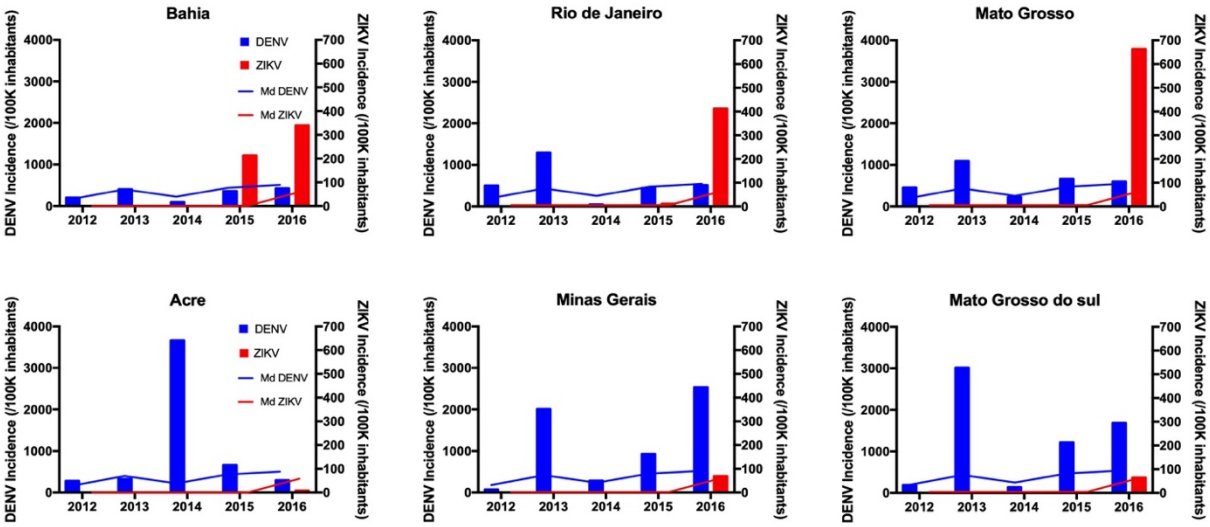


Figura 2. Incidência de dengue e zika nos estados do Brasil entre 2012 e 2016. Os valores das incidências estão representados por barras e as médias nacionais por linhas. Na parte superior estão os estados com baixas incidências de DENV e altas incidências de ZIKV. Na parte inferior, estão os estados com altas incidências de DENV e baixas incidências de ZIKV. Dados de DENV estão representados em azul; e ZIKV em vermelho.

2.1.2 Biologia e transmissão dos flavivírus

Flavivírus são vírus endêmicos e emergentes de importância clínica mundial. São considerados um problema de saúde pública relacionado a fatores ambientais e vetoriais. As constantes alterações no meio ambiente e ecossistemas são influenciadas pelo comportamento humano na natureza e vida silvestre, contribuindo para o aumento de doenças infecciosas causadas por vetores ali habitados (Donalisio, Freitas, & Von Zuben 2017; Fauci & Morens 2012; Frenk & Moon 2013). Esses, por sua vez, têm a capacidade de transmitir uma diversidade de patógenos, como bactérias, parasitas e vírus.

Os flavivírus pertencem a um grupo chamado “arbovírus” (expressão em inglês, “*arthropod-borne virus*”) que representa os vírus que são transmitidos por artrópodes, como mosquitos e carrapatos. Os arbovírus estão globalmente dispersos em áreas tropicais e são mantidos por meio de ciclos complexos na natureza, principalmente silvestres. Nesses ciclos, muitas espécies de artrópodes hematófagos agem como vetores, e animais vertebrados selváticos, como reservatórios. Alguns dos arbovírus mantêm ciclos urbanos, como dengue, zika, chikungunya e oropouche, e outros peri-urbanos, como mayaro e febre amarela, cujos hospedeiros acidentais envolvem

humanos (Batista et al. 2013; Cardoso et al. 2015; Chang et al. 2016; Moreli & da Costa 2013).

Na natureza, a manutenção dos flavivírus ocorre por meio de ciclos zoonóticos que envolvem espécies de mosquitos do gênero *Aedes* spp., e primatas não-humanos, como principais vetores e reservatórios, respectivamente. Enquanto que em ambientes urbanos, os flavivírus têm os mosquitos sendo os transmissores e o homem como principal reservatório (figura 3). Além de artrópodes e humanos, os flavivírus também são conhecidos por infectar uma diversidade de espécies animais e ameaçar animais domésticos economicamente importantes. Estudos vêm demonstrando a circulação desses vírus em outras espécies de mosquitos, como *Culex* spp., e de hospedeiros, como morcegos, aves silvestres e domésticas e equinos (Dick, Kitchen, & Haddow, 1952; Guzmán et al., 2020).

O modo de transmissão dos flavivírus para o ser humano ocorre, em sua maioria, por meio da picada de mosquitos fêmeas hematófagas (OMS, 2016), entretanto o ZIKV vem apresentando evidências de transmissões não-vetoriais. O contágio sexual foi o primeiro a ser descrito, por Foy e colaboradores (2011) que, em seguida, motivou estudos que relataram a infecção de ZIKV via sêmen, urina e outros fluídos corporais, por meio de contato pessoa-pessoa (Kurscheidt et al., 2019; Moreira, Peixoto, Siqueira, & Lamas, 2017; Musso et al., 2015).

A transmissão de ZIKV vertical (mãe para feto) foi reportada sendo mediada por vias intrauterina, transplacentária e amamentação (Besnard, Lastère, Teissier, Cao-Lormeau, & Musso, 2014; Colt et al., 2017; Salge et al., 2016). A infecção por transfusão sanguínea também foi demonstrada (Jimenez, Shaz & Bloch 2017; Marano et al. 2016). Outras rotas de transmissão vem sendo discutidas, como mordidas de macacos, ferimentos com agulhas, transplantes e hemodiálise (Runge-Ranzinger, Morrison, Manrique-Saide, & Horstick, 2019).

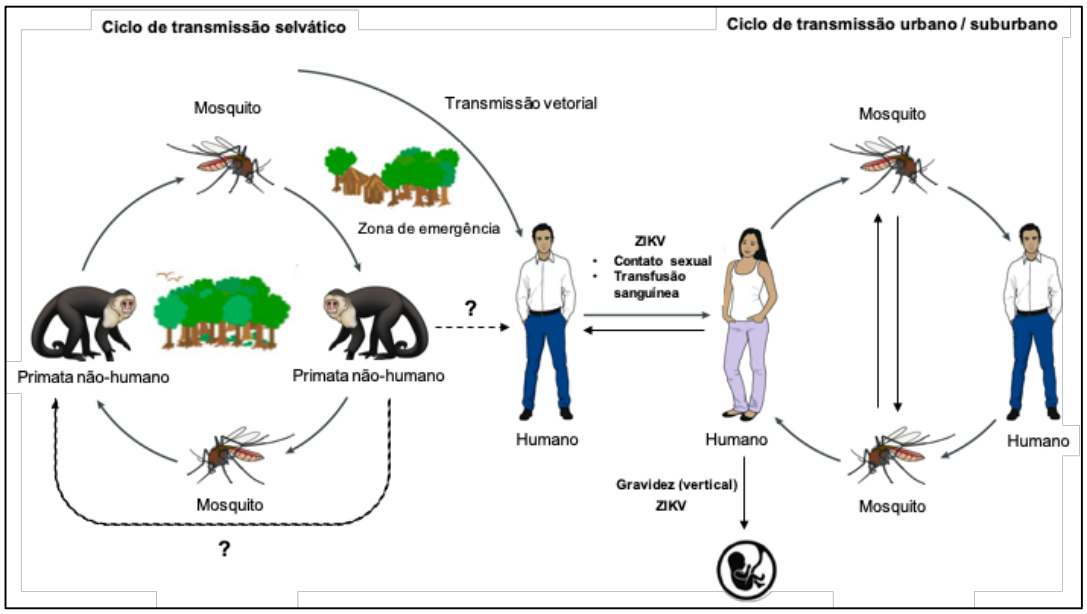


Figura 3. Ciclos de transmissão silvestre e urbano dos principais flavivírus (adaptado de Kurscheidt et al. 2019).

Os flavivírus são vírus de RNA (ácido ribonucleico) pertencente a Família *Flaviviridae*, gênero *Flavivirus*. O gênero *Flavivirus* compreende 53 espécies virais, dentre elas constam alguns importantes patógenos que causam doença em humanos, como os vírus Dengue (DENV), Zika (ZIKV), Febre Amarela (YFV), Encefalite Japonesa (JEV), Encefalite de Saint Louis (SLEV) e vírus do Oeste do Nilo (WNV), além de outros com potenciais emergentes como vírus Rocio (ROCV), Ilheus (ILHV) e Encefalite nascida de carrapato (TBEV) (ICTV, 2020).

A estrutura geral de um flavivírus consiste de uma partícula icosaédrica e envelopada, cujo genoma é um segmento de RNA de cadeia simples (ssRNA) (figura 4), polaridade positiva e de tamanho aproximado de 11 kb. O RNA genômico possui uma sequência da região de leitura aberta (expressão em inglês, "*open reading frame - ORF*") que codifica uma única poliproteína que é clivada pela combinação de proteases virais e da hospedeira para gerar componentes estruturais e não-estruturais (figura 5). Os três componentes estruturais incluem o capsídeo (C), a pré-membrana (prM) / membrana (M) e o envelope (E), esse último subdividido em três domínios (D-I, D-II e D-III), enquanto os sete não-estruturais são NS1, NS2a, NS2b, NS3, NS4a, NS4b e NS5 (Chang et al. 2016; Kuno et al. 1998; Kuno & Chang 2007; Pierson & Diamond, 2013).

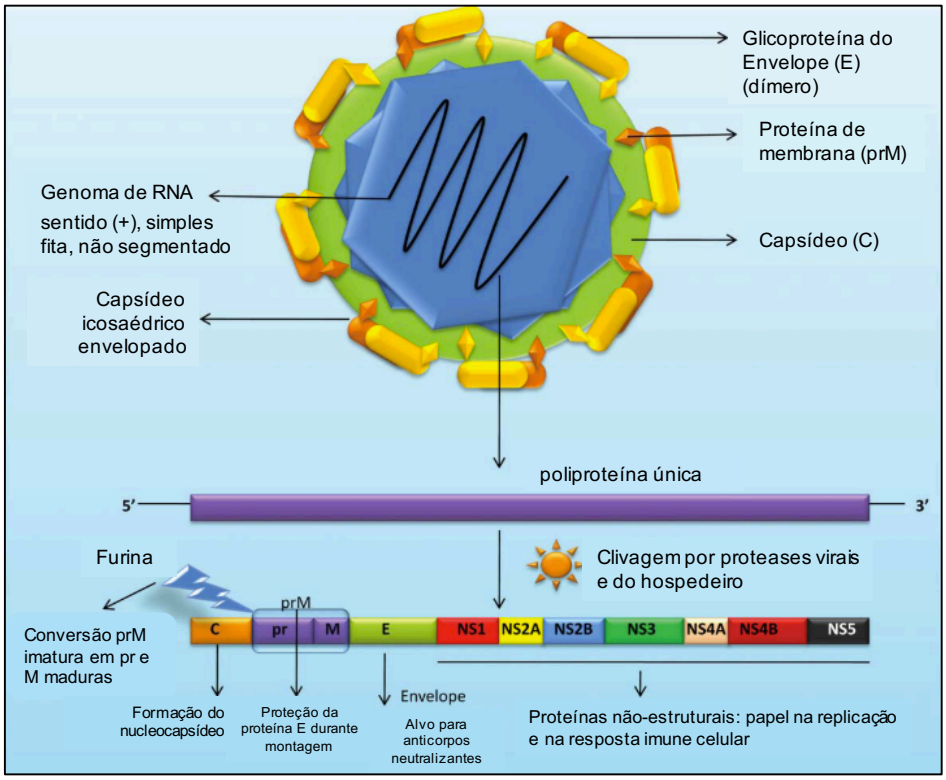


Figura 4. Representação esquemática da morfologia de uma partícula viral e da poliproteína do gênero *Flavivirus*. As proteínas estruturais do capsídeo (C), envelope (E) e membrana (prM) formam uma partícula icosaédrica envelopada, cujo genoma RNA tem uma fita positiva e não-segmentada. As proteínas não-estruturais desde NS1 a NS5. As funções básicas das proteínas estão mencionadas (adaptado de Singh et al. 2016).

A entrada dos flavivírus na célula hospedeira é mediada por receptores via endocitose a partir da interação do complexo glicoproteico do envelope viral e os receptores de superfície da célula hospedeira. O vírus se funde a membrana endossômica, devido ao baixo pH dentro do endossomo que modifica a conformação do envelope proteico, e libera seu genoma no citoplasma para replicação e síntese proteica. A progênese dos vírions ocorre no retículo endoplasmático, cujas invaginações esféricas atuam como vesículas induzidas pelo vírus para proteger os componentes virais e conduzir as partículas formadas ainda imaturas até o complexo de Golgi. Vírions recentemente sintetizados são transportados para maturação através da via secretória, onde ocorre a clivagem do sítio da prM pela furina, protease da hospedeira, seguido da liberação por exocitose para o ambiente extracelular (figura 5) (Lindenbach & Rice 2003; Lindenbach, Thiel & Rice 2007; Romero-Brey & Bartenschlager 2014).

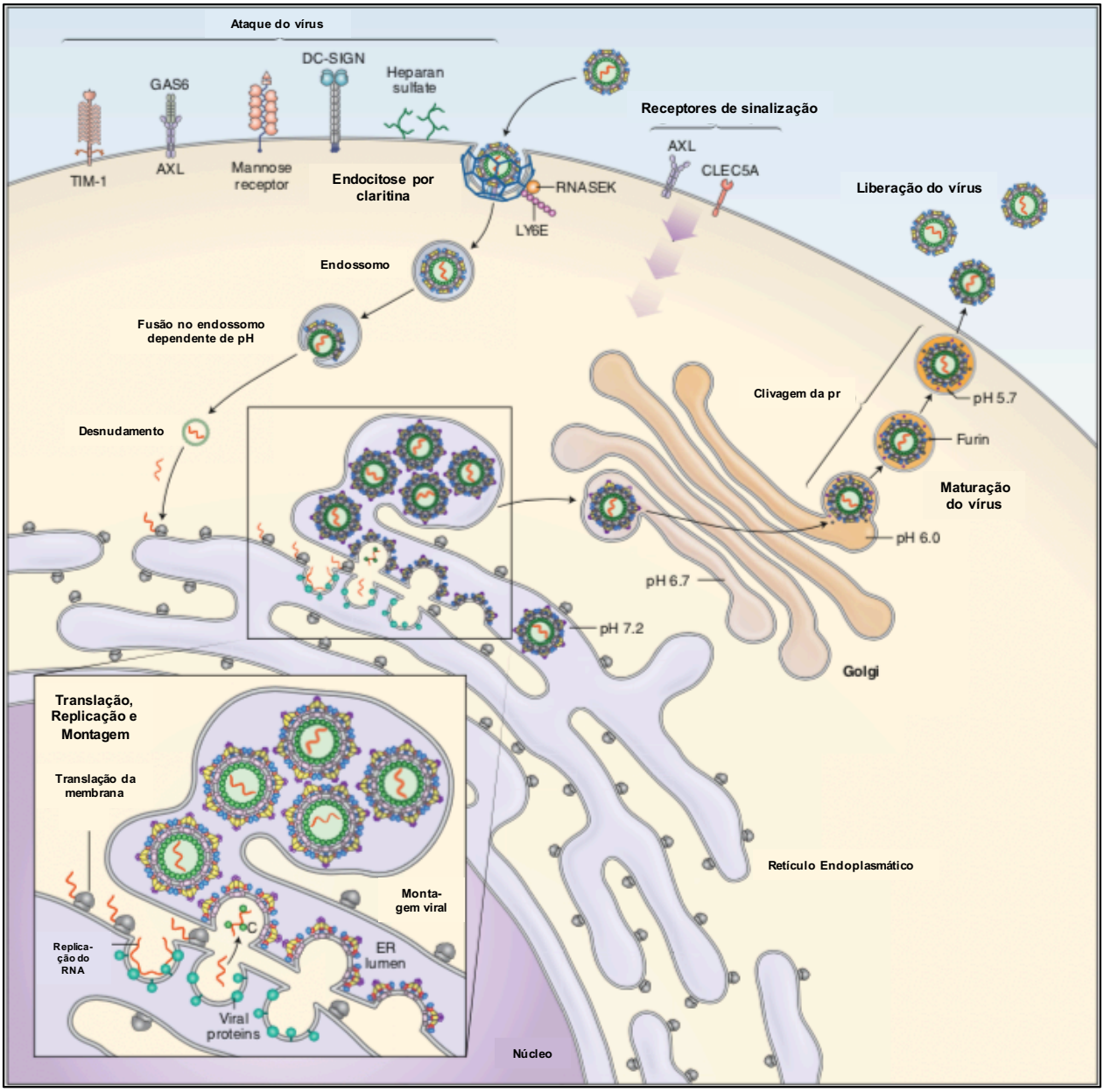


Figura 5. Representação esquemática do ciclo celular dos flavivírus (adaptado Pierson & Diamond 2020).

2.1.3 Patologia associadas a infecções por flavivírus

Os flavivírus podem causar diferentes e inesperadas manifestações clínicas, dependendo da espécie e do indivíduo afetado. O perfil clínico de infecções humanas agudas por flavivírus varia de doença leve (assintomática ou quadros febris autolimitados) a doença grave e com risco de vida (febre hemorrágica, síndrome do choque, encefalite, paralisia, defeitos congênitos, hepatite e insuficiência hepática). As principais doenças causadas por flavivírus podem ser agrupadas em viscerais e neurotrópicas, embora algumas espécies tenham características de ambas, como o

vírus zika (Pierson & Diamond 2020; Vasconcelos & Calisher 2016; Weaver & Reisen 2010).

Apresentação da doença entre os flavivírus pode diferenciar em função de algumas características individuais como o tropismo celular e tecidual, diferenças na capacidade de evadir ou antagonizar a imunidade do hospedeiro e a interação entre os efeitos patogênicos resultantes da infecção viral e a lesão causada pela resposta do hospedeiro. Aproximadamente 50–80% das infecções por flavivírus são assintomáticas ou causam doença de forma branda. As infecções sintomáticas por flavivírus resulta majoritariamente em síndromes febris não-específicas, manifestando febre, mialgia, artralgia, dor de cabeça, conjuntivite e exantemas de curta recuperação e poucas sequelas (Brasil et al. 2016; Chávez et al. 2013; Donalisio et al. 2017; Ioos et al. 2014).

Os fatores determinantes da evolução para a doença grave por flavivírus ainda não são totalmente elucidados, contudo alguns estudos vêm levantando hipóteses em relação a polimorfismos em genes do hospedeiro, idade, estado imunológico, comorbidades, imunidade prévia de flavivírus anterior, patogenicidade diferencial associadas a cepas de vírus e outros possíveis fatores adquiridos, incluindo o microbioma (J. K. Lim et al., 2008; Murray et al., 2006; Sakuntabhai et al., 2005; Trackray et al., 2019).

Os principais flavivírus causadores de doenças hemorrágicas e viscerais em humanos são DENV, YFV e ZIKV. DENV pode infectar células mieloides no sangue e nos tecidos que resulta em aumento de permeabilidade vascular, trombocitopenia, sangramento anormal, hemoconcentração e hipotensão (Nhan et al., 2001; Rothman, 2011). As altas taxas de replicação de YFV nas células do fígado resultam em hepatite grave, insuficiência renal, hemorragia, choque e morte (Kleinert et al., 2019; Monath & Vasconcelos, 2015). O ZIKV infecta células progenitoras, epiteliais e mieloides, bem como tecidos periféricos, causando lesões nos olhos e nos tratos reprodutivos masculino e feminino e também audição. Além disso, a sua capacidade de persistir em fluidos seminais permite a transmissão sexual (Mead et al. 2018; Medina et al. 2019; Miner & Diamond 2017; Yockey et al. 2016).

Os flavivírus neutrotrópicos importantes são os WNV, ZIKV, JEV, TBEV e vírus Powassan (POWV) e podem causar encefalite, deficiência cognitiva, convulsões e paralisia. Esses vírus causam lesões em neurônios ou células neuroprogenitoras, no

caso de ZIKV, de regiões como tronco encefálico, córtex cerebral, hipocampo, tálamo, cerebelo ou medula espinhal, por meio da infecção viral direta ou indiretamente por imunomediados. As sequelas neurológicas e funcionais provenientes das infecções por esses flavivírus são consideráveis, mesmo após a convalescência (Cain et al. 2019; Maximova & Pletnev 2018).

Além de seu amplo tropismo por diversas células e tecidos, o ZIKV também é teratogênico, devido a sua capacidade de infectar células da placenta e prejudicar o desenvolvimento embrionário e placentário (Martines et al. 2016; Miner et al. 2016; Miner & Diamond 2017). O tropismo pela placenta pode não ser exclusivo do ZIKV, uma vez que a inoculação de explantes placentários humanos ou camundongos grávidas com WNV ou POWV também resultou em infecção e lesão da placenta (Platt et al., 2018).

Estudos filogenéticos e estruturais sugerem que o ZIKV adquiriu mudanças em determinados motifs do envelope que podem ter influenciado na sua patogenia. Os polimorfismos encontrados nos isolados da linhagem asiática de ZIKV em torno dos padrões de glicosilacão da proteína do envelope, relacionados a infectividade e montagem, foram considerados determinantes comparado a outros flavivírus. Essas alterações estão associadas ao sítio de ligação do vírus às células hospedeiras, tropismo celular e patogenicidade, podendo assim correlacionar os efeitos como Síndrome de Guillain-Barré e microcefalia (Faye et al., 2014; Sirohi et al., 2016).

2.1.4 Resposta imune aos flavivírus

Ao realizar o repasto sanguíneo, os mosquitos transmissores inoculam os flavivírus. Estes vírus, por sua vez, são reconhecidos e entram nas células dos hospedeiros por meio de receptores de superfície celular de células da pele. Em resposta a esse estímulo, surge a primeira linha de defesa do hospedeiro contra infecções virais, denominado sistema imune inato. Os mecanismos rápidos de defesa incluem interferons do tipo I (IFNs-I), citocinas pró-inflamatórias, sistema complemento, atividade das células NK e apoptose. Os IFNs-I (IFNs α/β) são considerados importantes imunoreguladores, pois induzem células a expressarem genes internos com potencial antiviral, chamados de genes estimulados por interferon (ISGs) (Randall & Goodbourn 2008; Ye et al. 2013).

Ao passo que o patógeno ativa a imunidade inata, a resposta imune adaptativa pode também ser desencadeada. O sistema adaptativo consiste na resposta humoral, caracterizada pela produção de anticorpos por células B, e celular, destacada pelas atividades realizadas por células T, CD4+ e CD8+. A imunidade adaptativa é fundamental para limitar a disseminação e controlar a infecção por flavivírus. Estudos têm mostrado que as características particulares e compartilhadas entre os flavivírus são determinantes na patogênese da doença e no resultado da infecção por flavivírus (Ye et al., 2013), incluindo os meios de evasão imunológica utilizados por esses vírus.

Os flavivírus empregam diversas estratégias para evadir da imunidade do hospedeiro, como inibição e/ou atenuação dos IFNs, bloqueio das funções antivirais do sistema complemento, redução dos efeitos citotóxicos das células NK e escape das respostas de células T e das ações de anticorpos neutralizantes por variações antigênicas e maturação proteica parcial. Esses mecanismos de evasão são compartilhados em virtude da alta similaridade da poliproteína dos flavivírus, principalmente nas proteínas não-estruturais NS3 e NS5. Contudo, existem meios de escape importantes de alguns flavivírus que utilizam a proteína NS1 e o envelope para modular a resposta do hospedeiro contra a infecção viral (Ye et al., 2013).

Ainda que os flavivírus tenham essa capacidade de fugir do sistema imune do hospedeiro, a imunidade celular desempenha papéis importantes no combate à invasão viral. As respostas por células T, especialmente linfócitos TCD8+ ou T citotóxicas (LTC's), contribuem na proteção e imunopatologia contra flavivírus. Estudos vêm demonstrando o papel protetivo de LTC's em infecções primárias por DENV, WNV e ZIKV, bem como observaram um mecanismo de suporte em situações de insuficiência de anticorpos neutralizantes (nAcs) em animais adultos imunocompetentes selvagens (Brien et al. 2007; Ngono et al. 2017; Winkler et al. 2017; Yauch et al. 2009).

As respostas de células T heterólogas e de memória também podem ter funções protetoras. Células TCD8+ imunes a DENV podem limitar a infecção e o desenvolvimento da doença por ZIKV devido a uma reatividade cruzada, inclusive na gravidez. Reciprocamente, as células TCD8+ primadas por ZIKV podem proteger contra a infecção por DENV em camundongos (H. Huang et al., 2017; Regla-Nava et al., 2018; Wen et al., 2017). Dados recentes sugeriram atividade antiviral precoce de LTC's em infeções por ZIKV quando compararam grupos de camundongos deficientes

de TCD8+ ou de IFN-I e observaram paralisia dos membros apenas no último grupo, sugerindo que essa condição está relacionada a sinalização imune (Nazerai et al., 2018; Nazerai, Christensen, & Thomsen, 2019).

As células TCD4, por sua vez, podem proteger contra a infecção por flavivírus ativando para as respostas de anticorpos, mantendo as ações das LTC's que permitem a eliminação viral, produzindo citocinas antivirais e lisando células infectadas (Kumar et al., 2004). Um estudo mostrou a atividade citolítica *ex vivo* de células TCD4 específicas para DENV e sugeriu que o controle da infecção por DENV em humanos pode ser mediado por essas células (Weiskopf et al., 2015). Foram consideradas essenciais para a proteção de infecções primárias por WNV, YFV, JEV e ZIKV em modelo murino, esse último em camundongos imunocompetentes e imunocomprometidos (Aberle et al. 2018; Hassert et al. 2018; Lucas et al. 2018).

Células TCD4 de memória também mostraram ter efeito protetor quando há imunização com epítopos de células antes da infecção de DENV e resultaram na diminuição da carga viral após o desafio com DENV homólogo em camundongos (Yauch et al., 2010). Células T de memória induzidas por exposição prévia ao DENV reconheceram peptídeos derivados de ZIKV e influenciaram a magnitude e a qualidade da resposta de células T contra ZIKV em humanos (Grifoni et al., 2017).

2.1.4.1 Papel da imunidade humoral contra infecções por flavivírus

A resposta humoral por meio da produção de anticorpos antivirais também possui um papel fundamental bem-estabelecido na proteção contra infecções por flavivírus. Os anticorpos neutralizantes podem impedir a invasão em diversos pontos do ciclo celular do vírus, sendo uma das principais o bloqueio do ataque do vírus às células hospedeiras (Slon Campos et al 2018).

Em modelo *in vivo*, foi observado que a transferência passiva de anticorpos policlonais ou monoclonais protegeu contra o desafio letal com WNV, TBEV, DENV, JEV e YFV (Diamond et al. 2003; Heinz et al. 1983; Kaufman et al. 1989; Kimura-Kuroda & Yasui 1988; Schlesinger & Chapman 1995), inclusive foi capaz de salvar camundongos da infecção intracerebral letal por ZIKV (Nazerai et al., 2018, 2019). Adicionalmente, os camundongos deficientes de células B produtoras de anticorpos são mais vulneráveis à infecção flaviviral (Charlier et al., 2002; Diamond et al., 2003;

Halevy et al., 1994).

Os anticorpos anti-flavivírus reconhecem majoritariamente epítopos da proteína do envelope (E) viral. Entretanto, anticorpos contra a proteína NS1 também são essenciais e bastante conhecidos na proteção contra infecções por flavivírus (Falgout et al. 1990; Jacobs et al. 1992; Muller & Young 2013; Reyes-Sandoval & Ludert 2019; Schlesinger et al. 1986). Os mecanismos podem ser mediados por receptor Fcγ (FCγR) e/ou complemento, secretando formas reativas para regiões da NS1 (Chung, Thompson, Fremont, & Diamond, 2007). Durante a infecção por flavivírus, os anticorpos são induzidos predominantemente contra as proteínas E, prM e NS1, tornando-os bons alvos vacinais, enquanto a resposta das células T age especialmente em E, prM, NS3 e NS5 (Culshaw, Mongkolsapaya, & Screaton, 2018; F. X. Heinz & Stiasny, 2012; Stettler et al., 2016; Winkler et al., 2017).

A proteína E de flavivírus é o antígeno dominante dos anticorpos neutralizantes e desempenha um papel importante na indução de respostas imunológicas no hospedeiro infectado. Muitos estudos vêm mostrando a eficiência dos anticorpos contra a proteína E e seus três domínios (DI, DII e DIII) (figura 6) (Zhang et al., 2017).

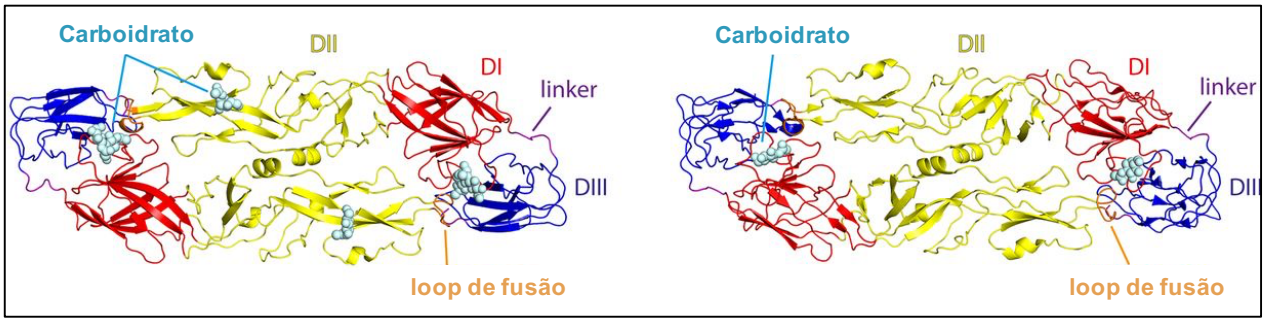


Figura 6. Domínios da proteína do envelope (adaptado de Heinz & Stiasny 2017).

A resposta imune gerada contra E ou um fragmento dessa proteína foram suficientes para prevenir a morte de filhotes e camundongos deficientes em IFN-I em modelos de desafio de ZIKV (D. Espinosa et al., 2018; Tai et al., 2018). Em um modelo murino de pós-exposição, anticorpos protetores foram identificados reconhecendo epítopos em amplos domínios da proteína E (Q. Wang et al., 2016). Complexos antigênicos das proteínas prM e E protegeram camundongos contra o desafio viral, resultando na ausência de detecção de viremia mediante transferência adotiva de anticorpos induzidos pelos antígenos (Larocca et al., 2016; Nazerai et al., 2019;

Richner et al., 2017; Tebas et al., 2017), e também exibiram papel protetor em macacos *rhesus* (Abbink et al., 2016). Uma outra combinação das proteínas NS1 e prM/E aumentou o efeito protetivo (X. Liu et al., 2018).

Dentro as regiões do envelope viral, o domínio III demonstrou ser uma proteína altamente antigênica e importante ferramenta diagnóstica para diferenciar espécies de *Flavivirus* (Libraty et al. 2015; Mathengtheng & Burt 2014). Além disso, é o principal alvo de anticorpos neutralizantes (Zhang et al., 2017). Em estudos químicos estruturais, foram identificados epítopos do domínio III com potencial para indução de anticorpo com função neutralizante (K. Wu et al., 2003). Essa região interagiu com receptores de células humanas e de mosquito e induziu produção de anticorpos que neutralizaram completamente sorotipos homólogos de DENV-1 e -2 e parcialmente sorotipos heterólogos (Chin et al. 2007). Anticorpos anti-E-DIII foram capazes de inibir infecções por WNV e JEV (Chu et al. 2005; Chu et al. 2007; Chua et al. 2013) e também de reduzir sintomatologia e a mortalidade por ZIKV em camundongos infectados (Robbiani et al., 2017; Zhao et al., 2016).

Em relação aos outros dois domínios, anticorpos monoclonais contra E-DI/II de WNV protegeram contra a infecção por WNV em camundongos, embora com menor eficiência neutralizante comparada aos anticorpos específicos contra o DIII (Oliphant et al., 2006). Esses dois domínios também se apresentaram reagindo cruzadamente e pouco neutralizantes, agravando a infecção por ZIKV e DENV com suposta influência de ADE (expressão em inglês "*antibody-dependent enhancement*") (Stettler et al., 2016). Os anticorpos direcionados às proteínas E de DENV e/ou E do WNV puderam se ligar e reagir de forma cruzada com as regiões E-DI/II de ZIKV, mas não neutralizaram o ZIKV (Dejnirattisai et al., 2016; Paul et al., 2016).

É importante compreender a reação cruzada de anticorpos contra flavivírus, uma vez que são vírus estritamente relacionados. Em regiões tropicais, existe a co-circulação de vários arbovírus. Dessa forma, é fundamental saber as consequências benéficas e prejudiciais de uma exposição prévia e as implicações na imunopatogênese de infecções subsequentes. Existe uma reatividade cruzada entre DENV e ZIKV em virtude da relação próxima entre as proteínas do envelope desses vírus (Barba-spaeth et al., 2016; Culshaw et al., 2018). Além disso, foi evidenciado anticorpos para epítopos da proteína E que reagiram cruzadamente com DENV e ZIKV (Barba-spaeth et al., 2016; Dejnirattisai et al., 2016; Swanstrom et al., 2016).

2.1.5 Fenômeno ADE e reação cruzada entre flavivírus

A imunidade humoral induzida por flavivírus também está associada ao desenvolvimento de manifestações graves da doença. A atividade de anticorpos de reação cruzada pode potencializar uma doença após infecções anteriores por vírus heterólogos. Este fenômeno amplamente conhecido e característico de determinados flavivírus é denominado de agravamento dependente de anticorpos (ADE) (Pierson & Diamond 2020; Slon Campos et al. 2018; Ye et al. 2013).

Isso ocorre frequentemente com DENV, pois mais de um sorotipo co-circula em muitas regiões. Há evidências epidemiológicas de que a infecção primária protege da reinfecção com o mesmo sorotipo. Entretanto, representa um fator de risco para o desenvolvimento de doença grave com prognóstico ruim após reinfecção com outro sorotipo, desencadeando alta carga viral e produção de citocinas (Halstead, 2014).

Inúmeros estudos identificaram anticorpos que são reativos de forma cruzada entre ZIKV e DENV. Foi demonstrado que uma imunidade pré-existente para DENV é capaz de aumentar a infecção de ZIKV em modelos *in vitro* e *in vivo* (Dejnirattisai et al., 2016; Masel et al., 2019; Paul et al., 2016; Priyamvada et al., 2016; Priyamvada, Hudson, Ahmed, & Wrammert, 2017). Um estudo em camundongo relatou transferência passiva de soro convalescente de DENV, aumentando a patogênese do ZIKV e sugerindo um papel de ADE prejudicial (Bardina et al., 2017).

Recentemente, foram descritos desfechos gestacionais severos após infecção por ZIKV *in vivo* em áreas endêmicas para DENV. A patogênese fetal mediada por ZIKV foi potencializada na presença de anticorpos reativos a DENV, aumentando a replicação viral e agravando a lesão na placenta, a interrupção no crescimento do feto e a reabsorção fetal (Brown et al., 2019).

Em contrapartida, anticorpos com especificidade para DENV não agravaram a doença por ZIKV em macacos *rhesus* (Pantoja et al., 2017). Outros trabalhos também reportaram que anticorpos contra DENV foram capazes de neutralizar a infecção por ZIKV *in vitro* (Barba-spaeth et al., 2016; Stettler et al., 2016) ou proteger camundongos em modelos de desafio e de gravidez (Robbiani et al., 2017; Sapparapu et al., 2016; Swanstrom et al., 2016).

Um estudo sintetizou a relação do ADE entre DENV e ZIKV, propondo uma redefinição desse evento com o intuito de oferecer perspectivas clínicas mais objetivas

após resultados de modelos experimentais *in vitro* e *in vivo*. ADE agrava as manifestações clínicas em uma infecção subsequente por DENV heterólogo em modelos *in vitro*, murinos, primatas não-humanos e humanos. Por outro lado, o fenômeno ADE de ZIKV após prévia exposição a DENV pode ocorrer *in vitro* e em camundongos imunocomprometidos, mas ainda sem evidências em primatas não-humanos ou em humanos (Sariol, Nogueira & Vasilakis 2018).

Outro resumo destacou que em camundongos imunes à DENV com infecção por ZIKV subsequente houve alta carga viral e produção de citocinas, mas sem mortalidade, enquanto em primatas não-humanos não houve quaisquer alterações clínicas. Entretanto, em animais infectados primariamente por ZIKV e, em seguida, por DENV houve carga viral e citocinas aumentadas, com óbito somente em camundongos (Langerak et al., 2019)

O mecanismo do ADE é um fenômeno importante a se considerar na patogênese de doença infecciosas, no entanto são necessários mais estudos sobre os mecanismos de ação de ADE durante um processo natural de infecção. Uma outra questão levantada é o tropismo celular de cada vírus e suas particularidades na replicação genômica. Uma vez que o ZIKV se replica em células diferentes daquelas preferenciais para DENV (Miner & Diamond 2017), existe a possibilidade de anticorpos específicos para um vírus não terem um impacto determinante da patogênese do outro (Culshaw et al., 2018).

Existe também a hipótese de que os sorotipos do DENV podem induzir diferentes respostas humorais, ou benéfica ou nociva, interferindo diretamente na reação cruzada entre ZIKV e DENV. Além disso, pouco se sabe sobre a influência da quantidade de exposições anteriores a DENV e o intervalo de tempo entre essas infecções (Culshaw et al., 2018). Uma única infecção prévia por DENV pode agravar a patogênese por ZIKV, enquanto a partir de duas exposições pode ocorrer uma resposta protetiva, como acontece em infecções consecutivas por DENV (Anderson et al., 2014).

A reação cruzada entre DENV e ZIKV têm mostrado implicações importantes na imunidade natural e em futuras vacinações. Alguns padrões de neutralização cruzada de anticorpos sugerem que o ZIKV parece estar relacionado antigenicamente ao DENV. Os níveis de anticorpos neutralizantes ao longo do tempo podem distinguir entre infecções por ZIKV e/ou por DENV, quando são analisados simultaneamente

(Montoya et al., 2018). Em análises espaciais, foi observado que títulos de anticorpos prévios contra DENV foram associados à redução do risco de infecção e sintomas do ZIKV, e que a imunidade atual do ZIKV pode influenciar no risco de transmissão futura (Rodriguez-Barraquer et al., 2019).

Em adultos saudáveis de uma região endêmica para DENV, anticorpos contra DENV neutralizaram cruzadamente ZIKV e sorotipos heterológos de DENV, e anticorpos anti-ZIKV protegeram parcialmente contra ao menos um sorotipo de DENV. Adicionalmente, indivíduos com exposição repetida a DENV exibiram maiores títulos de anticorpos neutralizantes contra E e NS1 de ZIKV comparados àqueles com uma única exposição a DENV (Hattakam et al., 2021). Além disso, a vacinação contra o ZIKV em indivíduos expostos a DENV pode ampliar a imunidade flaviviral pré-existente, protegendo contra ZIKV e DENV. Esse resultado destaca a vantagem da vacinação do ZIKV em áreas endêmicas para flavivírus (Dussupt et al., 2020).

Contrariamente, houve casos em que anticorpos anti-ZIKV ou anti-DENV neutralizaram, mas também intensificaram de forma cruzada a infecção por ROCV, SLEV, WNV, ILHV e ZIKV mediado por ADE em células que expressam receptores Fc-γ (Montecillo-Aguado et al., 2019; R. A. S. Oliveira et al., 2019). Também pode ocorrer uma baixa proteção cruzada para ZIKV entre soropositivos para DENV (Langerak et al., 2019). Além disso, a maioria das infecções por DENV não induz anticorpos duráveis e suficientes para neutralização cruzada de ZIKV. Os anticorpos específicos são produzidos pós-infecção por ZIKV, independentemente da exposição anterior ao DENV (Collins et al., 2017).

No contexto de imunidade celular, células T de DENV com reatividade cruzada são capazes de mediar a proteção contra proteínas não-estruturais de ZIKV. Isso é de grande relevância e sugere uma possibilidade de incluir antígenos de células T em possíveis vacinas contra o ZIKV (Subramaniam et al., 2020).

Essas evidências mostraram um cenário abrangente da resposta humoral aos flavivírus. No Amazonas, há a vacinação obrigatória de YFV, a múltipla exposição a DENV, a co-circulação dos quatro sorotipos de DENV e outros flavivírus com potencial epidêmico e emergente como WNV, JEV e ZIKV. Por isso, é de extrema relevância estudos epidemiológicos para flavivírus na região. É importante avaliar a dinâmica de circulação dos vírus e o conhecimento geral da resposta de anticorpos para futuros esquemas de vacinação e estratégias de controle e prevenção de doenças.

2.2 SARS-CoV-2

2.2.1 Histórico e Epidemiologia do SARS-CoV-2

Os coronavírus (CoV), descobertos em meados da década de 60, eram conhecidos como um grupo de vírus que geralmente provocava infecções respiratórias e intestinais em aves e mamíferos, e doenças respiratórias brandas como resfriados comuns em humanos, exibindo quadros mais graves somente em indivíduos imunocomprometidos (Cheng, Lau, Woo, & Kwok, 2007).

No final de 2002, um potencial mais patogênico surgiu quando um coronavírus causou surtos de uma síndrome respiratória aguda grave (SARS) em uma província na China e imediatamente se propagou para regiões vizinhas. A doença se manifestava inicialmente com febre alta e sintomas respiratórios leves, mas progredia em poucos dias para pneumonia. O agente infeccioso foi identificado em 2003 e chamado de coronavírus associado a SARS (SARS-CoV). Essa epidemia resultou em mais de 8000 casos entre 2002-2003 com uma taxa de mortalidade de 10% (Parry, 2003; Zhong et al., 2003). Acredita-se que a infecção humana pode ter ocorrido por contato com animais como civetas, cachorro-guaxinim e texugos-furões, descritos como hospedeiros intermediários, e morcegos como os reservatórios do vírus (Y. Guan et al., 2003; W. Li et al., 2005).

Em 2012, uma década depois da epidemia de SARS-CoV, um segundo coronavírus foi identificado provocando graves problemas respiratórios em regiões do Oriente Médio, ficando conhecido como episódios de Síndrome Respiratória do Oriente Médio (MERS) (Chan, Lau & Woo 2013). Esse vírus associado a MERS (MERS-CoV) foi reportado pela primeira vez em junho de 2012 na Árabia Saudita causando desconforto respiratório agudo em um paciente idoso que sucumbiu com falência múltipla de órgãos (Zaki, van Boheemen, Bestebroer, Osterhaus, & Fouchier, 2012). Desde 2012, cerca de 30 países relataram casos de MERS-CoV. A OMS estima que 35% dos pacientes confirmados em laboratório com MERS-CoV vão à óbito, embora possa ser uma taxa superestimada devido a casos leves subnotificados (WHO, 2019). Aparentemente, MERS-CoV é um vírus zoonótico transmitido primariamente por camelos e seus fluídos contaminados (Corman et al., 2016).

Recentemente, em dezembro de 2019, casos de pneumonia viral de causa desconhecida foram reportados em Wuhan, na China. Alguns dias depois, as autoridades chinesas identificaram que o agente infeccioso responsável pelos novos surtos era um novo coronavírus do gênero *Betacoronavirus*. Inicialmente este vírus foi nomeado como o novo coronavírus de 2019 (2019-nCoV) (Gralinski & Menachery, 2020; Zhu et al., 2020). Após análises filogenéticas, o nome passou a ser SARS-CoV-2, pois o novo vírus era geneticamente relacionado ao coronavírus responsável pelo surto de SARS em 2003. No mesmo dia, a OMS decretou que a doença causada pelo SARS-CoV-2 seria nomeada como doença do coronavírus de 2019 (COVID-19) (WHO, 2020b).

A preocupação com os níveis alarmantes da ligeira propagação do vírus e a gravidade da doença e dos desfechos, levou a OMS a caracterizar a COVID-19 como uma pandemia no dia 11 de março de 2020 (WHO, 2020a). Atualmente circulam variantes de SARS-CoV-2 classificadas como variantes de interesse (VOI) ou variantes de preocupação (VOC). As principais de grande importância clínica são as VOC, devido as suas capacidades em aumentar a transmissibilidade ou minimizar a eficácia de anticorpos ou reduzir a neutralização do vírus, tais como B.1.1.7 (Alpha), B.1.351 (Beta), B.1.617.2 (Delta) e P.1 (Gamma).

O mundo possui atualmente cerca de 185 milhões de casos (figura 7), com mais de 4 milhões de óbitos, sendo as Américas mais afetadas, responsáveis por 40% dos casos e 48% das mortes. O Brasil teve seu primeiro caso de COVID-19 no dia 26 de fevereiro de 2020. Até o momento, é o terceiro o país com mais casos (aproximadamente 19 milhões) e com mais de 530 mil vítimas fatais, ocupando o segundo lugar em números absoluto de óbitos por COVID-19 no mundo. Adicionalmente, é um dos países com as maiores taxas de incidência, 8.900 casos cumulativos por 100 mil habitantes (figura 8) (WHO, 2021b).

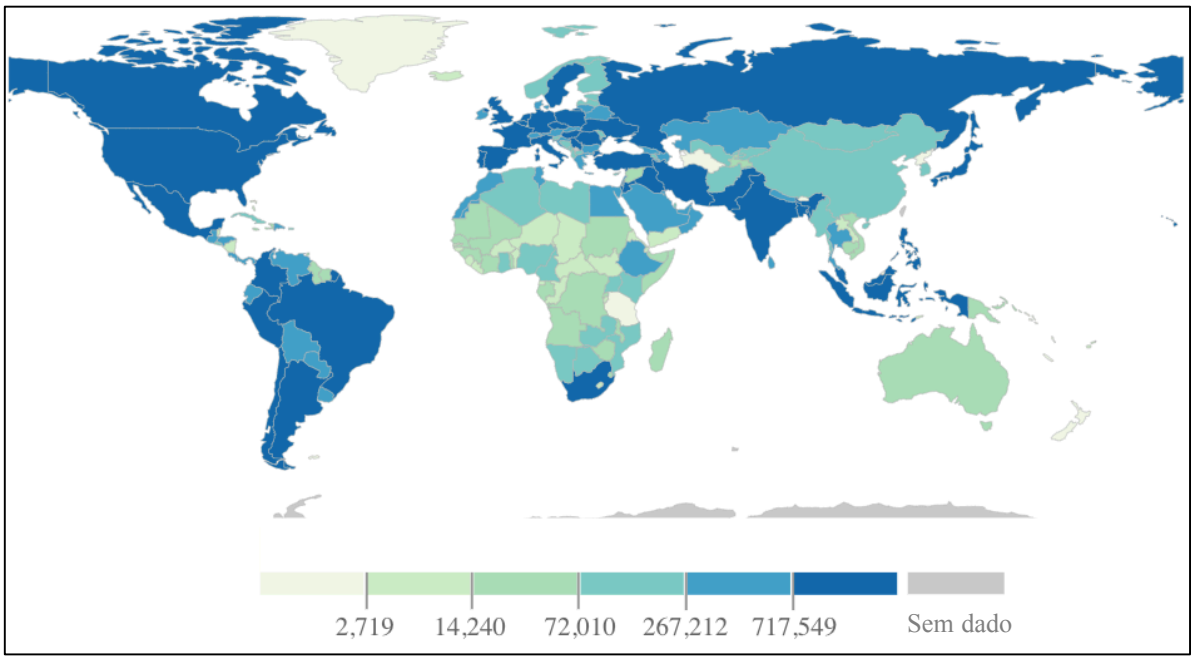


Figura 7. Figura. Casos cumulativos globais de COVID-19 reportados. Fonte: OMS, ilustrado em "COVID *Data Tracker*" pelo Centro de Controle e Prevenção de Doenças (CDC). Disponível em: <https://covid.cdc.gov/covid-data-tracker/#global-counts-rates>. Dados atualizados até 13 de julho de 2021, sujeitos à alteração.

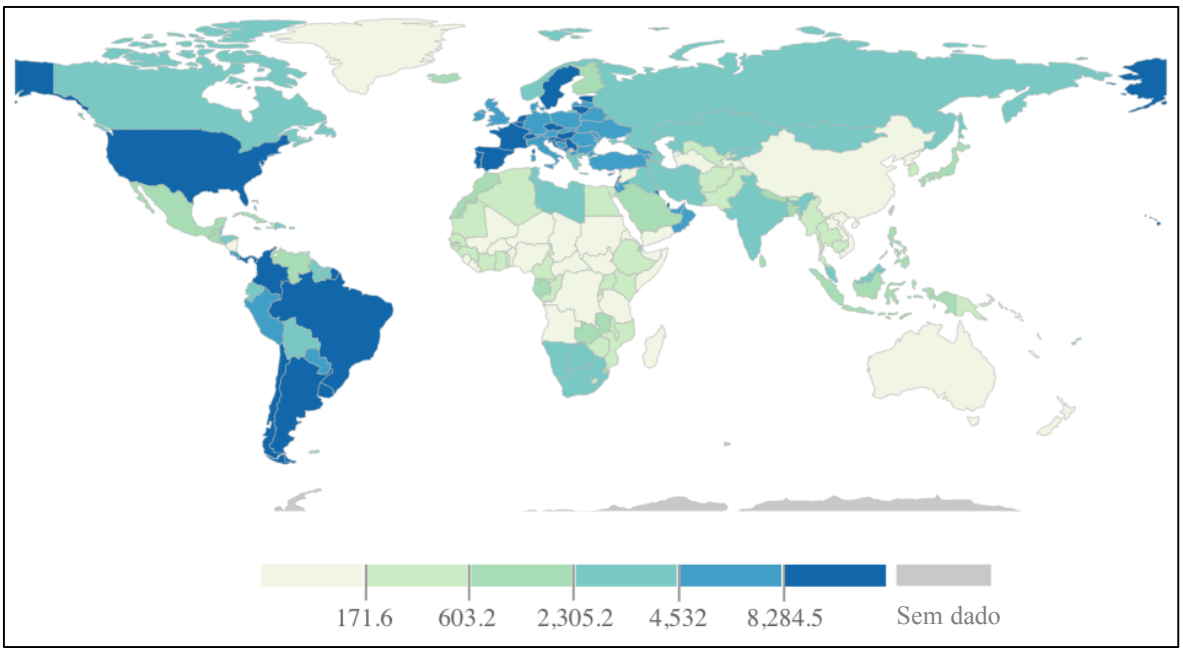


Figura 8. Casos cumulativos globais de COVID-19 reportados por 100.000 habitantes. Fonte: OMS, ilustrado em "COVID *Data Tracker*" pelo Centro de Controle e Prevenção de Doenças (CDC). Disponível em: <https://covid.cdc.gov/covid-data-tracker/#global-counts-rates>. Dados atualizados até 13 de julho de 2021, sujeitos à alteração.

O primeiro caso de COVID-19 no estado do Amazonas foi registrado dia 13 de março de 2020. Atualmente, acumula mais de 400 mil casos, com a incidência de 9.839 casos/100 mil habitantes (17º ranking nacional) com um incremento na média móvel nos últimos 14 dias de 2,5%, mas ainda com uma taxa dentro da estabilidade. Com mais de 13 mil óbitos até o momento, o estado apresenta uma taxa de letalidade de 3,3%, a terceira maior marca entre as unidades federativas, e também a quarta maior mortalidade pela doença no país, com uma taxa de 323 óbitos/100 mil habitantes. Mas a média móvel nos últimos 14 dias mostrou uma considerável redução com uma variação de -20%(FVS/MS, 2021; MS, 2021).

O Amazonas sofreu dois momentos de calamidade em saúde pública durante essa pandemia. O primeiro pico ocorreu entre abril e junho de 2020 e o segundo e pior pico entre janeiro e fevereiro de 2021. Na segunda onda epidêmica, caracterizada por casos mais críticos, rápidos e letais, além de toda a problemática do não-cumprimento das medidas preventivas e restritivas, houve o surgimento e a persistência de uma nova variante denominada P.1, identificada em dezembro de 2020 e associada a alta transmissibilidade e a alta carga viral (Naveca et al., 2021). No segundo evento, o estado chegou a ter 225 mortes em um único dia (figura 9), enquanto o Brasil, meses depois, estaria chegando a pior marca com de mais 4 mil óbitos em 24 horas (FVS/MS, 2021; MS, 2021).

A capital, Manaus, teve um cenário que acompanhou as condições do estado. Acumula cerca de 190 mil casos, com mais de 9 mil óbitos e a maior taxa de letalidade do estado, 4,8%. A taxa de incidência é de 217 casos/100 mil habitantes, com diferença de taxas entre os bairros, sendo Centro e Distrito Industrial I com as maiores incidências, 592,5 e 543,6 casos/100 mil habitantes, respectivamente (figura 10) (FVS/MS, 2021).

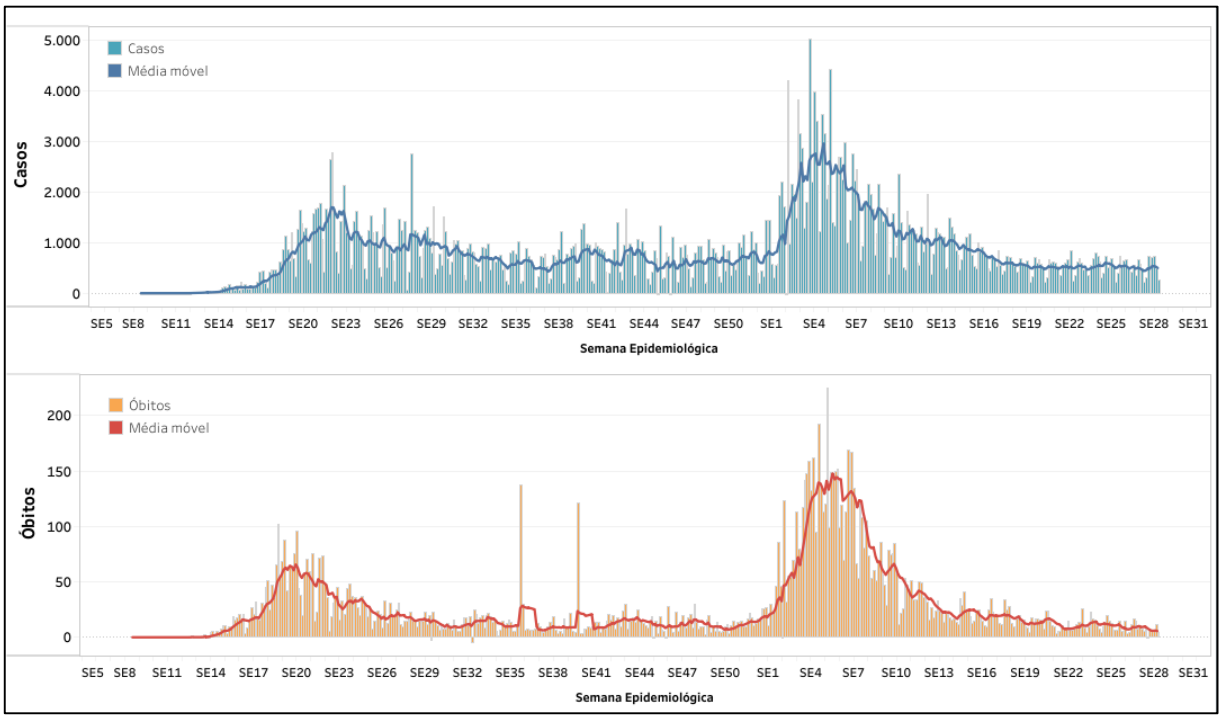


Figura 9. Casos e óbitos de COVID-19, e suas respectivas médias móveis, por semana epidemiológica, no estado do Amazonas, desde o início da pandemia. Fonte: Fundação de Vigilância em Saúde do estado do Amazonas (FVS/AM), Governo do Estado do Amazonas – Painel de monitoramento da COVID-19. Dados atualizados até 12 de julho de 2021, sujeitos à alteração.

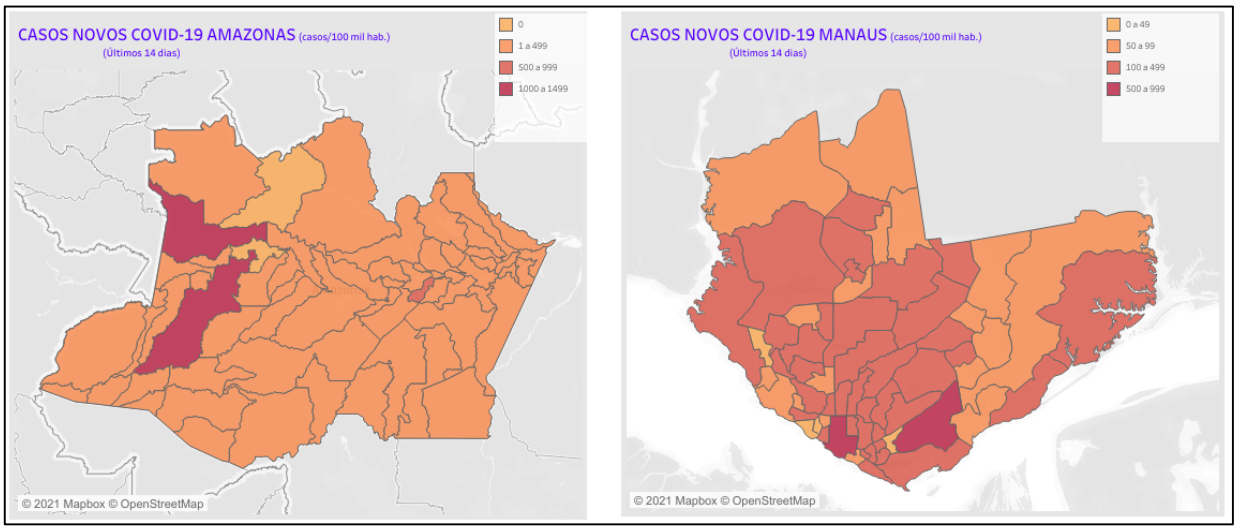


Figura 10. Incidência de COVID-19 no Amazonas (por município) e em Manaus (por bairro) nos últimos 14 dias. Fonte: Fundação de Vigilância em Saúde do estado do Amazonas (FVS/AM), Governo do Estado do Amazonas – Painel COVID-19 no Amazonas. Dados atualizados até 12 de julho de 2021, sujeitos à alteração.

2.2.2 Classificação e biologia do SARS-CoV-2

Os coronavírus (CoV) são um grupo complexo de vírus da subfamília *Orthocoronavirinae*, na família *Coronaviridae*, da ordem *Nidovirales*. Esta subfamília é composta de quatro gêneros: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus* e *Deltacoronavirus*. Desses, sete espécies de coronavírus são patogênicos para humanos: HCoV-229E e HCoV-NL63 (gênero *Alphacoronavirus*), e HCoV-HKU1, HCoV-OC43, MERS-CoV, SARS-CoV SARS-CoV-2 (gênero *Betacoronavirus*) (B. Chen et al., 2020; Y. Lim, Ng, Tam, & Liu, 2016).

Embora sejam fenotipica e genotipicamente diversos, as organizações do genoma são semelhantes para todos os CoVs. São vírus de RNA de fita simples de sentido positivo não-segmentado que variam entre 27 a 32 kb, contendo 14 ORFs (figura 11b). As ORFs 1a e 1b, localizadas na extremidade 5', codificam as poliproteínas pp1a e pp1ab, que são clivadas por proteases virais em 16 proteínas não estruturais (NSPs) (denominadas NSP1-NSP16) que auxiliam no processo de replicação viral (Fehr & Perlman, 2015; Y. T. Wang et al., 2020).

As ORFs na extremidade 3' codificam as proteínas estruturais spike (S), envelope (E), membrana (M) e também o nucleocapsídeo (N). Na maioria dos coronavírus, S é clivado por uma protease semelhante à furina da célula hospedeira em dois domínios, S1 e S2. A subunidade S1 constitui o grande domínio de ligação ao receptor (RBD), enquanto S2 forma o talo da molécula. Existe também proteínas acessórias (3a, 3b, 6, 7a, 7b, 8, 9b, 9c e 10) que demonstram exibir papel importante na patogênese viral, regulando o processo infeccioso (Fehr & Perlman, 2015; Satarker & Nampoothiri, 2020).

A estrutura viral dos CoVs é uma partícula envelopada e esférica com 60 a 140 nm de diâmetro e *spikes* de 9-12 nm, os quais são considerados os fatores mais proeminentes dos coronavírus, pois o formato de coroa sugeriu a origem do nome desses vírus (figura 11a) (Fehr & Perlman, 2015; Neuman et al., 2006).

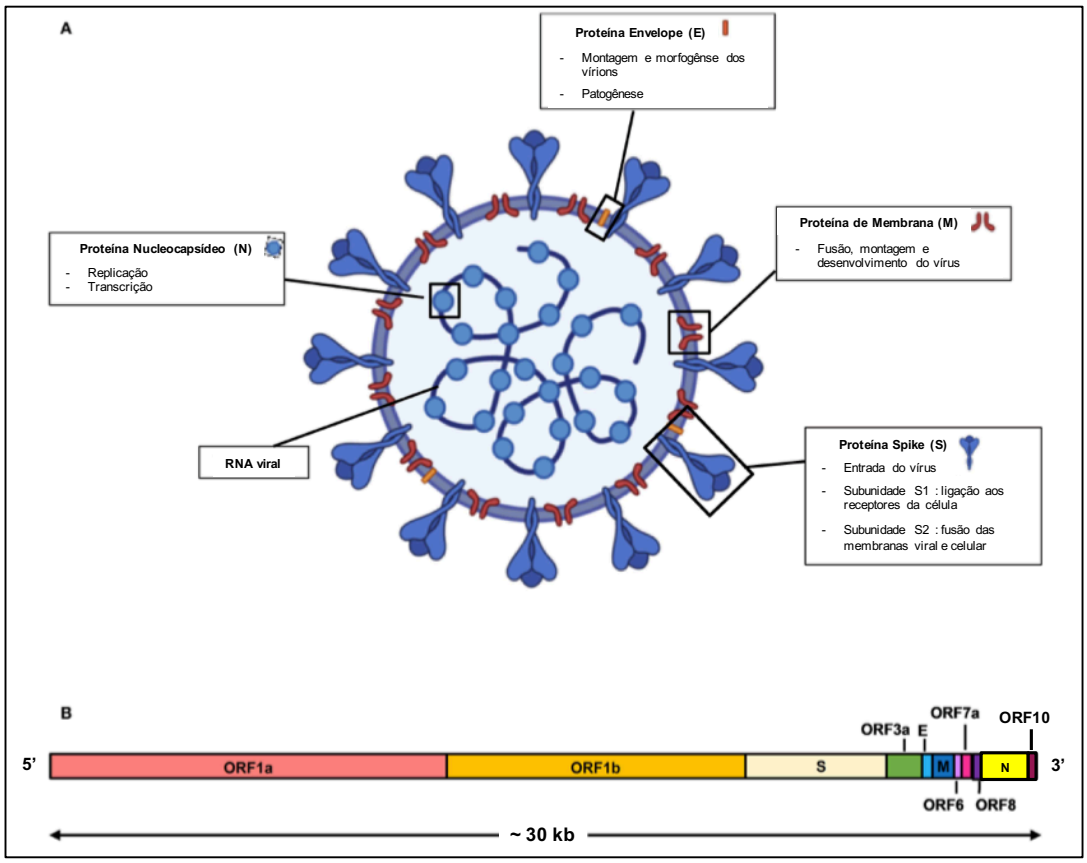


Figura 11. Representação esquemática da estrutura viral e organização do genoma dos coronavírus. **A.** As proteínas estruturais spike (S), envelope (E) e membrana (M) formam a partícula envelopada esférica e o RNA viral coberto pelo nucleocapsídeo (N). As funções básicas das proteínas estão mencionadas. **B.** A organização do genoma mostra na extremidade 5' as ORFs 1a/1b, e na extremidade 3' as ORFs que codificam as proteínas estruturais e acessórias. (adaptado de Lee et al. 2020).

A infecção por coronavírus (figura 12) inicia com a interação do domínio RBD da subunidade S1 da proteína S com a enzima conversora de angiotensina 2 (ACE2) como receptor cognato na superfície da célula hospedeira. Essa ligação, permite a entrada do vírus na célula por meio de endocitose. A exposição ao baixo pH no endossomo ativa proteases do hospedeiro, como a TMPRSS2, que clivam a proteína S, permitindo a fusão da membrana plasmática viral e celular. Então o genoma do RNA é liberado no citosol, onde é traduzido nas proteínas replicase (ORF1a/b). As poliproteínas pp1a e pp1ab são processadas pelo hospedeiro e proteases virais para gerar as proteínas não estruturais (NSPs), que formarão o complexo replicase. A replicação do RNA viral começa em vesículas de membrana dupla induzidas por vírus (DMVs) derivadas do retículo endoplasmático (RE), que protegem os genes virais (Den Boon & Ahlquist, 2010). O RNA viral genômico é replicado e o RNA subgenômico

é transcrito e traduzido para formar as proteínas estruturais. A proteína N interage com o RNA genômico para formar o complexo ribonucleoproteico e, junto com as outras proteínas estruturais (S, M e E) e acessórias, são recrutadas no compartimento intermediário RE-Golgi (ERGIC) para a montagem do vírion. Os genomas de RNA subsequentes são incorporados aos vírions sintetizados, e as novas partículas virais são transportadas para a superfície celular em vesículas e liberadas por exocitose (Harrison, Lin & Wang 2020; Lim et al. 2016).

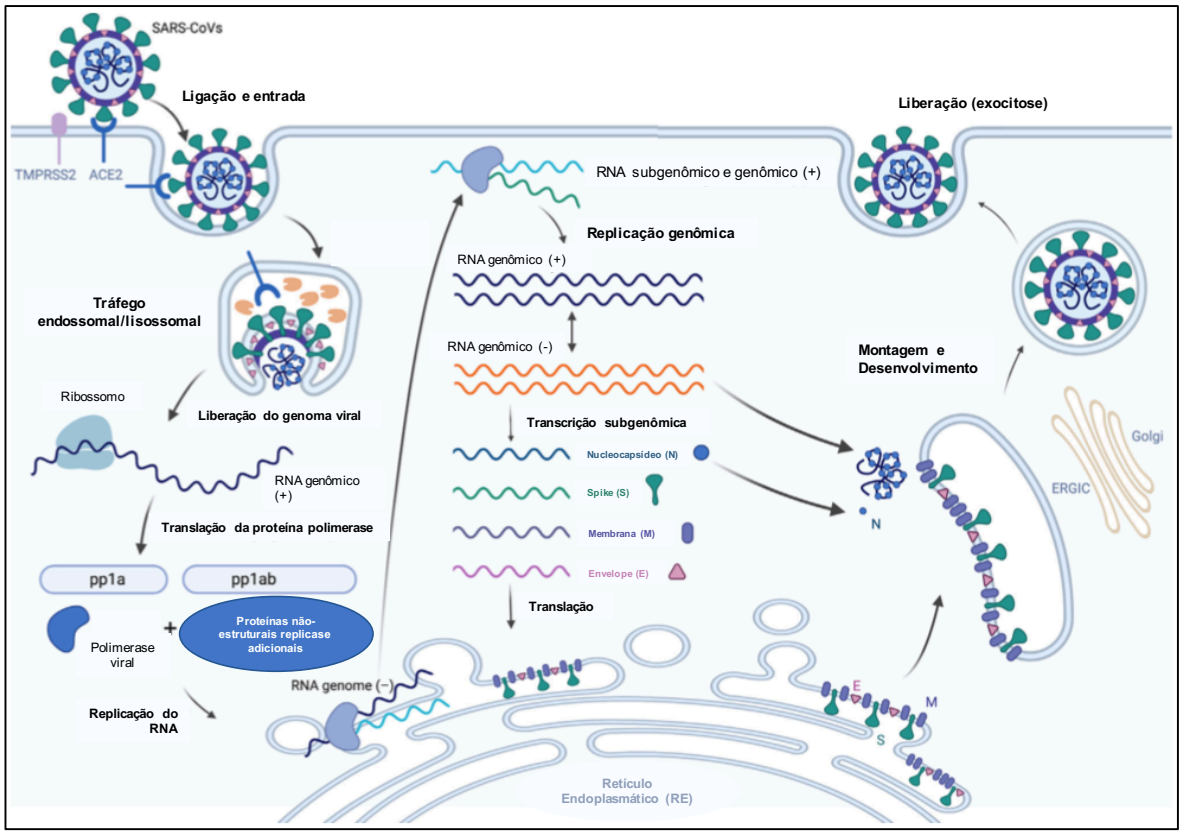


Figura 12. Representação esquemática do ciclo celular dos coronavírus. (adaptado de Harrison et al. 2020).

Ambos os SARS-CoVs utilizam o receptor de superfície ACE2 como porta de entrada na célula. Mas estudos mostraram mutações importantes no domínio RBD ou região C-terminal da proteína S de SARS-CoV-2 que elevaram a afinidade com ACE2, indicando maior força de ligação, consequentemente maior infectividade de SARS-CoV-2, comparada ao SARS-CoV (Lan et al., 2020; Walls et al., 2020; Q. Wang et al., 2020; Wrapp et al., 2020; Yan et al., 2020).

2.2.3 COVID-19

A COVID-19 é uma doença infecciosa contagiosa causada pelo novo coronavírus, que possui um grande potencial pandêmico. O novo coronavírus é transmitido no contato pessoa-pessoa por meio de gotículas respiratórias, aerossóis e indiretamente por superfícies contaminadas pelo vírus (J. F. W. Chan et al., 2020; Q. Li et al., 2020; Y. Liu et al., 2020).

Existem estudos promissores em andamento a respeito da potencial rota fecal-oral, pois foram isolados RNA do SARS-CoV-2 em amostras fecais de indivíduos infectados (Y. Wu et al., 2020; F. Xiao et al., 2020; Xu et al., 2020). Além disso, foram relatadas evidências de transmissão vertical transplacentária de SARS-CoV-2 de mães infectadas para recém-nascidos (H. Chen et al., 2020; Vivanti et al., 2020)

O vírus é introduzido no organismo ao inalar aerossóis ou levar as mãos contaminadas para regiões das vias aéreas, como boca, nariz e olhos (C. Lu, Liu, & Jia, 2020; Mukhra, Krishan, & Kanchan, 2020). Ao infectar o hospedeiro, o primeiro alvo de entrada do SARS-CoV-2 é o trato respiratório superior, seguindo para o trato respiratório inferior e depois a mucosa gastrointestinal (Y.-R. Guo et al., 2020; F. Xiao et al., 2020). Uma das características diferenciais de SARS-CoV-2 é a capacidade de infectar eficiente e produtivamente o trato respiratório superior (tecidos nasofaríngeos e orofaríngeos) devido a sua maior afinidade com ACE2, que é altamente expresso em tecidos humanos nasais e orais. Esses achados, conjuntamente, podem explicar a rápida disseminação geográfica de COVID-19 (Harrison et al., 2020).

A COVID-19 causa uma variedade clínica, desde uma infecção sem sintomas e quadros leves no trato respiratório superior a uma pneumonia viral grave, insuficiência respiratória, falência de múltiplos órgãos e morte. Um estudo mostrou que a carga viral entre pacientes assintomáticos e sintomáticos pode ser semelhante e que existe persistência na detecção do RNA viral em assintomáticos e poucos sintomáticos, sugerindo o potencial de transmissão desses indivíduos (Ling et al., 2020; Zou et al., 2020). O período de incubação do SARS-CoV-2 dura em média 8 dias, podendo atingir até 14 dias (Dhouib et al., 2021).

A maioria dos casos de COVID-19 são assintomáticos ou com sintomas leves como febre, tosse, fadiga e mialgia. Pode ocorrer também perda do paladar e do olfato, cefaleia, náuseas, vômitos, dor abdominal e diarreia. Os casos mais graves,

cerca de 20% dos infectados, podem apresentar dor do peito, dispneia, cianose, taquipneia, dificuldade respiratória, hipotensão, além da descompensação das doenças subjacentes morte (figura 13) (Chen et al. 2020; Cespedes & de Souza 2020). A infecção pode evoluir para uma pneumonia leve ou grave, exibindo um infiltrado inflamatório nos pulmões com produção exacerbada de citocinas e quimiocinas pró-inflamatórias, desencadeando uma lesão pulmonar aguda e até fibrose (Channappanavar & Perlman, 2017; Felsenstein, Herbert, McNamara, & Hedrich, 2020).

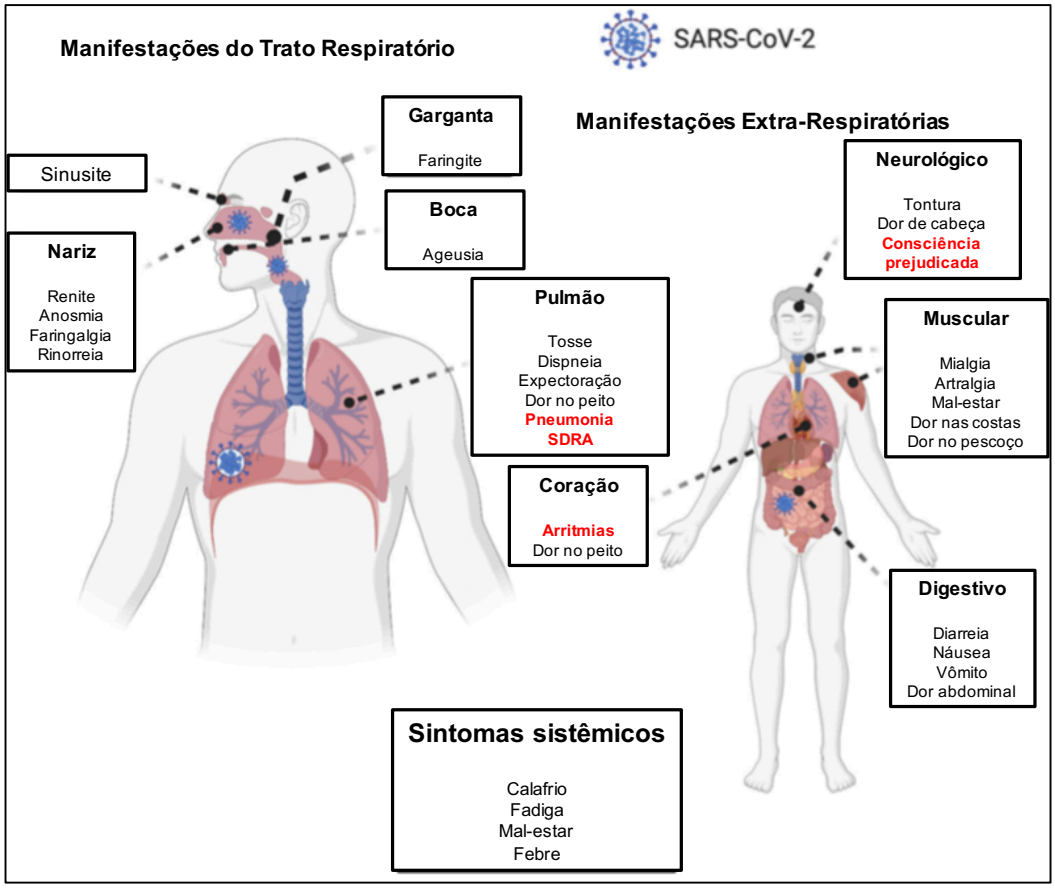


Figura 13. Sintomas clínicos da COVID-19. Sinais e sintomas destacados em vermelho tendem a ser apresentar em casos mais graves. SDRA: síndrome do desconforto respiratório agudo (adaptado de Harrison et al. 2020).

A COVID-19 também afeta sistemas extra-pulmonares (figura 13), comprometendo os sistemas cardíaco, hematológico, neurológico, gastrointestinal, hepatobiliar e renal. Os efeitos causados são multifatoriais, contudo, o estado hiper-inflamatório sistêmico mediado por citocinas inflamatórias e a citotoxicidade viral mediada por expressão de ACE2 nas células e tecidos são determinantes na

patogênese. A doença grave pode acompanhar hipóxia, choque, encefalites, miocardite, síndrome coronária aguda, leucopenia, linfopenia, disfunção coagulativa, disbiose intestinal e lesões hepática e renais agudas (Cascella, Rajnik, Aleem, Dulebohn, & Napoli, 2021). Além disso, pode progredir para uma síndrome de desconforto respiratório (SDRA), possivelmente fatal (W. Guan et al., 2020).

A princípio, existem populações que são mais susceptíveis às infecções graves por SARS-CoV-2 (Huang et al. 2020; Chen et al. 2020), consequentemente, maior índice de internação. A COVID-19 é caracterizada por alta taxa de morbidade e mortalidade para grupos de riscos específicos: pessoas idosas ou com doenças crônicas cardiovasculares ou respiratórios, histórico de diabetes ou uso de hipertensivos (Wang et al. 2020).

A taxa de letalidade aumenta consideravelmente em função da idade dos pacientes, atingindo 30% entre 60 e 80 anos e 36% com mais de 80 anos (Riou, Hauser, Counotte, & Althaus, 2020). Contudo, a COVID-19 grave não é restrita à população idosa, crianças e adultos jovens também estão em risco (Chao et al., 2020; Dong, Mo, HU, & Al, 2020), e casos infantis podem ser fatais (Xiaoxia Lu et al., 2020). Aparentemente, todas as idades são sensíveis à infecção por SARS-CoV-2, mas a idade média da infecção é de 50 anos (Harrison et al., 2020). No entanto, as manifestações clínicas podem diferenciar com a idade. Em geral, estudos mostraram que o sexo masculino com idade avançada (> 60 anos) e apresentando comorbidades têm maior probabilidade de desenvolver doenças respiratórias graves com uma tendência à hospitalização e ao óbito, enquanto a maioria dos jovens e crianças apresentam quadros brandos (não-pneumonia ou pneumonia leve) ou são assintomáticos (W. Guan et al., 2020; Xiaoxia Lu et al., 2020; Z. Wu & McGoogan, 2020).

Dentre os indivíduos infectados, cerca de 25% apresenta uma doença subjacente, no entanto, 60% a 90% das hospitalizações são de pacientes infectados exibindo alguma comorbidade (Garg et al., 2020; PAHO/WHO, 2021b). As comorbidades mais comuns em pacientes hospitalizados incluem hipertensão (48 a 57% dos pacientes), diabetes (17 a 34%), doença cardiovascular (21 a 28%), doença pulmonar crônica (4 a 10%), doença renal crônica (3 a 13%), malignidade (6 a 8%) e doença hepática crônica (<5%) (O. A. Espinosa et al., 2020) Estudos mostraram que as taxas de letalidade dentro dessas condições podem ser elevadas: 75% para

hipertensos, 50% para diabéticos, 40% para cardiopatas, 55% para obesos, 20% para nefropatas e 10% em malignidades (Ejaz et al., 2020). E muitos desses grupos de risco necessitam de medidas extremas como ventilação mecânica invasiva, posição pronada e oxigenação por membrana extracorpórea (Elezkurtaj et al., 2021)

Em um relato de mais de 70 mil casos na China, 81% foram classificados como leves, 14% foram casos graves que necessitaram de ventilação em uma unidade de terapia intensiva (UTI) e 5% foram críticos (casos de insuficiência respiratória, choque séptico e/ou disfunção ou falência de múltiplos órgãos) (Chen et al. 2020; Wu & McGoogan 2020). Em comparação com os pacientes que não necessitaram de terapia intensiva, os pacientes da UTI apresentaram níveis mais elevados de citocinas plasmáticas, o que sugere um processo imunopatológico causado por uma "tempestade de citocinas". Nesta coorte de pacientes, cerca de 2,3% das pessoas morreram em um período médio de 16 dias do início da doença (Chen et al. 2020; Huang et al. 2020; Yang et al. 2020).

Esquemas de manejo clínico-terapêutico foram elaborados pelo Instituto Nacional de Saúde (NIH), utilizando medicamentos sintéticos e produtos de anticorpos monoclonais de uso emergencial, para pacientes hospitalizados e não-hospitalizados, considerando a condição do paciente, a gravidade da doença, bem como indivíduos adultos (NIH, 2021). Atualmente a melhor estratégia de proteção contra a COVID-19 é a vacinação, junto às medidas de prevenção estabelecidas pela OMS, como o distanciamento social, uso de máscaras e higienização constante das mãos e do rosto (Cascella et al., 2021; WHO, 2021a). Essas estratégias são essenciais principalmente em locais, como o Brasil, que ainda apresenta baixas taxas de indivíduos vacinados e existe um descumprimento constante dos protocolos de prevenção no território nacional.

2.2.4 Resposta imune COVID-19

A história natural da COVID-19 possui três fases: a fase de viremia que ocorre até o 7º dia, a fase aguda entre 7º e 10º dia caracterizada pela presença de pneumonia, e a terceira fase que pode variar em função da capacidade do sistema imune de controlar a infecção viral de forma adequada (Lin, Lu, Cao, & Li, 2020). Na COVID-19, uma resposta inflamatória eficiente e equilibrada permite uma evolução

autolimitada e benigna da doença. Quando ocorre uma resposta exacerbada sem controle ao SARS-CoV-2, desencadeada por fatores ainda não completamente conhecidos, as formas graves da doença são desenvolvidas (Mehta et al., 2020; C. Wu et al., 2020; Zhou et al., 2020).

O vírus SARS-CoV-2 é um betacoronavírus cujo genoma está intimamente relacionado a outros vírus zoonóticos altamente patogênicos do mesmo grupo, como SARS-CoV e MERS-CoV, compartilhando 80% e 50% de similaridade na sequência genômica, respectivamente. SARS-CoV-2 exibe maior semelhança com SARS-CoV, por pertencerem ao mesmo sub-gênero *Sarbecovirus* (R. Lu et al., 2020). Estudos prévios realizados com SARS-CoV e MERS-CoV ofereceram muitas informações relevantes em relação a infecção pelo novo coronavírus e a resposta imunológica criada pelo hospedeiro (Prompetchara, Ketloy, & Palaga, 2020).

A resposta do hospedeiro e a eliminação de infecções virais dependem fortemente da expressão de IFN-I, característico da imunidade inata. A expressão de IFN-I e sinais *downstream* modulam as respostas celulares e reprogramam o sistema a um "estado antiviral" para promover o controle da infecção e a eliminação do patógeno (Lazear, Schoggins, & Diamond, 2019). No geral, os vírus são identificados por receptores de reconhecimento de padrões (PRRs) nas células do sistema imune. Vírus de RNAs, como SARS-CoV, SARS-CoV2 e MERS-CoV são detectados por PRRs no endossomo, incluindo receptores do tipo Toll (TLR) 3 e 7, ou no citosol, como gene I indutível por ácido retinóico (RIG-I) e proteína 5 associada à diferenciação de melanoma (MDA-5). A partir desses PRRs ativados, ocorre a ativação dos fatores de transcrição IFN-fator regulador 3 e 7 (IRF3 e IRF7) e NFκB. Por sua vez, isso desencadeia a expressão de IFN-I mediada por IRF3 e citocinas pró-inflamatórias inatas (IL-1, IL-6, TNF-α) mediada por NFκB (De Wit, Van Doremalen, Falzarano, & Munster, 2016).

Estudo *in silico* mostrou que a glicoproteína S de SARS-CoV-2 mostrou uma forte ligação e papel crucial de TLR4, e em menor intensidade com TLRs 1 e 6 (Choudhury & Mukherjee, 2020). É importante lembrar que SARS-CoV, MERS-CoV e provavelmente o SARS-CoV2 conseguem evitar o reconhecimento pelos PRRs por meio da supressão desses mecanismos, favorecendo a infecção e desenvolvendo fenótipos mais graves e pior prognóstico (Channappanavar & Perlman, 2017; Kindler, Thiel, & Weber, 2016; Xiaolu Lu, Pan, Tao, & Guo, 2011).

A COVID-19 é caracterizada por uma desregulação do sistema imunológico, sendo representada geralmente por citopenia e por uma hiperinflamação sistêmica mediada pela “tempestade de citocinas e quimiocinas”. Inclusive, essa é chave da patogênese da COVID-19. A resposta de citocinas induzida por SARS-CoV-2 é diferente em comparação a outros vírus respiratórios, pois o SARS-CoV-2 nem sempre induz citocinas específicas como outros coronavírus ou influenza, como IL-2, IL-10, IL-4 ou IL-5 (Olbei et al., 2021). Níveis séricos elevados de vários mediadores pró-inflamatórios foram reportados, tais como fator de necrose tumoral-alpha (TNF-α), interleucina-1beta (IL-1β), IL-1RA, IL-6, IL-7, IL-8, IL-9, IL-12, IL-13, IL-17, IFN-γ, IP10 (proteína 10 induzida por IFN-γ), fator estimulador de colônias de granulócitos-macrófagos (GM-CSF) e de macrófagos (M-CSF), fator de crescimento endotelial vascular (VEGF), proteína 1 quimiotática de monócito (MCP-1) e proteína 1 inflamatória de macrófago-alpha (MIP1-α) (Costela-ruiz et al. 2020; Giamarellos-Bourboulis et al. 2020; Huang et al. 2020; Mehta et al. 2020; Wang et al. 2020).

Relatos mostraram que as citocinas IL-6, IL-1β e TNF-α são as principais no estabelecimento desse desequilíbrio imune. O aumento dessas citocinas leva a quimiotaxia de várias células imunes circulantes, como macrófagos, neutrófilos e células T, ao local da infecção. Sem conseguir controlar o processo infeccioso, esse acúmulo celular causa lesões teciduais, a falha múltipla dos órgãos e morte (Aarestrup, 2020; Channappanavar & Perlman, 2017).

Como resultado desta ativação imune inata e consequente expressão de mediadores inflamatórias, as células imunes adaptativas se envolvem na defesa do hospedeiro contra infecções virais. Os linfócitos T desempenham um papel central na resposta antiviral, incluindo citocinas derivadas de células TCD4, citotoxicidade mediada por células TCD8+ e ativação de células B para a produção de anticorpos (Felsenstein et al., 2020). No entanto, os CoVs podem escapar desses mecanismos induzindo a apoptose de células T (Yi, Lagniton, Ye, Li, & Xu, 2020).

Na infecção por SARS-CoV-2, linfopenia é um fator determinante na evolução da doença. Serve como indicador eficaz de gravidade e hospitalização em pacientes com COVID-19 (L. Tan et al., 2020). Entretanto, vários padrões distintos de resposta de células T podem existir em diferentes pacientes, o que sugere a possibilidade de abordagens clínicas de acordo com o fenótipo particular do paciente (Z. Chen & John Wherry, 2020).

Wang e colaboradores (2020) observaram um perfil linfopênico, com uma diminuição gradativa dos números absolutos de células TCD4, células TCD8+ e células B em relação ao agravamento da doença. A porcentagem de células TCD8+ produtoras de IFN-γ aumentou em pacientes graves e extremamente graves em comparação com pacientes leves. A porcentagem de células T CD4+ produtoras de IFN-γ aumentou em pacientes extremamente graves, enquanto a porcentagem de células T reguladoras naturais diminuiu.

SARS-CoV-2 também pode induzir respostas de células T de memória robustas, amplas e altamente funcionais, sugerindo que a exposição natural ou infecção pode prevenir episódios recorrentes de COVID-19 grave. As células T específicas de SARS-CoV-2 de fase aguda exibem um fenótipo citotóxico altamente ativado que se correlaciona com marcadores clínicos de gravidade, enquanto as células T específicas de fase convalescente pode ser polifuncionais e exibir um fenótipo de memória (Sekine et al., 2020). Aparentemente, as respostas efetoras das células T na proteção contra a infecção por SARS-CoV-2 são importantes por serem mais estáveis e duradouras, quando comparado a resposta de anticorpos de curta duração (Altmann & Boyton, 2020).

2.2.4.1 Resposta imune humoral a COVID-19

A resposta imune humoral, especialmente a produção de anticorpos neutralizantes, desempenha um papel protetor, limitando a infecção (Bao et al., 2020) e podendo evitar uma futura reinfecção. Um dos pontos cruciais em relação a imunidade humoral é a magnitude e duração das respostas de anticorpos contra SARS-CoV-2.

Na COVID-19, indivíduos assintomáticos apresentam respostas de anticorpos mais fracas, com uma redução significativa na fase de convalescência, comparados a sintomáticos (Long, Tang, et al., 2020). Adicionalmente, pacientes com sintomas leves também apresentaram menor resposta comparados a casos mais graves de COVID-19 (Long, Liu, et al., 2020; N. M. A. Okba et al., 2020; Röltgen et al., 2020; W. Tan et al., 2020). Essas mesmo cenário foi verificado em SARS-CoV e MERS-CoV (Alshukairi et al., 2016; N. Okba et al., 2019). Contudo, baixas concentrações de

anticorpos neutralizantes são ainda eficazes na neutralização de SARS-CoV-2 (Robbiani et al., 2020).

A alta produção de anticorpos contra SARS-CoV-2 em pacientes hospitalizados com COVID-19 grave, enquanto assintomáticos ou com doença leve produzem uma resposta humoral transitória e tardia, podendo estimular a secreção da imunoglobulina A (IgA) específica de SARS-CoV-2 na mucosa, ou, até mesmo, não soroconvertem (Yongchen et al., 2020). A ausência na detecção de anticorpos pode ser consequência da eficiência da resposta imune inata local ou pela produção da IgA nas mucosas expostas a baixas cargas virais (Cervia et al., 2021). Dessa forma, a infecção seria resolvida precocemente por meio de fagócitos residentes e da presença de IgA mucosal (P. R. S. Oliveira & Tavares, 2020).

Em infecções por SARS-CoV, a soroconversão geralmente ocorre no quarto dia após o início da doença, com o pico do título de anticorpos IgG e anticorpos neutralizantes no quarto mês. Ambos foram altamente correlacionados e persistiram por 2 e 3 anos, pelo menos. (Cao, Liu, Zhang, Zhang, & Richardus, 2007; W. Liu et al., 2006). Para MERS-CoV, a seroconversão foi observada na segunda ou terceira semana pós-sintomas (W. J. Liu et al., 2017), apresentando anticorpos IgG detectáveis por até 1 ano após a infecção (Choe et al., 2017). Em ambas os coronavírus, foi observado que IgM e IgG aumentam nas primeiras semanas, com IgM diminuindo gradativamente no primeiro mês de infecção, ao contrário de IgG que se manteve. Anticorpos IgA anti-SARS-CoV apresentaram comportamento similar a IgM (Hsueh, Huang, Chen, Kao, & Yang, 2004; Spanakis et al., 2014).

Uma revisão sistemática avaliou que SARS-CoV-2 teve o tempo médio mais curto na detecção de anticorpos (11 dias; intervalo interquartil 7-14 dias), comparado a SARS-CoV (14 dias; 10–18) e MERS-CoV (15 dias; 12–18) (A. T. Huang et al., 2020). Estudos preliminares sobre o SARS-CoV-2 apontam para um padrão diferente dos outros coronavírus: anticorpos IgM aparecem ao mesmo tempo em casos graves e não graves, IgG aparece mais cedo em COVID-19 grave (W. Tan et al., 2020).

Anticorpos IgM anti-SARS-COV-2 podem ser detectados após três dias de infecção, com pico em duas ou três semanas e mantendo níveis detectáveis após um mês. (Theel et al. 2020; Xiao, Gao, and Zhang 2020). Já a resposta para anticorpos IgA tem se revelado mais intensa e persistente comparado aos anticorpos IgM, podendo surgir na primeira semana após a infecção, com o platô entre 20 e 22 dias e

mantendo esses altos títulos por mais 40 dias ((Lee et al., 2020; N. Okba et al., 2020; Padoan et al., 2020). Quanto à resposta de anticorpos IgG, apresentar-se com início tardio, precoce e também simultâneo (L. Guo et al., 2020; Padoan et al., 2020) em relação a IgA e IgM. Os anticorpos IgG tem atingido o pico de títulos em 17 dias, em média (Long, Liu, et al., 2020; P. R. S. Oliveira & Tavares, 2020). E estudos tem mostrado títulos elevedos de anticorpos IgG anti-SARS-CoV-2 em dois ou três meses após a infecção(Hou et al., 2020).

No SARS-CoV, os epítopos de células T e B foram extensivamente mapeados para as proteínas estruturais S, N, M e E (W. J. Liu et al., 2017). As glicoproteínas do capsídeo viral e nucleoproteínas são os principais antígenos na indução da respostas de anticorpos primária ou secundária IgG (e subclasses), IgM e IgA em infecções por SARS-CoV e SARS-CoV-2 (Oliveira & Tavares 2020; Poland, Ovsyannikova & Kennedy 2020). A imunogenicidade dessas duas proteínas é ainda discutida em termos de sensibilidade (Woo et al., 2005). Vários formatos têm sido usados em diagnóstico para infecção por SARS-CoV-2, inclusive tanto as proteínas de comprimento total quanto as truncadas.

Em primatas, a infecção por SARS-CoV-2 resultou na indução de anticorpos neutralizantes, e também respostas imunes celulares, específicos para S e ofereceu proteção em modelos de novo desafio de SARS-CoV-2 (Bao et al., 2020; Chandrashekar et al., 2020).

Estudos de coorte observaram índices de anticorpos IgG direcionados a proteína S e seus domínios mais elevados em comparação com ao antígeno N em pacientes com doença leve comparado aqueles gravemente doentes, especialmente na fase de convalescência (Atyeo et al., 2020; Röltgen et al., 2020). Além disso, níveis de anticorpos bloqueando a interação ACE2-RBD foram correlacionados com os títulos de IgG anti-RBD (Röltgen et al., 2020).

Estudos avaliando a resposta de subclasses de anticorpos IgG contra SARS-CoV-2, mostraram maiores reatividades de IgG1 e IgG3, seguida por IgG2, especialmente associadas a quadros graves de COVID-19, enquanto a subclasse IgG4 apresentou a menor reatividades dentre todos (Amanat et al., 2020; Dogan et al., 2021; Hunt et al., 2021; Iles et al., 2021). As respostas específicas para proteína N foram predominantemente anticorpos IgG3, enquanto que para a proteína S foram anticorpos IgG1, embora S também tenha exibido uma forte ligação para IgG3 (Iles et

al., 2021). Outro estudo relatou respostas IgG1 e IgG3 predominantes contra as subunidades S1 e S2 da *spike*, respectivamente (Yates et al., 2021). Subsequentemente, mostraram que a resposta de IgG1 era dominante para o nucleocapsídeo de comprimento total e subunidades proteica de S, enquanto IgG3 teve maior reatividades contra a proteína spike de comprimento total (Hunt et al., 2021).

Dogan et al. (2021) analisaram as correlações da neutralização de SARS-CoV-2 e os níveis de anticorpos pós-COVID-19. Eles observaram que todos os formatos de anticorpos, incluindo IgG anti-RBD, IgG anti-N, IgA anti-RBD e IgM anti-RBD, mostraram correlação significativa com os valores de neutralização. Entre as subclasses, todos os anticorpos estavam positivamente correlacionados a resposta específica contra o domínio RBD/S1, sendo IgG1 a maior correlação, seguida de IgG3, IgG2, e em menor grau a subclasse IgG4.

Em modelos de transferência adotiva, anticorpos humanos isolados de paciente de COVID-19 convalescente não somente neutralizaram especificamente SARS-CoV-2 *in vitro* como também inibiram a infecção por SARS-CoV-2 em macacos *rhesus* de forma profilática e de terapêutica (Shi et al., 2020). Da mesma forma, anticorpos IgG purificados de soros de macacos recuperados protegeram macacos *rhesus* não-imunes contra a infecção por SARS-CoV-2 (McMahan et al., 2021).

Uma das grandes questões agora enfrentadas na pandemia é a durabilidade dos anticorpos neutralizantes após contato com SARS-CoV-2. Em um estudo de modelagem, após 250 dias da imunização, pode ocorrer uma perda significante na proteção contra a infecção por SARS-CoV-2, embora ainda proteja contra as formas graves da COVID-19 (Khoury et al., 2021). Muitos estudos vêm mostrando a queda dos anticorpos neutralizantes em fases tardias de convalescência, consequentemente, permitindo que ocorram reinfecções (Cromer et al., 2021; Khoury et al., 2021). Dessa forma, é de extrema importância alcançar uma imunidade altamente protetora e duradoura mediada pela vacinação.

Considerando que a quantidade e a qualidade das respostas vacinais podem diferenciar, em conjunto com a capacidade de mutação do SARS-COV-2 e suas variantes, é importante compreender a dinâmica das respostas ao vírus para a implantação de estratégias para diferentes cenários futuros ao longo dessa pandemia.

3. OBJETIVOS

Geral

Investigar a distribuição e o papel dos anticorpos em infecções por flavivírus e por SARS-CoV-2.

Específicos

1. Analisar a distribuição de reatividade de anticorpos anti-flavivírus endêmicos e emergentes em população saudável.

2. Investigar a reatividade cruzada de indivíduos soropositivos para dengue contra o vírus zika.

3. Avaliar o perfil dos anticorpos IgA, IgG total e subclasses de IgG contra o nucleocapsídeo e a proteína spike em grupos de pacientes com sintomas leves e graves causados por COVID-19.

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Capítulo 2

ARTIGO 1 – PUBLICADO

Prevalence of arbovirus antibodies in young healthy adult population in Brazil

Parasites & Vectors

Prevalence of arbovirus antibodies in young healthy adult population in Brazil

--Manuscript Draft--

Manuscript Number:	PARV-D-21-00333R3
Full Title:	Prevalence of arbovirus antibodies in young healthy adult population in Brazil
Article Type:	Research
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Abstract:	Background: The emergence and re-emergence of infectious diseases are a cause for worldwide concern. The introduction of Zika and Chikungunya disease in the Americas has exposed unforeseen medical and logistical challenges for public health systems. Moreover, the lack of preventive measures and vaccination against known and emerging mosquito-transmitted pathogens, and the occurrence of unanticipated clinical complications, has produced an enormous social and economic impact in the affected populations. In this study, we aimed to measure the seroprevalence of endemic and emerging viral pathogens in military personnel stationed in Manaus, Amazonas state. Methods: We measured the seropositivity of antibodies against nineteen endemic and emerging viruses in healthy military personnel group using a Hemagglutination Inhibition Assay (HIA). Results: Overall, DENV positivity was 60.4%, and 30.9% of the individuals reacted against ZIKV. Also, 46.6%, 54.7%, 51.3% and 48.7% individuals reacted against West Nile virus (WNV), Saint Louis Encephalitis virus (SLEV), Ilheus virus (ILHV) and Rocio virus (ROCV), respectively. Individuals with high DENV HIA titre reacted more frequently with ZIKV or WNV compared to those with low HIA titres. Observed cross-reactivity between Flaviviruses varied depending on the virus serogroup. Additionally, 0.6% and 0.3% individuals were seropositive for Oropouche virus (OROV) and Catu virus (CATUV) from the family Peribunyaviridae, respectively. All samples were negative for Eastern Equine Encephalitis virus (EEEV), Western Equine Encephalomyelitis virus (WEEV), Mayaro virus (MAYV), Mucambo virus (MUCV) and CHIKV from the family Togaviridae . Conclusions: A high proportion of individuals in our high-risk population (~60%) lacked antibodies against major endemic and emerging viruses, which makes them susceptible for further infections. Military personnel serving in the Amazon region could serve as sentinels to strengthen global infectious disease surveillance, particularly in remote areas.
Corresponding Author:	Pritesh Lalwani ILMD Fiocruz Amazônia: Instituto Leonidas e Maria Deane Fiocruz Amazonia BRAZIL
Corresponding Author E-Mail:	pritesh.lalwani@fiocruz.br
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	ILMD Fiocruz Amazônia: Instituto Leonidas e Maria Deane Fiocruz Amazonia
Corresponding Author's Secondary Institution:	
First Author:	Bárbara Batista Salgado
First Author Secondary Information:	
Order of Authors:	Bárbara Batista Salgado Fábio Carmona de Jesus Maués Renato Lemos Pereira

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Response to Reviewers:	13/07/2021 Dr. Filipe Dantas-Torres Editor-in-chief, Parasites & Vectors Dear Editor, We are pleased to resubmit a revised version of our manuscript Salgado et al., Prevalence of arbovirus antibodies in young healthy adult population in Brazil" (PARV-D-21-00333R2), on behalf of all the authors. Below this letter we have included the summary of changes made to the manuscript. We thank you for your diligent changes and comments. We believe that the requested changes and the contributions of reviewers have additionally improved the quality and clarity of our manuscript, and we hope the revised manuscript is now acceptable for publication. Kind regards, Pritesh Lalwani Summary of changes Methods Ethics section moved to declarations section. Results Figure numbers verified and confirmed as per Parasite & Vectors format. Results Line 247: P value as per Parasite & Vectors format. Supplementary information Figure S1 legend modified as per Parasite & Vectors format, panel labels in lowercase. Additional File 1: Figure 1 panel label in lowercase. Additional File 2: Table S1 modified with one digit after decimal. Additional File 3: Table S2 title is inserted. Additional File 4: Table S3 references corrected. References Modified as per Parasite & Vectors format. Graphical abstract 600DPI picture inserted. Figure legends Formatting corrected and information verified. Table 1 was previously missing, which has been uploaded along with other files. Font changed to Times New Roman all throughout the manuscript.
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Prevalence of arbovirus antibodies in young healthy adult population in Brazil

Bárbara Batista Salgado[1†], Fábio Carmona de Jesus Maués[1†], Renato Lemos Pereira[1,2], Jannifer Oliveira Chiang[3], Maria Nazaré de Oliveira Freitas[3], Milene Silveira Ferreira[3], Lívia Caricio Martins[3], Pedro Fernando da Costa Vasconcelos[3], Christian Ganoza[4], Pritesh Lalwani[1*]

[1]Instituto Leônidas e Maria Deane (ILMD), Fiocruz Amazônia, Manaus, Amazonas, Brazil

[2] Centro de Instrução de Guerra na Selva (CIGS), Manaus, Amazonas, Brazil

[3]Instituto Evandro Chagas (IEC), Seção de Arbovirologia e Febres Hemorrágicas, Ananindeua, Pará, Brazil.

[4]Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano Heredia, Lima, Perú

[†]Bárbara Batista Salgado and Fábio Carmona de Jesus Maués contributed equally to this work

*Correspondence: pritesh.lalwani@fiocruz.br

BBS: barbarasalgadob@gmail.com

FCJM: fabiomaues@hotmail.com

RLP: renatocigs@gmail.com

JOC: janniferchiang@iec.gov.br

MNOF: maria_freitas17@hotmail.com

MSF: mileneferreira@iec.pa.gov.br

LCM: liviamartins@iec.gov.br

PFCV: pedrovasconcelos@iec.pa.gov.br

CG: cganoza@gmail.com

PL: Pritesh.lalwani@fiocruz.br

Abstract

Background: The emergence and re-emergence of infectious diseases are a cause for worldwide concern. The introduction of Zika and Chikungunya disease in the Americas has exposed unforeseen medical and logistical challenges for public health systems. Moreover, the lack of preventive measures and vaccination against known and emerging mosquito-transmitted pathogens, and the occurrence of unanticipated clinical complications, has produced an enormous social and economic impact in the affected populations. In this study, we aimed to measure the seroprevalence of endemic and emerging viral pathogens in military personnel stationed in Manaus, Amazonas state.

Methods: We measured the seropositivity of antibodies against nineteen endemic and emerging viruses in healthy military personnel group using a Hemagglutination Inhibition Assay (HIA).

Results: Overall, DENV positivity was 60.4%, and 30.9% of the individuals reacted against ZIKV. Also, 46.6%, 54.7%, 51.3% and 48.7% individuals reacted against West Nile virus (WNV), Saint Louis Encephalitis virus (SLEV), Ilheus virus (ILHV) and Rocio virus (ROCV), respectively. Individuals with high DENV HIA titer reacted more frequently with ZIKV or WNV compared to those with low HIA titers. Observed cross-reactivity between *Flaviviruses* varied depending on the virus serogroup. Additionally,

0.6% and 0.3% individuals were seropositive for Oropouche virus (OROV) and Catu virus (CATUV) from the family *Peribunyaviridae*, respectively. All samples were negative for Eastern Equine Encephalitis virus (EEEV), Western Equine Encephalomyelitis virus (WEEV), Mayaro virus (MAYV), Mucambo virus (MUCV) and CHIKV from the family *Togaviridae*.

Conclusions: A high proportion of individuals in our high-risk population (~60%) lacked antibodies against major endemic and emerging viruses, which makes them susceptible for further infections. Military personnel serving in the Amazon region could serve as sentinels to strengthen global infectious disease surveillance, particularly in remote areas.

Keywords: Arbovirus, seroprevalence, hemagglutination inhibition assay, public health, military personnel, cross-reactivity.

Background

Deforestation, urbanization and climate change have led to perpetual public health challenges of infectious diseases worldwide [1]. In the last few decades, viruses transmitted by arthropods have caused numerous outbreaks worldwide in humans and animals, causing diseases ranging from sub-clinical or mild, through febrile, encephalitic, hemorrhagic, or arthritogenic disease, with a significant proportion of fatalities [2, 3].

Dengue virus (DENV), one of the foremost studied arboviruses, is still responsible for millions of infections and numerous deaths worldwide [4]. Consecutive dengue epidemics have been occurring in Brazil since 1986, and more than four million cases of dengue fever (DF) had already been recorded to date [5, 6]. Moreover, all four dengue virus serotypes causing human disease have been detected in Brazil, and at least two serotypes co-circulate in most of the Brazilian states; please refer to Table S3 for details regarding dengue serotypes and Brazilian state [7, 8, 9]. In the absence of effective

therapeutics to treat dengue disease, and low adherence to ineffective mosquito control measures, there has been an increase in dengue-associated deaths along with increasing disease incidence [5, 9].

The social and economic impact attributed to the recent Zika virus (ZIKV) and Chikungunya virus (CHIKV) disease introductions, driven by the large sheer number of human infections and associated pathology in Brazil and the Americas are well documented [10, 11]. In addition, the recent isolation of West Nile virus (WNV) from equine hosts represents an additional emerging virus circulating in Brazil [12]. It is worth pointing out that in Brazil the co-circulation of several flaviviruses (like Dengue, Zika, Yellow Fever, Saint Louis encephalitis, Ilheus and others), complicates the serological diagnosis of these emerging infections, due to the extensive flavivirus cross-reactivity in the serological assays [13, 14, 15, 16]. Whilst many current arboviruses do not appear to cause pathology in humans or animals, this large number of widely different and highly adaptable arboviruses provides an immense resource for the emergence of new pathogens in the future [2, 11]. Epidemiological and molecular clock studies demonstrate that ZIKV and CHIKV introduction in Brazil happened up to a year before their detection, moreover these studies also point towards clinical misdiagnosis in some cases [17, 18]. Combating these pathogens has historically been driven by the circumstances: expecting the unexpected and being prepared to respond when the unexpected occurs. Therefore, understanding the epidemiology of these emerging and endemic pathogens is necessary to ascertain their public health impact and to respond efficiently to these epidemiologic and diagnostic challenges.

Furthermore, compared to civilian population, military personnel live in a communal nature, train in diverse locations like the Amazon rainforest and participate in humanitarian aid in adverse conditions, alongside suboptimal hygiene and stress in the

field, which increases their risk of contracting emerging infectious diseases. Hence, soldiers can act as a sentinel population to identify emerging pathogens. However, we have little data about the serological status before or during recent arbovirus outbreaks and the role of cross-reactive antibodies against these emerging viruses. Hence, in this study we evaluated the prevalence and antibody reactivity among military personnel participating in the jungle survival course using a cell-based assay against major endemic and emerging arboviruses from three different virus families. Additionally, we performed a literature review to understand the distribution of DENV prevalence in Brazil between 1980 and 2020.

Methods

Sample size calculation

Seroprevalence greatly varies depending on the study population, age, sex and the serological assay employed for antibody testing. Based on previous estimates of seroprevalence for arboviruses in Brazil, which ranged between <1 % to >50% depending on the geographical location [9, 19, 20, 21, 22]. A sample size of 285 individuals was calculated using an estimated prevalence of 25% and 95% confidence interval (334,500 is the current strength of the Brazilian Armed Forces) with a desired probability of 0.05. We recruited 300 individuals (assuming a maximum of 5% of participants excluded from analyses because of missing data or analysis). Sample size calculation was performed using the Epi Info software v5.5.3 (iOS mobile).

Study population and sample collection

The study population comprised of Brazilian army personnel participating in a Jungle survival course at the Jungle Warfare Training Center (CIGS, Centro de Instrução de

Guerra na Selva), located in Manaus, Amazonas State. Every year CIGS organizes up to four training camps in the Amazon rainforest, where recruits spend a maximum of three months inside the rainforest, training and performing exercises. We interviewed and sought ethical consent from participants before entering the rainforest and collected blood samples after the end of the jungle survival course. Number of participants varied with each training camp. Adults of both sexes ≥18 years were invited to participate. During the study period there were no female participants. Consecutive individuals were enrolled in this observational and cross-sectional study using convenience sampling until attaining calculated sample size of 300 between January 2014 and December 2015. A total of 4 mL of blood was drawn from each participant (using EDTA tubes, BD Vacutainer), subsequently tubes were centrifuged and plasma was separated and stored at -80 °C until further analysis.

Hemagglutination inhibition test

The serological tests were performed at the Instituto Evandro Chagas (IEC), (Belém, Pará). Plasma samples collected were subjected to an in-house hemagglutination inhibition assay (HIA) with a titration cut-off of 1:20 plasma dilution, as previously described [20, 23, 24]. Samples were tested by HAI test to detect antibodies reactive to the following viral families: *Flaviviridae* (*Flavivirus* genus): Yellow Fever virus (YFV), Dengue Virus (DENV) serotypes 1 to 4 (DENV-1, DENV-2, DENV-3 and DENV-4), Zika virus (ZIKV), Saint Louis Encephalitis virus (SLEV), West Nile virus (WNV), Ilheus virus (ILHV), Rocio virus (ROCV); *Togaviridae* (*Alphavirus* genus): Eastern Equine Encephalitis virus (EEEV), Western Equine Encephalomyelitis virus (WEEV), Mayaro virus (MAYV), Mucambo virus (MUCV), Chikungunya virus (CHIKV);

Peribunyaviridae (*Orthobunyavirus* genus): Oropouche virus (OROV), Tacaiuma virus (TCMV) and Catu virus (CATUV).

Spatial analysis and virus distribution

PubMed, Science Direct, LICACS, Web of Science and Medline databases were searched by keywords (Figure 5) to identify research papers with dengue virus seroprevalence data. QGIS Software version 2.18.26 for macOS was used to plot spatial distribution of dengue prevalence in Brazilian cities, Additional file 2: Table S1 lists studies included in this analysis between 1980 and June 2020. Hot spot detection maps were plotted using publicly available data for Dengue, Zika and Chikungunya virus incidences between 2014-2018 (Ministry of Health Brazil, https://www.saude.gov.br/boletins-epidemiologicos).

Data analysis

To analyze the clustering of study subjects according to their HIA plasma titers against all viral species analyzed, we normalized their serological dilution values using the $\log_2$ of the inverse titer value, calculated with the formula normalized_titer=Log_2[1/titer] [25]. We then constructed a heatmap plot of plasma HIA normalized titer levels with the Manhattan clustering method using the heatmap package version 1.0.12 (https://cran.r-project.org/package=pheatmap) in R for macOS with RStudio (R version 3.6.2, RStudio version 1.2.5033). The bubble plots depicting the percentages of seropositive individuals was done using Microsoft Excel 2019**.** Chi-Square test was used to examine the differences between observed and self-reported dengue virus infection rates (GraphPad Prism version 9.1.2, Mac OS)

Results

In the current study, we performed a cross-sectional analysis to determine serological reactivity against endemic and emerging viruses. We aimed to assess the pre-Zika and chikungunya epidemic serological status of individuals in a highly mobile group of individuals (Additional file 1: Figure S1), to better understand and estimate the size of the virus-exposed and susceptible populations. We recruited 300 individuals; however, two questionnaires were incomplete and removed from the analysis. Data for the 298 individuals included in the analyses is described in Figure 1 and Additional file 2: Table S1. Serological results are summarized in Table 1. All study participants were male, with a mean age of 27.98 years and median age of 26 years (IQR, 24-31 years). Study participants had served in the military since attaining eighteen years of age and had resided in several Brazilian states, hence, we could not ascertain the local of infection for the pathogens tested in this study. Among the three virus families tested, the majority of the individuals (251/298, 82.2%) reacted against the viruses from the *Flaviviridae* family (genus *Flavivirus*) (Table 1 and Figures 1 and 2). The prevalence for the *Peribunyaviridae*, family was 0.6% and 0.3% (2/298, OROV and 1/298 CATUV, respectively). Our entire sample was negative for all five viruses tested from the *Togaviridae* family (Table 1).

Antibodies to DENV were predominant among soldiers and varied between 48.3% and 58.4%, depending on the serotype of the virus (Table 1 and Figure 3). We observed, that during the ongoing 2014 ZIKV epidemic, 30.9% participants had anti-ZIKV antibodies (Table 1). Also 46.6% and 54.7% study participants were reactive for WNV and SLEV of the Japanese Encephalitis serogroup, respectively. ILHV and ROCV, from the Ntaya virus serogroup, had a seroreactivity of 51.3% and 48.7%, respectively. Furthermore, we observed an apparent relationship between increasing age and

percentage HIA positivity, however, no statistically significant differences were observed between seropositivity percentage data when stratified by age (Table 1, data not shown).

We then evaluated the clustering of individuals according to their serological titres against all viruses tested. We observed that our population clustered into three groups: A (n=70), B (n=59), and C (n=169) (Figure 2). Group A was a distinct group where most individuals had HIA titers against all flavivirus tested. Individuals in groups A and B showed a similar clustering pattern, with high HIA titers to DENV1-3 and YFV-17D (vaccine strain), but differed in their positivity to ZIKV (98.6% vs 35.6%, respectively), showing lower HIA titers than subjects in group A overall. Group C represented a group with 29.6% seropositivity to YF17D vaccine strain (compared to 98.6% and 100% in groups A and B, respectively), and a decreased fraction of flavivirus-seroreactive individuals overall (YFV 10%, vs 98.6 and 84.7 in groups A and B, respectively); only two individuals were seroreactive to ZIKV (1.2%). *Peribunyaviridae*-positive individuals clustered with groups A (n=1) and B (n=2).

Next, we plotted a bubble chart to understand the distribution of anti-DENV antibodies per serotype (Figure 3). A higher proportion of individuals were reactive against DENV-1 and DENV-2, ≥320 HIA titer. In addition, to assess the cross-reactivity between tested *Flaviviruses*, we analyzed the reactivity of DENV-1- or DENV-2-positive individuals with their corresponding reactivity for DENV-2 or DENV-1, WNV or ZIKV (Figure 4). We observed that individuals with higher HIA titre for DENV-1 or DENV-2 reacted with WNV or ZIKV more frequently, in contrast, individuals with lower DENV titers demonstrated low or no reactivity against WNV and ZIKV. Additionally, DENV-1 or DENV-2 positive individuals reacted more frequently with WNV compared to ZIKV (Figure 4).

Furthermore, 5% (15/295) and 17.2% (51/296) individuals self-reported previous malaria and dengue virus infection, respectively. We performed a chi-square test to assess if self-reported dengue infection rates were similar to results observed by serological diagnosis performed in this study. We observed that serological testing showed a higher percentage of positive individuals than self-reported disease (P<0.0001, data not shown).

To understand overall antibody reactivity and distribution of DENV in Brazil, studies between 1980 and 2020 were identified from scientific databases and spatial analysis was performed (Figure 5 and 6 and Additional file 3: Table S2 and Additional file 4: S3). Most of these studies used different sampling strategy, age groups and dengue detection assay that impede comparison between them (Additional file 2: Table S1). However, several large cities demonstrated an increase in DENV prevalence since first detection. Most Brazilian cities have more than one dengue serotype circulating, for examples Manaus in Amazonas state all four DENV serotypes have been detected. Hence, this prevalence data may be important for future DENV vaccination and arbovirus disease control strategies.

In summary, we observed that a major part of our tested population had antibodies to DENV, however, the absence or low reactivity against several arboviruses makes this population susceptible for endemic and emerging arboviral diseases.

Discussion

Emerging and re-emerging arbovirus infections pose a serious public health threat in tropical and subtropical regions worldwide [2, 3]. In this study, we observed that half of the study population was seropositive for dengue virus; however, over 90% of our study population was susceptible to endemic and emerging viruses in the absence of detectable antibodies. The presence of a large and susceptible population, and the abundant presence

of mosquito vector populations are important factors that contributed to the recent large outbreaks of ZIKV and CHIKV reported in Brazil and other Latin American countries, upon the introduction of the viral diseases in the continent. Also, a lack of adequate molecular diagnostic tools, and the antibody cross-reactivity between these endemic viruses, contributed to their spread and delayed diagnosis.

All four dengue serotypes are endemic in Brazil, and at least two of the four serotypes circulate in most of the Brazilian states, refer Table S3 [7, 9, 26, 27]. For example, in Manaus, Amazonas state, all four DENV serotypes have been detected [28]. Likewise, prevalence has been shown to be in the range of 15 to 80%, depending on the population and region; in addition, the prevalence of DENV infection has been shown to increase with age [5, 7, 9, 19, 29]. We observed a maximum positivity of 58.4% for DENV-2 among our study population. Also, HIA titers for DENV-1 and 2 antibodies were higher compared to DENV-3 and 4, which may be partially explained by the late introduction of these viruses in Brazil [7, 26]. However, we did not observe any correlation between age and prevalence frequencies as observed in other studies. Military personnel are dynamic populations, and they move constantly from one region to another for duty; this factor could explain the discrepancy observed between the lack of correlation between age and prevalence in our study population as compared with the general population that resides in the same location over a period of time. The evaluated information suggests that an important factor in the development of the overwhelming epidemic of ZIKV and CHIKV in Brazil could be attributed to the low level or absence of pre-existing antibody levels, combined with the ample presence of *Aedes aegypti* in the urban setting [11, 29, 30, 31].

More individuals had antibodies reactive against the Yellow Fever Vaccine (YFV) strain 17D compared to the wildtype strain. Moreover, the decreasing reactivity

toward wild type strain may be an indicator of gradual decrease of antibody titers overtime after vaccination. YFV booster doses at 10 years after primary vaccination in travelers to endemic region, endemic population, and high-risk individuals like military personnel are necessary to heighten the 17DD-YF specific immune response and to achieve efficient immunity [32].

The presence of heterologous neutralizing antibody titers is inversely correlated with the severity of patients with a second DENV infection [8, 33, 34, 35]. Additionally, over 30% of the individuals had antibodies against major endemic and emerging *Flaviviruses* tested in this study. We cannot rule out multiple flavivirus infections in the same individual. These findings, along with other reports, indicate that SLEV, WNV, ILHV and ROCV circulation in Brazil is largely unknown, and there may be epidemiological implications of the co-circulation of these arboviruses [16, 19]. Overall, *in vivo* or cohort studies are needed to ascertain role of multiple *Flavivirus* infection in cross-protection or induction of a severe disease [8, 13, 15, 36]. Regarding their role in disease and protection, low avidity antibodies against DENV have been shown to participate in severe disease, also poorly-neutralizing antibodies can participate in antibody-dependent enhancement (ADE) in DENV infections [37, 38, 39]. Recent studies have demonstrated a role of DENV antibodies in causing ADE during ZIKV infection [40, 41]. On the other hand, several studies have also demonstrated a lack of ADE like cytokine storm and partial protective role of these flavivirus cross-reactive antibodies upon ZIKV infection [42, 43]. Nevertheless, comprehensive *in vivo* studies are necessary to ascertain role of these cross-reactive antibodies in ADE during ZIKV and other *Flavivirus* infections.

MAYV is endemic in the Amazon region and there have been imported cases in other regions of Brazil [21, 44]. A prevalence of more than 40% for MAYV has been

described in some Amazonian communities [21]. However, we did not observe any positive samples for the *Alphavirus* tested in our study. Similarly, we observed very low prevalence to OROV belonging to *Peribunyaviridae*. OROV is still localized in the Amazon region and is responsible for causing neurological disease in urban and rural areas [45].

One of the limitations of the present study is that it was not feasible to perform a neutralization assay to distinguish between dengue serotypes and confirm or rule out multiple infections or cross-reactivity. HIA detects total reactive antibodies against the test antigen, previous studies have demonstrated a lower sensitivity of the HIA compared to ELISA. However, since no sensitivity and specificity values for each virus is available, we could not perform a statistical adjustment to estimate the true prevalence; here, we describe only the crude percentage reactivity for each virus tested in this study. Although, most study participants were young male adults, these results are in accordance with previous studies on DENV prevalence [9, 29]. Furthermore, given the size and geographical differences in Brazil, the estimates from one region or state cannot be used to understand epidemiology from the whole of Brazil. On the other hand, soldiers are a high-risk group because of the activities they are involved and their contact with endemic regions such as the Amazon rainforest. A very small proportion of the study population reported previous malaria infection, which suggests that most of the individuals lived in urban regions or have spent little time in the rural Amazon region, where 99.9% of the malaria cases are described [46]. This might explain the low level or absence of antibodies against arboviruses described in the Amazonian region, such as OROPV and MAYV [22, 47]. Moreover, only 17% of the individuals tested in this study self-reported dengue infection, which was three times lower than that observed by serology. These unreported dengue infections could be asymptomatic subclinical infections or self-limiting fever

without diagnosis or clinically not diagnosed as dengue. Our cluster analysis suggests that ~60% of our study population comprehended a low *Flavivirus*-positive population and therefore susceptible to infection. Theoretically, susceptible military personnel could act as disease-spreading agents when returning to civil life when the combination of a susceptible population and specific vectors are present. More detailed serological surveys together with vector population assessment and viral detection strategies are needed to further characterize the extent of favorable factors that can contribute to future outbreaks, and to forecast potential public health needs.

Overall, mosquito control measures and integrated vector management are essential for control of all arboviruses, and were effective in controlling ZIKV and CHIKV outbreaks in Brazil and worldwide [48, 49, 50]. However, vector control precedes the decrease in herd immunity and the increase in the availability of susceptible populations [11, 33]. Adaptions of these emerging viruses to urban vectors like *Aedes aegypti* or *Culex quinquefasciatus* and the decreasing herd immunity to them might facilitate further epidemics of endemic and emerging arboviruses [2, 3]. Currently, differential clinical diagnosis is a major challenge when multiple viruses that cause similar clinical symptoms co-circulate [13, 14]; moreover, the lack of adequate diagnostics tools can limit early identification and efforts to block outbreaks [11, 51]. Warmer weather conditions brought on by the El Niño phenomenon and the destruction of the Amazon native forest can encourage a faster breeding and maturation cycles for *Aedes* and *Anopheles* mosquito populations [52].

Conclusions

A high percentage of individuals lacked antibodies against major endemic and emerging arboviruses, which makes them susceptible for further infections. Hence, an improved

vector and febrile syndrome surveillance to identify emerging pathogens is essential to prevent future outbreaks.

Supplementary information

Additional file 1: Figure S1. Distribution of Dengue, Zika and Chikungunya virus cases in Brazil between 2014-2018. Hot spot detection maps were plotted using publicly available data for dengue (a and b), Zika (c and d) and Chikungunya (e and f) virus incidences between 2014-2018 (Ministry of Health Brazil, https://www.saude.gov.br/boletins-epidemiologicos). Please note the differences in the incidence rate scales for each virus. North region: Acre-AC, Amapá-AP, Amazonas-AM, Pará-PA, Rondônia-RO, Roraima-RR, Tocantins-TO; Northeast region: Alagoas-AL, Bahia-BA, Ceará-CE, Maranhão-MA, Paraíba-PB, Pernambuco-PE, Piauí-PI, Rio Grande do Norte-RN, Sergipe-SE; Midwest region: Goiás-GO, Mato Grosso-MT, Mato Grosso do Sul-MS, Distrito Federal (Federal District)-DF; Southeast region: Espírito Santo-ES, Minas Gerais-MG, Rio de Janeiro-RJ, São Paulo-SP; South region: Paraná-PR, Rio Grande do Sul-RS, Santa Catarina-SC.

Additional file 2: Table S1. Socio-demographic features of the study population.

Additional file 3: Table S2. Dengue seroprevalence in Brazil reported between 1980 and 2020.

Additional file 4: Table S3. Distribution of dengue virus serotypes in Brazilian states.

Abbreviations

HIA: Hemagglutination inhibition assay; YFV: Yellow Fever virus; DENV: Dengue Virus; ZIKV: Zika virus; SLEV: Saint Louis Encephalitis virus; WNV: West Nile virus; ILHV: Ilheus virus; ROCV: Rocio virus; EEEV: Eastern Equine Encephalitis virus;

WEEV: Western Equine Encephalomyelitis virus; MAYV: Mayaro virus; MUCV: Mucambo virus; CHIKV: Chikungunya virus; OROV: Oropouche virus; TCMV: Tacaiuma virus; CATUV: Catu virus.

Declarations

Ethics approval and consent to participate

This study was approved by the Universidade Federal do Amazonas (UFAM) and Universidade Nilton Lins (UNINILTONLINS) research ethics committee (Project number 96171218.7.0000.5020 and 83715618.0.0000.5015), in accordance with Brazilian law, which complied with the Helsinki declaration. All the study participants signed an informed consent prior to enrolment.

Consent for publication

Patients signed informed consent regarding publishing their data.

Competing interests

The authors have no conflicting financial interests.

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Authors' contributions

PL designed the study with inputs from BBS and RLP. BBS and RLP collected samples. BBS, FCJM, JOC, MNOF, MSF performed laboratory analysis. LCM and PFCV analyzed HIA data together with BBS, FCJM, CG and PL. BBS and PL performed meta-analysis of serological data. BBS, CG and PL wrote the manuscript with input from all authors. All authors approved corrected version of the manuscript.

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Availability of data and material

The dataset supporting the conclusions of this article is included within the main manuscript and supplementary material.

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Figure legends

Fig. 1 Sample collection flowchart (Strobes diagram)

Fig. 2 Seroreactivity against principal human flaviviruses. Patient samples (n=298) were tested for antibodies against flaviviruses by hemagglutination inhibition assay (HIA). Heatmap represents the normalized antibody titers of individuals positive for at least one Flavivirus, clustered by titer. Groups: A (n=70), B (n=59), and C (n=169).

Fig. 3 Distribution of dengue virus antibody titers. Dengue virus positive samples were plotted on a bubble chart as function of antibody titers and virus serotype. Bubbles show the column percentages for each virus and their sizes are proportional to their values.

Fig. 4 Antibody reactivity between flaviviruses. Dengue positive samples were used to evaluate cross-reactivity between emerging viruses. (a) DENV-1 or (b) DENV-2 HIA titers were compared with DENV-2 or -1, ZIKV or WNV antibody titers. Bubbles show the column percentages for each virus and their sizes are proportional to their values. All samples were considered positive with HIA titer $\geq$ 20 units. Bubbles with pie chart represent distribution of individuals with reference to DENV-1 or DENV-2 HIA reactivity.

Fig. 5 Preferred reporting items for systematic reviews and meta-analysis (PRISMA) flowchart for dengue prevalence studies.

Fig. 6 Summary of dengue prevalence studies reported between 1980 and 2020. QGIS software was used to plot tropical and subtropical areas along with study location, dengue prevalence and year of sample collection (Additional file 3: Table S2). All Brazilian states have confirmed DENV circulation but no prevalence data was available for some states (RO, RR, AP, PI, RN, PL, AL, SE, ES, DF and SC). *Manaus-AM 2015 data is from this study (refer Table 1).

Click here to access/download;Table;Table 1.docx

Table 1. Prevalence of antibodies against principal human arboviruses

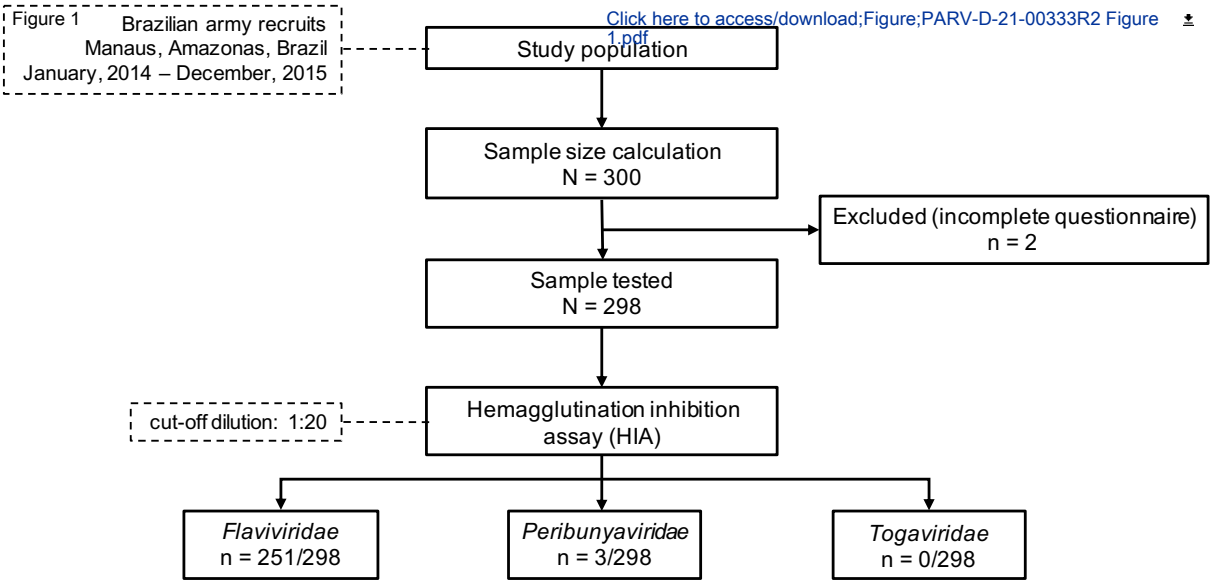
Classification	Virus*	Total %#	Age 18 - 25 years %#	Age 26 - 35 years %#	Age > 35 years %#
Flaviviridae					
Yellow fever virus group	YFV/17D	82.9 (247/298)	82.5 (113/137)	81.5 (97/119)	87.8 (36/41)
	YFV	45.6 (136/298)	42.3 (58/137)	50.4 (60/119)	41.5 (17/41)
Dengue virus group	DENV-1	56.7 (169/298)	51.8 (71/137)	62.2 (74/119)	56.1 (23/41)
	DENV-2	58.4 (174/298)	53.3 (73/137)	63.9 (76/119)	58.5 (24/41)
	DENV-3	56.4 (168/298)	50.4 (69/137)	61.3 (73/119)	61.0 (25/41)
	DENV-4	48.3 (144/298)	43.1 (59/137)	52.1 (62/119)	53.7 (22/41)
Spondweni group	ZIKV	30.9 (92/298)	27.7 (38/137)	31.9 (38/119)	36.6 (15/41)
Japanese encephalitis virus group	WNV	46.6 (139/298)	40.9 (56/137)	53.8 (64/119)	43.9 (18/41)
	SLEV	54.7 (163/298)	48.9 (67/137)	63.0 (75/119)	48.8 (20/41)
Ntaya virus group	ILHV	51.3 (153/298)	48.1 (66/137)	54.6 (65/119)	51.2 (21/41)
	ROCV	48.7 (145/298)	43.8 (60/137)	52.9 (63/119)	51.2 (21/41)
Peribunyaviridae					
Simbu serogroup	OROV	0.7 (2/298)	0.7 (1/137)	0.8 (1/119)	0 (0/41)
Guama serogroup	CATUV	0.3 (1/298)	0 (0/137)	0 (0/119)	2.4 (1/41)
Anopheles A serogroup	TCMV	0 (0/298)	0 (0/137)	0 (0/119)	0 (0/41)
Togaviridae					
Semliki forest virus complex	MAYV	0 (0/298)	0 (0/137)	0 (0/119)	0 (0/41)
	CHIKV	0 (0/298)	0 (0/137)	0 (0/119)	0 (0/41)
Eastern Equine Encephalitis Complex	EEEV	0 (0/298)	0 (0/137)	0 (0/119)	0 (0/41)
Western Equine Encephalitis Complex	WEEV	0 (0/298)	0 (0/137)	0 (0/119)	0 (0/41)
Venezuelan Equine Encephalitis Complex	MUCV	0 (0/298)	0 (0/137)	0 (0/119)	0 (0/41)
Negatives		15.8 (47/298)	16.1 (22/137)	16.8 (20/119)	12.2 (5/41)

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* *Flaviviridae* (Flavivirus genus): Yellow fever virus (YFV), Dengue virus (DENV) serotypes 1 to 4 (DENV-1, DENV-2, DENV-3 and DENV-4), Zika virus (ZIKV), Saint Louis Encephalitis virus (SLEV), West Nile virus (WNV), Ilheus virus (ILHV) and Rocio virus (ROCV); *Peribunyaviridae* (Orthobunyavirus genus): Oropouche virus (OROV), Catu virus (CATUV) and Tacaiuma virus (TCMV); *Togaviridae* (Alphavirus genus): Mayaro virus (MAYV), Chikungunya virus (CHIKV), Eastern Equine Encephalitis virus (EEEV), Western Equine Encephalomyelitis virus (WEEV) and Mucambo virus (MUCV).

Figure 1

Click here to access/download;Figure;PARV-D-21-00333R2 Figure 1.pdf



Click here to access/download;Figure;PARV-D-21-00333R2 Figure 2.pdf

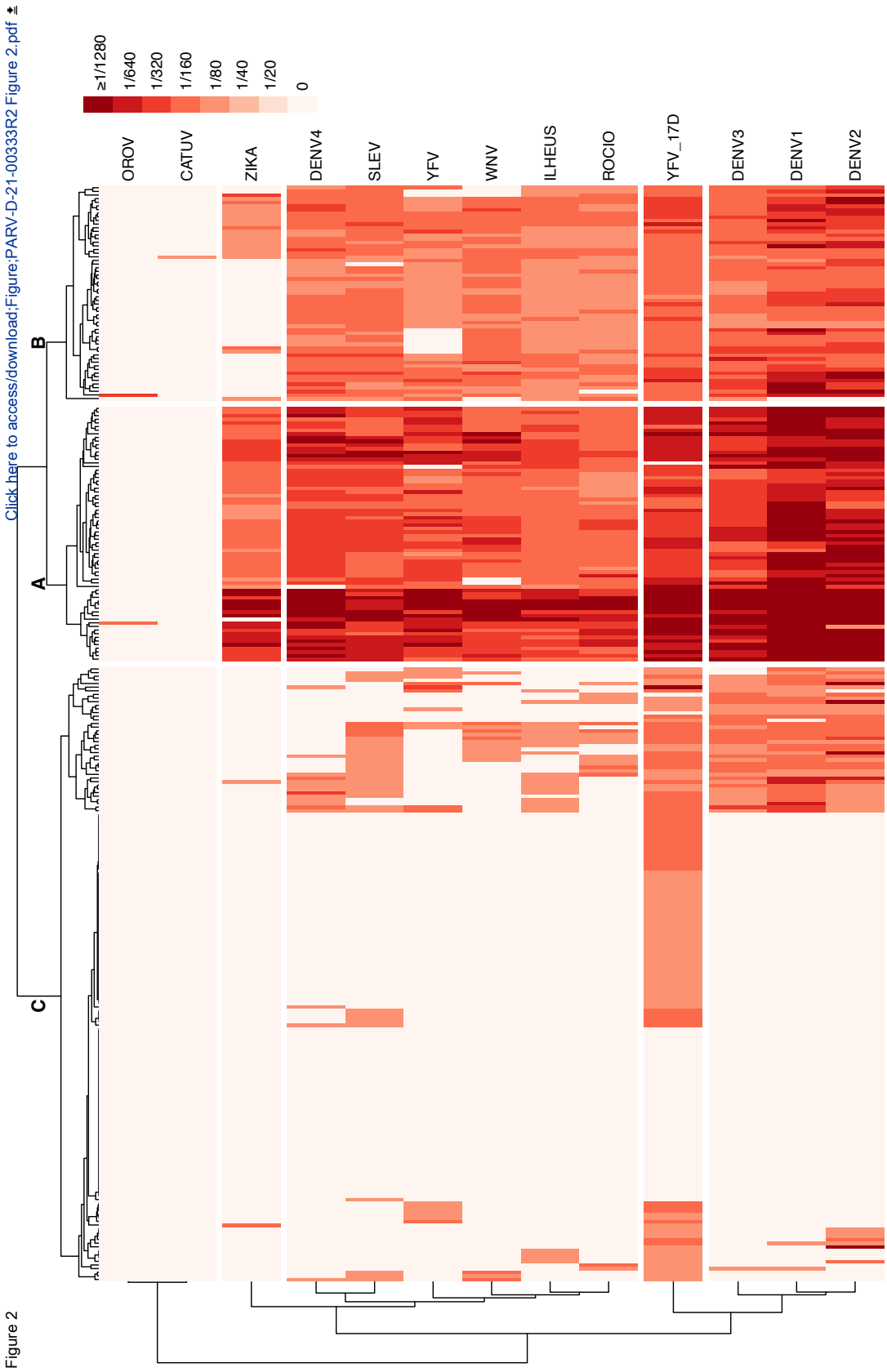


Figure 2

Figure 3

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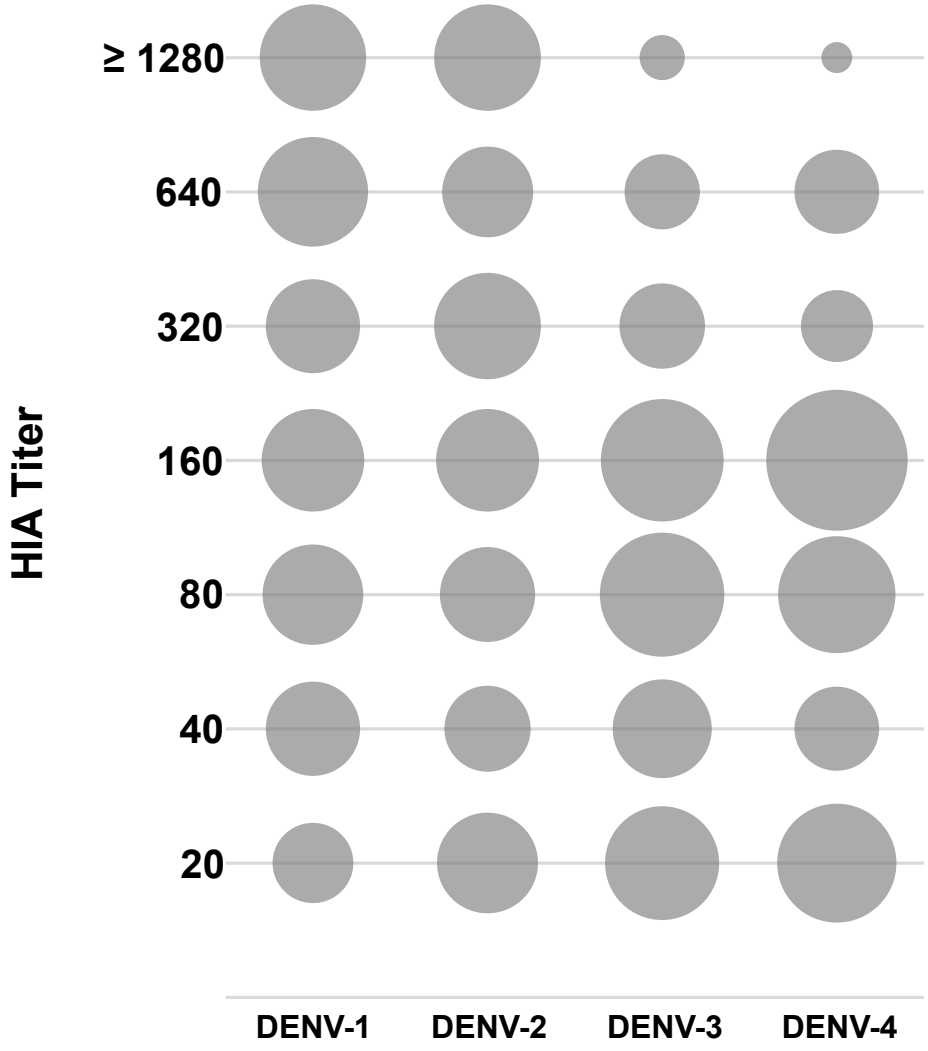


Figure 4

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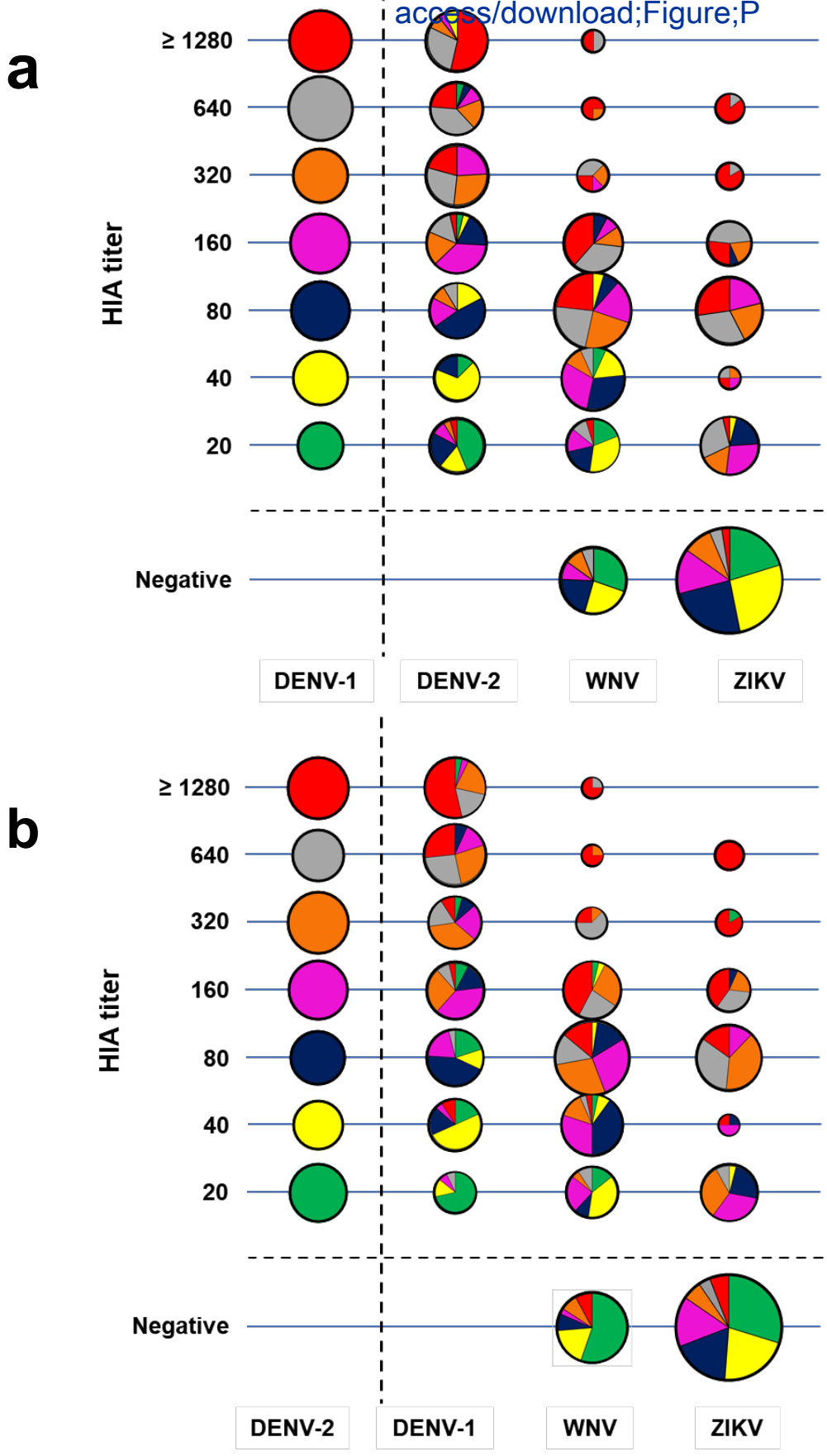
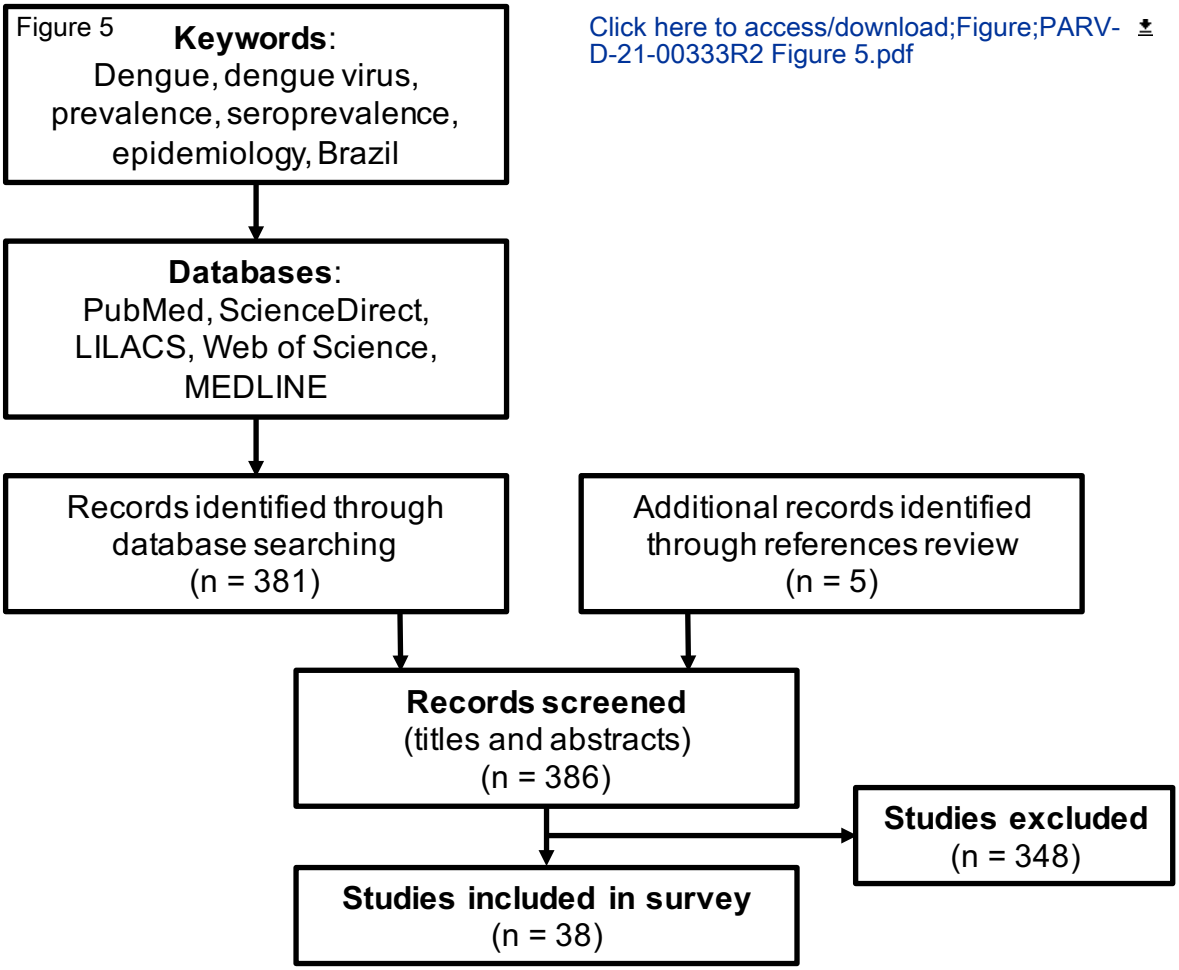


Figure 5



Click here to access/download;Figure;PARV-D-21-00333R2 Figure 5.pdf

Figure 6

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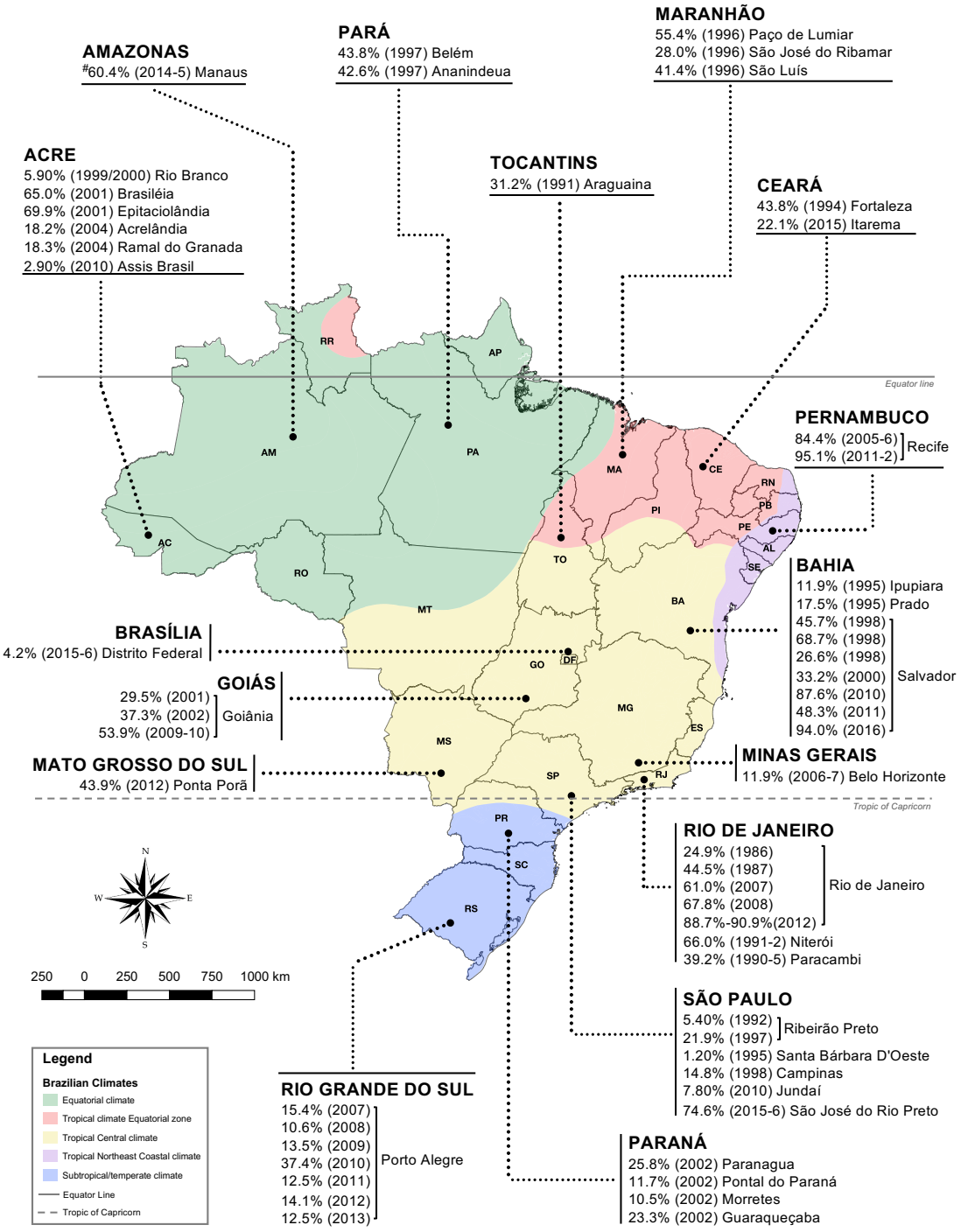


Figure S1

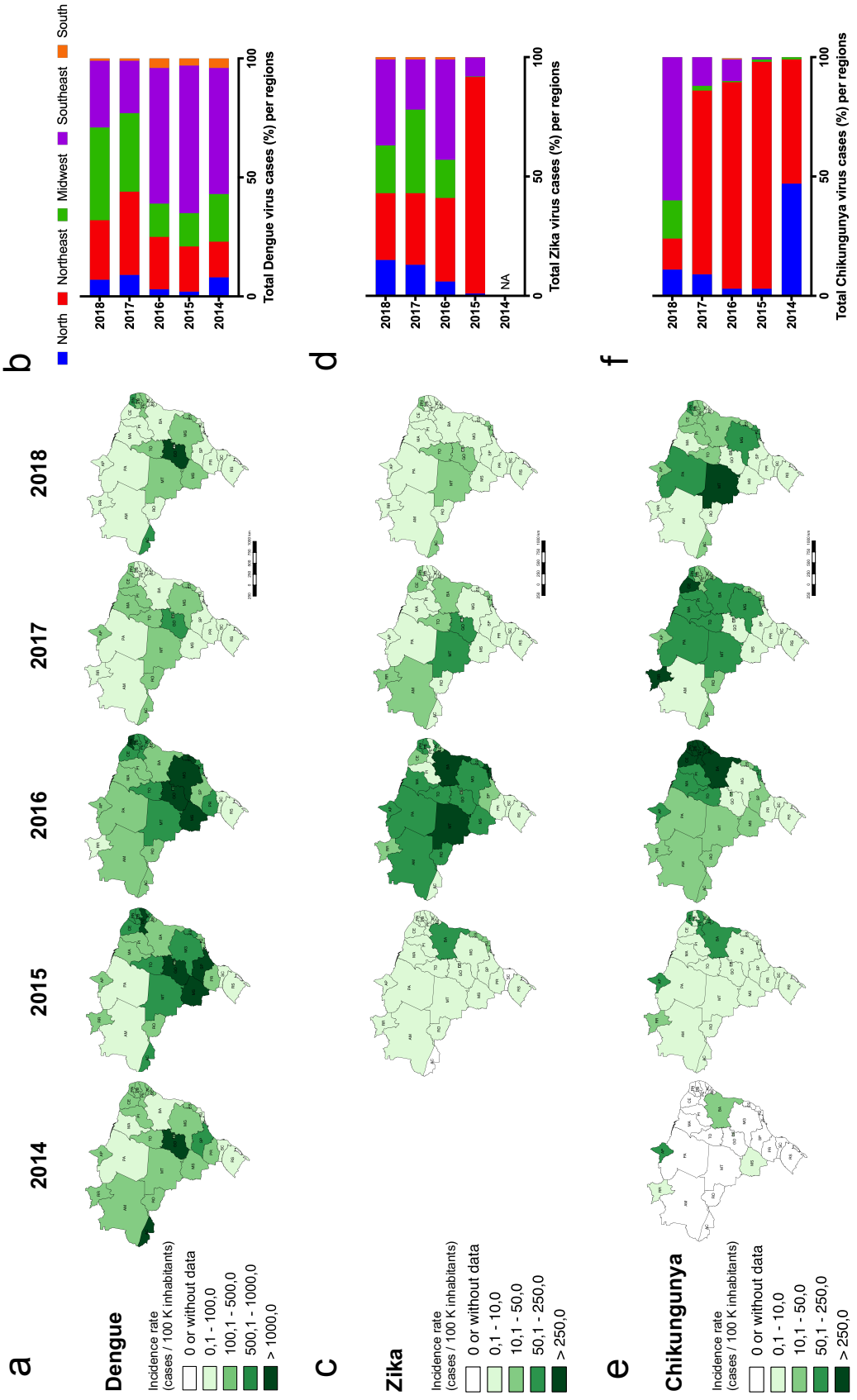

a
2014
2015
2016
2017
2018
Dengue
Incidence rate
(cases / 100 K inhabitants)
0 or without data
0,1 - 100,0
100,1 - 500,0
500,1 - 1000,0
> 1000,0
b
North
Northeast
Midwest
Southeast
South
2018
2017
2016
2015
2014
0
50
100
Total Dengue virus cases (%) per regions
c
Zika
Incidence rate
(cases / 100 K inhabitants)
0 or without data
0,1 - 10,0
10,1 - 50,0
50,1 - 250,0
> 250,0
d
2018
2017
2016
2015
2014
NA
0
50
100
Total Zika virus cases (%) per regions
e
Chikungunya
Incidence rate
(cases / 100 K inhabitants)
0 or without data
0,1 - 10,0
10,1 - 50,0
50,1 - 250,0
> 250,0
f
2018
2017
2016
2015
2014
0
50
100
Total Chikungunya virus cases (%) per regions

Table S1. Socio-demographic features of the study population

Characteristic		**Value %**
Age (years)	20-29	71.0
	30-39	22.0
	40-49	7.1
Race	Brown	45.2
	Black	9.5
	White	35.0
	Yellow	1.7
	Mixed	8.5
Family income	Minimum wage	1.0
	Two minimum wages	3.5
	Three minimum wages	9.7
	Four minimum wages	20.41
	Five minimum wages or more	65.4
Education level	Elementary school	0.3
	High school	54.0
	Graduate	45.6
Dengue infection [1]	Yes	17.9
Malaria infection [1]	Yes	5.0
Use Sistema Único de Saúde (SUS) [2]	Yes	14.3

[1]Self-reported, [2]Brazilian public funded health care system

Table S2. Dengue seroprevalence in Brazil reported between 1980 and 2020

State	City or location	Year of collection	N	Prevalence	Study population	Population description	Sorologic Test	Reference
NORTH								
Acre	Rio Branco	1999/2000	394	5.9%	202 females and 188 males Mean age 28.5 years	Inhabitants were tested in two times: before (august 1999) and three months (january 2000) after immunization with yellow fever 17D vaccine in October 1999.	Haemagglutination inhibition	Tavares-Neto et al., 2004
	Brasiléia	2001	177	IgM = 63.8% IgG = 65.0%	210 females and 110 males Age range: from 11 to over 65 years Mean age 38.7 years	Dwellers from two municipalities that border the endemic Bolivian area after first epidemic outbreak in Acre 2000.	IgG ELISA e IgM MAC-ELISA	Guimarães et al., 2006
	Epitaciolândia		143	IgM = 55.9% IgG = 69.9%				
	Acrelândia	2004	357	18.2%	Both genders Age range: from 5 to more than 60 years Mean age 27.5 years	Volunteers from a agricultural settlement in rural area of Acrelândia.	Haemagglutination inhibition	Nunes et al., 2006
	Ramal do Granada	2004	405	18.3%	193 females and 212 males P/A: 123 (5-14y), 130 (15-30y), 128 (31-60y) and 24 (>60y)	Volunteers from a agricultural settlement in rainy rural area with humid equatorial climate.	IgG ELISA Dengue serotypes 1, 2, 3 and 4 virus	Nunes et al., 2008
	Assis Brasil	2010	411	2.9%	200 females and 211 males Age range: from 6 months to 12 years Mean age 5.15 years and median age 3.9 years	Children living in urban area.	PANBIO Dengue IgG Indirect ELISA	Martins et al., 2014
Pará	Belém	1997	31,506	43.8%	20,199 females and 20,038 males Age range: from less than 5 to over 55 years	Febrile patients. The global prevalence in these two cities was 43.5%.	Haemagglutination inhibition and IgM MAC-ELISA	Travassos da Rosa et al., 2000
	Ananindeua		8,731	42.6%				
	Juriti	2007-2008	1,597	1.3%*	Not mentioned	People with and without symptoms of febrile illness living on the right bank of the Amazon River.	Haemagglutination inhibition and IgM MAC-ELISA	Cruz et al., 2009
Tocantins	Araguaina	1991	400	IgM = 27.8% IgG = 31.2%	215 females and 185 males P/A: 50 (0-4y), 128 (5-14y), 81 (15-24y), 48 (25-34y), 38 (35-44y), 29 (45-54y) and 25 (≥55y)	People from urban area belonging to three neighborhoods, in which were detected the first cases.	Haemagglutination inhibition and IgM MAC-ELISA	Vasconcelos et al., 1993
NORTHEAST								
Bahia	Ipupiara	1995	461	11.9%	273 females and 188 males Mean age of 38.1 years	Participants from urban area randomly selected with main productive activities as subsistence farming, mineral extractivism and commerce.	Haemagglutination inhibition	Vasconcelos et al., 2000
	Prado	1995	228	17.5%	148 females and 80 males Mean age of 44.4 years	Participants from urban area randomly selected with main productive activities as tourism, fishing and small commerce.		
	Salvador	1998	1503	45.7%	Not mentioned	Simple random sample divided into 30 delimited areas ("sentinel areas") of a urban complex which were classified into 7 different strata based on 3 levels of sanitation and 3 income levels of the population.	Haemagglutination inhibition	Teixeira et al., 1999
		1998	1515	68.7%	877 were females and 638 males P/A: 77 (< 5y); 317 (5–14 y) and 1121 (≥ 15 y)		Haemagglutination inhibition and IgG ELISA	Teixeira et al., 2002
		1998	625	26.6%	288 females and 337 males Age range: from 0 to 3 years	Random cohort study with preschool age children examined in 1998, and the non-reactive IgG children in this year were retested two years later to investigate seroconversion.	Haemagglutination inhibition and IgG ELISA	Teixeira et al., 2012
		2000	289	33.2%				
		2010	2,323	87.6%	1,286 females and 1,037 males P/A: 581 (5-14y), 491 (15-24y), 470 (25-34y), 339 (35-44y), 257 (45-54y) and 184 (≥55y)	Prospective cohort study with dwellers from a poor urban community analysed in 2010, and the non-reactive IgG participants in this year were reexamined one year later to investigate seroconversion.	PANBIO Dengue IgG Indirect ELISA	Tavares, 2014
		2011	240	48.3%	138 females and 102 males P/A: 116 (5-9y), 66 (10-14y), and 58 (≥15y)			
		2016	133	94%	133 pregnants females Mean age of 25 ± 6 years	In February 2016, a surveillance for congenital Zika virus infection and a cross-sectional study at a maternity hospital evaluate the prevalence of recent and previous infections by Zika, Dengue and Chikungunya virus in pregnants.	IgG ELISA	Oliveira et al., 2018
Ceará	Fortaleza	1994	1,341	43.8%	Both genders and wide age range	Random seroepidemiological survey with inhabitants of nine health districts of Fortaleza in response to the epidemic of March 1994.	Haemagglutination inhibition	Vasconcelos et al., 1998
	Itarema	2015	280	22.1%	164 females and 116 males P/A: 72 (<15y), 183 (15-59y) and 26 (>59y) Mean age 30.2 years	Indian Adult-young population from Tremembé ethnicity, resident in the village of Tapera.	PANBIO Dengue IgG Indirect ELISA	Sacramento et al., 2018
Maranhão	Paço de Lumiar	1996	101	55.4%	709 females and 508 males P/A: 144 (≤14y), 345 (15-24y), 298 (25-34y), 182 (35-44y), 103 (45-54y) and 145 (≥55y)	Residents from three cities that belong São Luis Island, after epidemic in Maranhão 1995. The global prevalence in the island showed 41.5%.	Haemagglutination inhibition	Vasconcelos et al., 1999
	São José do Ribamar		100	28%				
	São Luis		1,016	41.4%				
Pernambuco	Recife	2005-2006	2,819	84.4%	1,600 females and 1,219 males P/A: 1,282 (5-14y) and 1,537 (15-64y) Mean age 26.03 years	Residents from three different areas were selected to represent deprived, intermediate and high socio-economic areas. The prevalence by area: 91,1% in deprived, 87,4% in intermediate and 74,3% in high.	PANBIO Dengue IgG Indirect ELISA	Braga et al., 2010
		2011-2012	417	95.1%	417 females	Parturients from an ongoing birth cohort study from March 2011 to May 2012.	IgG ELISA	Leite et al., 2014
MIDWEST OR CENTRAL-WEST								
Goiás	Goiânia	2001	1,585	29.5%	1,066 females and 519 males P/A: 63 (5-14 years), 1,084 (15-49 years) and 438 (≥50 years)	Two surveys conducted from January-February 2001 and in the same period in 2002 in the Central Brazil. Due to DENV introduction in 1994 on the region, several serosurveys had been performed.	PANBIO IgM and IgG EIA Dengue	Siqueira et al., 2004 Siqueira-Junior et al., 2008
		2002	2,906	37.3%	1,967 females and 939 males P/A: 133 (5-14 years), 1,969 (15-49 years) and 794 (≥50 years)			
		2009-2010	505 (1)	IgG (1) = 53.9%	505 parturients Mean maternal age 25.8 ± 6.4 years	Cross-sectional study among pairs of (1) maternal and (2) corresponding umbilical cord blood samples in two public hospitals from December 2009 to May 2010. It yielded 99.3% co-positivity for placental antibody transfer.	PANBIO Dengue IgG Indirect ELISA	Argolo et al., 2013
			505 (2)	IgG(2) = 55.0%				
Mato Grosso do Sul	Ponta Porã	2012	396	43.9%	290 females and 106 males P/A: 6 (<10y), 40 (10-19y), 78 (20-29y), 62 (30-39y), 64 (40-49y), 75 (50-59y, 36 (60-69y) and 35 (≥70y)	Cross-sectional analytical design performed in urban residents of a city which borders an endemic Paraguayan area.	PANBIO Dengue IgG Indirect ELISA	Mahmoud, 2012
Brasília	Federal District	2015-2016	475	IgG = 4.2% IgM = 6.7%	160 females ans 315 males Mean age 35.1 ± 10.7 years	Pilot cohort study of blood donors during a large DENV outbreak from December 2015 to May 2016. It also assessed viral RNA presence and NS1 positivity, besides anti-CHIKV and anti-ZIKV IgM.	PANBIO Dengue IgM Capture ELISA and Euroimmun anti-DENV IgG ELISA	Slavov et al., 2019
SOUTHEAST								
Rio de Janeiro	Rio de Janeiro	1986	402	24.9%	243 females and 159 males Age range: from 4 to 14 years	Schoolchildren from elementary public schools of five districts of the city and were investigated at the beginning and end of a 12-month interval.	Haemagglutination inhibition	Figueiredo et al., 1990
		1987	384	44.5%	237 females and 147 males Age range: from 4 to 14 years			
	Niterói	1991-1992	450	66.0%	236 females and 214 males P/A: 140 (5-10 years), 218 (11-14 years) and 92 (15-19 years)	Schoolchildren from an endemic area of dengue fever in the summer 1991/1992, after epdemics by Dengue virus type 1 and 2.	Haemagglutination inhibition	Cunha et al., 1995
	Paracambi	1990-1995	370	IgM = 17.3% IgG = 39.2%	197 females and 173 males P/A: 102 (4-10 years), 161 (11-14 years) and 107 (15/16 years)	Schoolchildren from public schools of the city, area with environments conditions for natural breeding of *Aedes aegypti* and *A. albopictus.*	Haemagglutination inhibition and IgM MAC-ELISA	Cunha et al., 1997
	Rio de Janeiro	2007	326	IgM = 2.8% IgG = 61.0%	Both genders Median Age: (Area 1= 28.5y) (Area 2= 11y) (Area 3= 11y)	Random representatives from three districts of the city: Higienópolis (urban), Tubiacanga (suburban) and Palmares (suburban slum), differing in populational density, sanitation, vegetation cover and DENV history.	PANBIO Dengue IgM capture and Dengue IgG indirect ELISA	Honório et al., 2009
		2008	258	IgM = 9.3% IgG = 67.8%	Both genders Median Age: (Area 1= 42y) (Area2= 12y) (Area 3= 11y)			
		2012	494 (1)	IgG (1) = 88.7%	Both genders Age range: from 16 to 67 years	Blood donors of a study for determining the background rate of DENV and estimate IgG seroconversion: (1) February 15 to March 5 (outset) and (2) 8–14 June (end).	IgG ELISA Focus Diagnostics	Busch et al., 2016
			498 (2)	IgG(2) = 90.9%				
São Paulo	Ribeirão Preto	1992	662	5.4%	325 females and 337 males Age range: from 5 to 15 years	Participants randomly selected to investigate DENV distribution in different parts of the city due to outbreak in 1990.	IgG Enzyme immunoassay on infected cultured cells (EIA-ICC) using several flaviviruses strains	Figueiredo et al., 1995
	Santa Bárbara D'Oeste	1995	1,113	1.2%	Both genders and wide age range	Systematic random sampling of citizens five months after the end of the epidemics.	Haemagglutination inhibition	Lima et al., 1999
	Ribeirão Preto	1997	196	IgM = 21.4% IgG = 21.9%	Internals: 105, males only, aged 14-18 years Employees: 91 (24 females and 67 males) aged ≥ 20 years	Internals and employees from detention facility surrounded by a wide green area with low vegetation and small streams.	IgM MAC-ELISA and IgG ELISA using dengue serotypes: Hawaii (type 1) and TR-1751 (type 2)	Rodrigues et al., 2002
	Campinas	1998	1,260	14.8%	742 females and 514 males P/A: 127 (0-9y), 225 (10-19y), 192 (20-29y), 205 (30-39y), 178 (40-49y) and 327 (≥50y)	Participants randomly selected during a post-epidemic period.	EIA-ICC using Dengue serotypes 1 and 2 virus	Lima et al., 2007
	Jundaí	2010	876	IgG = 7.8% IgM = 7.3%	Not mentioned	Serological survey on January 2010 in the population of Novo Horizonte and São Camilo due to 2008 outbreak. The IgM prevalence was performed using the 68 IgG positive samples.	IgG ELISA	Arenas et al., 2012
	São José do Rio Preto	2015-2016	1,322	74.6%	801 females and 521 males P/A: 195 (10-19y), 457 (20-39y), 443 (40-59y) and 227 (60-91y)	A cohort with residents of Vila Toninho neighbourhood was enrolled from October 2015 to March 2016 (the cohort first wave).	Abcam IgG ELISA anti-DENV	Chiaravalloti-Neto et al., 2019
Minas Gerais	Belo Horizonte	2006-2007	709	11.9%	427 females and 284 males P/A: 227 (<15 or >60y) and 482 (15-60y) Mean age of 41.6 years	Inhabitants from different sanitary districts, the same investigated on serosurvey carried out in 1999/2000.	Seroneutralization for Dengue serotypes 1, 2 and 3 virus	Pessanha et al., 2010
SOUTH								
Rio Grande do Sul	Porto Alegre	2007	1,529	15.4%	Both genders Age range: from less than 1 to over 60 years	9,779 human samples and patients with clinically suspected DF were used to DENV surveillance diagnosis. This was the first time-series report on dengue epidemiology in the state. The global prevalence was 18.9%.	PANBIO Dengue IgM Capture and IgM MAC-ELISA	Tumioto et al., 2014
		2008	917	10.6%				
		2009	297	13.5%				
		2010	2,345	37.4%				
		2011	1,772	12.5%				
		2012	681	14.1%				
		2013	2,238	12.5%				
Paraná	Paranagua	2002	66	25.8%	Not mentioned	Cross-sectional study using serological survey carried out on residents from different localitons of the state's coast. The global prevalence was 18.8%.	PANBIO Dengue IgG Indirect ELISA	Mello, 2005
	Pontal do Paraná		77	11.7%				
	Morretes		38	10.5%				
	Guaraqueçaba		90	23.3%				

Table S3. Distribution of dengue virus serotypes in Brazilian states

Geographical regions and states	Serotypes confirmed[a,b]
North Region	
Acre (AC)	DENV-1
Amazonas (AM)[c]	DENV-1, DENV-2, DENV-3 and DENV-4
Amapá (AP)	DENV-1
Pará (PA)	DENV-1, DENV-2 and DENV-4
Rondônia (RO)	DENV-1 and DENV-4
Roraima (RR)	DENV-1, DENV-2, DENV-3 and DENV-4
Tocantins (TO)	DENV-1, DENV-2, DENV-3 and DENV-4
Northeast Region	
Alagoas (AL)	DENV-1 and DENV-4
Bahia (BA)	DENV-1 and DENV-4
Ceará (CE)	DENV-1, DENV-2, DENV-3 and DENV-4
Maranhão (MA)[d]	DENV-1, DENV-2, DENV-3 and DENV-4
Paraíba (PB)	DENV-1, DENV-2, DENV-3 and DENV-4
Pernambuco (PE)	DENV-1, DENV-2, DENV-3 and DENV-4
Piauí (PI)	DENV-1, DENV-2 and DENV-3
Rio Grande do Norte (RN)	DENV-1, DENV-2 and DENV-4
Sergipe (SE)	DENV-1 and DENV-4
Midwest Region	
Distrito Federal (DF)	DENV-1, DENV-2, DENV-3 and DENV-4
Goiás (GO)	DENV-1, DENV-2, DENV-3 and DENV-4
Mato Grosso (MT)[d,e]	DENV-1 and DENV-4
Southeast Region	
Espírito Santo (ES)	DENV-1 and DENV-4
Minas Gerais (MG)	DENV-1, DENV-2, DENV-3 and DENV-4
Rio de Janeiro (RJ)	DENV-1, DENV-2[d], DENV-3[f] and DENV-4
São Paulo (SP)	DENV-1, DENV-2, DENV-3 and DENV-4
South Region	
Paraná (PR)	DENV-1, DENV-2 and DENV-4
Rio Grande do Sul (RS)	DENV-1, DENV-2[e], DENV-3[e] and DENV-4
Santa Catarina (SC)	DENV-1

DENV serotypes reported in Brazil by the Ministry of Health. Data are organized by geographical regions and the states they include. [a]Data reported by Brazilian Ministry of Health until the epidemiological week 16-31/12/17 to 21/04/18. Source: http://portalsaude.saude.gov.br. [b]Data obtained in Fares et al. (2015) [6]. [c]Data obtained in Bastos et al. (2012) [28]. [d]DENV serotype (s) found only in year 2015. [e]DENV serotype (s) found only in year 2016. [f]DENV serotype (s) found only in year 2017.

Capítulo 3

ARTIGO 2 – NÃO PUBLICADO (DRAFT)

Antibody cross-reactivity and evidence for susceptibility against emerging Flaviviruses in dengue endemic Brazilian Amazon

Barbara Batista Salgado [1]*, Fábio Carmona de Jesus Maués[1]*, Maele Ferreira Jordão[1]*, Renato Lemos Pereira[1,2], Daniel A. Toledo-Teixeira[4], Pierina L. Parise[4], Fabiana Granja[4], Higo Fernando Santos Souza[3], Marcio Massao Yamamoto[3], Jannifer Oliveira Chiang[5], Livia Caricio Martins[5], Silvia Beatriz Boscardin[3], Pedro Fernando C Vasconcelos[5], Jaila Dias Borges Lalwani[6], José Luiz Proença-Modena[4], Pritesh Lalwani[1#]

[1]Leônidas e Maria Deane Institute (ILMD), Fiocruz Amazônia, Manaus, Amazonas, Brazil

[2]Centro de Instrução de Guerra na Selva (CIGS), Manaus, Amazonas, Brazil

[3]Biomedical Science Institute, University of São Paulo (USP), São Paulo, Brazil.

[4]Laboratory of Emerging Viruses, Department of Genetics, Microbiology and Immunology, Institute of Biology, University of Campinas, Campinas, São Paulo, Brazil.

[5]Evandro Chagas Institute (IEC), Arbovirology and Hemorrhagic Fever Sector, Ananindeua, Pará, Brazil.

[6]Pharmaceutical Science College (FCF), Federal University of Amazonas (UFAM), Manaus, Amazonas, Brazil

*Bárbara Batista Salgado, Fábio Carmona de Jesus Maués and Maele Ferreira Jordão contributed equally to this work

Corresponding author

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Contact Information: Instituto Leônidas e Maria Deane, Fiocruz Amazônia

Rua Terezina 476, Adrianópolis. Manaus - AM. CEP: 69.057-070.

Email: pritesh.lalwani@fiocruz.br

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Abstract

ZIKV is becoming increasingly prevalent in DENV-endemic regions, raising the possibility that pre-existing immunity to one virus could modulate the response to a heterologous virus, although whether this would be beneficial or detrimental is unclear.

Introduction

Emerging viruses is a growing health problem in Brazil and worldwide. Over the last decade, Brazil has endured zika virus (ZIKV), chikungunya virus (CHIKV) and yellow fever virus (UFV) epidemic. These pathogens have not only been associated with morbidity and mortality but also with additional healthcare cost. Arboviruses like DENV and YFV have been endemic in Brazil for several decades but not centuries. Recent studies demonstrate an increase in dengue-associated deaths in last decade. Yellow fever vaccine has been shown be efficient in preventing infections, recent YFV outbreak in Brazil demand better understanding of disease distribution in general population.

Additionally, introduction of ZIKV in Brazil caused huge epidemics in some regions of Brazil, which was associated with microcephaly in new-born and Guillen Barre syndrome in adults. New studies have been shedding light on the ever-growing pathologies associated with ZIKV infection. However, we still haven't understood the reasons for the proportion of ZIKV outbreak in Brazil. Several hypotheses have been considered but still little is understood. However, dengue endemic regions with YFV vaccination might have partially played a role in protecting individuals against ZIKV pathologies rather than participating in increasing ZIKV replication due to the presence of antigenically related immune memory.

In this study, we estimate seropositivity rates against nine different endemic or emerging *Flaviviruses* in Manaus capital of Amazonas state. Next, we assessed antibody cross-reactivity of DENV positive individuals with ZIKV using purified envelope antigens in ELISA, hemagglutination inhibition assay (HIA) and live-virus neutralization.

Methods

Ethics

This observational and cross-sectional study was approved by the Universidade Federal do Amazonas (UFAM) Research Ethics Committee, with approval number CAAE 96171218.7.0000.5020, in accordance with the Brazilian law, which complied with the Declaration of Helsinki. All the study participants gave oral and written informed consent prior to enrolment.

Study population and sample and data collection

The study population comprised of healthy individuals accessing services at the Centro de Controle de Zoonoses (CCZ), Manaus-Amazonas. Consecutive 450 random samples were collected between January 2015 and December 2015. The study included individuals of both sexes ≥18 years old who agreed to participate. Study participants signed the consent form and answered an epidemiological questionnaire. 4 mL of venous blood was drawn from each participant using EDTA tubes (BD Vacutainer). Soon after blood collection, collection tubes were centrifuged and plasma was separated and stored at -80 °C until further analysis.

Sample size calculation

A sample size of 398 individuals was calculated using an estimated prevalence of 50% and 95% confidence interval for a large population with a desired precision of 0.01 (Salgado, Maués et al. 2021). To avoid dropouts due to incomplete questionnaire and subsequent reduction in statistical power, we recruited 450 individuals. However, three questionnaires were incomplete and removed from analysis. Sample size calculation was performed using the Epi Info software version 7.2.

Maps and socioeconomic indicators from Brazil

Maps were created using the QGIS Software version 2.18.26 for macOS. Graphs displaying the Human Development Indexes and Sanitation Indicators for Brazilian capitals was plotted using public data available at Atlas Brasil from Census 2010 (https://atlasbrasil.org.br) and National System of Sanitation Information/Trata Brasil from 2018 (http://www.snis.gov.br), respectively.

Dengue and Zika cases in Manaus and Brazil

Supplementary Figure 3 compares DENV and ZIKV cases before and after the Zika epidemic in Manaus city and Brazil. Graphs were plotted using Dengue and Zika cases data by epidemiological week and year of First symptoms, between 2015-2017. These data are changeable and were available on Tabnet – DATASUS (Ministry of Health Brazil, https://datasus.saude.gov.br/informacoes-de-saude-tabnet/), and PLISA – PAHO/WHO (Health Information Platform for the Americas – Health Pan-American Organization, https://www3.paho.org/data/index.php/es/).

Dengue Rapid Test

Lateral flow Dengue virus antibodies detection test was performed using BioPix® Dengue IgM/IgG Rapid test in the Laboratory of Emerging Viruses at University of Campinas (LEVE - Unicamp). The serum samples were assayed to qualitatively detect IgG and IgM antibodies against all four Dengue virus serotypes. Assay had a sensitivity of 99% and specificity of 98%.

Hemagglutination inhibition test

To assess overall flavivirus reactivity, HIA was performed at Evandro Chagas Institute (IEC), Belém. Serum samples collected were subjected to a hemagglutination inhibition assay (HIA) and adapted to the microplate technique, with a titration cut-off point of 1/20 as previously described (Shope 1963). Plasma samples were tested for antibodies against the yellow fever vaccine strain (YFV-17D) and 10 arboviruses endemic or emerging in the Brazilian Amazon of the *Flavivirus* genus: Yellow Fever virus (YFV), Dengue Virus (DENV) serotypes 1 to 4 (DENV-1, DENV-2, DENV-3 and DENV-4), Zika virus (ZIKV), Saint Louis Encephalitis virus (SLEV), West Nile virus (WNV), Ilheus virus (ILHV) and Rocio virus (ROCV)].

For the HIA test, plasma samples were pretreated with acetone (P.A.) and NaCl (0.85%), hydrated with borate buffer saline (BBS) and adsorbed with goose (*Anser cinereus*) erythrocytes. In the screening step, they were added into a 96-well microtiter plate (“U” shape wells) together with a viral suspension containing four hemagglutinating unit (4 HAU) of the test antigen and a white goose erythrocyte suspension diluted in dextrose, gelatin, and barbital at 1:5 dilution (DGV). Initially, screening was performed at 1:20 dilution, positive samples were then serially diluted with bovine serum albumin (0.4%) up 1:1280 dilution.

Cloning, expression and purification of Dengue-2 and Zika virus recombinants envelope domain III proteins

The Dengue virus-2 envelope domain III protein sequence (E-DIII, residues 577-674, GenBank number HQ026763) was cloned and expressed using the pET28b vector [Silvia Beatriz Boscardin, University of São Paulo (USP)]. Zika virus envelope amino acid sequences from different isolates of Zika were obtained from GenBank and multiple sequence alignments was performed to identify an amino acid consensus sequence using MEGA software version 7.0.26. A polyhistidine (6x-His) tag was added at the N-terminal to facilitate downstream purification. Consensus ZIKV E-DIII sequence was codon-optimized and inserted into prokaryotic expression vector pGS21-a (GenOne Biotechnologies, Brazil). The recombinants vectors were transformed into electrocompetents BL21 (DE3) *Escherichia coli* cells, after incubation ampicillin-resistant colonies were grown at an optical density of 0.6-0.8 (600nm) at 30°C. The proteins expression was induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). The bacterial culture was harvested by centrifugation at 4,000 rpm for 20 minutes at 4°C. The pellets were resuspended in lysis buffer (lysozyme 1mg/mL and PMSF 100 mM) and lysed by ultrasonication; and debris were removed by centrifugation. The remaining sediment were subjected to another lysis round with lysis buffer, sonication and centrifugation. The bacterial lysate was resuspended in denaturing extraction buffer (NaH_2PO_4 100mM, Tris-HCl 10 mM, Urea 8M, pH 7,0), and debris were separated by centrifugation. Supernatants post-extraction containing E-DIII proteins were purified by metal affinity chromatography using an Ni-NTA column (HisTrap® HP column 1mL, GE Healthcare Life Science) coupled to an ÄKTA Purifier 10 system (GE healthcare) using a gradient; proteins were eluted in buffer containing NaH_2PO_4 100mM, Tris-HCl 20 mM, Urea 8M, 500 mM

imidazole, glycerol 10%, pH 7.0. Affinity purified proteins were then applied to Amicon® Ultra-2mL 3K Centrifugal Filters (MerckMillipore®) for desalting, diafiltration, concentration and buffer exchanging to phosphate buffer saline (PBS) pH 7.4, following the manufacture's recommendations.

SDS-PAGE and Western Blot analysis

The soluble fractions of purified DENV- or ZIKV-E-DIII proteins collected during the purification process were analyzed on an SDS-PAGE stained with Coomassie Blue. Western blotting (WB) was used to test antigenicity of the recombinant's proteins (Supp Fig 3). DENV or ZIKV-EDIII proteins were loaded onto a 12% polyacrylamide gels and transferred to a nitrocellulose membrane (0.2uM hybond ECL – GE Healthcare) by semi-dry electroblotting method. The transferred membranes were blocked with 5% skimmed milk in PBS for one hour at room temperature. Then, membranes were incubated for one hour at room temperature with anti-His tag mouse antibody (Sigma), or DENV or ZIKV positive and negative human samples at 1:1000 dilution. Membranes after incubation were washed three times with PBS-Tween 20 0.05% (PBS-T). Next, secondary anti-mouse or anti-human horseradish-labeled IgG antibodies at 1:1000 (KPL, Seracare, USA) were added and incubated for one hour at room temperature; after incubation of secondary antibody, excess unbound antibody was removed by washing membrane three times with PBS-T. The nitrocellulose membranes were then incubated with DAB (3,3',4,4' diaminobenzidine) (BioRad, EUA) plus hydrogen peroxide for 5 minutes, reaction was stopped by washing membrane with distilled water. Membranes were dried and protein band were then compared to protein molecular weight marker (Sigma).

ELISA to detect anti-DENV and ZIKV E-DIII-specific IgG antibodies

We standardized an indirect in-house Enzyme Linked Immunosorbent Assay (ELISA) to detect anti-DENV and anti-ZIKV IgG antibodies in plasma samples using recombinants E-DIII proteins as antigens. Checkerboard method was used to determine antigen concentration, sample dilution and secondary antibody concentration to obtain an optimal signal to noise ratio. To test patient samples 96-well ELISA plates (Immunoplate, SPL) were coated with 100 ng of DENV or ZIKV E-DIII protein in carbonate buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$ pH 9.6) at 4°C overnight and unbound proteins were removed by washing 5 times with PBS-T (0.5% Tween). Wells were then blocked with 10% non-fat dry milk in PBS for 90 minutes at room temperature and orbital shaking and plates were then washed 4 times with PBS-T. Plasma samples at 1:100 dilution were incubated at room temperature for 1 hour, followed by washing 5 times with PBS-T. Next, an anti-human horseradish peroxidase-labeled IgG antibody produced in goat (KP, Seracare, USA) at 1:10,000 concentration were added to each well, plates were incubated at room temperature and then washed 5 times with PBS-T. TMB (3,3′,5,5′-Tetramethylbenzidine) substrate (Thermo Fisher Scientific) was added to the antigen-antibody enzyme complex and incubated for 5 minutes at room temperature. Reaction was stopped adding 3M of sulfuric acid. The absorbance optical density was reading at 450 nm with a microplate reader (Hidex chameleon V microplate reader).

Zika virus neutralization test

Focus Reduction neutralization test (FRNT) was performed in collaboration with the Laboratory of Emerging Viruses at University of Campinas (LEVE – Unicamp). Serum samples were heated at 56°C for 30 minutes to inactivate the complement system. 4-fold plasma samples diluted with Earle minimum essential media (MEM) (Sigma-

Aldrich) were incubated with 100 focus forming units (FFU) of ZIKV (strain BeH823339-Asian). This virus plasma mixture was then added to monolayer of Vero-E6 cells (ATCC) cultivated in 96-well plates (Corning) and maintained at 37°C with CO2 5% for 2 hours. Semi-solid media (MEM, medium viscosity carboxymethylcellulose 0.75%, fetal bovine serum 5%, and penicillin and streptomycin 1%) was added to each well and plates incubated for another 48 hours at 37°C with CO_2 5%. 1% paraformaldehyde was added to the 96 well plates and fixed overnight at 4°C. Cells were next washed 3 times with PBS pH 7.4, and blocked and permeabilized by adding washing buffer (PBS 0.15M, bovine albumin serum 0.1%, Triton X-100 0.1%) and incubating for 30 minutes. Next, 1:1,000 anti-ZIKV NS1 antibody (Abcam) was added and plates were incubated for 2 hours at room temperature (20 to 25°C). Cells were subsequently washed and incubated with 1:2,000 anti-mouse IgG peroxidase-conjugated (Sigma-Aldrich) for 1 hour at room temperature. Cells were then washed and TrueBlue peroxidase substrate (KPL) was added following the manufacture's recommendations. FFU units were then counted for each plasma dilution and results tabulated.

Statistical Analysis

Descriptive statistics was used to describe the socio demographic features and mosquito preventive measures of the study population. Missing values were excluded from calculations. The bubble charts depicting the percentages of seropositives with HIA antibody titers was created Microsoft Excel 2019 software. All statistical analysis was performed using the Graphpad Prism software version 9.1.2. Paired T test was used to evaluated the ELISA results for DENV- and ZIKV-E-DIII. The linear correlation between variables was determined using the Pearson’s correlation. P values ≤0.05 were considered statistically significant.

Results

High Dengue Prevalence in Urban Manaus

A random convenience sampling strategy enrolled 450 individuals in this cross-sectional non-interventional study (Supplementary Figure 1). 447 healthy individuals of both sexes and ≥18 years were included in the final analysis; 3 questionnaires were incomplete and removed from the analysis. Demographic features of the study population are described in Table 1. We oversampled females (70.5%) and the median age of our study population was 38 years. 30% of the study participants auto declared that they were unemployed in last 12 months and 61.70% had completed high school (Table 1). 37.5% and 13.2 study participants reported a previous dengue and malaria infection, respectively. 80.8% reported vaccination for YFV and 75.9% use SUS-Brazil's public health system. A majority (94.9%) reported performing at least one preventive measure against mosquitos, avoiding standing water and disposing trash correctly followed by personal mosquito repellents were the most popular measures adopted to prevent mosquitoes (Table 2).

Manaus is a tropical metropolis of Brazil with about 2.5 million inhabitants in the center of the Amazon rainforest (Figure 1A). Manaus capital of the Amazonas state when compared to other Brazilian capitals demonstrates a low sociodemographic and human development index (HDI). In Manaus, the lack of sanitation is also widespread with wastewater facilitating vector proliferation and water-borne diseases, which affect the poorest (Figure 1B). Figure 1C compares our study population age distribution compared to the 2010 census data.

Next, we assessed prevalence of anti-DENV antibodies by lateral flow point of care (POC) tests and hemagglutination inhibition assay (HIA). 74.52% of participants were IgG positive (310/416) and 1.68% (15/416) (data not shown) had anti-DENV IgM

antibodies as estimated by POC tests. Overall, 93.7% of participants were seropositive (419/447) for at least one DENV serotype and the DENV seropositivity ranged between 84.8 and 91.0% as determined by HIA (Figure 1D). We observed a majority of our participants above 20 years of age had anti-DENV antibodies (Figure 1E-G and supplementary Figure 2).

Cross-reactivity between principal endemic and emerging *Flaviviruses*

We performed HIA a cell-based assay to assess the overall cross-reactivity by performing serial dilution of plasma samples; results are summarized in Table 3 and Figure 2. YFV is endemic in the Amazon region and vaccination is compulsory, here we observed 92.8% has anti-YFV-17D reactive antibodies compared to 80.5% towards the wild-type YFV (Table 3). Whereas, a total of 80.3% self-reported vaccinated for YFV (Table 2). 37.2% self-reported previous DENV infection, whereas we detected 93.7% individuals with anti-DENV antibodies towards at least one serotype. A chi-square test found a significant difference between the observed (laboratory confirmed) and expected (self-reported) dengue infection ($p<0.0001$, $\chi 2$ test, data not shown). Among the DENV serotypes DENV-2 followed by DENV-1, DENV-3 and DENV-4 had the highest seropositivity (Figure 2 and Table 3). Individuals with high titers of DENV correspondingly had higher reactivity towards other *Flaviruses* (Figure 2A). All samples were collected before or during the ZIKV epidemic in Manaus and we could observe ZIKV among all the viruses tested had the lowest seropositivity percentage (Figure 2B). Percentage cross-reactivity for other emerging viruses classified in serogroups is presented in Table 3. Overall, antibody reactivity was the lowest among the 18-19 age group and increasing rapidly with age for all tested arboviruses (Table 3, Figure 2 and supplementary Figure 2).

Low cross-reactivity between pre-existing DENV antibodies and Zika virus

Next, we evaluated cross-reactivity towards ZIKV among DENV positive individuals using HIA, ELISA and live virus neutralization assay. Firstly, modified bubble plots compared DENV-1, DENV-2 or ZIKV HIA titers and their distribution for each virus compared to the reference virus. Upon comparing HIA titers, we observed that a majority of the individuals positive for DENV with low titers failed to react with ZIKV. Whereas, only individuals with elevated titers for DENV-1 or DENV-2 reacted with ZIKV in the HIA assay (Figure 3).

Envelope domain III (E-DIII) is an important target for neutralizing antibodies among *Flaviviruses*, hence we performed ELISA using E-DIII proteins for DENV-2 or ZIKV and assessed antibody reactivities. Similarly to the HIA, we observed anti-DENV2 E-DIII IgG levels were elevated compared to ZIKV antibodies (Fig 4A). A paired analysis of the samples showed a significant decrease in the antibody reactivities between DENV and ZIKV (Fig 4B-C). As expected, anti-DENV E-DIII antibody levels estimated by ELISA significantly increased with age (Fig 4D); and stratified by sex, males showed highest reactivity towards DENV-2 EDIII (Fig 4E). A positive correlation between HIA antibody titers and ELISA reactivity was observed for DENV and ZIKV (Fig 4F).

Further, to understand the high DENV antibody reactivity and relation to a potential neutralizing antibody response towards the ZIKV, we performed a Focus Reduction Neutralization Test (FRNT) with live ZIKV with a small subset (n=57) of DENV positive samples chosen at random. Figure 5A compares DENV-2 or ZIKV ED-III ELISA results with ZIKV $FRNT_{50}$ values; samples were divided as higher or lower than median of reactivity towards DENV-2 ED-III antigen. Primarily, we observed that individuals with anti-DENV E-DIII OD > Median value (red) neutralized ZIKV more efficiently compared

to individuals with OD < median values (green). Figure B-D represents the neutralizing activity for each patient. Overall, DENV-2 E-DIII antibody levels are inversely proportional to ZIKV FRNT values and high DENV reactivity had potential neutralizing activity against ZIKV. Collectively, these results indicate that both the quantity and quality of DENV specific response is necessary to neutralize ZIKV.

These findings reinforce the elevated dengue prevalence in the Amazon region and cross-reactivity between flaviviruses. *Flavivirus* is a genus with highly related species. Since Manaus is endemic to dengue and presence of high mosquito density may have influenced the zika virus epidemic (Supplementary Figure 1). Here we observed low protection in DENV positive cases with ZIKV virus. Our results suggest that, individuals with high neutralizing titers against DENV could partially neutralize ZIKV. Further, cross-reactivity data may be important to understand the ZIKV epidemic evolution and also for future *Flavivirus* vaccination and disease control strategies.

Discussion

Endemic and emerging arboviruses are a huge public health concern worldwide not only due to their unexpected clinical manifestations and potential complications but also due to the lack of appropriate diagnostic assays and vaccines (Mota, Terzian et al. 2016, Musso and Desprès 2020, Pierson and Diamond 2020). In this study, we report one of the highest dengue seropositivity in Manaus-Brazil (Salgado, Maués et al. 2021); 74.52% by POC and 93.7% by HIA. This suggest that most residents of the region have likely been infected with DENV, if not with other arbovirus at least once. Evidence of varying degree of antibody cross-reactivity with principal emerging flaviviruses depends on the virus serogroup and pre-existing level of antibody titers. Thus, individuals with the highest DENV titers could strongly cross-neutralize ZIKV. Elevated dengue prevalence in Manaus and the Amazon region does not undermine the threat towards other arboviruses.

Dengue is endemic in Brazil and without adequate prevention and treatment, the number of reported cases of dengue has increased in Brazil and the Americas (Salles, da Encarnação Sá-Guimarães et al. 2018, Nunes, Daumas et al. 2019). In our previous study, we observed increasing seroprevalence in several Brazilian cities in the last decades (Salgado, Maués et al. 2021). On the contrary, dengue incidence levels have been the same over the last decade in the most regions; nevertheless, there has been a huge increase in severe dengue disease cases and deaths (Teixeira, João Bosco Siqueira et al. 2013, Fares, Souza et al. 2015, Salles, da Encarnação Sá-Guimarães et al. 2018, Nunes, Daumas et al. 2019). Three out of four Brazilian municipalities are heavily infested with the mosquito *Ae. aegypti*, which highlights the staggering epidemiological and economic burden in the endemic regions (Tapia-Conyer, Tapia-Conyer et al. 2012, Mota, Terzian et al. 2016). Thus, the prospects of controlling

dengue or other arbovirus transmitted disease are not promising. All four DENV serotypes have been detected in Manaus, with the late introduction of DENV-3 and DENV-4 (de Souza Bastos, Itapirema et al. 2012, Nava, Shimabukuro et al. 2017, Salgado, Maués et al. 2021). Our serological results demonstrate a low seropositivity towards DENV-3 and DENV-4 compared to DENV-2 and DENV-1. High DENV prevalence in urban Manaus observed in this study is in line with our previous observations from other Brazilian cities (Salgado, Maués et al. 2021). 37.2% of individuals self-reported a previous DENV infection compared to over 75% individuals with detectable antibodies in this study. This disparity between the observed and expected numbers highlight the need for more laboratory diagnosis of febrile cases.

Yellow fever vaccination is compulsory in Manaus and the Amazon region and 92.8% of the study participants had anti-YFV/17D reactive antibodies, whereas, 80.5% had anti-YFV reactive antibodies against the wild type virus. ILHV and ROCV had been endemic in Brazil, however their spread in the Amazon region and prevalence studies are lacking. In this present study, Ntaya virus group represented by ILHV and ROCV had a seropositivity rate of 85.7% and 75.8%, respectively. Recently, WNV (Martins, Silva et al. 2019, Costa, Giovanetti et al. 2021) and SLEV (Weaver, Rosa et al. 2013, Silva, Romeiro et al. 2014) cases have been reported among horses in Brazil but no human outbreaks cases have been reported. Co-circulation of these arboviruses which share B and T cell epitopes preludes the understanding of the role of pre-existing immunity essential to assess the clinical implications of the disease. Infection with DENV can protect against subsequent JEV, SLEV and WNV infections (Sather and Hammon 1970, Price and Thind 1971, Tarr and Hammon 1974), ZIKV infection confers protection against subsequent WNV infection (Vazquez-Calvo, Blazquez et al. 2017), JEV and SLEV infections can protect against WNV and DENV infections (Goverdhan,

Kulkarni et al. 1992, Tesh, Travassos da Rosa et al. 2002, Petrovsky, Larena et al. 2013, Li, Gao et al. 2016), and ILHV and SLEV can elicit cross-protection against a lethal ROCV challenge (Mossel, Amarilla et al. 2018). Compulsory YFV vaccination and high DENV prevalence in Manaus may partially explain the lack of evidence for co-circulation of other flaviviruses. This cross-protection may partly also explain why YFV is not present in India which is endemic to WNV, JEV and DENV. On the other hand, recent ZIKV outbreak in India demonstrate that cross-protection might be temporary and viruses may adapt to the epidemiological conditions (TOI 2021).

Samples used in this study were collected before or during the zika virus outbreak in Brazil. In this present study, 52.6% had anti-ZIKV antibodies, the lowest seropositivity rate among all the viruses tested by HIA. Also, ED-III ELISA demonstrated a weak reactivity towards ZIKV among DENV positive individuals. Thus, a substantial proportion of the participants showed evidence of exposure to DENV but not ZIKV (Hobman, Langerak et al. 2019). Moreover, DENV positive individuals with low HIA titers failed to react with ZIKV and only high titer DENV plasma samples react with ZIKV. Role of DENV antibodies in ADE has been controversial and there are arguments which support or deny its role in disease pathology. Patterns of antibody cross-neutralization suggest that ZIKV lies outside the DENV serocomplex (Montoya, Collins et al. 2018), similarly we observed that HIA was able to distinguish ZIKV from DENV infections when all viruses were analyzed simultaneously. We observed a lack of durable cross-neutralizing antibody response against ZIKV from DENV positive individuals as previously reported (Collins, McGowan et al. 2017). In this study, patients with highest DENV titers neutralized ZIKV more efficiently, in line with previous report demonstrating that individuals with elevated DENV titers due to repeated

exposure to dengue virus elicits robust cross neutralizing antibodies against Zika virus (Hattakam, Elong Ngono et al. 2021).

In Manaus a high prevalence of DENV serotypes and YFV vaccination together may provide a strong increase in homologous and heterologous cross-neutralizing antibodies. Zika vaccination in a DENV-experienced individual have been shown to boost pre-existing flavivirus immunity and elicit protective responses against both ZIKV and DENV (Dussupt, Sankhala et al. 2020). On the other hand, Tick borne encephalitis virus (TBE) vaccination in YFV vaccinated individuals caused a significant reduction in the TBE-specific neutralizing antibodies (Bradt, Malafa et al. 2019). T-cell responses seems to be robust between *flaviviruses* and T cell responses may, at least in part, explain the cross-protection seen against ZIKV from DENV infection (Subramaniam, Lant et al. 2020). Nevertheless, the durability of humoral and cellular response needs to be assessed in longitudinal studies to determine the role of the pre-existing immunity in protection or pathology.

Due to a large number of positive samples, we could not apply statistical test to understand epidemiological risk factors. Our convenience sampling does not allow to calculate the true dengue prevalence; however, our results demonstrates that urban Manaus DENV prevalence is high, majority of the individuals get infected before attaining thirty-years and male-sex had higher prevalence. We used DENV-2 ED-III protein in ELISA to compare with ZIKV ED-III response and did not test with other dengue serotypes, nevertheless, we believe that a similar relationship will be observed given the HIA results and since DENV and ZIKV belong to distinct serogroups. Although DENV neutralizing serotype-specific antibodies are mainly against E-DIII, there might be a sustainable antibody response against other targets such as domains I/II of the envelope protein, which were not evaluated in this study. We also did not

have history of hospitalization or severe disease caused by dengue among our study participants to correlate with the humoral response.

In conclusion, we observed a low cross-protection among dengue positive individuals to zika virus. However, further studies are needed on ADCC and the possible role of cross-reactive dengue virus antibodies in Zika virus pathogenesis. ZIKV and other emerging flaviviruses will become increasingly prevalent in DENV-endemic regions, raising the possibility that pre-existing immunity to one virus could modulate the response to a heterologous virus, although whether this would be beneficial or detrimental is unclear. COVID-19 pandemic has negatively affected mosquito control measures, additionally, financial resources have been diverted toward the pandemic which makes the control measures more difficult. Presently, Brazil and Amazonian region faces a complex epidemiological scenario characterized by simultaneous circulation of several arboviruses, hence a functional vector control program and febrile-syndrome surveillance is essential to identify unforeseen epidemiological threats.

Tables

Table 1 Socio demographic features of the study population-Manaus, Amazonas

Table 2 Mosquito prevention practices reported by study participants

Table 3 Prevalence of anti-*Flavivirus* antibodies

Figure Legends

Figure 1: Elevated Dengue seropositivity rates in Urban Manaus

(A) Maps depict location of Manaus. (B) Human Development Index (left) and Sanitation indicators (right) for the 27 Brazilian capitals was obtained from the 2010 census and a 2018 survey, respectively. (C) Study population distribution is compared to 2010 Census data obtained from the Brazilian Institute of Geography and Statistics (IBGE). (D-G) Plasma samples were tested for anti-Dengue virus antibodies using BioPix® Dengue Rapid Test IgG/IgM (n=416) and Hemagglutination Inhibition Assay (HIA, n=447) and seropositivity percentages with 95%CI were plotted. BioPix® Dengue Rapid Test IgG/IgM was qualitative. HIA was quantitative and samples with HIA titer ≥ 20 units were considered positive for the test virus.

Figure 2: Antibody reactivity profile against human emergent Flavivirus. Samples (n=447) were tested for antibodies against ten different endemic and emerging Flaviviruses and yellow fever vaccine strain using the Hemagglutination inhibition assay (HIA). (A) Colors depict the HIA titers, each column represents one patient and each row is one test virus in the heat map. Samples described in the heatmap were sorted as per DENV-2 HIA titers. (B) Samples with HIA titer ≥ 20 units were considered positive and plotted as percentage positives among the study age groups. (C) HIA titers were compared to participant age. Solid lines are splines for each test virus.

Figure 3: Low antibody cross-reactivity between Dengue with Zika virus.

Modified bubble chart compares cross-reactivity (n=447), (A) DENV-1 with DENV-2 or ZIKV and (B) DENV-2 with DENV-1 or ZIKV. All samples with HIA titer ≥ 20 units were considered positive. Bubble size is proportional to the percentage of individuals with

the corresponding HIA titer and each column adds up to 100%. HIA titers are color coded for the first reference virus column for comparison. Each pie-chart represents the composition of HIA titer for the reference test virus.

Figure 4: Reactivity between Dengue virus and Zika virus envelope domain III protein.

ELISA plates were coated with DENV-2 or ZIKV envelope domain III (E-DIII) proteins to detected IgG antibodies in human plasma samples (n=416). (A) Range of antibody reactivity towards EDIII antigens is depicted. Red horizontal line denotes median optical density (OD, 450nm) values. (B) Pair-wise comparison denotes reactivity towards DENV-2 and ZIKV. Mann Whitney or paired T test, ****p <0.0001. (C) DENV-2 and ZIKV anti-E-DIII antibody levels measured by ELISA were compared, red line in the graph represents the spline fitting for the distribution. (D) DENV-2 or ZIKV E-DIII reactivity was compared to participant (D) age or (E) sex. Solid lines represent Splines fit for the antibody distribution. Pearson's correlation and p values are denoted in the graphs. (F) HIA tiers for dengue virus serotypes is compared between DENV-2 or ZIKV E-DIII reactivity determined by ELISA. Each solid line is color coded and represents one test virus. Each dot represents one sample in all the graphs.

Figure 5: High titers of Dengue virus reactive antibodies neutralize Zika virus.

Focus Reduction Neutralization Test (FRNT) for Zika virus was performed to estimate neutralizing antibodies in patient plasma samples (n=57). (A) DENV-2 or ZIKV E-DIII protein specific IgG reactivity is depicted in first two columns. Box and whiskers indicate the median lines, 25 and 75- interquartile and min-to-max points. Red dots denote samples with anti-DENV-2 E-DIII reactivity values greater than the median

value and green dots denotes samples displaying anti-DENV-2 reactivity less than median value. Third column in the graph represents the 50-percent FRNT ($FRNT_{50}$) values for Zika virus. Right Y-axis is $FRNT_{50}$ values in log for Zika virus. Zika virus antibody neutralization dose-response curves were plotted with (B) high (OD_{450} > median) or (C) low (OD_{450} < median) DENV-2 E-DIII antibody reactivity. Each curve represents one patient sample diluted serially. Anti-ZIKV NS1 monoclonal antibody was used as control. (D) Representative neutralization dose-response curves were plotted based on DENV-2 E-DIII antibody reactivity.

Supplementary Figures

Supplementary Figure 1: Dengue and Zika cases evolution through and post-Zika virus epidemic in Manaus city and Brazil.

Dengue and Zika cases per epidemiological week between 2015-2017 in Manaus county (A) and Brazil (B) were obtained from the Ministry of Health (DATASUS - https://datasus.saude.gov.br/informacoes-de-saude-tabnet/) and Health Pan-American Organization (PLISA - https://www3.paho.org/data/index.php/es/), date accessed June 30, 2021. Grey area depicts the study population enrollment. ZIKV epidemic-related events depicted (text inserts) during the ZIKV epidemic were collated from epidemiological bulletins using Brazilian Health Ministry website (https://www.gov.br/saude/pt-br/assuntos/boletins-epidemiologicos) and from timeline of ZIKV emergence using Pan American Health Organization website (https://www3.paho.org/hq/index.php?option=com_content&view=article&id=11959:timeline-of-emergence-of-zika-virus-in-the-americas&Itemid=41711&lang=en) , date accessed June 30, 2021).

Supplementary Figure 2: Dengue-reactive antibody distribution in the Amazonian population.

Bubble chart is used to represent HIA dengue positive samples as function of antibody titers and age groups. Bubbles show the column percentages for each age group and their sizes are proportional to their values. All samples were considered positive with HIA titer ≥ 20 units.

Supplementary Figure 3: Characterization of Dengue-2 and Zika rE-DIII protein.

Codon optimized-consensus ZIKV domain III-envelope (E-DIII) sequence, containing a polyhistidine (6x-His) tag at the N terminus, was expressed in *Escherichia coli* strain BL21, purified by affinity and dialysed as previously described. Western blotting (WB) was used to test the antigenicity of the recombinant protein. A total of 1 μg of ZIKV E-DIII were loaded onto 12% polyacrylamide gels and transferred to a nitrocellulose membrane using standard procedures. The membranes were blocked with 5% skimmed milk in phosphate buffer saline (PBS) for 1 hour at room temperature (RT). Blotted membranes were incubated for one hour at RT with 1:1000 dilution with 5% milk in PBST (PBS + 0.05% Tween-20) of primary antibodies, which were ZIKV positive plasma, Flavivirus negative plasma sample pool and DENV positive plasma. Next, membranes were washed three times with PBST then incubated with goat anti-human IgG HRP-labelled (0.1μg/mL KPL, Seracare, USA) for one hour at RT. The membranes were again washed three times with PBST and the proteins were visualized using an enzymatic reaction by adding DAB (3,3',4,4' diaminobenzidine) (BioRad, EUA) and H_2O_2 mixture for 5 minutes. Reaction was blocked by washing membrane five-times with distilled water and membranes were dried and protein band were then compared to protein molecular weight marker (Sigma).

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Table 1. Socio-demographic features of study population- Manaus, Amazonas

	Characteristic	Value %
Age (years)	18 - 19	7.80
	20 - 29	23.10
	30 - 39	24.70
	40 - 49	23.60
	≥ 50	20.80
median (IQRs) range	38.0 (28.0 – 49.5) 18 – 77	
Sex	Female	70.50
Family size	1	3.50
	2	14.80
	3	24.20
	4	25.60
	≥ 5	31.90
Number of children in house	0	52.90
	1	29.00
	2	11.30
	3	3.90
	≥4	2.90
Occupation[1]	Unemployed	31.40
	Domestic service workers and manual workers	14.00
	Professionals with university education	10.60
	Professionals or technicians	10.10
	Administrative service workers	10.10
	Poorly specified occupations of informal work	9.60
	Service and commercial workers	8.70
	Member of the armed forces, police and military firefighters	2.50
	Senior government officials or senior corporate officials	1.60
	Art Professional	1.40
Family Income	Minimum wage	22.60
	Two minimum wages	29.80
	Three minimum wages	20.50
	Four minimum wages or more	27.10
Education level	Illiterate	1.30
	Literate	7.40
	Elementary school	9.80
	High school	61.70
	Graduate	19.80

[1] Last 12 months

Table 2. Mosquito prevention practices reported by study participants

Characteristic		Value %
Dengue infection [1]	Yes	37.5
Malaria infection [1]	Yes	13.2
Vaccinated against Yellow fever [1]	Yes	80.8
Use Sistema Único de Saúde (SUS) [2]	Yes	75.9
Neighbours with dengue fever	Yes	43.3
Mosquito problem in neighbourhood	Yes	43.1
Perform preventive measures against mosquitos	Yes	94.9
Mosquito prevention measures [3]:		
	Avoid standing water	64.30
	Disposing trash correctly	62.50
	Personal mosquito repellents: cream and spray	54.90
	Personal and household hygiene	33.70
	Fans	28.10
	Close windows and doors	21.80
	Mosquito coil, mats and liquid vaporisers	12.60
	Mosquito net for door and window	10.90
	Protective clothing	9.40
	Mosquito bed nets	5.80
	Others	4.30

[1] Self-reported, [2] Brazilian public funded health care system, [3] Multiple responses

Table 3. Prevalence of anti-Flavivirus antibodies

Serogroup	Virus*	Total# %	Age (years)				
			18-19	20 - 29	30 - 39	40 - 49	≥ 50
Yellow Fever Vaccine	YFV/17D	92.8 (415/447)	67.6 (23/34)	98.0 (99/101)	95.4 (103/108)	93.2 (96/103)	92.3 (84/91)
Yellow Fever Virus Group	YFV	80.5 (360/447)	61.8 (21/34)	77.2 (78/101)	83.3 (90/108)	79.6 (82/103)	86.8 (79/91)
Dengue Virus Group	DENV@	93.7 (419/447)	79.4 (27/34)	95.0 (96/101)	95.4 (103/108)	94.2 (97/103)	95.6 (87/91)
	DENV-1	90.6 (405/447)	76.5 (26/34)	93.1 (94/101)	91.7 (99/108)	88.3 (91/103)	93.4 (85/91)
	DENV-2	91.0 (407/447)	82.3 (28/34)	93.1 (94/101)	92.6 (100/108)	88.3 (91/103)	92.3 (84/91)
	DENV-3	85.5 (382/447)	82.3 (28/34)	86.1 (87/101)	87.0 (94/108)	84.5 (87/103)	83.5 (76/91)
	DENV-4	84.8 (379/447)	64.7 (22/34)	87.1 (88/101)	88.0 (95/108)	84.5 (87/103)	84.6 (77/91)
Spondweni Group	ZIKV	52.6 (235/447)	32.3 (11/34)	48.5 (49/101)	55.5 (60/108)	54.4 (56/103)	59.3 (54/91)
Japanese Encephalitis Virus Group	WNV	81.9 (366/447)	55.9 (19/34)	84.2 (85/101)	88.0 (95/108)	78.6 (81/103)	84.6 (77/91)
	SLEV	86.3 (386/447)	64.7 (22/34)	87.1 (88/101)	89.8 (97/108)	84.5 (87/103)	90.1 (82/91)
Ntaya Virus Group	ILHV	85.7 (383/447)	64.7 (22/34)	85.1 (86/101)	90.7 (98/108)	83.5 (86/103)	89.0 (81/91)
	ROCV	75.8 (339/447)	70.6 (24/34)	74.3 (75/101)	72.2 (78/108)	79.6 (82/103)	78.0 (71/91)
Negatives		3.4 (15/447)	11.8 (4/34)	4.0 (4/101)	0.9 (1/108)	3.9 (4/103)	2.2 (2/91)

Hemagglutination Inhibition (HI) titre ≥ 1:20

@ Positive for at least one DENV serotype

* *Flaviviridae* (Flavivirus genus): Yellow Fever virus (YFV), Dengue Virus (DENV) serotypes 1 to 4 (DENV-1, DENV-2, DENV-3 and DENV-4), Zika virus (ZIKV), West Nile virus (WNV), Saint Louis Encephalitis virus (SLEV), Ilheus virus (ILHV), Rocio virus (ROCV).

Figure 1. Elevated Dengue seropositivity rates in Urban Manaus.

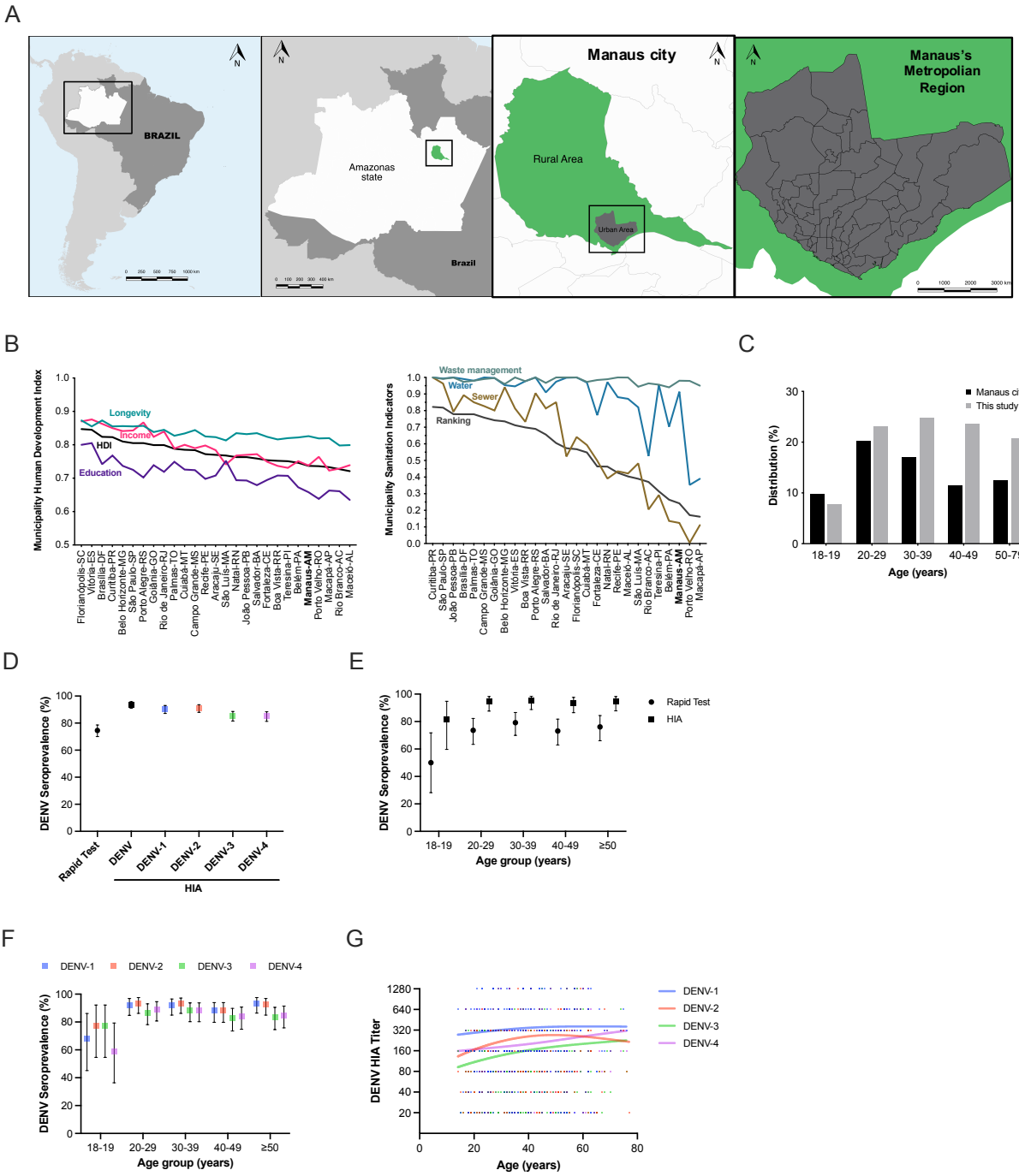


Figure 2. Antibody reactivity profile against human emergent Flavivirus.

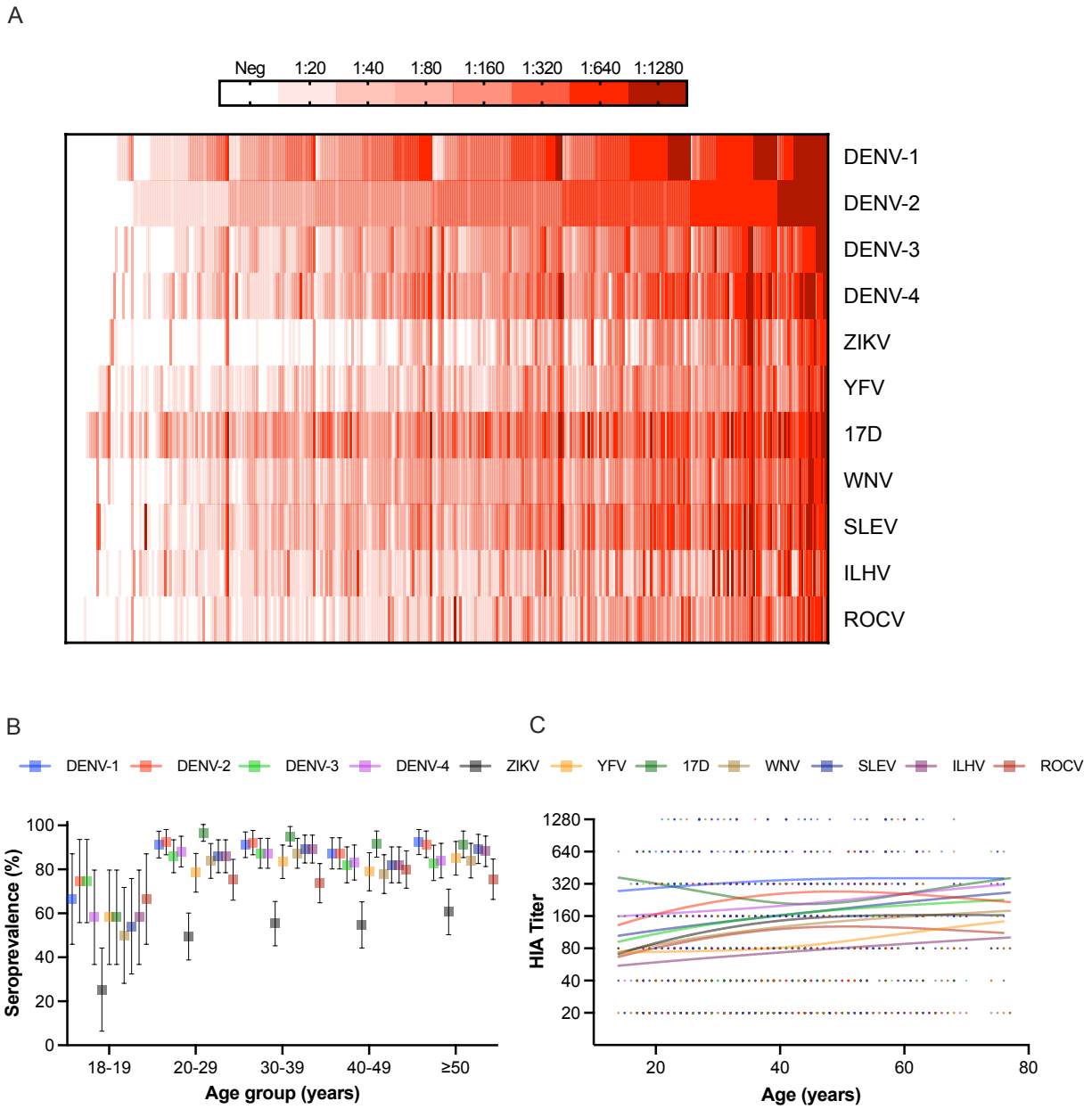


Figure 3. Low antibody cross-reactivity between Dengue with Zika virus .

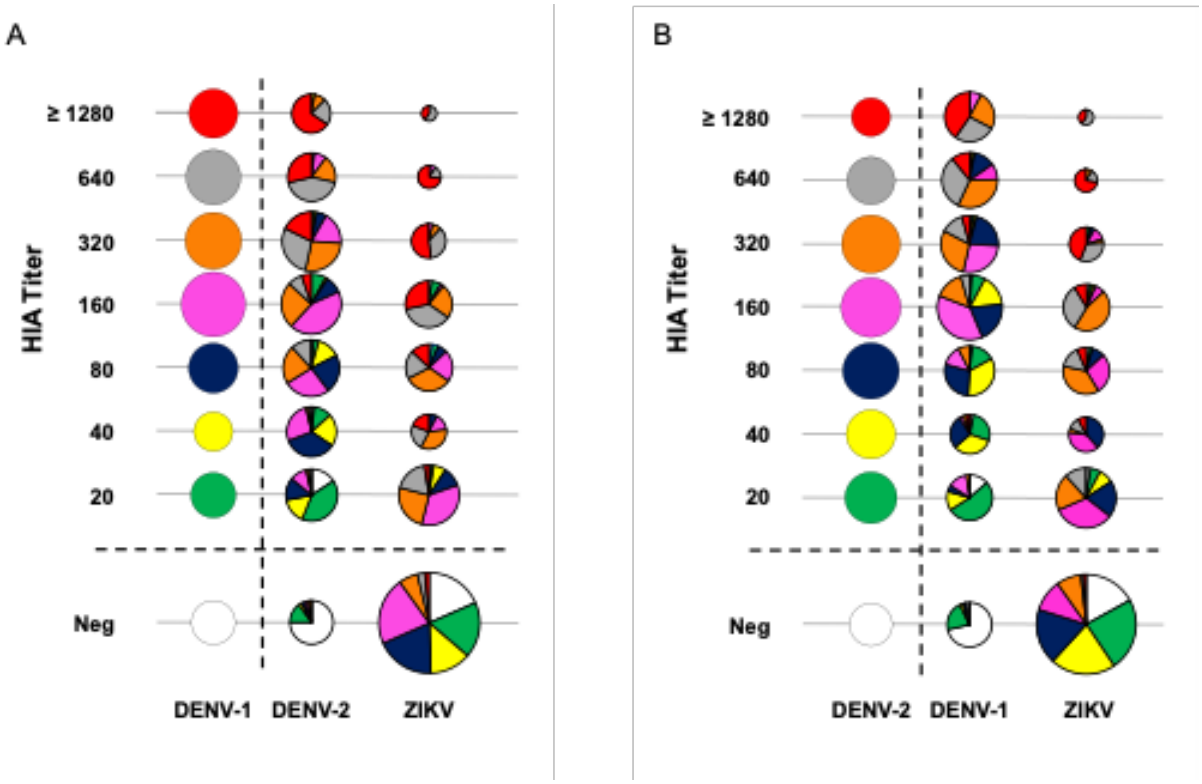


Figure 4. Reactivity between Dengue virus and Zika virus envelope domain III protein.

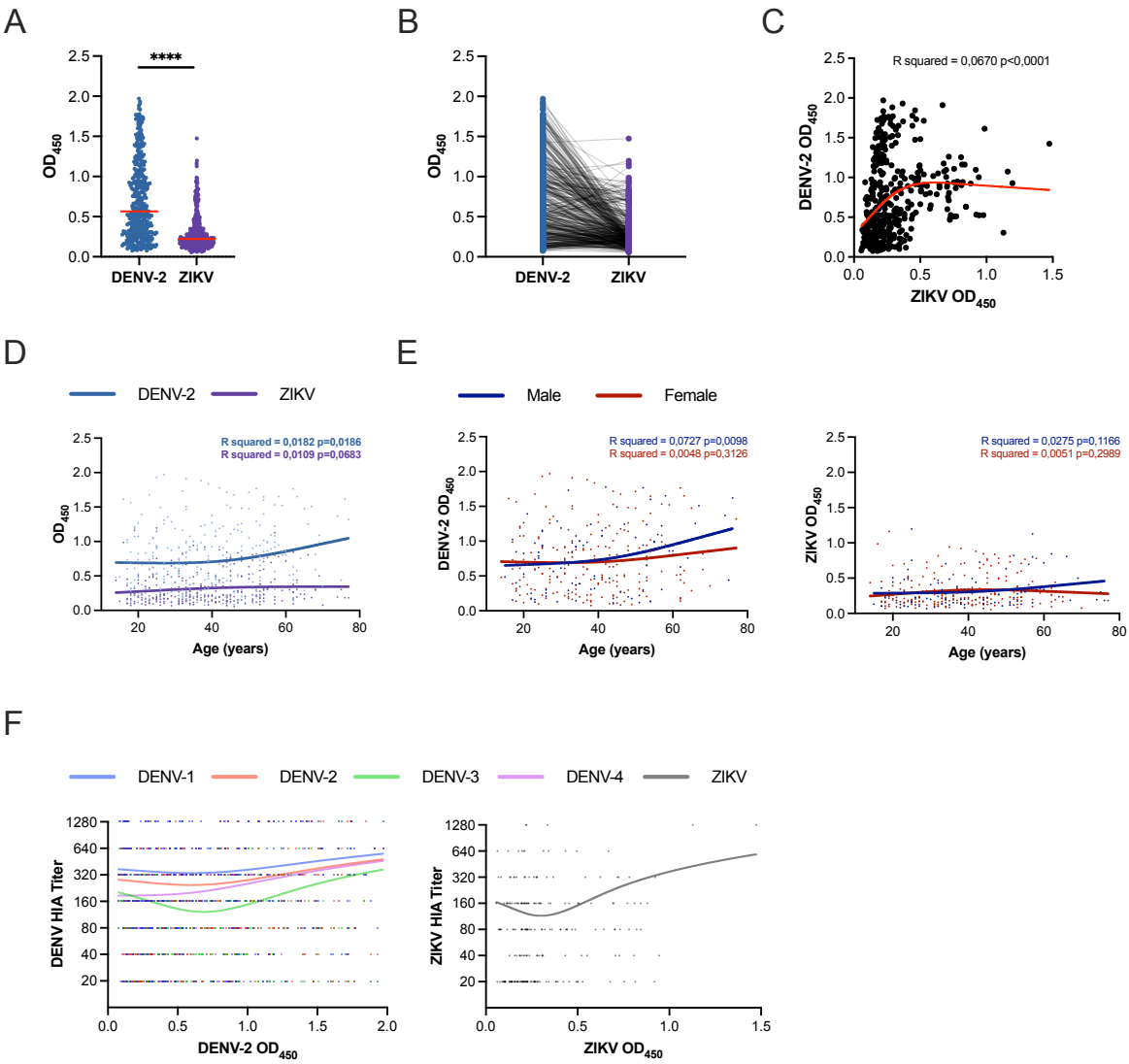
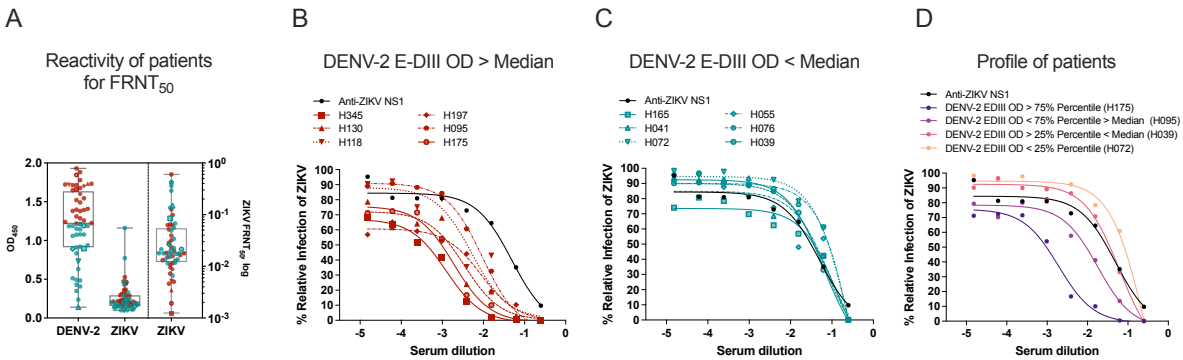
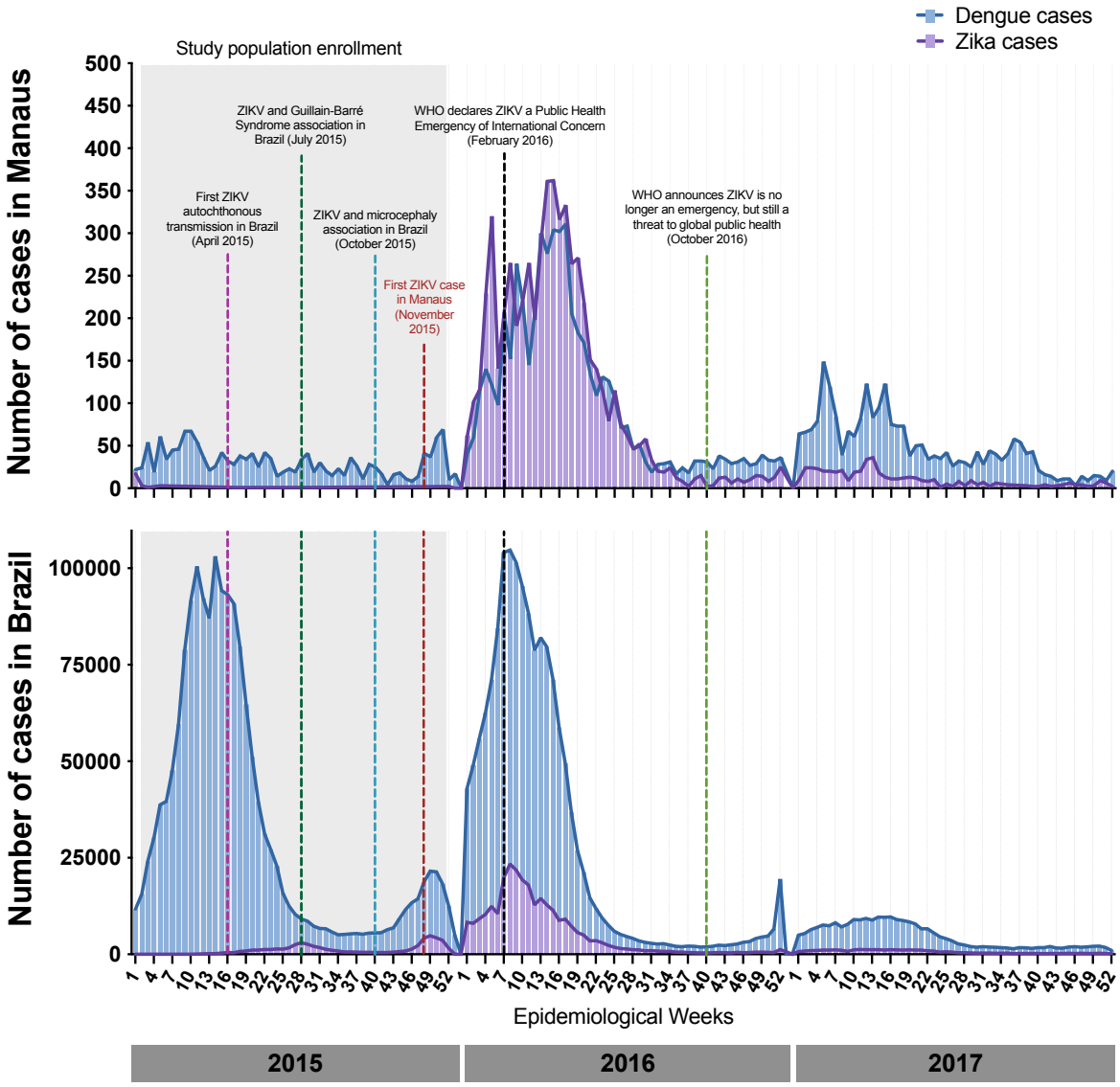


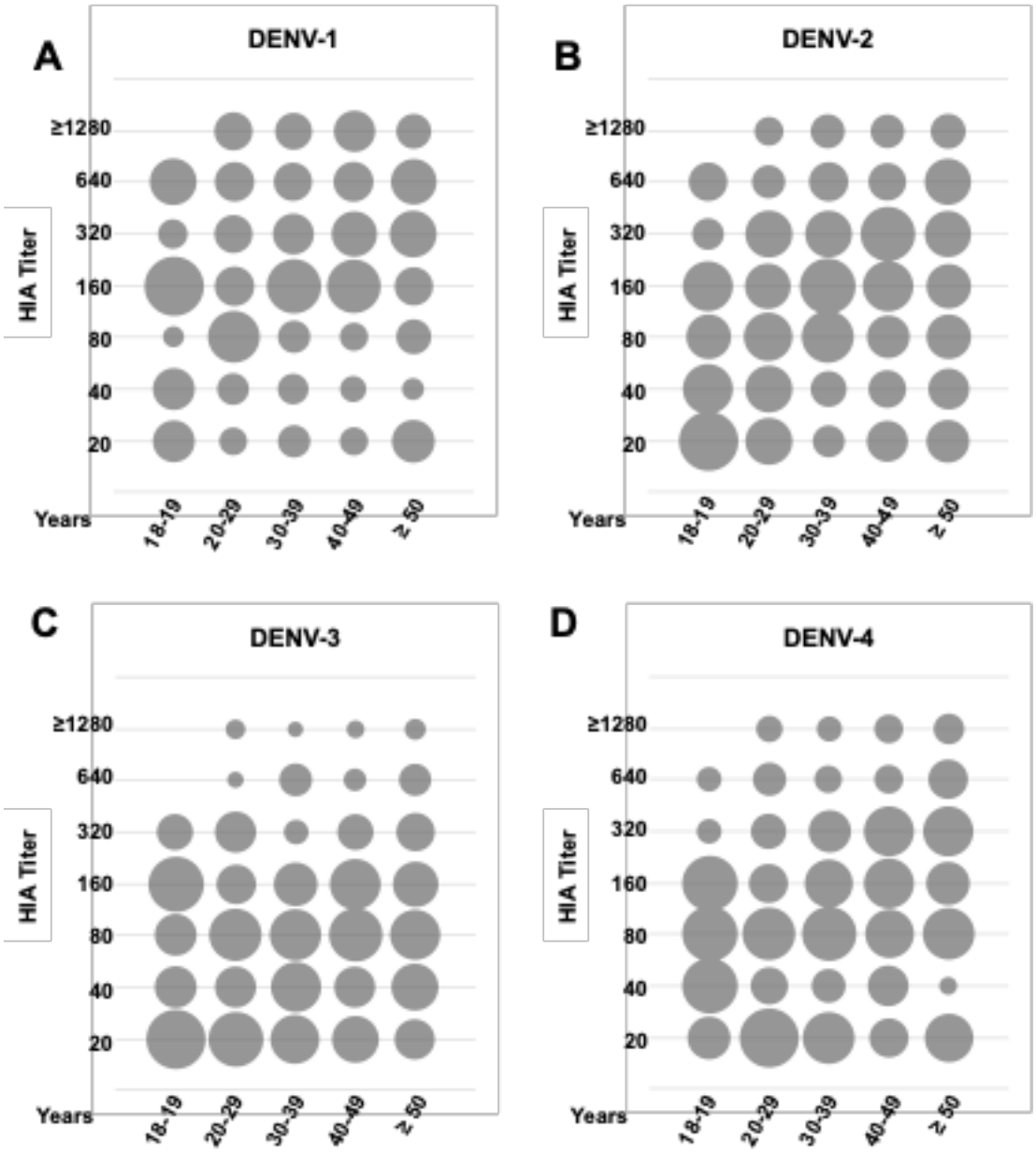
Figure 5. High titers of Dengue virus reactive antibodies neutralize Zika virus.



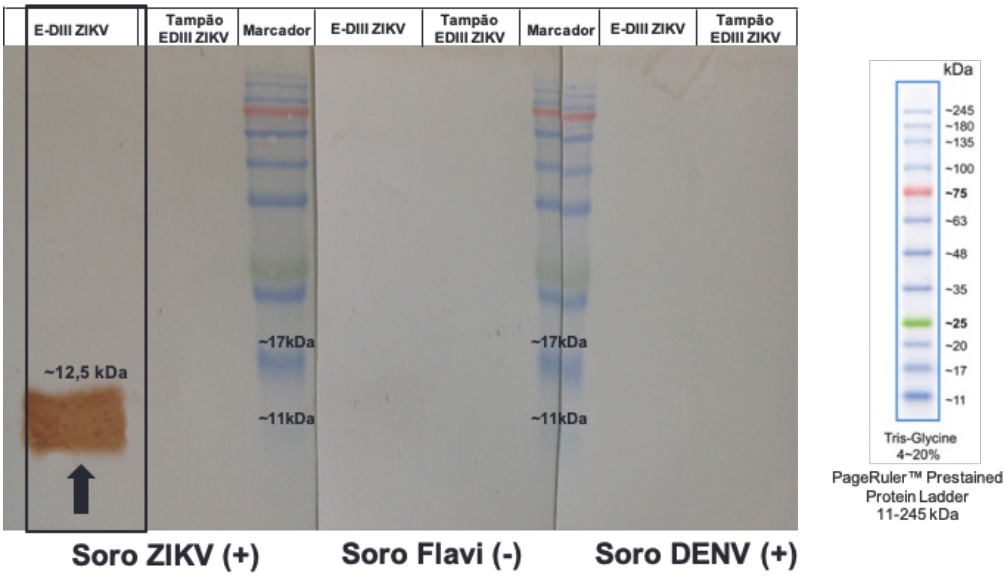
Supplementary Figure 1. Dengue and Zika cases evolution through and post-Zika virus epidemic in Manaus city and Brazil.



Supplementary Figure 2. Dengue-reactive antibody distribution in the Amazonian population.



Supplementary Figure 3. Characterization of Zika rE-DIII protein.



Capítulo 4

ARTIGO 3 – NÃO PUBLICADO (DRAFT)

SARS-CoV-2 Infection Severity Is Linked to Superior Humoral Immunity

Running title: XXX

Bárbara Batista Salgado[1§], Maele Ferreira Jordão[1§], Thiago Barros do Nascimento de Morais[1], Danielle Severino Sena da Silva[1], Ivanildo Vieira Pereira Filho[1], Wlademir Braga Salgado Sobrinho[1], Nani Oliveira Carvalho[1], Rafaella Oliveira dos Santos[1], Kerollen Runa Pinto[2], Ingrid Silva Correia[2], Isabelle Bezerra Cordeiro[2], Júlio Nino de Souza Neto[2], Enedina Nogueira de Assunção[2], Fernando Fonseca Almeida Val[7], Gisely Cardoso Melo[7], Vanderson de Souza Sampaio[7], Wuelton Marcelo Monteiro[7], Leda dos Reis Castilho[3], Fabiana Granja[6], William Marciel de Souza[4], Jaila Dias Borges Lalwani[2], Spartaco Astofi Filho[2], José Luiz Proença-Modena[6], Christian A Ganoza[7], Marcus Vinícius Guimarães de Lacerda[1,8], Paulo Afonso Nogueira[1#], Pritesh Lalwani[1#]

[1] Instituto Leônidas e Maria Deane (ILMD), Fiocruz Amazônia, Manaus, Amazonas, Brazil;

[2] Universidade Federal do Amazonas (UFAM), Manaus, Amazonas, Brazil;

[3] Laboratório de Engenharia de Cultivos Celulares (LECC) – Coppe, Universidade Federal do Rio de Janeiro (UFRJ), Rio de Janeiro, Brazil;

[4] Faculdade de Ciências Farmacêuticas, Universidade de São Paulo (USP), São Paulo, Brazil;

[5] Instituto René Rachou (IRR), Fiocruz Minas Gerais, Belo Horizonte, Minas Gerais, Brazil;

[6] Universidade de Campinas (UNICAMP), Campinas, São Paulo, Brazil;

[7] Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano Heredia, Lima, Perú

[8] Fundação de Medicina Tropical, Dr. Heitor Vieira Dourado (FMT-HVD), Manaus, Amazonas, Brazil;

[§]Equal contribution

[#]Corresponding author

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Contact Information:

Pritesh Lalwani

Instituto Leônidas e Maria Deane, Fiocruz Amazônia

Rua Terezina 476, Adrianópolis. Manaus - AM. CEP: 69057-070.

E-mail pritesh.lalwani@fiocruz.br

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Contributors

PL and JDBL conceived the study with input from PAN, CAG, MVGL and SAF. Protein expression and purification was performed by BBS, IBC, JNSN, ENA and SAF. Sample collection was led by JDBL with assistance from CFC, PES, BCA. The laboratory set-up and sample processing were coordinated by PL, BBS and MFJ. BBS, IVPF, DSSS, TBNM, MFJ, JVO, ARCB, WBSS processed blood samples, performed laboratory testing, collected data and approved test results with PL supervision. PL, BBS and JDBL coordinated data acquisition and management. Data was cleaned and prepared by PL and RVA. MVGL coordinated the clinical study and data collection with FFA, GCM, VSS and WMM. PL, JDBL, CAG, BBS, PAN and MFJ analysed the results. FG, WMS and JLPM performed live virus neutralization assays and analysed the data. Statistical analyses and data visualization were done by PL and BBS. PL and CAG wrote the manuscript with input from all authors. All authors revised and approved the final version of this manuscript.

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Abstract

First time we show subclass response to full-length and other regions of N protein.

Introduction

The ongoing coronavirus diseases 2019 (COVID-19) pandemic has caused immense mortality and morbidity, and has also placed huge social and economic burdens on society. Around 18 months after the start of the pandemic, the global case count of infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has passed 180 million, with more than 3.9 million confirmed deaths due to the infection (WHO 2021). Noncompliance of non-pharmaceutical interventions (NPIs) and evolution of new variants of concern (VOC) and their dissemination maintains a perpetual challenge to control the ongoing pandemic worldwide. SARS-CoV-2 vaccines have been effective in reducing mortality and clinical symptoms associated with the infection, however growing concerns remain on the durability of the immune response and its ability to neutralize emerging SARS-CoV-2 variants of concern (VOC) (Earle, Ambrosino et al. 2021).

SARS-CoV-2 is an envelope, single-stranded, positive-sense RNA virus with a ~29.9 kb genome, member of the genus *β-Coronavirus* (CoV) and the subgenus *Sarbecovirus*. Genome comprises 14 open reading frames (ORFs), encoding 16 nonstructural proteins that make up the replicase complex, nine accessory proteins (ORF), and four structural proteins: spike (S), envelope (E), membrane (M), and nucleocapsid (N) (Lu, Zhao et al. 2020, Wu, Zhao et al. 2020, V'Kovski, Kratzel et al. 2021). The fundamental function of the N protein is to package the viral genome RNA into a long helical ribonucleocapsid (RNP) complex and to participate in the assembly of the virion through its interactions with the viral genome and membrane protein M. The N protein is highly conserved among the CoVs and is one of the most abundant structural proteins in virus-infected cells (Jayaram, Youn et al. 2005, Bojkova, Klann et al. 2020). N-terminal domain (NTD) is responsible for RNA binding, middle or linker

region comprises the Ser/Arg(SR)-rich flexible linker region (SRD) and the C-terminal domain (CTD) has an important role in protein dimerization. The spike (S) protein of SARS-CoV-2 is glycosylated, which plays a key role in the receptor recognition and cell membrane fusion process, is composed of two subunits, S1 and S2. The S1 subunit contains a receptor-binding domain that recognizes and binds to the host receptor angiotensin-converting enzyme 2 (ACE2), while the S2 subunit mediates viral cell membrane fusion by forming a six-helical bundle via the two-heptad repeat domain.

It is well documented that S and N proteins are the dominant antigens of SARS-CoV and SARS-CoV-2 that elicit IgA, IgG, and IgM antibodies (Poland, Ovsyannikova et al. 2020). There have been conflicting reports on which of the two proteins is most immunogenic; both as full-length or truncated proteins have been used for serological diagnosis of SARS-CoV-2 infection (Deeks, Dinnes et al. 2020). Current recombinant new generation vaccines are based on spike protein, with an aim to illicit high level of neutralizing antibody response to inhibit virus entry. SARS-CoV-2 infection in primates results in the induction of neutralizing antibody (NAb) and cellular immune responses including S-specific CD4+ and CD8+ T cells responses, and provided near-complete protection against SARS-CoV-2 re-challenge (Chandrashekar, Liu et al. 2020). In latter adoptive transfer studies, it was demonstrated that adoptive transfer of purified IgG from convalescent macaques protected naïve recipient rhesus macaques against SARS-CoV-2 infection in a dose dependent fashion (McMahan, Yu et al. 2020). New vaccine constructs are incorporating N protein to provide a robust T cell response accompanied by a robust neutralizing anti-spike antibody response. Humoral response to current vaccine constructs is half to four times that of natural infection (Folegatti,

Ewer et al. 2020, Keech, Albert et al. 2020, Walsh, Frenck et al. 2020, Cromer, Juno et al. 2021, Khoury, Cromer et al. 2021, Zhang, Zeng et al. 2021), however, the durability of the vaccine response could be different among the vaccines based on their design and further boosting might be necessary to maintain the high neutralizing antibody levels (Cromer, Juno et al. 2021, Khoury, Cromer et al. 2021). Clearly the T cell response to SARS-CoV-2 infection seems to be more stable, although their role in immune protection is not yet clear (Altmann and Boyton 2020). As more and more individuals get infected and immunized worldwide current focus remains on the understanding of immune correlates of protection to protect from reinfection in the medium to long term (Cromer, Juno et al. 2021, Jin, Li et al. 2021).

In this study, we performed a comprehensive analysis of full-length and truncated, nucleocapsid and spike protein specific IgA, total IgG and IgG-subclass antibody response, together with RBD-ACE2 competitive assay and SARS-CoV-2 live virus neutralization assay in plasma samples of RT-PCR SARS-CoV-2 positive individuals. We used healthy controls together with COVID-19 patients with mild-illness recruited in an outpatient clinic and severe hospitalized patients, and compared their humoral response to their disease outcome. Multidimensional analysis revealed a temporal pattern of progressive increase of antibody response toward different immunogenic regions. The robust response was directly proportional to the disease severity; however, the robust antibody response was not sufficient to protect individuals with severe infection and control of inflammation and other confounding parameters may dictate the disease outcome.

Materials and Methods

Ethics

This study was conducted in accordance with Brazilian law, and principles of the Helsinki Declaration. Samples analysed in this study received ethical clearance and informed consent forms were approved by the Comissão Nacional de Ética em Pesquisa (CONEP), approval number 062.00967/2020 and 3.929.646/2020 prior to study implementation (Borba, Val et al. 2020). All patients provided oral and written consent.

Patient recruitment and samples

A total of 141 patients (≥18 years) were enrolled between April to August 2020 in the city of Manaus, capital of Amazonas state-Brazil. Healthy controls (n=13) did not present any symptoms in the last 14 days and were negative for SARS-CoV-2 at enrolment. Outpatients (n=80) were recruited at the UPA Campos Sales a tertiary clinic and had a mild disease, with or without fever, mostly without the need of hospitalization. Inpatients or hospitalized patient (n=48 patients with n=131 samples) were recruited at the Hospital e Pronto-Socorro Delphina Rinaldi Abdel Aziz. Severe hospitalized patients presented with severe symptoms such as dyspnea, respiratory discomfort, peripheral oxygen saturation was lower than 94% and presented with severe acute respiratory syndrome (SARS). Hospitalized patients were followed up longitudinally for 15 days after enrolment and sequential blood samples were collected. All COVID-19 patients included in this study were RT-qPCR positive for SARS-CoV-2. Patient demographic, clinical and laboratory data was collected and stored using the REDCap (Research Electronic Data Capture) software.

Four millilitres of venous blood was drawn from each participant using a EDTA vacutainer (BD Vacutainer). Blood tubes were transported to laboratory and immediately centrifuged at 2,000 rpm. Plasma was then aliquoted and frozen at -70°C or lower in a Biorepository. All study samples were transported, handled and tested following good clinical (GCP), laboratory practice (GLP) standards and in accordance to the Brazilian Health Regulatory Agency (ANVISA) regulations.

SARS-CoV-2 proteins

Codon optimized full length nucleocapsid (N) protein (residues: 1–419) of SARS-CoV-2 (Gen Bank QHD43423.2) and its truncated fragments N1 (residues: 1–182), N2 (residues: 115–304) and N3 (residues: 245–419), containing a polyhistidine (6x-His) tag at the N terminus of each protein was expressed in *Escherichia coli* strain BL21 and purified by affinity and size-exclusion chromatography as previously described (Lalwani, Salgado et al. 2021). Spike (S) full length protein was expressed in Hek cells and produced by Prof. Leda Castilho (UFRJ) as previously described (Alvim, Lima et al. 2020). S1 region of Spike protein expressed in Hek cells was obtained from Native Antigen.

Measurement of IgA, IgG and IgG-subclass response

Indirect in-house ELISA was performed using SARS-CoV-2 full-length and truncated proteins to detect of IgG or IgA antibodies in plasma samples. 100ng of antigen per well was coated overnight in carbonate buffer pH 9.6. Next, ELISA plates were blocked with 10% skimmed milk in PBS at room temperature; plates were then washed and incubated with 1:101 dilution of patient plasma for 90 minutes. ELISA plates were then washed with 0.5% Tween 20 in PBS (PBST) and anti-human HRP-conjugated antibody

was added [goat anti-human IgG-1:40000 (SeraCare-KPL) and rabbit anti-human IgA-1:2000 (Sigma-Aldrich)]. The reaction was developed using Ultra-sensitive TMB (3,3′,5,5′-Tetramethylbenzidine) (Thermo Fischer); assay was stopped by adding 3M sulfuric acid and absorbance measured at 450 nm with a microplate reader (Chameleon V plate reader).

For detection of IgG subclass (IgG1, IgG2, IgG3 and IgG4) ELISA plates coated with 100ng of nucleocapsid or spike antigens and incubated with 1:101 dilution of patient plasma. Subsequently after washing step, plates were incubated with anti-human IgG subclass antibodies at 1:1000 dilution [mouse monoclonal anti-human IgG1, IgG2, IgG3 and IgG4; Sigma-Aldrich]. After incubation, plates were washed and incubated with 1:1000 goat anti-mouse IgG labelled with HRP. Upon incubation plates were washed and ultra-sensitive TMB was added to the ELISA plates, enzymatic reaction was stopped by adding 3M sulfuric acid and absorbance measured at 450 nm with a microplate reader.

Optical density of samples was divided by optical density of negative controls (pre-pandemic samples) to obtain reactivity index (RI). As previously described, we used pre-pandemic samples and performed ROC analysis to obtain cut-off, sensitivity and specificity values (Lalwani, Salgado et al. 2021). Samples with RI above 1.5 (cut-off) were defined as positives or reactive, and for RI below 1.5 were considered negative or non-reactive.

RBD-ACE2 competitive assay

NeutraLISA (Euroimmun) a commercial semi-quantitative assay was used as per manufacturer's instructions to estimate potentially neutralising antibodies inhibiting the binding of SARS-CoV-2 S1/RBD to ACE2 receptors of the host cells. Patient plasma was diluted (1:5) in sample buffer or controls and added together with biotinylated-ACE2 protein to the microtiter plates coated with S1-RBD protein. If antibodies that inhibit RBD-ACE2 interaction are present in the patient sample, they will compete for binding S1/RBD proteins together with biotinylated-ACE2. Unbound ACE2 is removed in subsequent washing steps. To detect the bound biotinylated-ACE2 to S1/RBD peroxidase-labelled with streptavidin is added together with the conjugate. The intensity of the reaction is inversely proportional to the concentration of antibodies blocking RBD-ACE2 interaction in the sample. Assay results were expressed as in percentage inhibition (IH) of RBD-ACE2 interaction: %IH = 100% - [Optical density of patient sample X 100% / optical density of blank (mean)]. Upper threshold of the normal range (cut-off value) was set at 25% IH as per manufacturer's instructions.

Statistics and Data analysis

Chi-square tests or Fisher´s exact for two-by-two contingency tables were used to examine the statistical significance and association between study variables. One-way ANOVA test with Tukey's post hoc test or Two-way ANOVA with Šidák post hoc correction for multiple comparisons was used. Spearman correlation was performed for study variables to understand correlation. All statistical analysis was performed using the GraphPad Prism software (v9.1.2 for Mac OS).

Results

Study design and patient demographics

A total of 128 individuals with a nasopharyngeal swab positive for SARS-CoV-2 by real-time RT-PCR (rRT-PCR) were included in this study. This included 80 symptomatic patients with or without fever attending an outpatient clinic; samples were collected only at enrolment. Hospitalized patients diagnosed with SARS were longitudinally followed for two weeks after admission. Among the 48 inpatients included in the study, 24 were discharged and 24 succumbed to the disease. Table 1 summarises the demographic characteristics of the study population, associated comorbidities and clinical symptoms at recruitment. The percentage of males and those with chronic heart disease or hypertension or diabetes comorbidities was elevated in the inpatient or hospitalized patient group. Symptoms of anosmia and dysgeusia were significantly higher in outpatients compared to the inpatients. In addition, a larger percentage of outpatients reported headache, nasal congestion, chills, myalgia and arthralgia compared to the hospitalized patients. A significant decrease in peripheral leukocytes was observed in inpatients compared to outpatients. Severe hospitalized patients had elevated urea, LDH and c-reactive protein levels (Table 1). A marked difference in all haematological parameters was observed among inpatients compared to the outpatients. Median days with symptoms before hospitalization was similar for discharged or deceased patients. However, median hospital stay was 19 days for deceased patients compared to 13.50 days for discharged. 20% of discharged patients were admitted to ICU and 20% required mechanical ventilation, whereas, 23 of 24 deceased patients required mechanical ventilation (Table 1).

Robust humoral response against the nucleocapsid and spike, full length and truncated proteins

Full-length and truncated SARS-CoV-2 proteins used in this study to assess humoral response are described in Figure 1A. Antigenicity of the N, N1, N2 and N3 proteins was confirmed by western-blot assay as described in Supplementary Figure 1. Spike S proteins was previously described to be antigenic and suitable for immunoassays (Alvim, Lima et al. 2020) and S1 protein was commercially obtained. COVID-19 positive patient plasma had significantly elevated IgA, IgG and IgG-subclass (IgG1, IgG2, IgG3 and IgG4) response towards the SARS-CoV-2-specific full-length and truncated proteins compared to the healthy plasma controls (Figure 1 and supplementary Figure 2-3). Robust IgA response was observed towards the C-terminal N3 protein compared to full-length N, N-terminal N1 and middle-domain N2. We failed to detect anti-N2 specific IgA response at 1:100 plasma dilution. Besides, lower plasma dilution (1:5 or 1:10) failed to detect anti-N2 IgA response (Supplementary Figure 4). Plasma S-specific IgA response was elevated among COVID-19 patients compared to the N-terminal S1 protein. Median N3- and S-specific IgA response was similar among the COVID-19 patients. Overall, C-terminal N3-specific IgG and IgG-subclass response was significantly higher compared to N, N1 and N2 proteins. Whereas, anti-S protein IgG response was most robust among all the antigens tested. Among the IgG subclass, breadth of the IgG1 response was the highest followed by IgG2, IgG3 and IgG4 (Figure 1B and Supplementary Figure 4). Reactivity towards SARS-CoV-2 antigens increased with the number of days after the onset of the disease. Dominant N3 and S protein response increased gradually within first days after the infection. Notably three weeks after onset of symptoms a peak humoral IgA and IgG response was observed. SARS-CoV-2 specific IgA response appears to be short-lived compared

to the IgG response and starts to decline towards three weeks after onset of symptoms (Figure 1C).

SARS-CoV-2 disease severity linked to superior humoral response

Hospitalized inpatients had a robust IgA, IgG and IgG-subclass response to nucleocapsid and spike proteins compared to the outpatients at recruitment (Figure 2A). Among all the test antigens N2 was the least immunoreactive. Upon comparing inpatients based on the disease outcome, we did not observe significant difference in the IgA response to N, N1, N2 and N3 proteins. However, an elevated IgA response against S and S1 proteins was observed in discharged patients compared to the deceased patients at recruitment. On the other hand, IgG-specific response to N2 and S1 was elevated in deceased compared to the discharged inpatients. Among all the subclass antibodies tested against the nucleocapsid and spike antigens, only S-specific IgG1 response was marginally elevated in discharged patients at recruitment (Figure 2B).

A robust SARS-CoV-2 specific IgA response was observed among severe inpatients during the first week after onset of symptoms, which further increased in breadth subsequently. Only, S-specific IgA response among outpatients was elevated between ≤7 days and >7 days groups. Whereas, N3 and S1-specific IgA response increased after first week of infection among the inpatients. SARS-CoV-2 specific total IgG response was similar among outpatients and inpatients during the first week of the disease. But after the first week a significant increase in IgG response to SARS-CoV-2 nucleocapsid and spike proteins was observed among inpatients compared to outpatients. IgG1, IgG2 and IgG3 response was significantly elevated after the first week of infection among inpatients compared to outpatients. IgG1 and IgG3 reactivity

index significantly increased in patients >7 days after infection (Figure 3). Overall, severe patients showed a steady increase in antibody reactivity index as disease progressed. Longitudinal follow-up of hospitalized patients illustrates sustained N, N1 and N3 specific IgA response over three weeks post onset of symptoms. S and S1-specific IgA response showed a decline around four weeks after starting symptoms. Total IgG response towards nucleocapsid and spike proteins steadily increased since the start of symptoms reaching a high around three weeks. Among the IgG subclasses, SARS-CoV-2 specific IgG1 and IgG3 was responsible to elevate the total IgG response. Whereas, IgG4 response was weak among inpatients (Figure 4).

Neutralizing antibodies are increased in inpatients compared to outpatients, and correlate with RBD-ACE2 blocking and S1 IgG titres

A competitive assay was setup to estimate potentially neutralizing antibodies that block RBD-ACE2 interactions among COVID-19 patients. We observed significantly elevated blocking antibodies among inpatients compared to outpatients, which steadily increased after the onset of the symptoms (Figure 5A-B). Median inhibitory antibodies were similar among deceased and discharged inpatients at recruitment. Longitudinal hospitalized data demonstrated an increase in RBD-ACE2 blocking antibodies among discharged patients compared to deceased patients during the first week of the disease (Figure 5C). A positive correlation was observed between IgA, IgG, IgG1 and IgG3 reactive for N, N3, S and S1 SARS-CoV-2 specific protein and RBD-ACE2 inhibitory antibodies for outpatients or inpatients (Figure 5D). However, the slopes for the correlations among inpatients were steeper compared to the outpatients.

Next, we compared the peripheral lymphocyte percentage with the humoral response among COVID-19 patients. Lymphocytes had a negative correlation with N, N3, S or

S1-specific, IgA or IgG response among all COVID-19 patients (Figure 6A). Also, a negative correlation was observed among peripheral lymphocyte and RBD-ACE2 inhibitory antibodies (Figure 6B). This negative correlation between lymphocytes and RBD-ACE2 inhibitory antibodies was also evident among outpatients and inpatients (Figure 6C-D). A principal component analysis using antibody reactivity data against nucleocapsid and spike full-length and truncated protein was used to understand the clustering among the different patient groups. We observed a tight cluster among outpatients with a mild or moderate diseases, however, discharged or deceased inpatients did not form clear clusters (Figure 6E). Overall, this clustering effect is line with other observations that there is little difference in terms of antibody response among inpatients and control of inflammation among inpatients leads to recovery from disease. Figure 6F summarizes the humoral response observed among the study participants. Overall, individuals with low N3 or S1 specific response had low levels of RBD-ACE2 inhibitory antibodies and robust humoral response was associated with disease severity but not associated with disease outcome.

Discussion

Clinical role of antibodies in modulating disease severity during infection, duration and persistence of the immune response and the protective role of these antibodies against reinfections have been the keen questions since the start of this pandemic. Comparison of vaccine-induced immune response to natural SARS-CoV-2 infection, and those who become reinfected will help in improving our understanding of the immunological correlates of protection. Since most vaccines are based on Spike protein, monitoring N-specific antibodies may be of utility in distinguishing vaccine-related antibodies from those stimulated by natural SAR-CoV-2 infection.

Thus, we evaluated serological responses in RT-PCR SARS-CoV-2 positive outpatients to hospitalized deceased patients, with detailed analysis of antibody response to SARS-CoV-2 full length and truncated nucleocapsid and spike proteins, and performed functional assays to identify neutralizing antibodies by RBD-ACE2 competitive assay and live-virus neutralization assay. At early time point post-onset of symptoms, we observed low IgA, IgG and IgG-subclass reactivity and hence no evidence of pre-existing antibodies that recognize SARS-CoV-2 or previous infection with other coronaviruses. However, these findings do not preclude the possibility that memory B cells stimulated by prior coronavirus exposure may be cross-reactive and form part of the response to SARS-CoV-2.

Our results confirm previous findings that IgA and IgG antibodies specific for SARS-CoV-2 antigens typically become detectable in patients' blood around three weeks after onset of symptoms. Here we also observed a low level of peripheral humoral response to SARS-CoV-2 antigens in outpatients with mild illness compared to severe

hospitalized inpatients, consistent with prior publications (Long, Liu et al. 2020, Röltgen, Powell et al. 2020, Guthmiller, Stovicek et al. 2021) and reports of other coronavirus infections (Drosten, Meyer et al. 2014, Alshukairi, Khalid et al. 2016, Okba, Raj et al. 2019). Patients with more severe disease have somewhat higher viral loads than patients with milder illness, suggesting that larger initial amounts of viral antigen may contribute to their greater serological responses.

SARS-CoV-2 infection can cause a multi-facetted disease, which is not only characterized by pneumonia and overwhelming systemic inflammatory immune responses, but which can also directly affect the digestive system and infect intestinal epithelial cells. IgA presence in the peripheral plasma samples was directly proportional to the disease severity. A robust antigen-specific IgA was observed early after onset of infection among hospitalized patients along with a robust IgG response. However, outpatients early in the disease had an IgG response but a weak IgA response. Nasopharyngeal swabs or saliva from outpatients have been shown to have a lower viral load compared to severe patients, which may correlate to higher amount of viral antigen in mucosal tissue. Additionally, lung scans have shown pneumonia or viral focus in severe patients compared to COVID-19 patients with mild illness. This apparent high viral load in mucosal tissues might explain a higher antigen-specific IgA presence in the plasma. Here we observed that the C-terminal N3 protein and full-length S protein were the most immunogenic. Moreover, a gradual increase in antigen-specific IgA response in the plasma of inpatients post onset of disease demonstrates a persistence or presence of virus antigen that stimulates IgA production. This antigen-specific plasma IgA response declines rapidly compared to IgG response as shown previously (Anna, Goyard et al. 2020, Dobaño, Santano et al. 2020, Moncunill, Mayor

et al. 2020, Nilsson, Holm et al. 2020). However, we know very little about the role and mechanism by which IgA antibodies participate in virus clearance or disease pathology. Evidence from secretory antibodies from mothers' milk have demonstrated a potent neutralizing antibody response.

Nucleocapsid C-terminal N3- and S-specific IgG response was detected very early in the patients compared to other antigens. Our data are consistent with results reported from a panel of antibody assays applied to single time point samples from COVID-19 patients who recovered or died of their disease, which found higher values of spike-targeting responses in the convalescents (Atyeo, Fischinger et al. 2020, Röltgen, Powell et al. 2020). In our study, we observed that C-terminal domain was more sensitive in identifying COVID-19 patients in the first week of infection. However, the highest sensitivity of IgG detection was three weeks post onset of disease. This low level of antibody response to most antigens during the first week of infection can affect detection sensitivity among the mild and moderate cases. Especially, majority of the vaccine breakthrough patients who have received two doses of vaccines develop mild illness. However, we need more studies to understand the utility of serological tests in diagnosis of vaccine breakthrough cases.

We observed an imbalanced IgG subclass response associated with disease severity. Robust IgG1 subclass response was followed by IgG2, IgG3 and IgG4. In our study, among the total COVID-19 patients the predominant humoral immune response to nucleocapsid protein was IgG1. Specifically, C-terminal nucleocapsid N3 protein was the most immunogenic compared to other full-length and truncated proteins tested in this study. IgG2 response was dominated by the N3 protein. However, IgG3 responses

did not have any specific dominant response. Lles et.al. reported a predominant humoral immune response to nucleocapsid was IgG3, whilst against spike it was IgG1 (Iies, Zmuidinaite et al. 2021). Whereas, Yates et.al. reported IgG1 and IgG3 predominant against Spike S1 and Spike S2 proteins, respectively (Yates, Ehrbar et al. 2021). In a subsequent study from the same lab they observed IgG1 response was dominant for full-length nucleocapsid and spike proteins, and IgG3 most dominant against full-length spike (Hunt, Yates et al. 2021). We did not observe any differences between subclasses and antigen among the outpatients with mild-illness. But upon comparing antigen specific IgG1 reactivity among hospitalized patients, we observed median nucleocapsid full-length N, N-terminal N1 and C-terminal N3 dominated this humoral response. N3-specific IgG2 response was the most dominant among the hospitalized patients. On the contrary, IgG3 median response was similar among the nucleocapsid and spike proteins tested in this study. Interestingly, our study demonstrated an increased S-specific IgG1 response among hospitalized patients who were discharged compared to deceased patients. Observed differences between the subclass humoral response might be due to differences in the geographical regions where the study was carried out and the circulating SARS-CoV-2 variant, associated coinfection among hospitalized patients and composition of the patient study groups.

Our results confirm a reduced peripheral blood lymphocyte count among hospitalized COVID-19 patients compared to patients with mild-illness. A negative correlation between lymphocyte and peripheral humoral response for antibody isotypes and RBD-ACE2 blocking antibodies was noted. Despite low lymphocyte counts severe patients had several fold higher responses compared to outpatients with higher lymphocytes count. Among the lymphocytes, CD3+ T cells, CD4+ T cells, CD8+ T cells, and natural

killer cells have been shown to significantly decrease in patients with COVID-19. In contrast, the decrease in B cell counts among severe COVID-19 patients is not as consistently observed as the decrease in T cell counts (Huang, Berube et al. 2020, Liu, Long et al. 2020). Probably, the heightened humoral response in severe patients could be due to the high availability of virus antigen since the start of the disease as observed by high RT-PCR viral loads among the severe patients. Alternatively, a robust B cell stimulation before the lymphocyte decline among the severe patients. The waning humoral response has been described for nucleocapsid and spike proteins over the first year after infection, suggesting that reinfections may become more frequent. However, the relationship between low lymphocyte counts and a robust B cell memory response induced during primary infection and the contribution of different aspects of immune memory to the protection against SARS-CoV-2 reinfection in humans remains unclear.

Neutralization and pathology

Functional RBD-ACE2 interaction inhibiting antibodies and live SARS-CoV-2 virus neutralization by patient antibodies appears with a similar time course to IgA and IgG, but were most closely correlated in magnitude with IgG titers. Neutralization, RBD-ACE2 blocking, and S1-specific IgG were all highly correlated in patients with high antibody levels, but RBD-ACE2 blocking was less sensitive than the neutralization assay, potentially because of antibodies that can neutralize by binding to non-RBD regions of the spike, or lower affinity antibodies that can neutralize in the cell culture assay but do not compete as well with binding of ACE2 under the blocking assay conditions (Röltgen, Powell et al. 2020). It is currently unclear, however, which of these assays will be the best predictor of *in vivo* immunological protection from SARS-CoV-

2 infection or reinfection in vaccinated or previously infected individuals. In the detailed serological time courses of the hospitalized patients in this study it was evident that the patterns of antibody responses could not fully explain patient outcomes, including death. Similarly, individuals with moderate antibody production were seen across the full spectrum of inpatient disease severity, and many patients who died of their disease generated high levels of antibodies, RBD-ACE2 blocking activity, and neutralizing titers. Differences between individuals in other aspects of the immune response or disease course, such as production of inflammatory mediators, T cell responses, host cell and tissue vulnerability to the damage during viral infection, underlying comorbidities, and secondary bacterial or fungal infections, are all likely to contribute to patient outcomes.

A limitation of our study is that, we did not test for IgM responses, since we observed higher cross-reactivity with pre-pandemic dengue positive sample during our assay standardization (data not shown). Also, we detected very early IgA and IgG responses and the classical class switch from IgM to IgG has not been evident among the SARS-CoV-2 infection compared to other arboviral infections endemic in the study region. We were not able to perform a longitudinal follow-up of outpatients as we did for inpatients due to logistical challenges at the peak of the pandemic. Follow-up of study participants beyond the study period could bring more information on the persistence of antibody responses for the different immunogenic regions of SARS-CoV-2 antigens. Additionally, we cannot rule out previous infection with other coronavirus among our study population or medication used during treatment, which may influence the antibody responses. Observed decrease in IgA antibody levels do not necessarily indicate a complete loss of immunity and local memory response in the mucosa might prevent and impede SARS-CoV-2 infection upon re-exposure.

In conclusion, the C-terminal nucleocapsid region of SARS-CoV-2 is immunogenic and might be suitable for seroprevalence and diagnostic purposes. Additional cohort studies might be necessary to evaluate the persistence of antibodies to different antigens and its role in protective immune response. This immunogenic C-terminal nucleocapsid region can be incorporated together with S protein as a promising vaccine candidate. Comparing the magnitude of immune responses to vaccination and infection, the currently licensed vaccines have shown to produce peak neutralizing antibody responses that range from approximately half to four-times those seen in convalescent patients (Folegatti, Ewer et al. 2020, Keech, Albert et al. 2020, Walsh, Frenck et al. 2020, Cromer, Juno et al. 2021, Khoury, Cromer et al. 2021, Zhang, Zeng et al. 2021). Additionally, we don't have data on the waning response for the current vaccines, to maintain antibody levels above the theoretical threshold of protection might need boosting. Hence, further detailed study of the generation of memory B cell populations, short- or long-lived plasma cells, and T cell memory to SARS-CoV-2 as well as other coronaviruses should begin to clarify some of these key mechanistic points to improve antibody persistence.

Table

Table 1: Demographic, clinical and haematological characteristics of COVID-19 patients.

Figure Legends

Figure 1: SARS-CoV-2 nucleocapsid and spike proteins elicits a robust IgA and IgG response in COVID-19 patients.

(A) Sketch depicts size of SARS-CoV-2 full length and truncated Nucleocapsid (N) and Spike (S) proteins. Complete nucleocapsid (N) and three truncated proteins (N1, N2 and N3) were expressed in *E. coli*, whereas the full-length Spike (S) and truncated protein S1 was expressed in eukaryotic cells. (B) 100ng of purified protein and 1:100 patient serum dilution was used in an indirect ELISA to estimate serum IgA, IgG and IgG-subclass response. Reactivity index (RI) values were calculated as a ratio between sample optical density and pre-pandemic negative samples. Horizontal red line denotes the median antibody range. (C) COVID-19 patient RI is compared to days after onset of symptoms. Each curve in the graph represents reactivity towards each antigen, Splines were plotted using the Fit Spline program GraphPad Prism software. ANOVA test with Tukey's post hoc test was used to compare differences in the RI between different antigen groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Figure 2: Severity of COVID-19 disease associated with a robust humoral response.

100ng of purified full-length and truncated SARS-CoV-2 proteins were coated on ELISA plates and 1:100 patient serum dilution was used in an indirect ELISA to estimate serum IgA, IgG and IgG-subclass response. (A) RT-PCR confirmed COVID-

19 patients (n=128) with a mild/moderate disease (outpatient) and severe disease (inpatient) were tested at recruitment. (B) Inpatients or hospitalized severe patients were then classified on the disease outcome as discharged or deceased and humoral response was compared at recruitment. Horizontal red or black lines denote the median antibody range. Two-way ANOVA with Šidák post hoc correction for multiple comparisons. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Figure 3: Robust humoral response is elicited one week after infection.

100ng of purified full-length and truncated SARS-CoV-2 proteins were coated on ELISA plates and 1:100 patient serum dilution was used in an indirect ELISA to estimate serum IgA, IgG and IgG-subclass response. RT-PCR confirmed COVID-19 patient's outpatients and inpatients were compared <=7 days or >7 days are the onset of symptoms. Horizontal red line denotes the median antibody range. Two-way ANOVA with Šidák post hoc correction for multiple comparisons. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Figure 4: Longitudinal humoral response dynamics in hospitalized patients.

100ng of purified full-length and truncated SARS-CoV-2 proteins were coated on ELISA plates and 1:100 patient serum dilution was used in an indirect ELISA to estimate serum IgA, IgG and IgG-subclass response. (A) RT-PCR confirmed hospitalized inpatient humoral response was compared in plasma samples collected longitudinally after hospital admission. Each line represents one patient and its antibody binding trajectories. Green colour denoted discharged inpatients and pink denotes deceased inpatients. Solid line in black represents the tendency for the

inpatients created by Fit Spline program GraphPad Prism software. X-axis denotes the RI and Y-axis days after onset of symptoms.

Figure 5: Potentially neutralizing antibodies that block ACE2-RBD interactions increase with symptomatic days and correlates with disease severity.

Commercial ACE2-RBD competitive assay was used to assess inhibitory antibodies in COVID-19 patient serum samples and compared between (A) Healthy controls and COVID-19 patients, (B) COVID-19 positive outpatients and inpatients or (C) discharged and deceased inpatients. Horizontal line denotes the median inhibitory antibodies range. Solid lines represent the tendency for the inhibitory antibodies created by Fit Spline program GraphPad Prism software. (D) Inhibitory antibody levels were correlated with IgA, IgG and IgG-subclass reactivity against the complete and truncated SARS-CoV-2 proteins. Each graph compares outpatient and inpatient with correlation and 95% CI. Inset depicts R squared values obtained by Spearman correlation. Unpaired t test with Welch's correction or Mann-Whitney U test or Two-way ANOVA with Šidák post hoc correction for multiple comparisons was applied. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Figure 6: Robust humoral response in severe patients despite low peripheral lymphocyte counts.

Lymphocyte data obtained by whole blood cell count was correlated with (A) anti-SARS-CoV-2 IgA or IgG response among COVID-19 patients or (B) potentially neutralizing antibodies that block ACE2-RBD interactions. Each dot represents one patient sample. Lymphocyte levels are compared between outpatients or inpatients

with (C) blocking antibodies or (D) days after onset of symptoms. (E) A principal component analysis was performed using all the available antibody reactivity variables, demographic and clinical characteristics of the patients. (F) Coloured heat-map depicts relationship between patient characteristics, N3 and S1 binding antibodies and ACE2-RBD inhibitory antibodies. Spearman correlation was performed and R square values depicted in the inset.

Supplementary Tables

Supplementary Table 1. Summary of patients and tests performed.

Supplementary Table 2. Pre-pandemic blood donor samples and antigen testing for specificity analysis

Supplementary figures

Supplementary Figure 1: Nucleocapsid proteins and antigenicity

Codon optimized full length nucleocapsid (N) protein of SARS-CoV-2 (Gen Bank QHD43423.2, residues: 1–419) and its domains N1 (residues: 1–182), N2 (residues: 115–304) and N3 (residues: 245–419), containing a polyhistidine (6x-His) tag at the N terminus of each protein was expressed in *Escherichia coli* strain BL21 and purified by affinity and size-exclusion chromatography as previously described. Western blotting (WB) was used to test the antigenicity of all the recombinants proteins. (A) A total of 1 μg of SARS-CoV full length N, N1, N2 and N3 proteins, with or without urea, were loaded onto 12% polyacrylamide gels and transferred to a nitrocellulose membrane using standard procedures. The membranes were blocked with 5% skimmed milk in phosphate buffer saline (PBS) for 1 hour at room temperature (RT). Blotted

membranes were incubated for one hour at RT with 1:1000 dilution of primary antibodies. Next, (B) anti-histidine tagged mouse monoclonal antibody (Sigma-Aldrich), (C) pool of pre-pandemic COVID-19 negative plasma sample pool and (D) RT-PCR positive COVID-19 patient plasma sample with 5% milk in PBST (PBS + 0.05% Tween-20) was added to the membranes. After incubation, membranes were washed three times with PBST then incubated with goat anti-mouse IgG HRP-labelled (0.2μg/mL KPL, Seracare, USA) or goat anti-human IgG HRP-labelled (0.1μg/mL KPL, Seracare, USA) secondary antibody for one hour at RT. The membranes were again washed three times with PBST and the proteins were visualized using an enzymatic reaction by adding DAB (3,3',4,4' diaminobenzidine) (BioRad, EUA) and H_2O_2 mixture for 5 minutes. Reaction was blocked by washing membrane five-times with distilled water and membranes were dried overnight to be photographed.

Supplementary Figure 2: Humoral response among COVID-19 patients

100ng of purified protein and 1:100 patient serum dilution was used in an indirect ELISA to estimate serum IgA, IgG and IgG-subclass response. Reactivity index (RI) values were calculated as a ratio between sample optical density and pre-pandemic negative samples. Each graph compares healthy and asymptomatic individuals with RT-PCR positive COVID-19 patients. Horizontal red line denotes the median antibody range.

Supplementary Figure 3: IgG4 response in COVID-19 patients

100ng of purified protein and 1:100 patient serum dilution was used in an indirect ELISA to estimate serum IgA, IgG and IgG-subclass response. Reactivity index (RI) values were calculated as a ratio between COVID-19 positive sample optical density

and pre-pandemic negative samples. (A) Full-length and truncated SARS-CoV-2 nucleocapsid and spike proteins antibody binding is compared. (B) Antibody reactivity is compared as a function of days after onset of symptoms. (C) Outpatients and inpatients or (D) discharged and deceased inpatients are compared. (E) IgG4 antibody response is compared between outpatients and inpatients =<7 days or >7 days post infection. ANOVA test with Tukey's post hoc test was used to compare differences in the RI between different antigen groups. Two-way ANOVA with Šidák post hoc correction for multiple comparisons. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Supplementary Figure 4: Comparison of full-length nucleocapsid and truncated N2 protein reactivity among COVID-19 patients.

(A) Outpatient and (B) inpatient plasma samples were serially diluted and tested against 100ng of full-length nucleocapsid or truncated N2 protein in an indirect ELISA. Y-axis shows the optical density (OD) measured for these test samples against IgA or IgG. Each coloured line represents one patient.

Supplementary Figure 5: Representative COVID-19 patients and their humoral response

100ng of purified SARS-CoV-2 protein and 1:100 patient serum dilution was used in an indirect ELISA to estimate serum IgA, IgG and IgG-subclass response. Each column represents one patient and each row represents specific response to one antibody isotype or RBD-ACE2 inhibitory antibodies. Each graph has reactivity index for SARS-CoV-2 full-length or truncated antigens tested. Outpatient samples were collected only at recruitment, whereas inpatients were followed longitudinally after hospitalization. Red area depicts ICU time and grey area depicts the hospitalization

time until diseae outcome. Symbols illustrate intubation/extubation time and outcomes (discharge or decease) to each patient. Horizontal line denotes the median inhibitory antibodies range.

Supplementary Figure 6: Correlation matrix for study variables

A correlation analysis was performed for all variables described in the figure. Scale denotes spearman r value for each variable compared.

Supplementary Figure 7: C-terminal domain of Nucleocapsid protein has the highest sensitivity to detect anti-SARS-CoV-2 antibodies.

Indirect ELISA was performed with full-length or truncated nucleocapsid and spike proteins, and commercial RBD-ACE2 competitive assay with RT-PCR positive COVID-19 patients serum samples. Reactivity index (RI) values were calculated as a ratio between sample optical density and pre-pandemic negative samples. ELISA cut-off was set at equal or above 1.5 RI as per the ROC analysis previously performed on COVID-19 and pre-pandemic serum samples (Lalwani et.al. IJID in press). All samples with equal or above 1.5 RI were considered positive. (A) Anti-SARS-CoV-2 IgA and IgG detection sensitivity or probability (95% CI) is depicted as function of days after onset of symptoms. Each solid line is splines fitting for each antigen represented in the graph. (B) COVID-19 patient samples were divided as per the days after onset of symptoms into three groups (<= 7 days, >7 days and >14 days) and sensitivity (95% CI) determined for IgA and IgG detection of anti-SARS-CoV-2 antibodies for each antigen. (C) Detection probability was then calculated for outpatients and inpatients at <= 7 days, >7 days and >14 days after onset of symptoms. (D) ROC analysis was performed to compare RI between outpatient and inpatients. AUC values with 95% CI

are represented for each antigen and antibody class tested. (E) Detection sensitivity or probability (95% IC) of inhibitory antibodies by RBD-ACE2 competitive assay is expressed as function of days after onset of symptoms, COVID-19 patients or outpatients and inpatients at <= 7 days, >7 days and >14 days after onset of symptoms. Solid lines represent the tendency for the inhibitory antibodies created by Fit Spline program GraphPad Prism software.

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TABLE 1. Demographic, clinical and hematological characteristics of COVID-19 patients

Characteristics		Outpatients n=80	Inpatients n=48	P value	Inpatients Discharged n=24	Inpatients Deceased n=24	P value
Age median (IQR)		40.5 (33 - 47)	55.5 (45 - 63)		52.5 (41.25 – 62.75)	58.5 (47.0 – 64.0)	
range		21 - 64	20 - 81		27 - 81	20 – 79	
Gender	Male	36 (45)	34 (70.8)		17 (70.8)	17 (70.8)	
	Female	44 (55)	14 (29.2)		7 (29.2)	7 (29.2)	
Race	Brown	63 (78.7)	37 (77.1)		18 (75.0)	19 (79.2)	
	Black	4 (5.0)	5 (10.4)		3 (12.5)	2 (8.3)	
	White	9 (11.3)	6 (12.5)		3 (12.5)	3 (12.5)	
	Yellow	4 (5.0)	0		0	0	
BMI median (IQR)		29.2 (25.9 - 33.1)	29.25 (25.9 - 32.4)		29.25 (25.75 – 32.38)	29.70 (26.05 – 35.00)	
range		17.9 - 49.9	22.8 - 58.8		22.6 – 58.8	23.3 – 43.4	
Symptoms Days median (IQR)		9 (5 - 33)	10.5 (8 - 22)		10.5 (8.0 – 13.0)	10.5 (7.25 – 15.0)	
Comorbidities*	Chronic heart disease	3/80 (3.75)	6/48 (12.5)	0.0789	3/24 (12.5)	3/24 (12.5)	
	Hypertension	23/80 (28.75)	21/48 (43.75)	0.5876	8/24 (33.3)	13/24 (54.2)	
	Pulmonary disease	4/80 (5.0)	4/48 (8.3)	0.4721	3/24 (12.5)	1/24 (4.17)	
	Tuberculosis	2/80 (2.5)	1/48 (2.1)	0.9999	1/24 (4.17)	0/24 (0)	
	Diabetes	12/80 (15.0)	14/48 (29.16)	0.0698	8/24 (33.3)	6/24 (25.0)	
	Obesity	36/80 (45.0)	21/48 (43.75)	0.9999	10/24 (41.7)	11/24 (45.8)	
	Alcoholism	48/80 (60.0)	20/48 (41.67)	0.8525	10/24 (41.7)	10/24 (41.7)	
	HIV/AIDS	1/80 (1.25)	0/48 (0)	0.9999	0/24 (0)	0/24 (0)	
	Kidney disease	5/80 (6.25)	0/48 (0)	0.1562	0/24 (0)	0/24 (0)	
	Neurological disease	8/80 (10.0)	3/48 (6.25)	0.5345	1/24 (4.17)	2/24 (8.3)	
	Rheumatic disease	5/80 (6.25)	5/48 (10.42)	0.5	5/24 (20.8)	0/24 (0)	
	Smoking habit	21/80 (26.25)	14/48 (29.16)	0.8384	6/24 (25.0)	8/24 (33.3)	
	Liver disease	3/80 (3.75)	0/48 (0)	0.2913	0/24 (0)	0/24 (0)	
	Chronic hematological disease	4/80 (5.0)	0/48 (0)	0.2692	0/24 (0)	0/24 (0)	
Clinical symptoms*	Fever	73/80 (91.25)	43/48 (89.6)	0.7623	22/24 (91.6)	21/24 (87.5)	
	Dry cough	71/80 (88.75)	42/48 (87.5)	0.9999	21/24 (87.5)	21/24 (87.5)	
	Sore throat	51/80 (63.75)	22/48 (45.8)	0.0649	12/24 (50.0)	10/24 (41.6)	
	Nasal congestion	61/80 (76.25)	18/48 (37.5)	0.0001	10/24 (41.6)	8/24 (33.3)	
	Nasal discharge	49/80 (61.25)	19/48 (39.6)	0.0276	12/24 (50.0)	7/24 (29.17)	
	Earache	28/80 (35.0)	7/48 (14.6)	0.014	6/24 (25.0)	1/24 (4.17)	
	Wheezing	31/80 (38.75)	17/48 (35.4)	0.8506	5/24 (20.8)	12/24 (50.0)	
	Chest Pain	62/80 (77.5)	31/48 (64.6)	0.1513	16/24 (66.7)	15/24 (62.5)	
	Myalgia	72/80 (90.0)	30/48 (62.5)	0.0005	18/24 (75.0)	12/24 (50.0)	
	Arthralgia	64/80 (80.0)	24/48 (50.0)	0.0007	13/24 (54.17)	11/24 (45.8)	
	Fatigue	75/80 (93.75)	42/48 (87.5)	0.3285	21/24 (87.5)	21/24 (87.5)	
	Shortness of breathe	70/80 (87.5)	45/48 (93.7)	0.3683	22/24 (91.6)	23/24 (95.8)	
	Intercostal circulation	41/80 (51.25)	20/48 (41.6)	0.3614	12/24 (50.0)	8/24 (33.3)	
	Nasal Wing Beating	23/80 (28.75)	12/48 (25.0)	0.687	8/24 (33.3)	4/24 (16.67)	
	Anosmia	66/80 (82.5)	28/48 (58.3)	0.0001	15/24 (62.5)	13/24 (54.17)	
	Dysgeusia (loss of taste)	66/80 (82.5)	23/48 (47.9)	0.0001	15/24 (62.5)	8/24 (33.3)	
	Dehydration	44/80 (55.0)	14/48 (29.2)	0.0058	7/24 (29.17)	7/24 (29.17)	
	Headache	72/80 (90.0)	35/48 (72.9)	0.0146	21/24 (87.5)	14/24 (58.3)	
	Chill	60/80 (75.0)	26/48 (54.2)	0.0197	15/24 (62.5)	11/24 (45.8)	
	Dizziness	51/80 (63.75)	22/48 (45.8)	0.0649	15/24 (62.5)	7/24 (29.17)	
	Convulsions	4/80 (5.0)	4/48 (8.3)	0.4721	2/24 (8.33)	2/24 (8.33)	
	Altered Consciousness	19/80 (23.7)	5/48 (10.4)	0.0665	2/24 (8.33)	3/24 (12.5)	
	Abdominal pain	42/80 (52.5)	23/48 (47.9)	0.7154	14/24 (58.3)	9/24 (37.5)	
	Vomiting	15/80 (18.7)	14/48 (29.2)	0.195	7/24 (29.17)	7/24 (29.17)	
	Nausea	52/80 (65.0)	20/48 (41.6)	0.0163	11/24 (45.8)	9/24 (37.5)	
	Diarrhea	41/80 (51.2)	22/48 (45.8)	0.5876	13/24 (54.17)	9/24 (37.5)	
	Conjunctivitis	3/80 (3.75)	4/48 (8.3)	0.4237	1/24 (4.17)	3/24 (12.5)	
	Rash	8/80 (10.0)	1/48 (2.1)	0.1519	1/24 (4.17)	0/24 (0)	
	Lymphadenopathy	22/80 (27.5)	4/48 (8.3)	0.1978	0/24 (0)	4/24 (16.67)	
	Sputum	37/80 (46.2)	27/48 (56.2)	0.3614	13/24 (54.17)	14/24 (58.3)	
	Itching	11/80 (13.7)	1/48 (2.1)	0.0305	1/24 (4.17)	0/24 (0)	
	Skin Injuries	3/80 (3.75)	0/48 (0)	0.2913	0/24 (0)	0/24 (0)	
	Bleeding (Hemorrhage)	1/80 (1.25)	2/48 (4.2)	0.5557	1/24 (4.17)	1/24 (4.17)	
Blood count#, mean (95% CI)	Hemoglobin (g/dL)	13.44 (12.95 – 13.94)	12.69 (12.13 – 13.25)	0.053	13.17 (12.50 – 13.85)	12.22 (11.33 – 13.11)	0.087
	Leukocytes (mm^3)	8.39 (7.54 – 9.24)	11.79 (9.93 – 13.64)	0.0002	10.62 (8.39 – 12.85)	12.91 (9.86 – 15.94)	0.2184
	Lymphocytes (%)	21.17 (18.69 – 23.64)	10.15 (8.08 – 12.23)	<0.0001	11.93 (8.67 – 15.19)	8.45 (5.79 – 11.11)	0.092
	Neutrophils (%)	70.61 (67.49 – 73.72)	81.75 (77.87 – 85.63)	<0.0001	81.28 (77.39 – 85.17)	82.20 (75.25 – 89.16)	0.8146
	Hematocrit (%)	46.87 (43.77 – 49.97)	39.51 (38.01 – 41.00)	0.0006	40.72 (38.96 – 42.49)	38.34 (35.92 – 40.77)	0.1099
	Platelets ($10^3/mm^3$)	259.94 (240.2 – 279.7)	301.04 (267.7 – 334.4)	0.025	301.7 (257.6 – 345.7)	300.5 (247.1 – 353.8)	0.9718
	ALT (U/L)	52.61 (44.30 – 60.91)	85.5 (62.34 – 108.70)	0.001	97.64 (62.39 – 132.9)	69.32 (38.91 – 99.73)	0.2207
	AST (U/L)	43.74 (37.76 – 49.71)	87.46 (57.49)	<0.0001	77.16 (54.78 – 99.55)	101.2 (31.88 – 170.5)	0.4260
	Glucose (mg/dL)	173.14 (110.1 – 236.2)	208.58 (173.7 – 243.4)	0.3775	214.4 (151.6 – 277.1)	203.4 (161.7 – 245.2)	0.7559
	Creatinine (mg/dL)	3.6 (-1.74 – 8.94)	1.57 (1.15 – 1.99)	0.5719	1.22 (0.85 – 1.59)	1.90 (1.15 – 2.64)	0.1059
	Urea (mg/dL)	27.92 (26.10 – 29.74)	56.04 (41.63 – 70.45)	<0.0001	42.18 (28.33 – 56.03)	69.27 (44.47 – 94.06)	0.0569
	LDH (U/L)	441.32 (144.8 – 737.9)	972.68 (619.9 – 1,326.0)	0.1573	796.7 (648.8 – 944.6)	1,149 (408.7 – 1,889)	0.3112
	C-reactive protein (mg/dL)	60.5 (0.1 – 120.9)	101.84 (76.87 – 126.8)	0.2028	94.20 (55.32 – 133.1)	109.50 (74.99 – 144.0)	0.5428
Hospitalization#	Symptoms days before hospitalization median (IQR)		8.0 (6.0 – 13.0)		8.0 (6.0 – 12.0)	8.5 (6.0 – 13.0)	0.3991
	Number of days in hospital median (IQR)		16.0 (13.0 – 23.75)		13.50 (11.0 – 23.0)	19.0 (14.25 – 24.50)	0.0711
	Symptoms days until outcome median (IQR)		28.0 (20.0 – 34.0)		24.0 (18.0 – 33.0)	29.50 (24.0 – 37.5)	0.0356
	ICU access*		29/48 (60.4)		5/24 (20.8)	24/24 (100)	<0.0001
	Number of days on ICU median (IQR)		16.0 (11.5 – 21.5)		19.0 (8.5 – 27.0)	16.0 (12.25 – 20.0)	0.9259
	Mechanical ventilation use*		28/48 (58.3)		5/24 (20.8)	23/24 (95.83)	<0.0001
	Number of days on ventilation median (IQR)		16.0 (12.0 – 20.0)		12.0 (5.5 – 20.5)	17.0 (14.0 – 20.0)	0.1191

The data from comorbidities and clinical symptoms are represented as number of individuals with the finding in question among total and the relative percentage as the proportion of these individuals in the group. #P value from Unpaired T test. *P value from Fisher's exact test. IQR: interquartile ranges; BMI: Body mass index; ALT: Alanine transaminase; AST: Aspartate transaminase; LDH: lactate dehydrogenase; ICU: Intensive Care Unit.

FIGURE 1

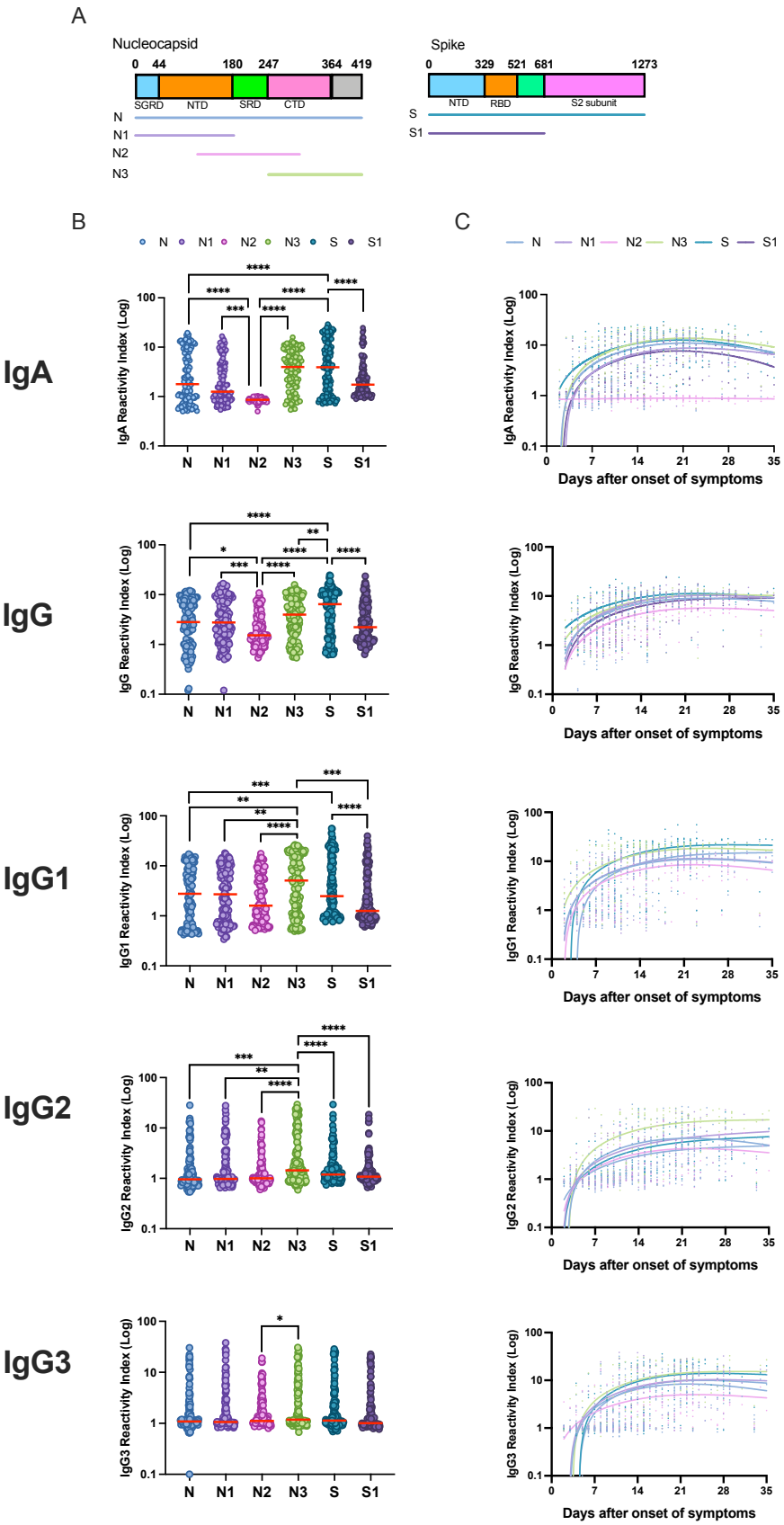

A
Nucleocapsid
0 44 180 247 364 419
SGRD NTD SRD CTD
N
N1
N2
N3
Spike
0 329 521 681 1273
NTD RBD S2 subunit
S
S1
B
C
N N1 N2 N3 S S1
IgA
IgG
IgG1
IgG2
IgG3
IgA Reactivity Index (Log)
IgG Reactivity Index (Log)
IgG1 Reactivity Index (Log)
IgG2 Reactivity Index (Log)
IgG3 Reactivity Index (Log)
Days after onset of symptoms

FIGURE 2

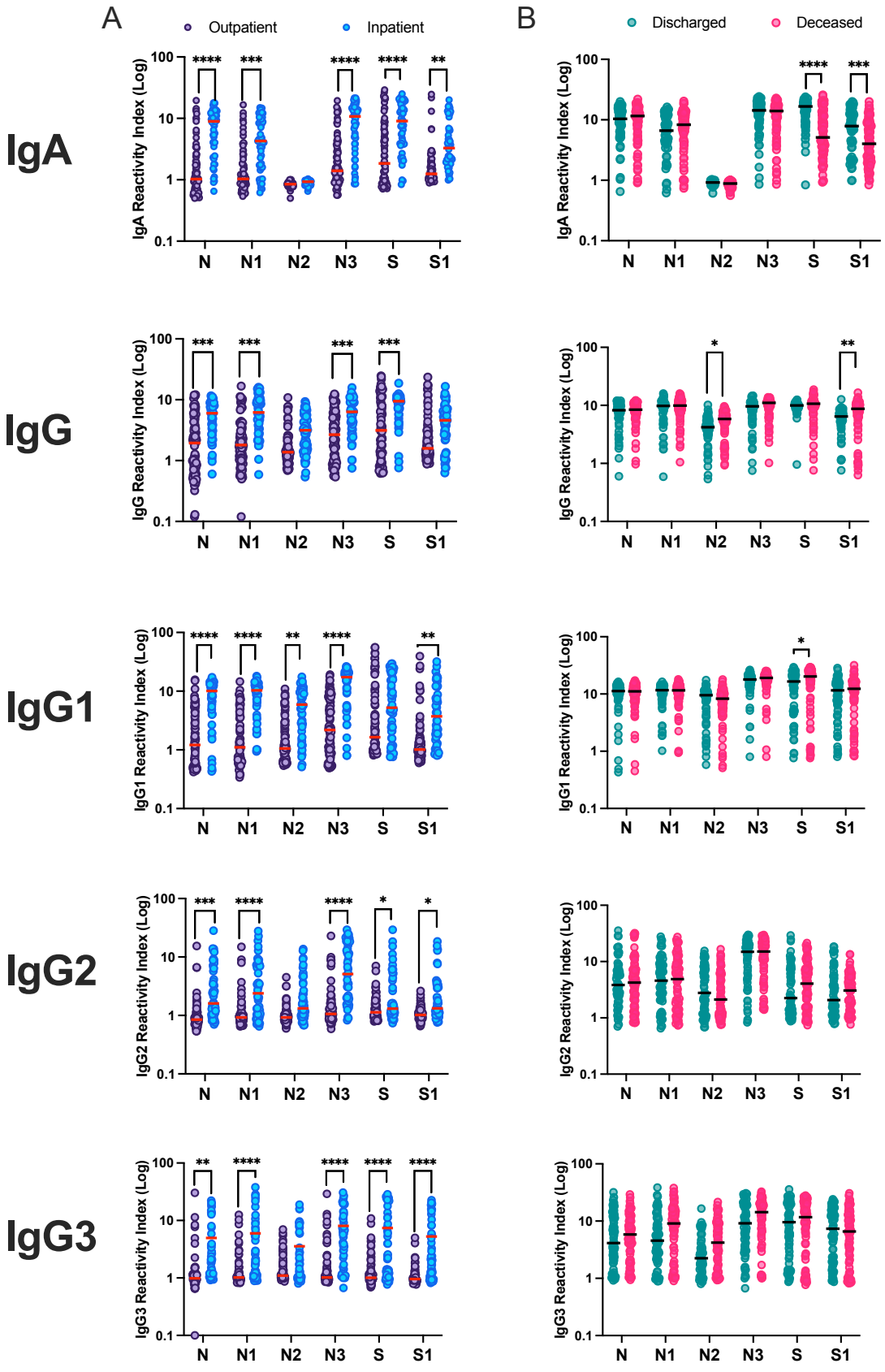

A
Outpatient
Inpatient
B
Discharged
Deceased
IgA
IgG
IgG1
IgG2
IgG3
IgA Reactivity Index (Log)
IgG Reactivity Index (Log)
IgG1 Reactivity Index (Log)
IgG2 Reactivity Index (Log)
IgG3 Reactivity Index (Log)
100
10
1
0.1
N
N1
N2
N3
S
S1

FIGURE 3

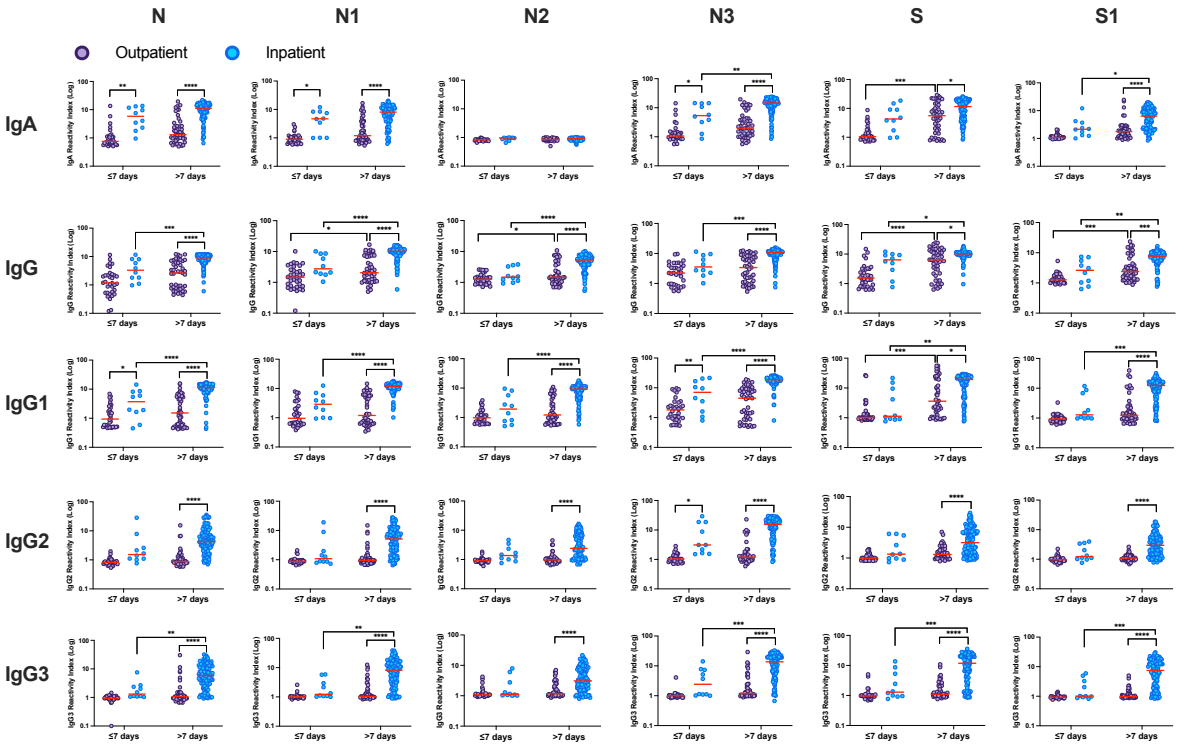

N
N1
N2
N3
S
S1
Outpatient
Inpatient
IgA
IgG
IgG1
IgG2
IgG3
≤7 days
>7 days

FIGURE 4

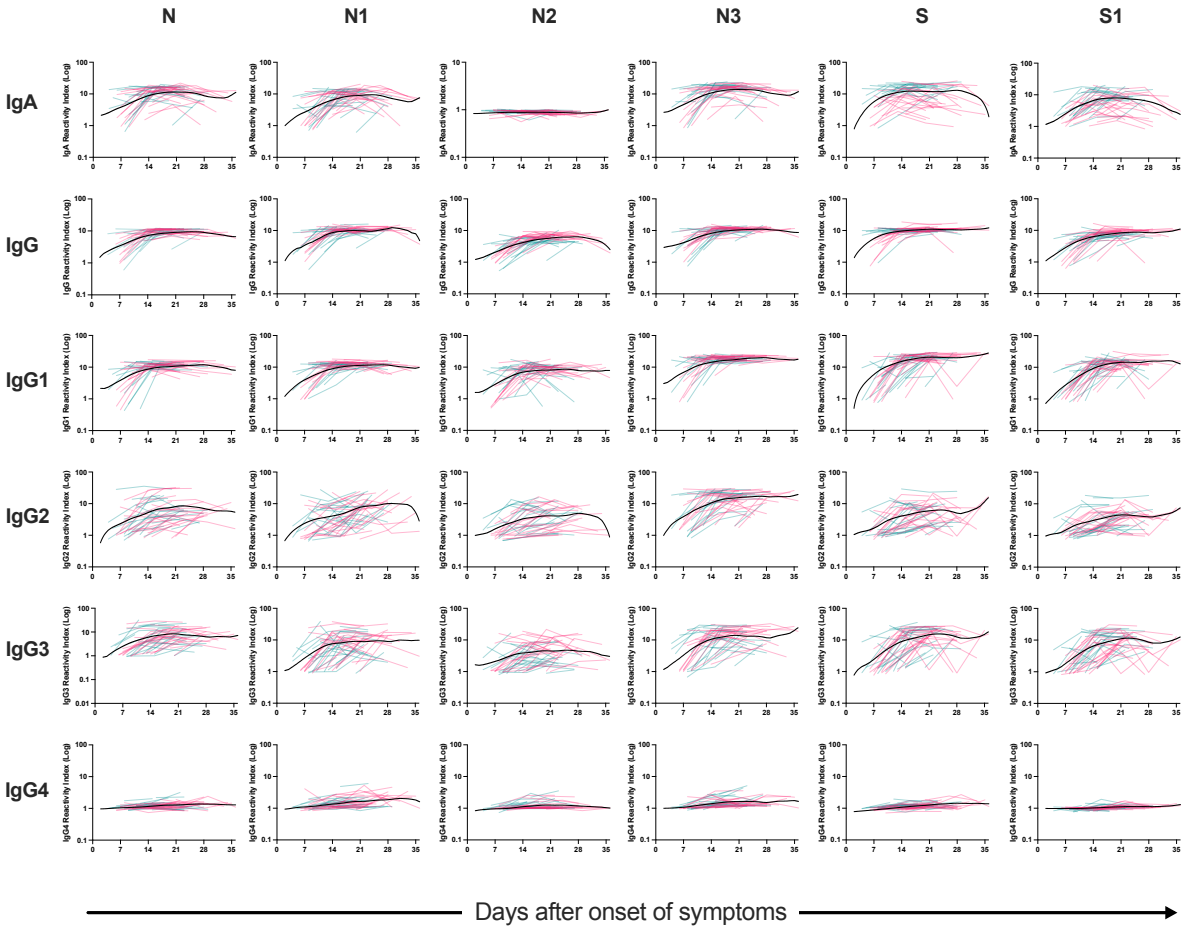

N
N1
N2
N3
S
S1
IgA
IgG
IgG1
IgG2
IgG3
IgG4
Days after onset of symptoms

FIGURE 5

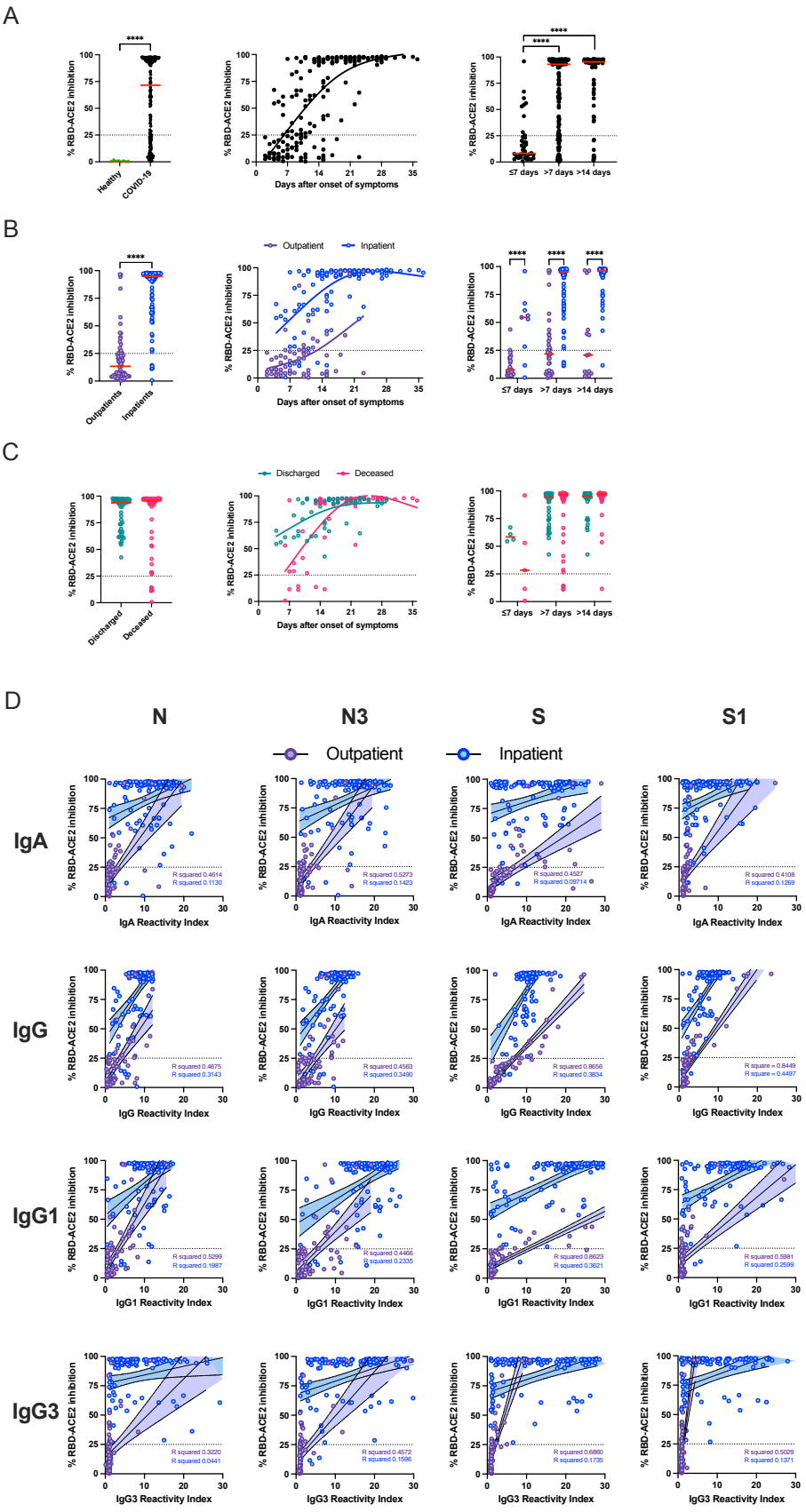
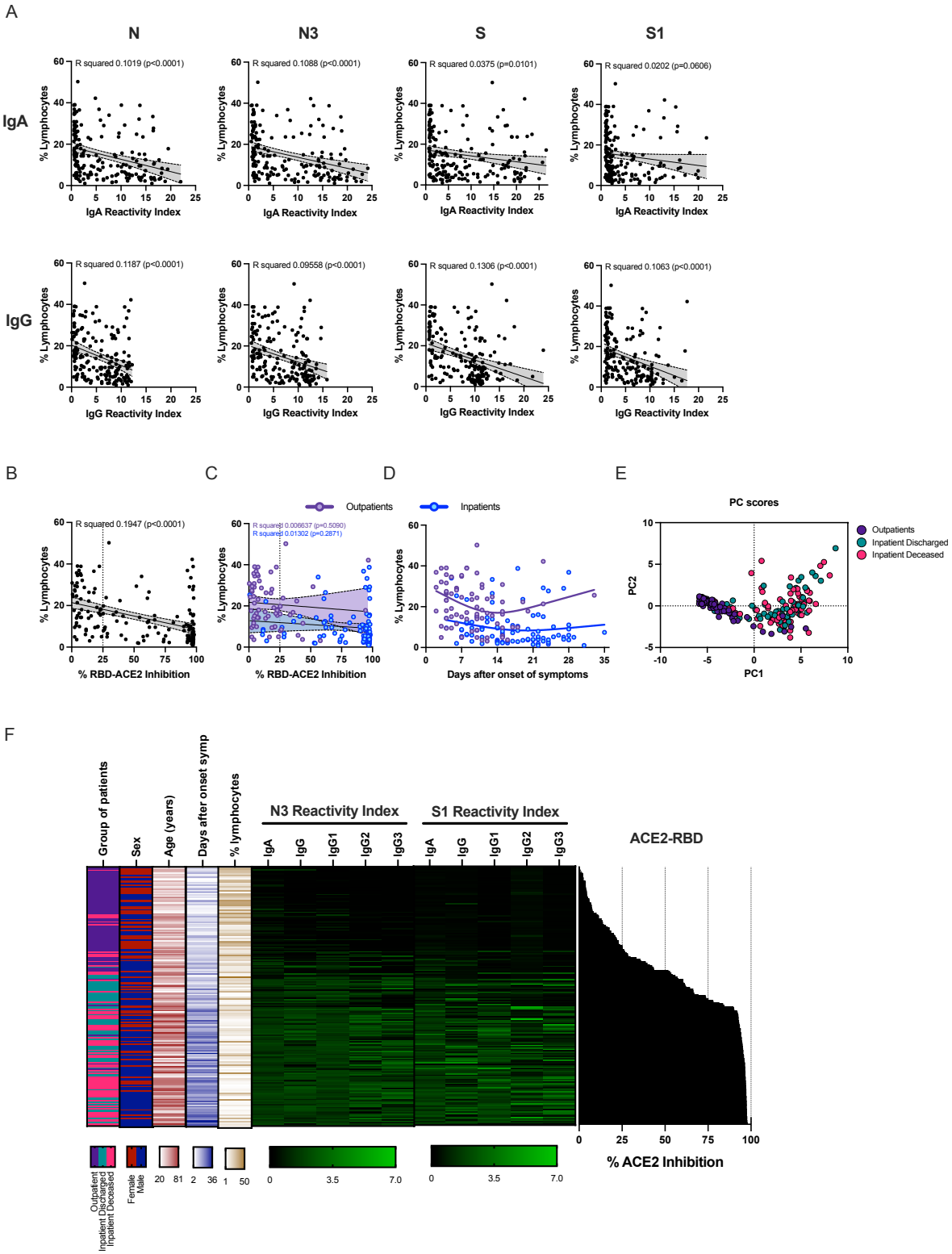

A
B
C
D
N
N3
S
S1
Outpatient
Inpatient
Discharged
Deceased
IgA
IgG
IgG1
IgG3
% RBD-ACE2 inhibition
Healthy
COVID-19
Outpatients
Inpatients
Deceased
Days after onset of symptoms
≤7 days
>7 days
>14 days
IgA Reactivity Index
IgG Reactivity Index
IgG1 Reactivity Index
IgG3 Reactivity Index

FIGURE 6


A
N
N3
S
S1
IgA
IgG
% Lymphocytes
IgA Reactivity Index
IgG Reactivity Index
R squared 0.1019 (p<0.0001)
R squared 0.1088 (p<0.0001)
R squared 0.0375 (p=0.0101)
R squared 0.0202 (p=0.0606)
R squared 0.1187 (p<0.0001)
R squared 0.09558 (p<0.0001)
R squared 0.1306 (p<0.0001)
R squared 0.1063 (p<0.0001)
B
C
D
E
% RBD-ACE2 Inhibition
R squared 0.1947 (p<0.0001)
Outpatients
Inpatients
R squared 0.006637 (p=0.5090)
R squared 0.01302 (p=0.2871)
Days after onset of symptoms
PC scores
PC1
PC2
Outpatients
Inpatient Discharged
Inpatient Deceased
F
Group of patients
Sex
Age (years)
Days after onset symp
% lymphocytes
N3 Reactivity Index
S1 Reactivity Index
IgA
IgG
IgG1
IgG2
IgG3
ACE2-RBD
% ACE2 Inhibition
Female
Male

SUPPLEMENTARY TABLE 1. Summary of patients and tests performed

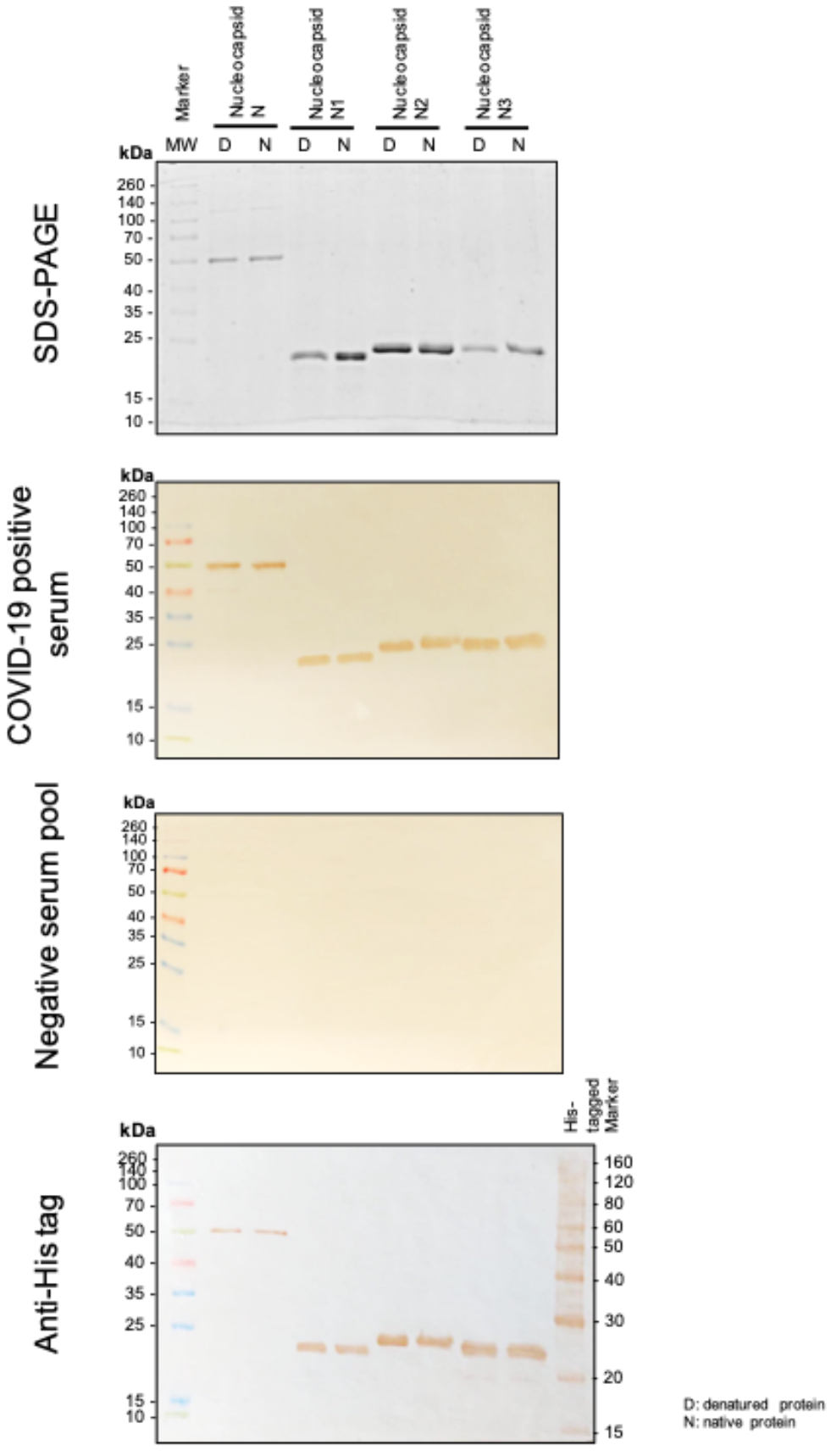
Method	Group of patients	Number of patients	Number of samples
ELISA* IgA IgG IgG1 IgG2 IgG3 IgG4	Healthy[#]	13	224
	Outpatients[§]	80	
	Inpatients Discharged[^]	24 (59[@])	
	Inpatients Deceased[^]	24 (72[@])	
NeutraLISA	Healthy[#]	13	203
	Outpatients[§]	70	
	Inpatients Discharged[^]	20 (54[@])	
	Inpatients Deceased[^]	21 (66[@])	

*All antibodies detection assays were assessed for N, N1, N2, N3, S and S1 antigens;
[#]People without symptoms and SARS-CoV-2 negative;
[§]Symptomatics, with or without fever and SARS-CoV-2 positive;
[^]Symptoms as dyspnea, respiratory discomfort, O2 saturation < 94%, Severe Acute Respiratory Syndrome and SARS-CoV-2 positive.
[@]Total number of samples, including longitudinal samples of patients

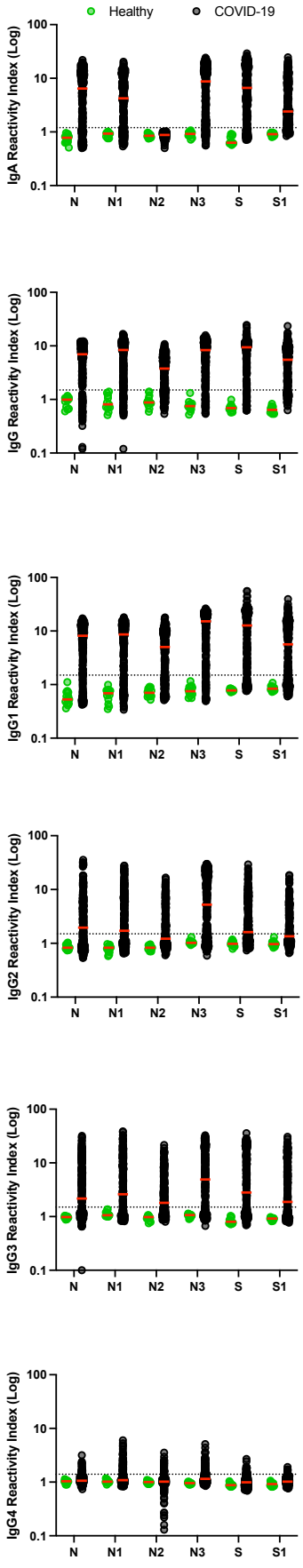
SUPPLEMENTARY TABLE 2. Pre-pandemic blood donors samples and antigen testing for specificity analysis

	Pre-pandemic Blood donors (2014-2016) **N = 78** Specificity % (95% CI)	
Antigen	IgG	IgA
N	91.00% (83.16 – 95.54)	98.91% (93.24 – 99.94)
N1	91.00% (83.16 – 95.54)	97.85% (91.71 – 99.63)
N2	95.79% (88.96 – 98.64)	100.00% (94.95 – 100.00)
N3	97.85% (91.71 – 99.63)	98.91% (93.24 – 99.94)

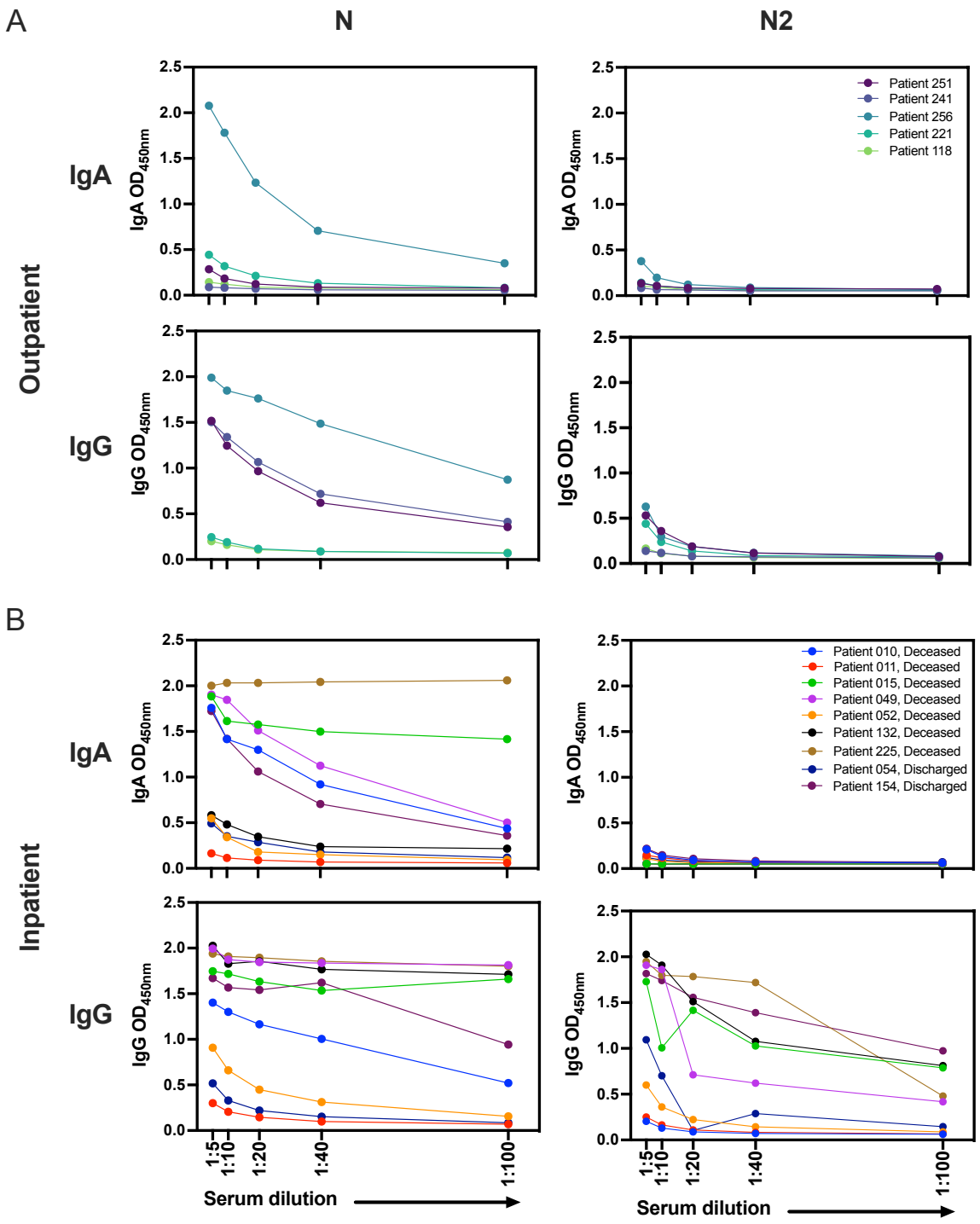
SUPPLEMENTARY FIGURE 1



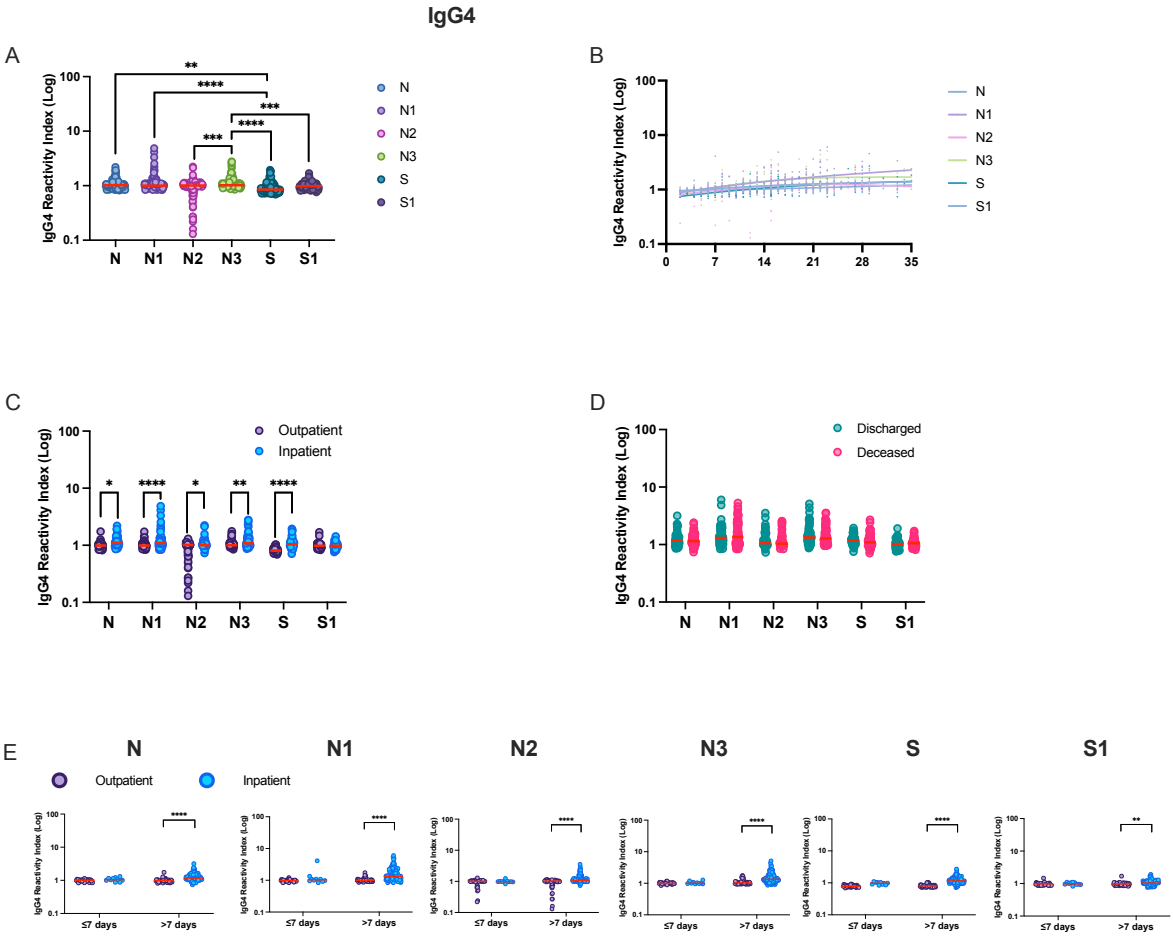
SUPPLEMENTARY FIGURE 2



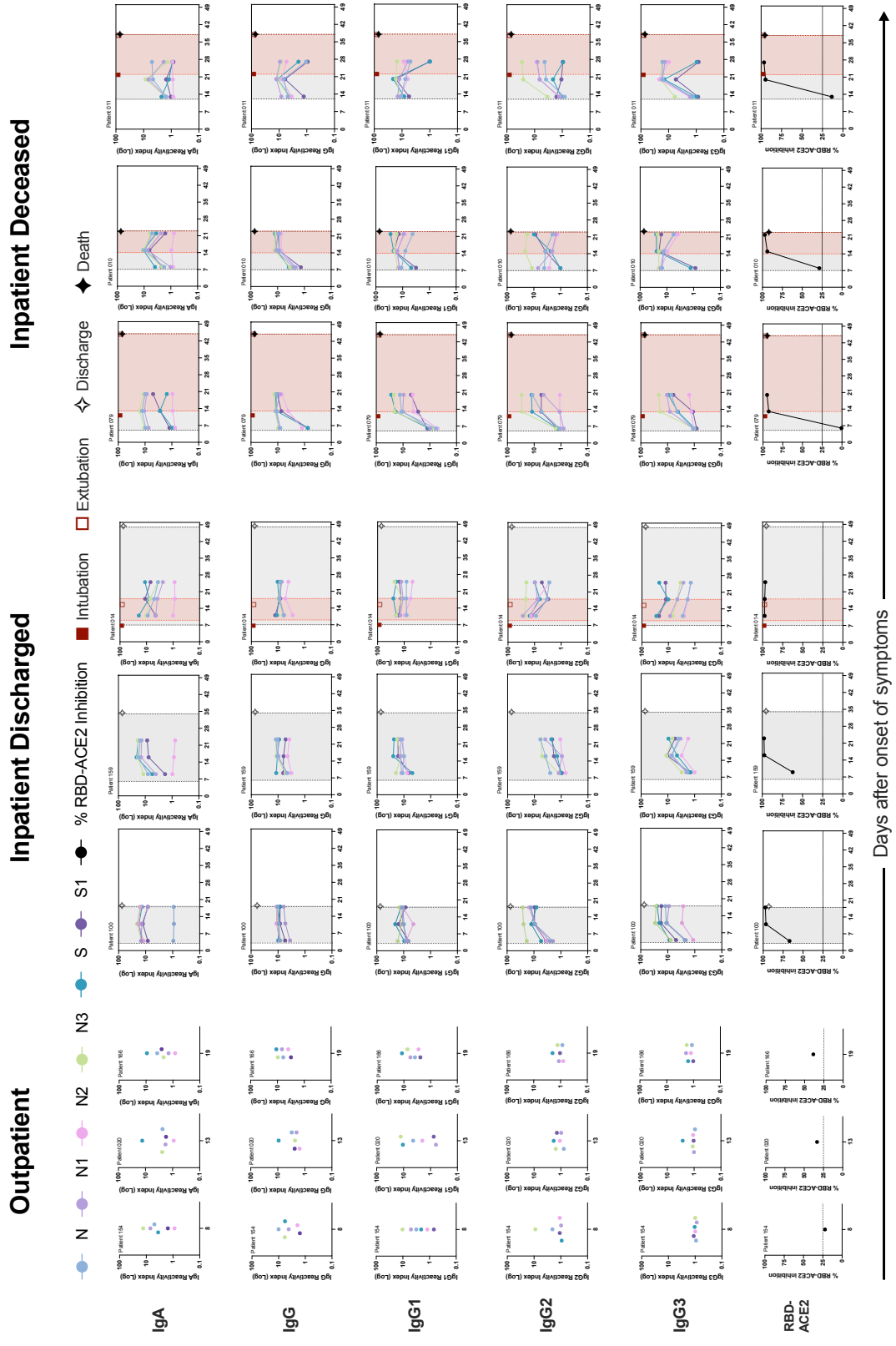
SUPPLEMENTARY FIGURE 3



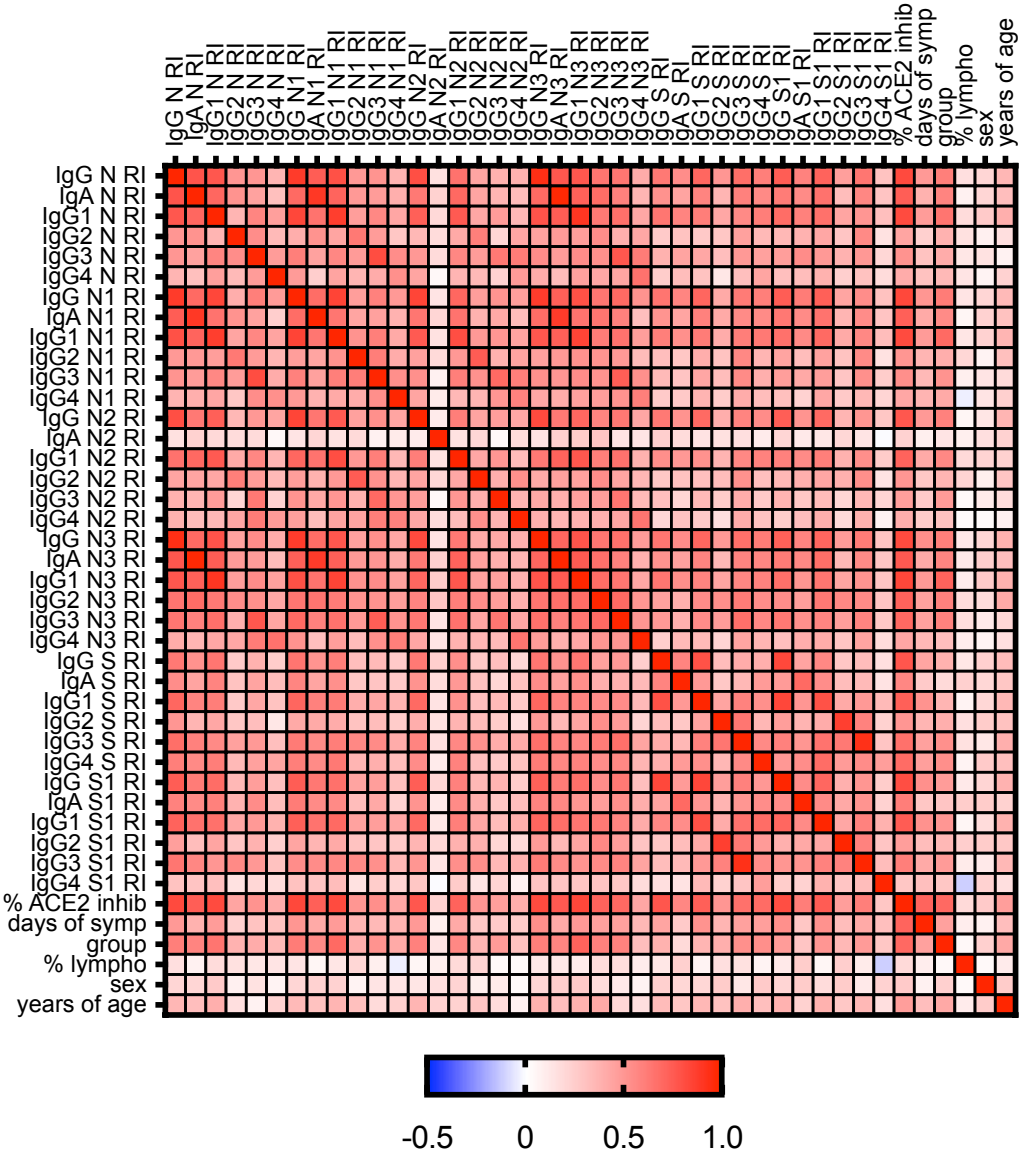
SUPPLEMENTARY FIGURE 4



SUPPLEMENTARY FIGURE 5

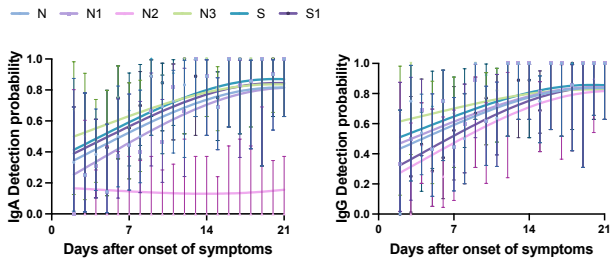


SUPPLEMENTARY FIGURE 6

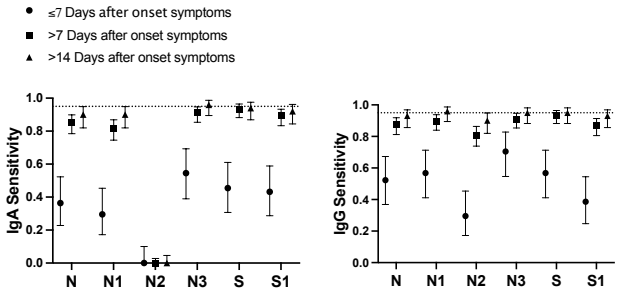


SUPPLEMENTARY FIGURE 7

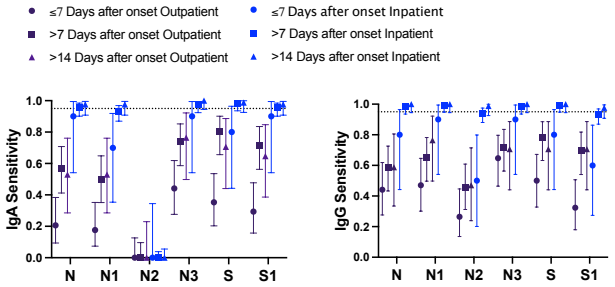
A



B



C

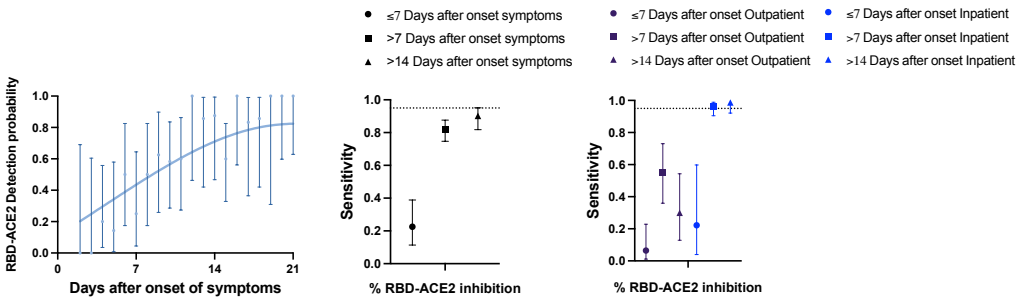


D

Outpatients vs Inpatients AUC 95% CI	N	N1	N2	N3	S	S1
IgA	0.90	0.88	0.67	0.90	0.77	0.86
	0.86 - 0.95	0.83 - 0.93	0.60 - 0.75	0.86 - 0.95	0.70 - 0.84	0.81 - 0.92
IgG	0.87	0.88	0.85	0.86	0.75	0.80
	0.81 - 0.92	0.83 - 0.93	0.79 - 0.91	0.81 - 0.92	0.67 - 0.84	0.73 - 0.88

All AUC presented p<0.0001

E

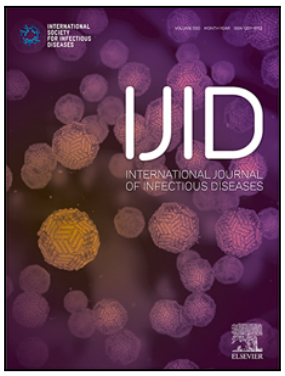


Capítulo 5

ANEXOS

ANEXO 1 – Artigo 1 publicado do nosso grupo de uma coorte em Manaus para COVID-19

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SARS-CoV-2 seroprevalence and associated factors in Manaus, Brazil: baseline results from the DETECTCoV-19 cohort study

Pritesh Lalwani , Bárbara Batista Salgado , Ivanildo Vieira Pereira Filho , Danielle Severino Sena da Silva , Thiago Barros do Nascimento de Morais , Maele Ferreira Jordão , Aguyda Rayany Cavalcante Barbosa , Isabelle Bezerra Cordeiro , Júlio Nino de Souza Neto , Enedina Nogueira de Assunção , Rafaella Oliveira dos Santos , Nani Oliveira Carvalho , Wlademir Braga Salgado Sobrinho , Cristiano Fernandes da Costa , Pedro Elias de Souza , Bernardino Claudio de Albuquerque , Christian A. Ganoza , Roger V. Araujo-Castillo , Spartaco Astofi Filho , Jaila Dias Borges Lalwani , The DETECTCoV-19 study team

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Highlights

- Prevalence of anti-SARS-CoV-2 antibodies was 29.10% (19/08/2020 to 02/10/2020).
- COVID-19 is shaped by socioeconomic inequalities.
- Transmission of SARS-CoV-2 within households is high.
- A large population of Manaus remains susceptible to SARS-CoV-2 after the first wave.

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SARS-CoV-2 seroprevalence and associated factors in Manaus, Brazil: baseline results from the DETECTCoV-19 cohort study

Pritesh Lalwani[1#], Bárbara Batista Salgado[1§], Ivanildo Vieira Pereira Filho[1,2§], Danielle Severino Sena da Silva[1§], Thiago Barros do Nascimento de Morais[1§], Maele Ferreira Jordão[1§], Aguyda Rayany Cavalcante Barbosa[1,3§], Isabelle Bezerra Cordeiro[3], Júlio Nino de Souza Neto[4], Enedina Nogueira de Assunção[4], Rafaella Oliveira dos Santos[1], Nani Oliveira Carvalho[1], Wlademir Braga Salgado Sobrinho[1], Cristiano Fernandes da Costa[5], Pedro Elias de Souza[6], Bernardino Claudio de Albuquerque[7], Christian A. Ganoza[8*], Roger V. Araujo-Castillo[9*], Spartaco Astofi Filho[3,4*], Jaila Dias Borges Lalwani[2#], and The DETECTCoV-19 study team.

[1]Instituto Leônidas e Maria Deane (ILMD), Fiocruz Amazônia, Manaus, Amazonas, Brazil

[2]Faculdade de Ciências Farmacêuticas (FCF), Universidade Federal do Amazonas (UFAM), Manaus, Amazonas, Brazil

[3]Instituto de Ciências Biológicas (ICB), Universidade Federal do Amazonas (UFAM), Manaus, Amazonas, Brazil

[4]Centro de Apoio Multidisciplinar (CAM), Universidade Federal do Amazonas (UFAM), Manaus, Amazonas, Brazil

[5]Fundação de Vigilância em Saúde do Amazonas (FVS/AM), Manaus, Amazonas, Brazil

[6]Programa de Pós - Graduação em Cirurgia, Faculdade de Medicina, Universidade Federal do Amazona (UFAM), Manaus, Amazonas, Brazil

[7]Faculdade de Medicina (FM), Universidade Federal do Amazonas (UFAM), Manaus, Amazonas, Brazil

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[8]Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano Heredia, Lima, Peru

[9]Facultad de Ciencias de la Salud, Universidad Peruana de Ciencias Aplicadas, Lima, Peru

[#]Authors contributed equally

[§]Authors contributed equally

*Authors contributed equally

Key words: Seroprevalence, SARS-CoV-2, Amazon, risk factors, epidemiology

Running Title: Seroprevalence and COVID-19 associated factors

Corresponding author

Jaila Dias Borges Lalwani, PhD

Universidade Federal do Amazonas (UFAM), Faculdade de Ciências Farmacêuticas (FCF), Av. General Rodrigo Octavio Jordão Ramos, 1200 - Coroado I, Manaus - AM, 69067-005

E-mail: jaila@ufam.edu.br

Pritesh Lalwani, Dr. rer. nat.

Instituto Leônidas e Maria Deane (ILMD), Fiocruz Amazônia

Rua Terezina 476, Adrianópolis. Manaus - AM. CEP: 69057-070.

E-mail: pritesh.lalwani@fiocruz.br

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The DETECTCoV-19 study team (Alphabetical order)

Aldina Iacy Paulain Holanda, Ana Lúcia Silva Gomes , Ana Paula Souza de França , Anna Paula de Carvalho, André Victor Rabelo Monteiro, Andressa dos Passos Santos, Antonia de Sousa Teixeira, Antônio Vinicius Soares de Souza, Beatriz Pinheiro, Bianca Pires dos Santos, Brenda Pereira Farias, Bruno Nicolau Paulino, Caio Lúcio Andreola da Silva, Carlos Eduardo Faresin e Silva, Cinthya Iamile Frithz Brandão de Oliveira, Dalila de Alcântara Martins, Diego Castro Squinello, Eline Araújo de Oliveira, Elisson Denny da Costa Carvalho, Evillyn Fernandes Da Costa, Fernanda Rodrigues Fonseca, Fernanda Guilhon Simplicio, Fernanda Serrão Pereira, Gabriele Pimentel Sinimbu, Giane Alves da Silva, Iago Sampaio Fernandes da Costa, Ingrid Silva Correia, Ilia Gilmara Carvalho dos Santos, Jackeline Vieira Guimarães, Jessica Vanina Ortiz, Juliana Correa Romana, José Renato Pereira Cavallazzi, Josineide de Oliveira Novo França, Kerollen Runa Pinto, Marcos Victor Silva Oliveira, Maria Clara Ramos da Silva, Maria Fiamma Farias Freitas, Maria Francisca de Souza Rodrigues, Marne Carvalho de Vasconcellos, Mariza Quercio Machado, Marizete Candido Moraes, Matheus da Silva Damasceno, Michelle Araújo Ruiz, Milena Maria Cardoso de Lemos, Neila Soares Picanço, Patrícia Danielle Oliveira de Almeida, Priscilla Mendes Cordeiro, Rayara Gonzaga Maia, Rebeca Cavalcante Pires, Regiane Carneiro Bezerra, Romeu Santos de Souza, Susy Cavalcante Harjani, Tamiris Moraes Siqueira, Valdenora Patrícia Rodrigues Macedo, Vitor Batista de Souza and Wellington Barbosa de Melo

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Abstract

Background: Manaus located in the Brazilian rainforest has twice experienced a health system collapse due to the SARS-CoV-2 pandemic. However, little is known about which groups among the general population have been more affected.

Methods: A convenience sampling strategy via online advertising recruited 3046 adults between 19/08/2020 and 02/10/2020. Sociodemographic characteristics, COVID-19-related symptoms, COVID-19 testing, self-medication and prescribed medications were recorded. Serum anti-SARS-CoV-2 nucleocapsid IgG antibodies were measured with an ELISA. Prevalence ratios (PR) were obtained using cluster-corrected and adjusted Poisson's regression models.

Results: A crude positivity rate among asymptomatic and symptomatic individuals, was estimated at 29.10%, with a maximum possible seroprevalence of 44.82% corrected by test characteristics and an antibody decay rate of 32.31%. Regression models demonstrated a strong association towards marginalized low-income and vulnerable residents with limited health access. Presence of a COVID-19 case (PR 1.39, 1.24-1.57) or death (PR 2.14, 1.74-2.62) in a household increased greatly the risk of other household members acquiring infection. SARS-CoV-2 seroprevalence was higher among those who self-medicated to prevent infection (PR 1.36, 1.27-1.46).

Conclusions: A disproportionate social and economic disparity was observed among the study participants. The syndemic nature of COVID-19 in the Amazon region needs differential policies and urgent solutions to control the ongoing pandemic.

Key words: Seroprevalence, Amazon, epidemiology, risk factors, SARS-CoV-2 and COVID-19.

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Introduction

Infectious diseases are remarkable for their ability to have a profound impact on humans, particularly vulnerable populations (Fauci and Morens, 2012). The emergence of acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and a lack of effective treatment and non-pharmaceutical interventions to curb transmission have led to an exponential increase in the burden of coronavirus disease 2019 (COVID-19) worldwide (Bo et al., 2021, Hsiang et al., 2020, Kraemer et al., 2020).

In April 2020, the mayor of Manaus declared that the health system had collapsed due to the high volume of severe and critical patients; moreover, the failure in Manaus also meant a state-wide collapse. Since early 2021, Manaus is experiencing a reprise of the healthcare collapse of April 2020, with an astounding increase in COVID-19 reported cases and deaths. As of 25 of January 2021, Brazil has reported over 8.9 million confirmed cases with a 2.5% lethality rate, whereas Amazonas state has reported over 205 thousand cases with 2.88% lethality rate (COVID-19, 2021). Manaus registered 110,689 cases and 4,911 deaths as of 25 of January 2021. (COVID-19, 2021, FVS, 2021) Overall, an adjusted seroprevalence of 14% among general population in June 2020 measured by Wondfo rapid serological test and 44% in October 2020 among blood donors tested by Abbott chemiluminescence assay was estimated in Manaus; however, it is still not clear how different sociodemographic characteristic, health access, and already reported risk factors influenced seroprevalence (Buss et al., 2020, Hallal et al., 2020). Identifying these factors is important to aid in the formulation of targeted public health measures (Aragona et al., 2020). To better understand SARS-CoV-2 epidemiology and

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associated risk factors we designed a prospective cohort (DETECTCoV-19) in Manaus, Brazil. This closely monitored cohort will provide a unique opportunity to determine disease attack rates and to monitor the serological status of Manaus residents, and the relevance of demographic and socioeconomical factors and their association with the prevalence of infection in a setting of high transmission and low non-pharmacological containment measures. Here, we present the overall prevalence of SARS-CoV-2 infection and associated factors obtained from the DETECTCoV-19 cohort.

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Methods

Ethics approval

The research ethics committee of Federal University of Amazonas (UFAM) approved this study (CAAE:34906920.4.0000.5020) in accordance with Brazilian law, and the Helsinki declaration. All the participants gave oral and written consent prior to enrolment.

Study design and participants

A longitudinal study (DETECTCoV-19) was designed to follow-up Manaus citizens for up to 6 months after recruitment with a sample collection every 8-12 weeks. The study included people of both sexes ≥18 years old resident of Manaus who agreed to participate. A convenience sampling strategy was adopted for recruitment, and 3046 individuals were recruited between 19/08/2020 and 02/10/2020. DETECTCoV-19 was advertised on social media and a university website. All participants registered online and presented at the blood collection centre (Nursing School, Federal University of Amazonas), where they signed the consent form, filled out an electronic questionnaire and donated a blood sample for testing (Supplementary Figure 1).

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Data and sample collection

Firstly, sociodemographic data, including age, sex, occupation, and residential address of each participant was collected. Secondly, information related to COVID-19 disease, symptoms since the start of the pandemic, prior diagnosis, preventive self-medication and prescribed medication used for treatment of symptoms was recorded. Finally, an independent form was used to record the laboratory results.

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Participant data was recorded using the Research Electronic Data Capture (REDCap) software, and all the data was stored on the local ILMD/Fiocruz Amazônia server. Details of the blood collection, sample processing and storage are described in the supplementary material and methods.

ELISA to detect anti-SARS-Cov-2 nucleocapsid IgG antibodies

We developed and used an indirect Enzyme Linked Immunosorbent Assay (ELISA)-based serological assay to measure anti-SARS-CoV-2 nucleocapsid IgG antibody titres in serum samples using recombinant full-length SARS-CoV-2 nucleocapsid protein (N, residues 1 to 419, GenBank QHD43423.2) expressed in *E. coli* and purified by affinity and size exclusion chromatography (Supplementary Figure 7). We determined antigen concentration, sample dilution, and secondary antibody concentration by a checkerboard method to achieve optimal signal to noise ratio. Antigen lot and inter-operator variations were assessed throughout the assay's development stage, before the analysis of the study samples. The sensibility and specificity of the assay were determined using sera from SARS-CoV-2 RT-PCR-positive patients (n=293) and pre-pandemic controls (n=229, Table S1). The anti-SARS-CoV-2 nucleocapsid IgG ELISA had a sensitivity of 89.07% (95% CI, 84·79 – 92.30) and 94.28% (95% CI, 89.44 – 97.07) for patient with ≥ 7 days and ≥14 days after onset of COVID-19 symptoms, respectively. A specificity of 97.03% (95% CI, 93.72 – 98.69) was estimated using the pre-pandemic serum samples. An anti-SARS-Cov-2 N IgG antibody reactivity index (RI) was expressed as the ratio between optical density of patient sample and negative control. All samples with a RI value equal or above 1.5 (assay cut-off) were considered positive. Further details of

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the in-house ELISA protocol and performance evaluation are described in the supplementary material.

Data analysis

This report evaluated the cohort baseline data, constituting a cross-sectional analysis. Crude seroprevalence was further adjusted by ELISA test characteristics (Diggle, 2011) and antibody decay, defined as the proportion of patients who had a current positive ELISA over the number of participants who had any positive serologic test at any point before the study, regardless of symptoms or time elapsed. Chi-square tests or Fisher´s exact for two-by-two contingency tables were used to examine the statistical significance and association between study variables. The presence or absence of antibodies to SARS-CoV-2 was the primary dependent variable, and characteristics that were identified in the descriptive analysis were the independent variables. Statistically significant variables were then used in a Poisson regression model with robust variance to estimate prevalence ratios. Variance was corrected per clusters (administrative areas) and the models were adjusted per sex, age, family income, presence of household members with COVID-19, use of preventive self-medication, and prior COVID-19 diagnosis.

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Results

Using online advertising, 4394 individuals agreed to participate in the study by making an appointment via the study website. From these, 3600 attended the interview (Supplementary Figure 1). In total, 554 (15.38%) individuals were ineligible or refused to participate in the longitudinal study, and were not included. Table 1 details the demographic characteristics and SARS-CoV-2 seropositivity rate estimated by anti-SARS-Cov-2 nucleocapsid IgG ELISA. Of the 3046 individuals included in the cohort, 60.80% comprised of women. Supplementary Figure 2 compares DETECTCoV-19 study cohort features with the census data from Manaus. Supplementary Figure 3 describes the distribution of patients with previous flu-like symptoms before recruitment compared to the COVID-19 epidemic curve of confirmed SARS-CoV-2 cases in Manaus.

A total of 886 individuals, symptomatic and asymptomatic, tested positive for SARS-CoV-2 specific IgG antibodies with an estimated crude IgG seroprevalence of 29.10% (95% exact CI 27.5 - 30.7). Since study participants presented with flu-like symptoms for at least one week before recruitment, we adjusted the crude seropositivity by the sensitivity and specificity of an ELISA determined for at least seven days of symptoms. With a sensitivity at 89.07% and specificity of 97.03%; the adjusted seroprevalence was 30.34% (95% CI 28.5 – 32.3) using the method described by Diggle (Diggle, 2011).

In our study, more than two thirds of the patients with prior COVID diagnosis (either by PCR or serology) were still positive at the cohort baseline, regardless of time since symptoms onset. Of the 141 participants who had a positive serology before

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the study, 38 tested negative. Using these data, we estimated a 26.95% antibody decay for the whole cohort over a median time of 131.5 days (interquartile range: 63-152) between both tests. Scientific literature suggests that antibody decay rate is higher among asymptomatic patients (Yang et al., 2021), possibly up to 2.08 times based on Chia et al (Chia et al., 2021). Considering 163 of the 886 IgG positive participants were asymptomatic (18.4%), our antibody decay proportion could be as high as 32.31%. Extrapolating those values, the actual seroprevalence in our cohort could oscillate between 41.53% and 44.82%. Anti-SARS-CoV-2 antibody levels estimated by reactivity index were elevated in symptomatic patients compared to asymptomatic patients (Supplementary Figure 5) and positively correlated with patient age (Supplementary Figure 6). Supplementary Figure 7 describes the distribution of COVID-19 positive individuals stratified by days after onset of flu-like symptoms, self-declared by study participant.

Seropositivity of SARS-CoV-2 antibodies was significantly higher in men compared to women (Table 1). The highest and lowest age-stratified seroprevalence was observed in individuals aged between 50 to 59 years and ≥ 60 years of age, respectively. The prevalence rates were inversely correlated with occupation-type and family income. Prevalence was higher among the poorest (35.50%) in relation to 24.45% among the wealthiest (Table 1). In general, individuals living in detached or conjugated houses had an overall higher SARS-CoV-2 antibody prevalence compared to individuals living in condo houses and apartment buildings. Moreover, increase in the number of adult or children in the family household significantly increased the seropositivity rates. Among the administrative zones of Manaus,

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Centro-Sul (Centre-South) had the lowest prevalence of 28.29%, whereas Leste (East) zone had the highest prevalence at 39.80% (Table 1).

Table 2 describes COVID-19 related risk factors in the study population. COVID-19 disease in a distant family member living locally or in another town had no influence on the serological status of the study participant. On the other hand, COVID-19 disease in a household member significantly increased the prevalence from 25.18% to 44.89% (Table 2). Moreover, a death in the family household due to COVID-19 further increased the SARS-CoV-2 antibody prevalence to 56% among the study participants (Table 2). Individuals with or without comorbidities had similar antibody prevalence. Overall, we observed one fourth of our study population self-medicated to prevent against SARS-CoV-2 infection. However, individuals taking self-medication of over-the-counter drugs or controlled drugs to prevent SARS-CoV-2 infection had a prevalence of 38.64% compared to 25.99% group that did not self-medicate (Table 2).

Table 3 summarizes individual symptoms reported by study participants since the start of the pandemic and access to COVID-19 testing in the city of Manaus. Overall, 77% of the study cohort reported at least one symptom since March 2020 and 17% in the last seven days before recruitment. Less than ten percent of symptomatic individuals were tested by RT-PCR test, and prior COVID-19 diagnosis increased SARS-CoV-2 antibody prevalence to 61.60% among these individuals. Around half of COVID-19 diagnosed patients reported not taking any over-the counter or prescribed medicine. Azithromycin followed by ivermectin and paracetamol were mainly prescribed to COVID-19 diagnosed patients (Table 3).

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All above-described statistically significant variables were used in a Poisson regression model described in Table 4. Data was corrected and adjusted for variables that were not controlled experimentally to identify true risk factors (Table 4 and Table S2). Firstly, men had an increased risk compared to women with an estimated adjusted prevalence ratio of 1.22 (95% CI, 1.08-1.38). Secondly, individuals with a well-paid job, good-housing quality and private insurance had a lower prevalence ratio and subsequently had a reduced risk of acquiring COVID-19. Moreover, having four or more adults, or three or more children in the family household significantly elevated the risk of acquiring COVID-19 infections. Most importantly, SARS-CoV-2 infection of a household member increased risk of acquiring infection among other household members and had an adjusted prevalence ratio 1.39 (95% CI 1.24-1.57). Furthermore, prevalence ratio elevated to 2.14 (95% CI 1.74 – 2.62) when a household had a confirmed death associated with COVID-19 disease. Overall, seroprevalence ratio was significantly higher if the individual had flu-like symptoms since March 2020 or a prior COVID-19 diagnosis (Table 4).

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Discussion

We estimated the relative frequency of anti-SARS-CoV-2 nucleocapsid antibodies and associated factors six months after the start of the pandemic in Manaus, Brazil. Our results indicate a high seropositivity of 29.10% (30.34% if adjusted by the sensitivity and specificity of the test), which includes asymptomatic and symptomatic individuals. Furthermore, our analyses showed that the pandemic disproportionately affected low-income families and those with limited access to health care. Our data demonstrates that living in the same household with a suspected/confirmed patient increased enormously the risk of contracting the disease, questioning if individuals adopted any home isolation measures. Additionally, preventive self-medication was associated with higher SARS-CoV-2 infection prevalence, probably because it is employed by people at higher contagion risk.

Anti-SARS-CoV-2 Nucleocapsid ELISA

We developed and validated a robust in-house indirect IgG ELISA using nucleocapsid protein, as a tool to estimate seropositivity. Test performance for the in-house assay was similar to other tests reported in other countries and regions (Cota et al., 2020, Deeks et al., 2020). We tested the performance of our assay using a wide range of samples, including outpatient or patients with mild symptoms, and inpatients or hospitalized patients with severe disease for determining the sensibility of the assay. Hence, our approach maximized the range of detection of serum IgG antibody concentrations that can be expected in an exposed heterogeneous population. The performance of our test improved markedly 14 days post onset of symptoms (Cota et al., 2020). Additionally, our specificity analysis

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included samples that were tested for other tropical diseases and infections to evaluate cross-reactivity. Pre-pandemic plasma samples positive for dengue, leptospirosis and malaria cross-reacted using our in-house assay (Cota et al., 2020, Masyeni et al., 2021). However, we did not have access to other (seasonal) coronavirus-positive samples and could not evaluate cross-reactivity against the other coronaviruses nucleocapsid protein. In-house assays with robust validation and good performance as ours can be a viable alternative to commercial assays in low-income countries.

Manaus Seroprevalence

Seropositivity rate crude and adjusted by test performance were high. The estimated number of affected people in this study suggests that the disease burden could be several times higher the one estimated using RT-PCR (FVS, 2021). In part, this difference could be due to the inclusion of asymptomatic and oligosymptomatic patients, who usually did not seek medical attention or SARS-CoV-2 viral testing and because of the lower testing rates in Brazil. A population-based household survey in the Maranhão capital region between July and August reported 40.4%, one of the highest seroprevalence estimates in Brazil (Silva et al., 2020). A repeated cross-sectional survey observed an increasing prevalence of SARS-CoV-2 antibodies in the Amazon (North) and Northeast region of Brazil between May and June 2020 (Hallal et al., 2020). The high seroprevalence observed by us and others, in areas of Brazil characterized by higher poverty rates, is in agreement with the recent evidence that COVID-19 has disproportionately affected marginalized populations where the human-development-index is the lowest (Horta et al., 2020). Buss et.al. estimated an attack rate of 66% in June rising to 76% in October based on

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mathematical estimates of antibody decline among blood donors in Manaus (Buss et al., 2020); they assumed constant antibody decay rates towards nucleocapsid protein, which may have overestimated the attack rate (Buss et al., 2020, Prowse et al., 2020). In our study, 61.6% (154/250) patients with prior COVID diagnosis (either by PCR or serology) were still positive at the cohort baseline, regardless of time since symptoms onset. Using patient reported data, we can estimate up to 32.31% as antibody decay proportion for the whole cohort, putting a maximum disease prevalence at 44.82%. Our maximum seroprevalence estimate is high, but also suggests that a large proportion of the vulnerable population is still susceptible (Aschwanden, 2020). Therefore we propose that the high proportion of susceptible population may partially explain the recent resurgence of SARS-COV-2 infections in Manaus (Ferrante et al., 2020).

To date, the role of antibodies in controlling disease severity during infection, the duration of serological responses, and the extent to which patient antibody responses may be protective against re-exposure remain to be fully elucidated. Antibody measurements do not necessarily reflect protection after infection, nor do they fully indicate the efficacy of active immunization (Addetia et al., 2020, Fontanet and Cauchemez, 2020, McMahan et al., 2020). Therefore, inferring immunity or protection from a single biomarker, at the individual or population levels, can be misleading. We acknowledge this limitation and interpret our serological findings and their implications with caution. Future studies comparing vaccine-induced immune responses to those stimulated by viral infection, and those of individuals who become re-infected, will help clarify the immunological correlates of protection (Anderson et al., 2020).

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Associated Factors

It is true that male sex, older age and comorbidities are associated to higher complication rates and mortality; however, their role in acquiring the infection is less clear (Giannouchos et al., 2020, Petrakis et al., 2020). We found that male sex was associated with higher seropositivity, which correspond to other studies. (Elmore et al., 2020) However, other population-based studies have failed to identify gender differences and risk of SARS-CoV-2 infection (Amanat et al., 2020, Hallal et al., 2020, Poustchi et al., 2020). We found an increasing seroprevalence with age until the group between 50 and 59 years old, with a marked decreased for the group older than 60. This could be explained by an increased risk of acquiring the infection with age (Elmore et al., 2020, Hallal et al., 2020, Stringhini et al., 2020), which was attenuated in the older group by a state mandate for elderly people to stay at home and stop performing presential work activities.

Our data also demonstrates a strong association between socioeconomic status and risk of acquiring SARS-CoV-2 infection. Additionally, we observed that people living in condo houses and apartment buildings have less seroprevalence, probably because they live in closed communities with strict COVID-19 rules. There was even marked differences when comparing different geographic areas. East Manaus which corresponds to the most crowded and poorer area of the city has seroprevalence up to 38.8%, almost double than the most affluent areas (South-Centre Manaus). When adjusting for all these factors, family income was the stronger factor predicting infection, with a 33% reduction in higher income houses compared with the lowest group. Access to primary health for the economically vulnerable group has always

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been a limiting factor worldwide; during the ongoing pandemic, this inequality has been even more evident (Bambra et al., 2020, Hallal et al., 2020, Orellana et al., 2020). All these studies establish an undemocratic distribution of SARS-CoV-2 virus and its consequences.

Households with four or more members, and with three or more children had a staggering proportion of positive cases, higher than 40%. In addition, our data showed that having a household member diagnosed with COVID increased 40% the chance of being positive, and in case of having a deceased member, the probability was more than double. These findings reveal that SARS-CoV-2 positive index cases may have not followed directives on home isolation. If we considered that household isolation is voluntary and not strictly enforced, many of the newly infected family members could spread the disease, making these houses, hotbeds for infection. A possible solution is to employ centralized isolation in government sponsored facilities. Additionally, we recommend strict follow-up of the diagnosed patients, their family members, and contacts to reduce virus transmission.

In our study, we observed that people who self-medicated as prophylaxis had higher seroprevalence of COVID-19. Evidence suggests that most of these drugs used as prophylaxis may not be effective (Mega, 2020). Moreover, taking a preventive self-medication could produce a false sensation of safety and security from the disease, leading to neglect other well-established preventive measures. Nonpharmaceutical interventions have been a source of debate in Brazil, hampering the SARS-CoV-2 control efforts. Additionally, conflicting stances between the state and federal

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government in Brazil on the strategies to face the pandemic could have played a role in its course (Ferrante et al., 2020, Hsiang et al., 2020).

Limitations and Strengths

A convenience sampling strategy was adopted instead of a populational approach due to financial and logistical constrains. The sampling was based on on-line and university website advertising, which potentially excluded individuals who did not have access to this information; higher education and university employees were oversampled. We had only one collection centre and this could have possibly excluded individuals that did not have resources to travel to the study centre for recruitment or lived far from the recruitment centre. The recruited cohort may not completely represent the general population of Manaus city and may vary with their health seeking and social distancing behaviour, immune response to infection and disease exposure risk; hence, results related to prevalence between study groups and associated risk factors should be interpreted with caution and not extrapolate to the Manaus population. Additionally, it is possible that participants approached the study to know their serological status for COVID-19, meaning they considered themselves at higher risk or having a higher percentage of flu-like symptoms since March 2020, leading to overreporting. Therefore, our seroprevalence estimates should be confirmed and extended by other studies, including serosurveys that use probabilistic sampling to enrol more representative populations. Regarding our in-house assay, we acknowledge that while Nucleocapsid and Spike proteins are abundantly expressed in SARS-CoV-2-infected cells and tissue, and that antibody responses towards both are highly correlated (Jiang et al., 2020, Noval et al., 2021),

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our in-house approach that included only Nucleocapsid protein as an antigen to detect seropositivity may potentially underestimate true disease seroprevalence.

On the contrary, our study and analysis has numerous strengths. Study cohort has representation of both the sexes, all age and economic groups and we have participation of individuals from all administrative zones of Manaus. Additionally, we have clinical, pharmacological and SARS-CoV-2 testing data from the symptomatic and asymptomatic recruits. Data collection was performed using electronic forms with internal check to improve the quality of the data and have few missing values. We also used a highly specific and sensitive immunoassay. To the best of our knowledge DETECTCoV-19 is one of the first longitudinal studies in the North of Brazil that aims to understand SARS-CoV-2 epidemiology and associated risk factors.

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Conclusion

Our baseline analysis of the DETECTCoV-19 cohort revealed a high seroprevalence in Manaus and demonstrated a disproportionate social and economic disparity among the study participants. Further prospective analyses of the cohort will allow us to determine rates of seroconversion over time, behavioural aspects of virus transmission and importantly the role of declining antibody titres and subsequent reinfection with SARS-CoV-2. In Amazonas and worldwide social and economic disparities, as well as inequalities in access to primary health, have amplified the impact of the COVID-19 pandemic. Governmental policies that do not take into account the syndemic nature of COVID-19 will have disproportionately long-term economic, social and health consequences on those who are already

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disadvantaged. Taken together, mass molecular testing and contact tracing, strict enforcement of voluntary isolation rules and non-pharmaceutical interventions, as part of the community control measures, are urgently needed to reduce SARS-CoV-2 transmission and its health and social impact.

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Contributors

PL and JDBL are the principal investigator of this study and acquired necessary funding. PL and JDBL conceived the study with input from CFC, PES, BCA, CAG and RVA. Antigen design and protein purification was performed by PL, BBS, IBC, JNSN, ENA and SAF. Sample collection was led by JDBL with assistance from CFC, PES, BCA. The laboratory set-up and sample processing were coordinated by PL and BBS. BBS, IVPF, DSSS, TBNM, MFJ, ARCB, ROS, NOC, WBSS processed blood samples, performed laboratory testing, collected data and approved test results with PL supervision. PL, BBS and JDBL coordinated data acquisition and data management. Data was cleaned and prepared by PL, BBS and RVA. Statistical analyses and data visualization were done by PL and CAG, and RVA lead the statistical analyses. PL, JDBL, CAG and RVA wrote the manuscript. All authors revised and approved the final version of this manuscript.

Conflict of interests

The authors declare no conflict of interest.

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Ethics approval

The research ethics committee of Federal University of Amazonas (UFAM) approved this study (CAAE:34906920.4.0000.5020) in accordance with Brazilian law, and the Helsinki declaration. All the participants gave oral and written consent prior to enrolment.

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Declaration of interests

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

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Table 1: Study population and health access according to SARS-CoV-2 seroprevalence and symptoms in Manaus, Amazonas state of Brazil

Characteristics	Categories	Total	Column %	IgG positive	Prevalence Row %	p value Chi2 test	IgG positive and Symptoms[1]	Prevalence Row %	p value Chi2 test
Total		**3,046**		**886**	**29.10**		**725**	**23.84**	
Demographic structure									
Sex	Female	1,852	60.80	508	27.43	0.012	427	23.08	0.220
	Male	1,194	39.20	378	31.68		298	25.02	
Age	18-29	703	23.08	197	28.06	0.025	167	23.82	0.015
	30-39	758	24.89	205	27.04		165	21.83	
	40-49	692	22.72	207	29.91		171	24.71	
	50-59	558	18.32	191	34.23		158	28.37	
	>=60	335	11.00	86	25.67		64	19.10	
Marital status	Married/Stable Union	1,424	46.98	431	30.27	0.382	353	24.79	0.512
	Divorced/Widow	275	9.07	81	29.45		65	23.64	
	Single	1,332	43.95	371	27.87		305	22.92	

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Sexual Orientation	Heterosexual	2,694	90.92	789	29.30	0.057	647	24.03	0.188
	Homo-/Bi-/Transsexual	269	9.08	64	23.79		55	20.45	
Occupation	Professional Higher	1,655	54.91	437	26.42	0.004	365	22.08	0.090
	Professional Middle	353	11.71	114	32.29		91	25.93	
	Worker/Informal	663	22.00	221	33.33		171	25.79	
	Unemployed	343	11.38	104	30.32		91	26.61	
Family Income	0-3 minimum wages	1,080	36.27	383	35.50	<0.001	310	28.73	<0.001
	4-6 minimum wages	712	23.91	197	27.67		164	23.03	
	>6 minimum wages	1,186	39.83	290	24.45		239	20.15	
Housing	Detached house	1,524	50.33	492	32.28	<0.001	399	26.18	<0.001
	Conjugated house	285	9.41	98	34.39		85	29.82	
	Condo house	293	9.68	79	26.96		61	20.82	
	Apartment	926	30.58	215	23.24		179	19.35	
Number Adults in	1	282	9.35	71	25.18	<0.001	57	20.21	0.001
residence	2	1,119	37.11	294	26.30		237	21.20	
	3	756	25.07	215	28.44		181	23.94	
	4+	858	28.46	296	34.50		243	28.32	
Number Children in	0	1,758	58.64	482	27.43	0.003	403	22.94	0.380

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residence	1	781	26.05	235	30.09		194	24.84	
	2	345	11.51	108	31.30		86	24.93	
	3+	114	3.80	49	42.98		33	28.95	
Administrative zones	Centro-Sul (Centre-South)	960	31.52	230	23.98	<0.001	183	19.10	<0.001
	Centro-Oeste (Centre-West)	258	8.47	73	28.29		64	24.81	
	Leste (East)	384	12.61	149	38.80		118	30.81	
	Norte (North)	568	18.65	175	30.81		145	25.57	
	Oeste (West)	344	11.29	99	28.78		85	24.78	
	Sul (South)	532	17.47	160	30.08		130	24.44	

§Participants who had both IgG SARS-CoV-2 positive test and reported symptoms prior to recruitment since March 2020.

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Table 2: COVID-19 Risk Factors and preventive self-medication according to SARS-CoV-2 seroprevalence and symptoms in Manaus, Amazonas state of Brazil

Characteristics	Variables	Total	Column %	IgG positive	Prevalence Row %	p value Chi2 test	IgG positive and Symptoms[1]	Prevalence Row %	p value Chi2 test
Total		**3,046**		**886**	**29.10**		**725**	**23.84**	
Private Insurance	No	1,235	40.68	408	33.04	<0.001	329	26.64	0.003
	Yes	1,801	59.32	476	26.44		396	21.94	
Influenza Vaccine	Last time 2020	1,441	47.53	398	27.62	0.272	326	22.62	0.481
	Last time 2019	698	23.02	220	31.52		175	25.07	
	Prior to 2019	586	19.33	170	29.01		142	24.23	
	Never	307	10.13	94	30.72		79	25.82	
Risk Factors									
[1]Family members with COVID-19	No	1,305	43.01	377	28.91	0.346	299	22.93	0.264
	Yes, alive	1,395	45.98	419	30.04		351	25.16	
	Yes, deaths	334	11.01	87	26.05		73	21.86	

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Household members	No	2,428	80.37	611	25.18	<0.001	486	20.02	<0.001
with COVID-19	Yes, alive	568	18.80	255	44.89		224	39.44	
	Yes, deaths	25	0.83	14	56.00		11	44.00	
Pregnancy	No	3,030	99.47	884	29.18	0.175*	723	23.90	0.387*
	Yes	16	0.53	2	12.5		2	12.50	
Comorbidities (any)	No	1,995	65.58	593	29.74	0.286	478	23.97	0.815
	Yes	1,047	34.42	292	27.89		247	23.59	
Which Comorbidities	Asthma	198	18.91	58	29.29	0.696	50	25.25	0.688
	Diabetes	186	17.77	64	34.41	0.184	51	27.42	0.294
	Hypertension	447	42.69	131	29.31	0.856	111	24.83	0.701
	Obesity	201	19.20	56	27.86	0.578	49	24.38	0.898
	Cardiopathy	60	5.73	18	30.00	0.965	16	26.67	0.630
	Cancer	17	1.62	5	29.41	0.977	4	23.53	1.000*
	Other	257	24.55	61	23.74	0.046	55	21.40	0.361
Preventive Self-medication (any)	No	2,293	75.38	596	25.99	<0.001	460	20.06	<0.001
	Yes	749	24.62	289	38.64		265	35.43	
Which medication	Nitazoxanide	28	3.74	7	25.00	0.905	7	25.00	0.517

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	Azithromycin	161	21.50	67	41.88	<0.001	65	40.63	<0.001
	Hydroxy/Chloroquine	10	1.34	5	50.00	0.085	5	50.00	0.019
	Corticosteroids	33	4.41	14	43.75	0.023	13	40.63	0.004
	Ivermectin	268	35.78	83	31.09	0.074	73	27.34	0.006
	Paracetamol	268	35.78	121	45.15	<0.001	116	43.28	<0.001
	Other	260	34.71	104	40.00	<0.001	96	36.92	<0.001

* Fisher's Exact Test

§Participants who had both IgG SARS-CoV-2 positive test and reported symptoms prior to recruitment since March 2020.

Family members with COVID-19 implies family members outside the household.

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Table 3: Symptoms and Diagnostic Tests according to SARS-CoV-2 seroprevalence and symptoms in Manaus, Amazonas state of Brazil

Characteristics	Variables	Total	Column %	IgG positive	Prevalence Row %	p value Chi2 test	IgG positive and Symptoms[3]	Prevalence Row %	p value Chi2 test
Total		**3,046**		**886**	**29.10**		**725**	**23.84**	
Symptoms									
Flu-like Symptoms last 7 days	No	2,479	82.61	716	28.89	0.474	---	---	
	Yes	522	17.39	159	30.46				
Flu-like Symptoms since March (any)	No	695	22.52	163	23.80	0.001	---	---	
	Yes	2,357	77.48	722	30.65				
Which Symptoms	Anosmia	525	22.27	341	64.95	<0.001	---	---	
	Body aches	971	41.20	372	38.35	<0.001	---	---	
	Chest Pain	337	14.30	138	41.07	<0.001	---	---	
	Chills	343	14.55	156	45.48	<0.001	---	---	

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	Conjunctivitis	61	2.59	19	31.15	0.200	---	---	
	Cough	842	35.72	304	36.10	<0.001	---	---	
	Diarrhoea	655	27.79	206	31.45	0.002	---	---	
	Fever	717	30.42	318	44.41	<0.001	---	---	
	Headache	1,633	69.28	508	31.13	<0.001	---	---	
	Lack of appetite	339	14.38	178	52.66	<0.001	---	---	
	Palpitations	239	10.14	76	31.80	0.015	---	---	
	Shortness Breath	487	20.66	175	35.93	<0.001	---	---	
	Side chest pain	612	25.97	224	36.66	<0.001	---	---	
	Skin rashes	130	5.52	45	34.62	0.009	---	---	
	Sore throat	1,049	44.51	315	30.06	0.004	---	---	
	Vomiting	140	5.94	44	31.43	<0.001	---	---	
Prior COVID-19 Diagnosis									
Prior SARS-COV-2 RT-PCR	No	2,788	92.35	798	28.63	<0.001	647	23.21	<0.001
	Yes, Negative	172	5.70	40	23.26		36	20.93	
	Yes, Positive	59	1.95	42	71.19		37	62.71	
Prior Serological test	No	2,349	77.76	660	28.11	<0.001	528	22.49	<0.001

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	Yes, Negative	531	17.58	116	21.85		103	19.40	
	Yes, Positive	141	4.67	103	73.05		89	63.12	
Prior COVID-19 Diagnosis§	No	2,771	91.72	728	26.28	<0.001	588	21.23	<0.001
	Yes	250	8.28	154	61.60		135	54.00	
Took medications after COVID-19 diagnosis	No	109	43.60	51	46.79	<0.001	43	39.45	<0.001
	Yes	141	56.40	103	73.05		92	65.25	
Which Medications	Nitazoxanide	11	7.80	10	90.91	0.008*	10	90.91	0.002*
	Azithromycin	108	76.60	78	72.22	<0.001	70	64.81	<0.001
	Hydroxy/Chloroquine	27	19.15	19	70.37	0.028	17	62.96	0.028
	Corticosteroids	36	25.53	28	77.78	0.001	26	72.22	0.001
	Ivermectin	77	54.61	52	67.53	0.005	47	61.04	0.004
	Paracetamol	59	41.84	46	77.97	<0.001	44	74.58	<0.001
	Others	38	26.95	28	73.68	0.004	25	65.79	0.005

* Fisher's Exact Test

§Participants who had both IgG SARS-CoV-2 positive test and reported symptoms prior to recruitment since March 2020.

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Table 4: Crude and Adjusted Regression Models for SARS-CoV-2 IgG positivity in Manaus, Amazonas state of Brazil

Variables		Crude Models*			Adjusted Model**		
		cPR	95%CI	p value	aPR	95%CI	p value
Sex	F	Ref			Ref		
	M	1.15	1.00-1.33	0.049	1.22	1.08-1.38	0.001
Age	18-29	Ref			Ref		
	30-39	0.96	0.84-1.11	0.610	1.04	0.97-1.12	0.256
	40-49	1.07	0.87-1.31	0.543	1.16	0.97-1.39	0.106
	50-59	1.22	1.02-1.45	0.026	1.35	1.18-1.55	<0.001
	>=60	0.91	0.68-1.24	0.566	1.01	0.81-1.26	0.926
Occupation	Professional Higher	Ref					
	Professional Mid	1.22	0.97-1.54	0.086	N/A		
	Worker/Informal	1.26	1.12-1.42	<0.001			
	Unemployed	1.15	0.89-1.49	0.297			
Family Income	0-3 minimum wages	Ref			Ref		
	4-6 minimum wages	0.78	0.65-0.93	0.006	0.79	0.66-0.94	0.009
	>6 minimum wages	0.69	0.55-0.86	0.001	0.66	0.53-0.82	<0.001
Housing	Detached house	Ref					

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	Conjugated house	1.07	0.84-1.35	0.603	N/A		
	Condo house	0.84	0.71-0.98	0.029			
	Apartment	0.72	0.58-0.89	0.002			
Number Adults in	1	Ref			Ref		
residence	2	1.04	0.87-1.25	0.633	1.00	0.80-1.26	0.981
	3	1.13	0.94-1.35	0.182	1.09	0.88-1.34	0.448
	4+	1.37	1.19-1.58	<0.001	1.24	1.02-1.51	0.032
Number Children in	0	Ref			Ref		
residence	1	1.10	0.99-1.22	0.081	1.08	0.96-1.20	0.194
	2	1.14	1.00-1.30	0.053	1.11	0.95-1.31	0.194
	3+	1.57	1.30-1.89	<0.001	1.28	1.06-1.54	0.009
Private Insurance	No	Ref					
	Yes	0.80	0.63-1.02	0.071	N/A		
Household members with COVID-19	No	Ref			Ref		
	Yes, alive	1.78	1.60-1.99	<0.001	1.39	1.24-1.57	<0.001
	Yes, deaths	2.22	1.49-3.32	<0.001	2.14	1.74-2.62	<0.001
Preventive Self-medication (any)	No	Ref			Ref		

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	Yes	1.49	1.38-1.60	<0.001	1.36	1.27-1.46	<0.001
Flu-like Symptoms since March	No	Ref			Ref		
	Yes	1.25	1.13-1.38	<0.001	1.13	1.02-1.25	0.018
Prior COVID-19 Diagnosis	No	Ref			Ref		
	Yes	2.34	2.09-2.63	<0.001	1.92	1.71-2.17	<0.001

cPR: Crude Prevalence Ratio. aPR: Adjusted Prevalence Ratio 95%CI: 95% Confidence Interval Ref: Reference Value (1.00).

* Poisson Regression Model with robust variance corrected per clusters (administrative areas).

** Poisson Regression Model with robust variance corrected per clusters (administrative areas) and adjusted per sex, age, family income, number of household adults, number of household children, presence of household members with COVID-19, use of preventive self-medication, presence of flu-like symptoms since March, and prior COVID-19 diagnosis.

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ANEXO 2 – Artigo 2 publicado do nosso grupo de uma coorte em Manaus para COVID-19

The Lancet Global Health

High anti-SARS-CoV-2 antibody seroconversion rates before the second wave in Manaus, Brazil, and the protective effect of social behavior measures: results from the prospective DETECTCoV-19 cohort.

--Manuscript Draft--

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Corresponding Author:	Pritesh Jaychand Lalwani, Dr. rer. nat. ILMD Fiocruz Amazônia: Instituto Leonidas e Maria Deane Fiocruz Amazonia Manaus, Amazonas BRAZIL
First Author:	Pritesh Jaychand Lalwani, Dr. rer. nat.
Order of Authors:	Pritesh Jaychand Lalwani, Dr. rer. nat.
	Roger V Araujo-Castillo
	Christian A Ganoza
	Barbara B Salgado
	Ivanildo Viera Pereira Filho
	Danielle Severino Sena da Silva
	Thiago Barros do Nascimento de Morais
	Maele F Jordão
	Jessica Vanina Ortiz
	Aguyda Rayany Cavalcante Barbosa
	Wlademir Braga Salgado Sobrinho
	Isabelle Bezerra Cordeiro
	Júlio Nino de Souza Neto
	Enedina Nogueira de Assunção
	Cristiano Fernandes da Costa
	Pedro Elias de Souza
	Bernardino Claudio de Albuquerque
	Spartaco Astofi-Filho
	Jaila Dias Borges Lalwani
Manuscript Region of Origin:	BRAZIL
Abstract:	Background: The city of Manaus, located in the Brazilian rainforest, has twice experienced a health system collapse due to the COVID-19 pandemic. We report anti-SARS-CoV-2 nucleocapsid IgG seroconversion rates and associated risk factors from an adult convenience population cohort, DETECTCoV-19, between August and November 2020, before the pandemic second wave in Brazil. Methods: 2496 adult participants enrolled in the DETECTCoV-19 cohort returned for a follow-up visit. We measured serum anti-SARS-CoV-2 nucleocapsid IgG antibodies at both visits using an in-house ELISA assay. From them, we studied a subgroup that were seronegative and uninfected at baseline. Sociodemographic characteristics, health access, comorbidities, COVID-19 exposure, protective behavior, symptoms, and COVID-19 testing were evaluated. We calculated the incidence of seroconversion during the study period. Incidence rate ratios were obtained using cluster-corrected

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	and adjusted Poisson regression models to analyze association with seroconversion. Findings: Among 1638 seronegative and uninfected participants at baseline, we observed an overall seroconversion incidence of 13·06% (95% CI 11·52–14·79); and 6·78% (95% CI 5·61–8·10) for symptomatic seroconversion over a median follow-up of 57 days. We estimated that 48·1% of seroconversion events were asymptomatic. The sample had a higher proportion of affluent and higher-educated people than reported for Manaus city. Risk factors for seroconversion were: having a COVID-19 case in the household, not wearing a mask during contact with a case, relaxation of protective measures, and non-remote work. Interpretation: We observed an intense infection transmission period that preceded the second wave of COVID-19 in the DETECTCoV-19 cohort in Manaus, and identified several modifiable behaviors that increased the risk of seroconversion as having a COVID-19 case in the household and non-compliance with NPIs measures, including not wearing a mask during contact, relaxation of protective measures, and non-remote work. Increased testing can provide timely information about ongoing transmission levels and can aid mitigating the pandemic by informing adequate public health measures and non-pharmaceutical interventions in areas of high transmission like Manaus and elsewhere.

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High anti-SARS-CoV-2 antibody seroconversion rates before the second wave in Manaus, Brazil, and the protective effect of social behavior measures: results from the prospective DETECTCoV-19 cohort.

Pritesh Lalwani[1#], Dr. rer. nat.; Roger V. Araujo-Castillo[2*], MD; Christian A. Ganoza[3*], MD; Bárbara Batista Salgado[1§], MSc; Ivanildo Vieira Pereira Filho[1,4§], BPharm; Danielle Severino Sena da Silva[1§], BSc; Thiago Barros do Nascimento de Morais[1§], MSc; Maele Ferreira Jordão[1§], MSc; Jessica Vanina Ortiz[1§], MSc; Aguyda Rayany Cavalcante Barbosa[1,5§], PhD; Wlademir Braga Salgado Sobrinho[1], MSc; Isabelle Bezerra Cordeiro[5], PhD; Júlio Nino de Souza Neto[6], PhD; Enedina Nogueira de Assunção[6], PhD; Cristiano Fernandes da Costa[7], MSc; Pedro Elias de Souza[8], BM; Bernardino Claudio de Albuquerque[9], MD; Spartaco Astofi-Filho[5,6*], PhD; The DETECTCoV-19 study team and Jaila Dias Borges Lalwani[4#] PhD.

[1]Instituto Leônidas e Maria Deane (ILMD), Fiocruz Amazônia, Manaus, Amazonas, Brazil
[2]Facultad de Ciencias de la Salud, Universidad Peruana de Ciencias Aplicadas, Lima, Peru
[3]Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano Heredia, Lima, Peru
[4]Faculdade de Ciências Farmacêuticas (FCF), Universidade Federal do Amazonas (UFAM), Manaus, Amazonas, Brazil
[5]Instituto de Ciências Biológicas (ICB), Universidade Federal do Amazonas (UFAM), Manaus, Amazonas, Brazil
[6]Centro de Apoio Multidisciplinar (CAM), Universidade Federal do Amazonas (UFAM), Manaus, Amazonas, Brazil
[7]Fundação de Vigilância em Saúde do Amazonas (FVS/AM), Manaus, Amazonas, Brazil
[8]Programa de Pós - Graduação em Cirurgia, Faculdade de Medicina, Universidade Federal do Amazona (UFAM), Manaus, Amazonas, Brazil
[9]Faculdade de Medicina (FM), Universidade Federal do Amazonas (UFAM), Manaus, Amazonas, Brazil
[#]Authors contributed equally
*Authors contributed equally
[§]Authors contributed equally

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Corresponding author
Pritesh Lalwani, Dr. rer. nat.
Instituto Leônidas e Maria Deane (ILMD), Fiocruz Amazônia
Rua Terezina 476, Adrianópolis. Manaus - AM. CEP: 69057-070.
E-mail: pritesh.lalwani@fiocruz.br

Jaila Dias Borges Lalwani, PhD
Faculdade de Ciências Farmacêuticas (FCF), Universidade Federal do Amazonas (UFAM), Av. General Rodrigo Octavio Jordão Ramos, 1200 - Coroado I, Manaus - AM, 69067-005
E-mail: jaila@ufam.edu.br

The DETECTCoV-19 study team (alphabetical order)
Aldina Iacy Paulain Holanda, Ana Lúcia Silva Gomes, Ana Paula Souza de França, André Victor Rabelo Monteiro, Andressa dos Passos Santos, Antônia de Sousa Teixeira, Antônio Vinicius Soares de Souza, Beatriz Pinheiro, Bianca Pires dos Santos, Brenda Pereira Farias,

Bruno Nicolau Paulino, Caio Lúcio Andreola da Silva, Cinthya Iamile Frithz Brandão de Oliveira, Dalila de Alcântara Martins, Eline Araújo de Oliveira, Elisson Denny da Costa Carvalho, Evillyn Fernandes Da Costa, Fernanda Guilhon Simplicio, Fernanda Serrão Pereira, Gabriele Pimentel Sinimbu, Genilton de Oliveira Cardenes, Giane Alves da Silva, Iago Sampaio Fernandes da Costa, Ingrid Silva Correia, Ilia Gilmara Carvalho dos Santos, Jackeline Vieira Guimarães, Jessica Samile Batista Pinheiro, Juliana Correa Romana, Josineide de Oliveira Novo França, Kerollen Runa Pinto, Maria Fiamma Farias Freitas, Marne Carvalho de Vasconcellos, Marizete Candido Moraes, Matheus da Silva Damasceno, Michelle Araújo Ruiz, Milena Maria Cardoso de Lemos, Neila Soares Picanço, Rayara Gonzaga Maia, Regiane Carneiro Bezerra, Romeu Santos de Souza, Susy Cavalcante Harjani, Vitor Batista de Souza and Wellington Barbosa de Melo

Abstract

Background: The city of Manaus, located in the Brazilian rainforest, has twice experienced a health system collapse due to the COVID-19 pandemic. We report anti-SARS-CoV-2 nucleocapsid IgG seroconversion rates and associated risk factors from an adult convenience population cohort, DETECTCoV-19, between August and November 2020, before the pandemic second wave in Brazil.

Methods: 2496 adult participants enrolled in the DETECTCoV-19 cohort returned for a follow-up visit. We measured serum anti-SARS-CoV-2 nucleocapsid IgG antibodies at both visits using an in-house ELISA assay. From them, we studied a subgroup that were seronegative and uninfected at baseline. Sociodemographic characteristics, health access, comorbidities, COVID-19 exposure, protective behavior, symptoms, and COVID-19 testing were evaluated. We calculated the incidence of seroconversion during the study period. Incidence rate ratios were obtained using cluster-corrected and adjusted Poisson regression models to analyze association with seroconversion.

Findings: Among 1638 seronegative and uninfected participants at baseline, we observed an overall seroconversion incidence of 13·06% (95% CI 11·52–14·79); and 6·78% (95% CI 5·61–8·10) for symptomatic seroconversion over a median follow-up of 57 days. We estimated that 48·1% of seroconversion events were asymptomatic. The sample had a higher proportion of affluent and higher-educated people than reported for Manaus city. Risk factors for seroconversion were: having a COVID-19 case in the household, not wearing a mask during contact with a case, relaxation of protective measures, and non-remote work.

Interpretation: We observed an intense infection transmission period that preceded the second wave of COVID-19 in the DETECTCoV-19 cohort in Manaus, and identified several modifiable behaviors that increased the risk of seroconversion as having a COVID-19 case in the household and non-compliance with NPIs measures, including not wearing a mask during contact, relaxation of protective measures, and non-remote work. Increased testing can provide timely information about ongoing transmission levels and can aid mitigating the pandemic by informing adequate public health measures and non-pharmaceutical interventions in areas of high transmission like Manaus and elsewhere.

Funding**:** Ministry of Education (MEC), Brazil; Fundação de Amparo à Pesquisa do Estado do Amazonas (FAPEAM); Pan American Health Organization (PAHO)/World Health Organization (WHO).

Research in context

Evidence before this study

A search of published and preprint literature with the combination of keywords, "incidence, risk factors, SARS-CoV-2, COVID-19, cohort, Amazon and Brazil", was performed. We found few cross-sectional studies that reported seroprevalence, and very few longitudinal studies that examined incidence of seroconversion in adult population; we also failed to identify studies that examined the effect of risk factors or Non-Pharmaceutical Interventions (NPIs) on SARS-CoV-2 incidence in the adult population. Previously, from our baseline DETECTCoV-19 cohort analysis, we observed that low-socioeconomic status and household case clustering increased the risk of acquiring SARS-CoV-2 infection during the first COVID-19 pandemic wave in Manaus. Intrafamilial transmission may have fueled the spread of disease among the population when voluntary isolation and protective measures were not appropriately adopted.

Added value of this study

The DETECTCoV-19 prospective cohort allowed us to study the incidence of seroconversion —a proxy for disease attack rate— in Manaus, between August and November 2020, before the second COVID-19 pandemic wave in Brazil. From our initial recruitment cohort, a total of 2496 participants returned for a second follow-up visit between 19/10/2020 and 27/11/2020. The cohort had a larger proportion of high earning, professionally employed individuals than Manus City population since it was recruited through a university website. Among these, the crude anti-SARS-CoV-2 nucleocapsid IgG seropositivity at baseline was 27·72%, which increased to 34·33% at the second visit. Test sensitivity- and specificity-adjusted antibody prevalence increased from 28·70% to 36·40% after follow-up. Among 1638 seronegative and uninfected participants at baseline, we observed an overall incidence of 13·06% and 6·78% for symptomatic seroconversion, indicating that 1% of the sample seroconverted every 4·5 days. We observed that having a COVID-19 case in the household and behavioral non-compliance with NPIs measures –including not wearing a mask during contact, relaxation of protective measures, and non-remote work– were associated with an increased risk of seroconversion. In addition, we estimate that ~50% of seroconversion events were asymptomatic during the study period.

Implications of all the available evidence

The seropositivity at the second visit and the seroconversion rate estimate suggest that a large proportion of this convenience sample was still susceptible to the SARS-CoV-2 virus after the first pandemic wave; even after accounting for reported antibody decay rates, at least 50% of the population represented by our sample would not have developed antibodies for SARS-CoV-2. Therefore, it is probable that an intense infection transmission period preceded the second pandemic wave in Manaus. We emphasize that NPIs and protective behavioral measures are crucial to reduce the risk of acquiring the disease. Serosurvey-informed public health interventions need to be rapidly implemented, maintained, and scaled in areas of high transmission like Manaus. This study provides timely evidence to inform public health policies and to formulate effective communication strategies to enforce public health measures aimed at reducing the risk of acquiring SARS-CoV-2 infection.

Introduction

Currently, Brazil ranks second in the reported number of COVID-19 deaths since the start of the pandemic [1]. The healthcare system in Manaus has collapsed twice in less than eight months [2,3]. The number of deaths and cases of the second epidemic wave have surpassed those from the first [4]. The current high toll in morbidity and mortality is aggravating the precarious state in which the first wave left the region, with important consequences for the families and communities affected [5]. In September 2020, health authorities were alerted of a possible second wave of infections if social distancing and mobility measures weren't appropriately enforced[3]. Additionally, non-pharmaceutical interventions (NPIs) were further relaxed, with consequences on behavior, mobility, and adherence to protective measures, thus hindering previous public health policies' achievements[6]. Concurrently, a sequential serological survey of anti-SARS-CoV-2 antibodies among several Amazonian cities observed an increasing trend in seropositivity[7], and a blood-donor-based report indicated a high attack rate in the region[8,9], indicating high levels of transmission. These findings implied that the effect –or adherence to– public health measures and NPIs, or natural immunity, were insufficient to control the rising cases. To better understand the disease dynamics in the adult population, we established a prospective observational cohort based on convenience sampling in Manaus. In our baseline cross-sectional assessment during August 2020, we observed that seropositivity was associated with unfavorable socioeconomic status and household clustering as previously reported [7,10]. In this study we estimated the incidence and risk factors associated with SARS-CoV-2 seroconversion from the DETECTCoV-19 cohort, before the start of the second wave of infections in Manaus.

Methods

Ethics and Study design

The research ethics committee of Federal University of Amazonas (UFAM) approved this study (CAAE:34906920·4·0000·5020) in accordance with Brazilian law, and the Helsinki declaration. All the participants gave oral and written consent prior to enrolment. This study followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guideline for cohort studies (Supplementary Table S8). The longitudinal study DETECTCoV-19 in Manaus was designed to follow-up adults of both sexes ≥18 years for up to 6 months after recruitment with a sample collection every 8-12 weeks as previously described. For the baseline study, a sample size of 2399 individuals was calculated such that the binomial 95% confidence interval around the point estimate of antibody test positivity was less than +/- 2%[10].

Recruitment and follow-up

A convenience sampling strategy, based on on-line and university website advertising, was used to recruit adult residents of Manaus to participate in a longitudinal study (DETECTCoV-19) [10]. First visit occurred between 19/08/2020 and 02/10/2020; and the second visit between 19/10/2020 and 27/11/202). All participants filled out an electronic questionnaire and donated a blood sample for SARS-CoV-2 serological testing (Supplementary Figure 1). At both visits we collected information related to social distancing and protective practices, information related to COVID-19 disease, symptoms since the start of the pandemic, prior diagnosis, auto-medication and prescribed medication used for treatment of symptoms. Study participants provided results of oropharyngeal SARS-CoV-2 RT-PCR or antigen test performed by the local government or private laboratories when available. We also recorded information about

COVID-19 cases diagnosed in the family and in the participant's residence. Finally, an independent form was used to record the SARS-CoV-2 serological assay results. Trained interviewers collected participant data using Research Electronic Data Capture (REDCap, project-redcap.org), an online web application with integrated quality checks (e.g., range and valid values, date formats, skip logic).

The study used an online appointment system to reduce unnecessary gathering and followed all state and federal COVID-19-related regulations. The collection center was equipped with state-compliant measures for appropriate social distancing, and all members of the DETECTCoV-19 team used appropriate personal protective equipment. All state and federal COVID-19-related regulations were followed during the course of the study. All test results were communicated by email or via phone messaging to the study participants.

Serological testing

An indirect Enzyme Linked Immunosorbent Assay (ELISA)-based serological assay was used to measure anti-SARS-CoV-2 nucleocapsid IgG antibody titers in serum samples using recombinant full-length SARS-CoV-2 nucleocapsid protein (N, residues 1 to 419, GenBank QHD43423.2) expressed in *E. coli* and purified by affinity and size exclusion chromatography, as previously described [10]. The sensibility and specificity of the assay was determined using sera from RT-PCR-positive SARS-CoV-2 patients (n=293, 87 outpatients and 113 inpatients) and pre-pandemic controls (n=229). The anti-SARS-CoV-2 nucleocapsid IgG ELISA had a sensitivity of 89·07% (95% CI, 84·79 – 92·30) and 94·28% (95% CI, 89·44 – 97·07) for patient with ≥ 7 days and ≥14 days after onset of COVID-19 symptoms, respectively. A specificity of 97·03% (95% CI, 93·72 – 98·69) was estimated using the pre-pandemic serum samples. An anti-SARS-CoV-2 N IgG antibody reactivity index (RI) was calculated as the optical density (OD) ratio between the sample and the negative control for each assay plate. A cut-off value of 1·5 was obtained using a receiver operating curve (ROC) analysis performed on the pre-pandemic controls and SARS-CoV-2 RT-PCR-positive patients. All samples with a RI value above 1·5 (assay cut-off) were considered positive for anti-SARS-CoV-2 nucleocapsid IgG antibodies [10].

Cohort definitions for calculating incidence of seroconversion

Inclusion criteria: Patients of both sexes, 18 years old or above, who accepted to participate in the study and have at least two evaluations separated for at least four weeks.

Exclusion criteria: Patients who reported having a COVID-19 diagnosis prior to the first visit, either by PCR, serology, radiology, or clinical diagnosis. Patients who have no data or did not answer questions regarding COVID-19 diagnosis prior to the first visit. Patients who had IgG ELISA reactivity index >1·5 at the first visit.

Seroconversion Definition: Patients meeting the selection criteria who had IgG ELISA reactivity index ≤1·5 at first visit, IgG ELISA reactivity index >1·5 at the second visit, and a reactivity index ratio (visit 2/visit 1) >2.

Data analysis

Statistical analysis was performed using Stata version 15·0 (College Station, Texas). Figures data plots were generated with GraphPad Prism for MacOS (version 9·1 ·0, graphpad.com) and RStudio (version 1·4·1106, rstudio.com; R version 4·0·4, cran.r-project.org) using the package ggplot2 (version 3·3·3, cran.r-project.org/web/packages/ ggplot2). Participants who had an indeterminate seroconversion status were not included in the statistical analysis (Supplementary Figure 1). We estimated the seropositivity as the proportion of participating individuals with a positive antibody test result. We adjusted the crude seropositivity estimates

for test sensitivity (0·89) and specificity (0·97) using the Rogan-Gladen estimator[11] and computed 95% confidence intervals using Blaker's method [12]. Number of people in each category were listed in the first table column to show incidence denominators and account for variations due to missing data. Missing values were excluded from all calculations, therefore were not part of the incidence denominators, or were listed as a separate category for each variable. Percentage of missing values for each variable were calculated.

Frequency distribution, cumulative incidence, and incidence density (considering follow-up time) for seroconversion and for symptomatic seroconversion were calculated for the whole group and according to the study variables. Chi-square or Fisher´s exact tests were used to evaluate association between seroconversion incidences and independent variables. Also, Poisson regression models with robust variance corrected per clusters (administrative areas) were used to estimate crude Relative Risk (RR) and crude Incidence Rate Ratios (IRR) that considered follow-up time for each variable. A multivariate model estimating adjusted IRRs was constructed including age, sex, and all the variables that had significative association in the crude models. P values ≤0·05 were considered statistically significant.

Since the sample was not random, nor stratified, neither done by clusters, a sensitivity analysis was performed to test if the results will change in case the sample would have had a sociodemographic distribution similar to Manaus. Two adjustment characteristics were chosen, Administrative Zone and Family Income (0-3, 4-6, >6 times minimum wages), considering that geographical location will probably balance other variables as health access, housing, and social behavior. Using data from Manaus 2010 Census obtained from the Instituto Brasileiro de Geografia e Estatística (IBGE, https://censo2010.ibge.gov.br), a weight factor was calculated for each family income in each zone, totaling 18 clusters. The weight factor was the probability of belonging to the Manaus cluster over the probability of having been sampled in the first visit. The descriptive analysis, bivariate comparisons, and regression models were repeated using the 18 clusters and weighting factors. Chi^2 test with Rao-Scott adjustment, and Poisson regression models with Taylor linearization and robust variances per clusters were used. A 5% variation in the descriptive percentages, and variations higher than one standard error in seroconversion incidence rates were considered pronounced. To contrast the multivariate model before and after weighting, we used a Wald Test to compare regression coefficients with their 95%CI for each variable of the model.

Role of the funding source
The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.
All authors had full access to the raw data and accept responsibility to submit for publication.

Results

A total of 2496 DETECTCoV-19 cohort participants returned for a second follow-up visit between 19/10/2020 and 27/11/2020 (Figure S1). This cohort had a larger proportion of high earning, highly educated, and professionally employed people than the whole Manaus city (Sensitivity Analysis Table 1) since a third of the participants lived in the region close to the university study site. Among these individuals, the crude anti-SARS-CoV-2 nucleocapsid IgG seropositivity at baseline was 27·72% (95% CI 25·98 –29·53), which increased to 34·33% (95% CI 32·47–36·24) at the second visit. Test sensitivity- and specificity-adjusted antibody prevalence increased from 28·70% (95% CI 26·70–30·80) to 36·40% (95% CI 34·30–38·60) after the follow-up (Figure 1A, top panel). These findings indicate that within the returning

participants, the number of seropositive individuals and their IgG anti-nucleocapsid antibody RI levels were higher at follow-up. Considering an antibody waning rate of 27.2% for symptomatic patients and double for asymptomatic patients over a 180 days follow-up period[13], at least 50% of the population represented by our sample would not have developed antibodies for SARS-Cov-2 at the second visit.

Next, we calculated the incidence of SARS-CoV-2 seroconversion. Figure 1B depicts the serological results of the 2496 participants at both visits. From the total participants evaluated at follow-up, 204 reported having COVID-19 infection prior to the first visit, and 24 had no data regarding prior disease status. From the remaining 2268, 1709 were IgG seronegative at first visit. Of them, 1424 had a RI ≤1·5 at the second visit and deemed still negative. As per protocol definition, 214 had a RI >1·5 with a RI ratio between the second and first visit >2·0, and were considered as seroconversion (Figures 1C and S1). Seventy-one participants had a second visit positive IgG, but did not meet the criteria of doubling the RI, thus were considered indeterminate, and were not included in the analysis (Figure S1). Percentage of missing values for each variable is shown in Table S7. Except for antigen test, each variable has 2.1% or less missing values, so no further adjustment was deemed necessary. The estimated incidence of seroconversion was 13·06% (95% CI 11·52–14·79) with a median follow-up duration of 57 days (IQR, 54–61 days); in other words, 1% of the sample seroconverted every 4·5 days. Of these cases, 48·1% were asymptomatic; therefore, the incidence of symptomatic seroconversion was 6·78% (95% CI 5·61–8·10). Between the 1st and 2nd visit; only 2·93% of the sample had a positive RT-PCR test, and 5·43% developed symptoms and were diagnosed as COVID-19 by the local government or private laboratories (Figure 1D and Table S1).

We then analyzed the effect of sociodemographic, health-related, behavioral, case clustering, and COVID-19 testing on seroconversion (Tables S2-S4). Statistically significant variables were then tested in a multivariate regression model for SARS-CoV-2 seroconversion (Table S5). Figure 2 illustrates incidence rate ratios (IRR) obtained via Poisson regression, considering follow-up time with robust variance corrected by clusters. IRRs are shown unadjusted and adjusted per all model variables (detailed in Table S5). We observed that working remotely was associated with lower incidence (IRR 0·74; 95% CI 0·56–0·97). Whereas relaxation or flexibilization of social distancing measures (IRR 1·31; 95% CI 1·05–1·64), having household member with COVID-19 infection (IRR 1·49; 95% CI 1·21–1·83), and direct contact with COVID-19 cases without a mask (IRR 1·25; 95% CI 1·09–1·45) were independently associated with an increase in incidence. Additionally, having flu-like symptoms (IRR 1·79; 95% CI 1·23–2·59), and a COVID-19 diagnosis between 1st and 2nd visit (IRR 3·57; 95% CI 2·27–5·63), were associated with increased IRR (Figure 2 and Table S5).

In the sensitivity analysis we observed changes in frequencies after applying weights in the descriptive sensitivity analysis (SA) tables (Supplementary Sensitivity analysis Tables 1 and 2). People living in the Center-South area (where the study collection center is located) dropped from 35·47% to 11·48%, whereas people living in lower socioeconomic level areas increased from 9·95% to 18·83% (East Zone), and from 16·73% to 26·29% (North Zone). Similarly, people with family income >6 times minimum wages dropped from 45·57% to 26·63%, and people with income 0-3 times minimum wage increased from 30·11% to 45·70%. Regarding seroconversion incidence rates, they increased after weighting for: males, white people, east-Asians, people who self-medicated with over-the-counter drugs, people who had COVID-19 contact using mask, PCR negative, and negative serology. Incidence dropped for African-Brazilians, and people with COVID-19 diagnosis (Supplementary Sensitivity analysis Tables 4 and 5). Regarding the multivariate model, there were no significant changes in the regression coefficients of any variable, and the calculated IRRs maintained remarkable similar values. However, social distance flexibilization, remote work, and having a COVID-19 contact lost significance due to widening of the 95% CI (Supplementary Sensitivity analysis Table 5).

Discussion

In this study, we found that the anti-SARS-CoV-2 seropositivity rate increased from 27·72% (95% CI 25·98–29·53) to 34·33% (95% CI 32·47–36·24) in the DETECTCoV-19 cohort prior to the second wave of COVID-19 in Manaus. We observed a high incidence of seroconversion at 13·06% (95% CI 11·52–14·79), with a median follow-up duration of 57 days (IQR, 54–61 days). Our regression models showed that seroconversion was associated with modifiable behaviors and case clustering in households. The risk factors for seroconversion outlined in this study provide evidence supporting that adherence to social distancing and NPIs decrease the risk of infection with SARS-CoV-2. Since this cohort oversampled wealthier and more educated people, we explored the effect of adjusting the sample to Manaus city demographics, we found a slight increase in the seroconversion 13·39% (95% CI 10·47–16·97). Seroconversion rates ranged between 10·22 and 19·53 for the different regions of the city, which reflects that least affluent portions of the city had higher seroconversion rates. This highlights the need to have a differentiated health surveillance and response system for areas of the city with marked disparities, especially in the context of the P.1 variant emergence [14,15] and the second epidemic wave.

To our knowledge, this is the first study that reports SARS-CoV-2 seroconversion rates prospectively in Brazil. Between the start of the 1st round collection and 2nd round ending (August 19th to November 27th), Manaus city Fundação de Vigilância em Saúde do Amazonas (FVS Amazonas) reported 28,550 adult COVID-19 cases confirmed by laboratory testing (http://www.fvs.am.gov.br, date accessed 28 February, 2021), which correspond to a case reporting rate of 1·97% (95% CI 1·95–1·99) in the study period. The difference between the seroconversion rate in our convenience sample and the official reporting rate highlights the variations obtained when using different case definition criteria; whereas it is seroconversion, symptomatic seroconversion, clinical diagnosis, diagnosis by PCR, or reporting to health agencies. Overall, it is important to timely identify these divergent incidences and their dynamics during the pandemic to adequately plan resources allocation and mitigation strategies. Crucially, we observed that a considerable volume of COVID-19 transmission during the study period consisted of asymptomatic cases. This finding underlines the importance of continuous disease monitoring to inform appropriate and timely disease control measures, especially when incidence rates are driven by a relatively high proportion of undocumented infections [16-19].

In contrast with our previous report, which evaluated prevalence of seropositivity during August 2020, this study examined factors that affected incidence over a median two-month period between August and November 2020. While in our first report we found that prevalence was strongly associated with sociodemographic characteristics (male sex, older age, lower income, occupation, and number of household members), these were no longer associated with the emergence of new cases during the study period. In contrast, we found that the main risk factors were related to the social behavior of the participants, such as not keeping social distance before August, relaxing social distancing after that, on-site working, and having contact with COVID-19 patients without a mask. These results show that, between the first and second epidemic waves in Manaus, behavioral risk factors that increase exposure to SARS-CoV-2 were more important than biological or structural characteristics as sex or poverty. This could be due to change in epidemic dynamics; some studies have shown that patients were younger and healthier during the second wave, thus reducing the effect of age and comorbidities over infection. [20,21] Conversely, protective measures compliance increased after the first wave, as shown in some countries, [22,23] acquiring a more prominent role in the control

of the epidemic. Having a person with COVID-19 diagnosis in the household affected both the prevalence in August and the incidence in the following two-three months with similar magnitude. Importantly, independent of the moment of the pandemic, our findings confirm that having a COVID-19 contact in the household remains a robust predictor for acquiring the disease [24]. Not surprisingly, having symptoms or having been diagnosed during the observation period were strongly associated with seroconversion, more so than in the cross-sectional study, underlying the inherent correlation between these events.

Since the start of the pandemic, disease control strategies that include effective surveillance, availability and ease of access to testing, well-implemented NPIs, and the public health response measures linked to testing –contact tracing, quarantine, and isolation– have been fundamental in controlling disease transmission [25-27]. Our findings reveal that COVID-19 index cases may have likely driven seroconversion –a representative proxy for infection burden– within the household, as observed in other high transmission settings [28]. Our data suggests that household close contacts of COVID-19 patients (survivors or deceased) should be tested regardless of symptoms and advised voluntary isolation [29]; strict follow-up of cases and contacts is essential to reduce virus transmission [25,30]. NPIs including social distancing, mask use and hygiene have been the pillar to reduce community SARS-CoV-2 transmission worldwide, therefore, abruptly lifting NPIs diminishes the gains accumulated by previously implemented policies [26,31]. In our study, individuals who relaxed social distancing measures or had contact with COVID-19 individuals without mask [32,33] had the highest risk of acquiring SARS-CoV-2 infection.

Our work has some limitations. A convenience sampling strategy based on on-line and university website advertising potentially excluded individuals who did not have access to this information and may not completely represent the adult population of Manaus. To test if this limitation has any impact in our results, we performed a thorough sensitivity analysis using weighting to make the sample more similar to the actual Manaus population. Truly, there were marked differences in socioeconomic and geographical distribution between the unweighted and weighted samples. Our sample has a disproportionate number of people with higher income, higher education, and residence around the university campus. After correcting for those, some seroconversion incidence rates changed, but without significantly modifying the crude IRRs, therefore no new variable became significant, nor the previous ones lost significance. This is reflected in the final multivariate model, in which the regressions coefficients remained remarkably similar after weighting. Social distance flexibilization, remote work, and having a COVID-19 contact lost significance due to widening of the 95%CI, but we attribute this to the expanded number of clusters from 6 to 18, and not to changes in the coefficient themselves. In conclusion, while it is true that there were marked differences in geographical and socioeconomic distribution between our sample and the actual Manaus population, these differences did not alter our final risk factors model nor our study conclusions. Additionally, although our in-house serological assay highly correlates with commercial SARS-CoV-2 tests, it is possible that performance variation among asymptomatic patients may potentially affect our seropositivity estimates.

We also had shortcomings in performing active surveillance to identify symptomatic infections and positive RT-PCR tests results. Additionally, most symptomatic participants had a mild or moderate infection, but our surveillance method cannot exclude that severe illness cases and deaths occurred among the non-returning participants, underestimating total seroconversion events. Although anti-SARS-CoV-2 IgG seropositivity is a robust indicator of prior infection [34,35], antibody response kinetics and their variability among populations limit our interpretations [36]. Despite these, our longitudinal serosurvey approach to assess exposure and burden, and the size of our cohort, made possible in-depth statistical analysis to identify the risk factors associated with seroconversion in a setting of high transmission and low NPI

containment measures. Unfortunately, we could not evaluate the role of the infecting SARS-CoV-2 strains. The study period between August and November 2020 theoretically predates the surge and dominance of the P·1 variant strain in the region; however, we cannot rule out that the high rate of seroconversion in our cohort could have been influenced partially by the emergence of a more infectious strain, like P.1 in Manaus[14,15]. We hypothesize that the role of the P.1 variant could be ascribed to accelerating the transmission rate observed after November, but we consider that the high seroconversion incidence found in our cohort during the study period might be explained by case clustering and host-related behavioral factors, as observed in high transmission settings [24,28,37,38].

Rampant spread of SARS-CoV-2 infection in settings with low compliance to behavioral and NPI containment measures is a cause of concern due to its high human costs, elevated burden imposed on healthcare systems, and a possible impact on the emergence of new variants – favored in high transmission settings– which can negatively impact the effectiveness of available and future countermeasures including diagnostics, vaccines and therapeutics. Our study shows that increased testing can provide timely information about ongoing transmission levels and can contribute to mitigate the pandemic by informing adequate public health measures and non-pharmaceutical interventions in areas of high transmission like Manaus and elsewhere.

Contributors

PL and JDBL are the principal investigator of this study and acquired necessary funding. PL and JDBL conceived the study with input from CFC, PES, BCA, CAG and RVA. Protein expression and purification was performed by BBS, IBC, JNSN, ENA and SAF. Sample collection was led by JDBL with assistance from CFC, PES, BCA. The laboratory set-up and sample processing were coordinated by PL and BBS. BBS, IVPF, DSSS, TBNM, MFJ, JVO, ARCB, WBSS processed blood samples, performed laboratory testing, collected data and approved test results with PL supervision. PL, BBS and JDBL coordinated data acquisition and data management. Data was cleaned and prepared by PL and RVA. Statistical analyses and data visualization were done by PL and CAG; RVA lead the statistical analyses. PL, JDBL, CAG and RVA analyzed the results and wrote the manuscript. All authors revised and approved the final version of this manuscript.

Declaration of interests

The authors declare no conflict of interest.

Acknowledgement

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Data Sharing

De-identified participant data can be made available to researchers after approval from the research ethics committee. The requests should be directed to the corresponding author (pritesh.lalwani@fiocruz.br or jaila@ufam.edu.br).

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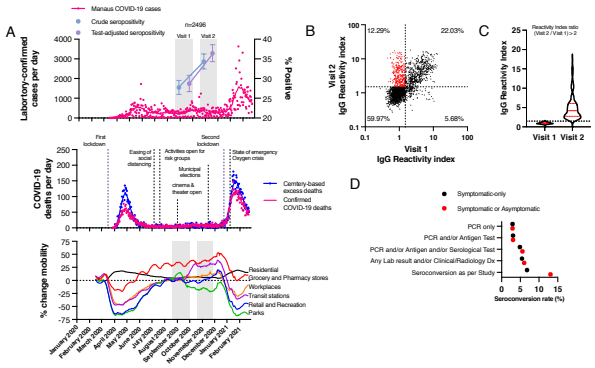


Figure 1: Anti-SARS-COV-2 nucleocapsid IgG antibody seropositivity and seroconversion before the second wave of infection from the DETECTCoV-19 cohort in Manaus.

A-upper plot: COVID-19 confirmed laboratory cases obtained from the Fundação de Vigilância em Saúde do Amazonas (FVS-AM, http://www.fvs.am.gov.br, date accessed 28 February, 2021). SARS-CoV-2 testing rates were low at the beginning of the epidemic and hence the infections were substantially sub-notified. Grey areas depict the sample collection periods for visit 1 and visit 2, along with the crude and test-adjusted anti-SARS-COV-2 nucleocapsid antibody seropositivity prevalence, depicted as percentage of seropositive individuals for both study visits (bars depict 95% CI). Right Y-axis denotes the seropositivity percentage. A-middle plot: Confirmed COVID-19 deaths published by the FVS-AM and cemetery-based excess deaths published by the municipality of Manaus. Excess deaths exceeded confirmed deaths during the epidemic, as it includes all sub-notified COVID-19 deaths and non-COVID-19 deaths due to health system collapse (data extracted by Marcelo Oliveira https://github.com/capyvara from ARPEN civil registry; source https://drive.google.com/drive/folders/1DYmuzzOJwHrB3LtXMrmguc1mIauJH4V_). NPI-related, public health measures, and pandemic-related events depicted (text inserts) during the SARS-CoV-2 pandemic were collated from the Amazonas state government website (http://www.amazonas.am.gov.br, date accessed 28 February, 2021). A-lower plot: Google mobility data for Manaus city (https://www.google.com/covid19/mobility/, date accessed 01 March 2021) plotted as percentage mobility change. (B) Anti-SARS-CoV-2 nucleocapsid IgG antibody reactivity index (RI) results from visit 1 and 2 (n=2496). Each dot represents one patient; dotted line denotes assay cut-off. Red dots represent seroconverters (individuals negative at first visit and positive at second visit). (C) Paired anti-SARS-CoV-2 nucleocapsid IgG RI of seroconverters as per study protocol (n=214); red horizontal line depicts median RI. (D) Seroconversion rates according to case definitions for symptomatic and asymptomatic (red) or asymptomatic-only individuals (black), case definitions in Supplementary table S1

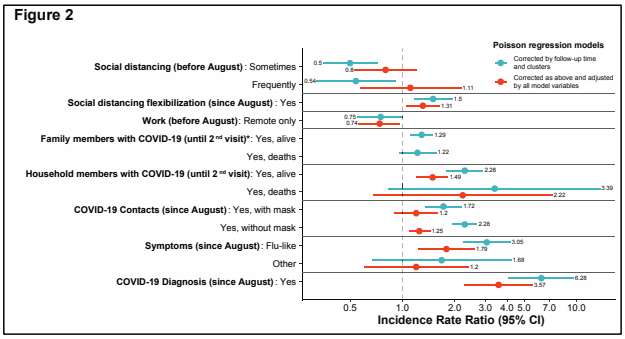


Figure 2: Multivariate regression model for SARS-CoV-2 seroconversion in an adult population cohort from Manaus, Amazonas state of Brazil (N=1,618). Forest Plot showing Incidence Rate Ratios (IRR) obtained via Poisson Regression considering follow-up time with robust variance corrected by clusters. IRRs are shown unadjusted and adjusted per all model variables (please refer Supplementary Table 5 for details). *Not included in the multivariate model due to collinearity with the Household contact variable.

Figure 1

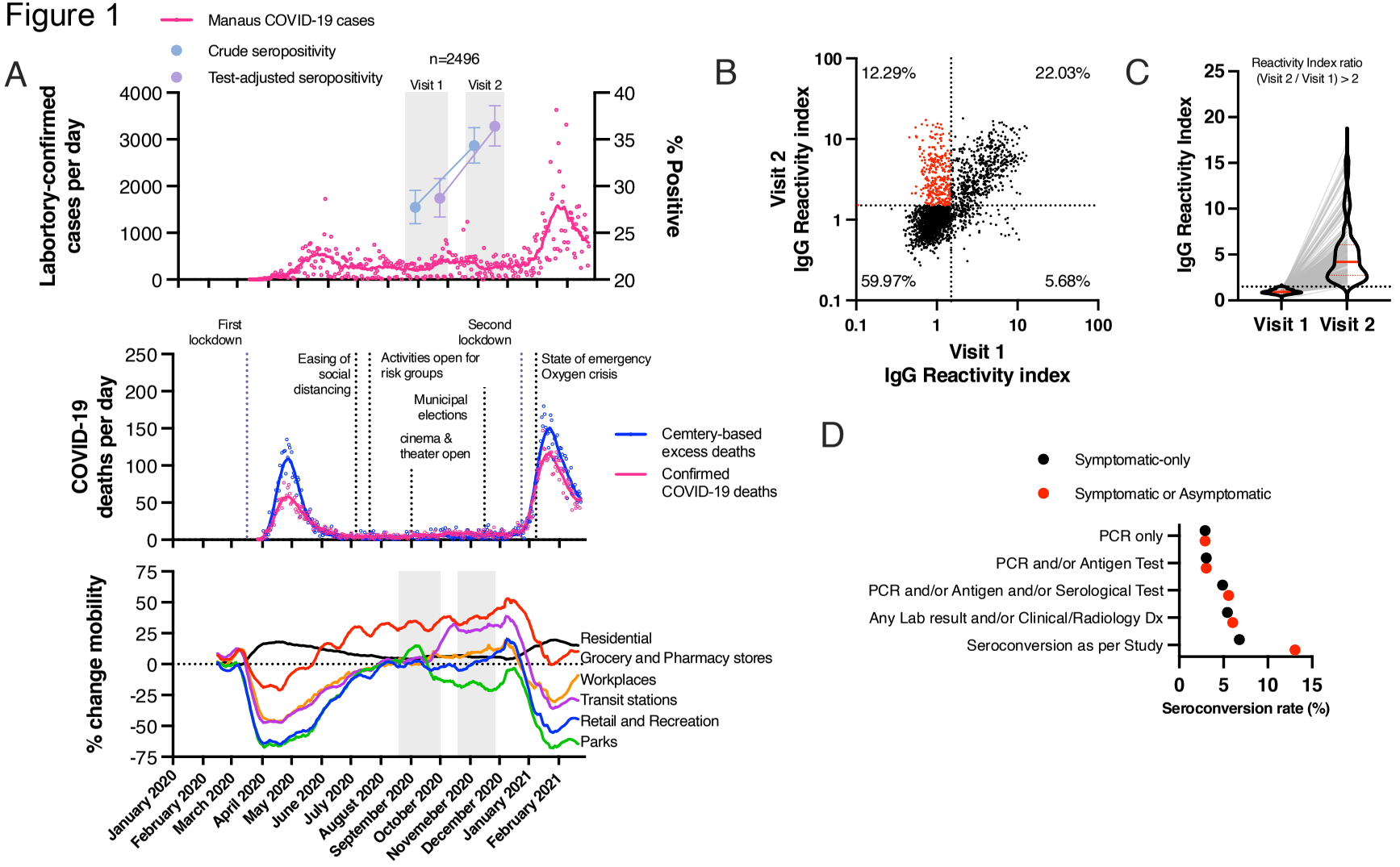

A
Manaus COVID-19 cases
Crude seropositivity
Test-adjusted seropositivity
n=2496
Visit 1
Visit 2
Labortory-confirmed cases per day
% Positive
First lockdown
Second lockdown
Easing of social distancing
Activities open for risk groups
Municipal elections
cinema & theater open
State of emergency Oxygen crisis
COVID-19 deaths per day
Cemtery-based excess deaths
Confirmed COVID-19 deaths
% change mobility
Residential
Grocery and Pharmacy stores
Workplaces
Transit stations
Retail and Recreation
Parks
January 2020
February 2020
March 2020
April 2020
May 2020
June 2020
July 2020
August 2020
September 2020
October 2020
Novemeber 2020
December 2020
January 2021
February 2021
B
12.29%
22.03%
59.97%
5.68%
Visit 2 IgG Reactivity index
Visit 1 IgG Reactivity index
C
Reactivity Index ratio (Visit 2 / Visit 1) > 2
IgG Reactivity Index
Visit 1
Visit 2
D
Symptomatic-only
Symptomatic or Asymptomatic
PCR only
PCR and/or Antigen Test
PCR and/or Antigen and/or Serological Test
Any Lab result and/or Clinical/Radiology Dx
Seroconversion as per Study
Seroconversion rate (%)

Figure 2

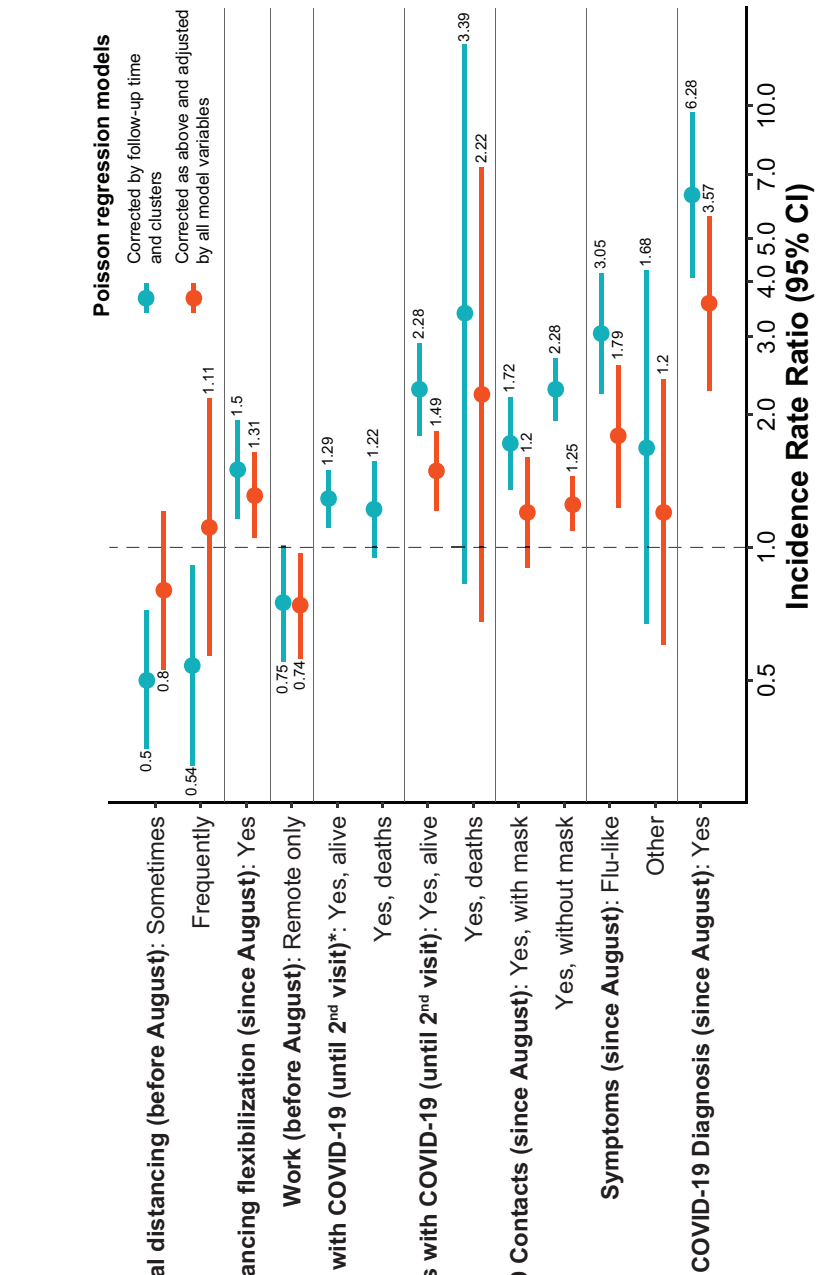


Figure 2

ANEXO 3 – Documento de aprovação do projeto pelo Comitê de Ética em Pesquisa (CEP) da Universidade Federal do Amazonas (UFAM)

UNIVERSIDADE FEDERAL DO AMAZONAS - UFAM

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: DESENVOLVIMENTO DE UM TESTE SEROLÓGICO MULTIPLEX PARA DETECÇÃO SIMULTÂNEA DE PATÓGENOS

Pesquisador: Pritesh Jaychand Lalwani

Área Temática:

Versão: 2

CAAE: 96171218.7.0000.5020

Instituição Proponente: CENTRO DE PESQUISAS LEONIDAS E MARIA DEANE - FUNDACAO

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 2.914.127

Apresentação do Projeto:

Plasmodium vivax, vírus Zika (ZIKV) e Dengue vírus (DENV) são endêmicos no Brasil e responsável por alta morbimortalidade materna e infantil. Na região amazônica onde existe expressiva desigualdade econômica e social, existe também uma grande carga de doenças febris malária negativo que nunca é diagnosticada devido à falta de ferramentas adequadas. Testes diagnostico atuais são muito difíceis de implementar em áreas rurais da Amazônia, pois exigem técnicos especializadas e grande investimento. Além disso, não existe vacinas ou medicamentos para tratar ZIKV e DENV, portanto, o diagnóstico preciso contribui para a identificação precoce da doença e uma intervenção eficaz para o melhor manejo do paciente.Nosso objetivo é desenvolver um único teste sorológico para identificar, simultaneamente, infecção por P.vivax, ZIKV e DENV. O maior vantagem deste

teste é o detecção simultânea de múltiplo infecções em mesmo amostra, rapidez e baixo custo, além disso, janela de detecção sorológico é maior comparado ao teste moleculares (PCR, polymerase chain reaction). Também, a flexibilidade deste imunoensaio permitirá no futuro ampliar a detecção do teste para até 8 patógenos, simultaneamente.O financiamento deste projeto permitirá criar uma plataforma única para a detecção de múltiplos agentes patogênicos e ajudará o SUS e o Ministério da Saúde a reduzir milhões de reais por ano em diagnóstico. Além disso, este imunoensaio capacitará os médicos a decidir estratégias de intervenção corretas e reduzir morbimortalidade materna e infantil.

Endereço: Rua Teresina, 495
Bairro: Adrianópolis **CEP:** 69.057-070
UF: AM **Município:** MANAUS
Telefone: (92)3305-1181 **E-mail:** cep.ufam@gmail.com

ANEXO 4 – Documento de aprovação do projeto pelo Comitê de Ética em Pesquisa (CEP) do Centro Universitário Nilton Lins (UNI-NILTON LINS)

CENTRO UNIVERSITÁRIO
NILTON LINS



PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: ECO-EPIDEMIOLOGIA DE PATÓGENOS ENDÊMICOS NA REGIÃO AMAZÔNICA
Pesquisador: Pritesh Jaychand Lalwani
Área Temática:
Versão: 2
CAAE: 83715618.0.0000.5015
Instituição Proponente: CENTRO DE PESQUISAS LEONIDAS E MARIA DEANE - FUNDACAO
Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 2.636.421

Apresentação do Projeto:
Trata-se de um projeto com utilização de material biológico de pessoas adultas (mais de 18 anos) em que usará amostras armazenadas em biorepositório e já utilizadas na pesquisa "Soroprevalência para Hantavírus em humanos e animais do Estado do Amazonas", realizada por pesquisadores da Universidade Federal do Amazonas (UFAM), cujo estudo foi aprovado pelo CEPUFAM (CAAE: 19328313.7.0000.5020) para armazenamento e futuras pesquisas com dispensa prévia de novo TCLE pelos participantes da pesquisa. O tamanho da amostra será de 2.851 (doadores de sangue 987, soldados 600, Coari 675, Manaus 582). Os experimentos serão conduzido em Laboratório de doenças infecciosas e imunologia, 1º andar, Bloco ICB-FCA pós graduação, UFAM, Mini campus ou em Laboratório multiusuário do Instituto Leônidas e Maria Deane (ILMD), Fundação Oswaldo Cruz (Fiocruz), Manaus, sob responsabilidade do coordenador Dr. Pritesh Lalwani. Quando houver necessidade, somente testes de Inibição da Hemaglutinação (HI) serão realizados em Instituto Evandro Chagas (IEC), Belém do Pará. Também, serão enviados amostras para Laboratório de Referência Nacional para Leptospirose, do Instituto Oswaldo Cruz (IOC/FIOCRUZ) para estudar anticorpos contra Leptospirose.

Objetivo da Pesquisa:
Objetivo Primário
-Avaliar a prevalência e os fatores de risco associados aos patógenos endêmicos na região

Endereço: Av. Profº Nilton Lins, 3259 - Bloco: UNICENTER --> Sala 147/CEP Conj. Parque das Laranjeiras
Bairro: Flores **CEP:** 69.058-040
UF: AM **Município:** MANAUS
Telefone: (92)3643-2170 **Fax:** (92)3643-2073 **E-mail:** waltergarciaparra@gmail.com

ANEXO 5 – Documento de aprovação do projeto pelo edital FAPEAM (PCTI-EMERGESAÚDE-AM)

Projeto

1. Plano de Trabalho

Edital:	EDITAL N. 005/2020 - PCTI-EMERGESAÚDE - AM
Título:	DESENVOLVIMENTO E AUTOMATIZAÇÃO DE TESTE SOROLÓGICO PARA DETECÇÃO DE ANTICORPOS CONTRA O SARS-CoV-2 EM AMAZONAS
Protocolo:	48134.UNI788.30289.23042020
Coordenador:	Pritesh Jaychand Lalwani
RG:	V890214-U
CPF:	557.518.572-91
Endereço:	Tv. Sargento Abrahim, 107 - Flores
Telefone:	-
E-mail:	lalwanipritesh@yahoo.com
Área de Conhecimento 1:	Ciências Biologicas » Imunologia
Área de Conhecimento 2:	
Área de Conhecimento 3:	
Tema de interesse:	
Instituição Executora:	CPqLMD/FIOCRUZ - Fundação Oswaldo Cruz - Centro de Pesquisa Leônidas e Maria Deane
Unidade Executora:	[Amazonas/AM] Sede/Campus Principal - CPqLMD
Início Previsto:	01/11/2020
Duração:	22 Meses
Cotação da Moeda Estrangeira:	0,00
Linhas Temáticas:	Linha Temática 2: Pesquisa, serviço e desenvolvimento de protocolos de análises

1.1. Arquivos

Nome	Tipo
Pritesh_CPF.pdf	CPF
Pritesh_Documento de Identidade.pdf	Documento de Identidade - frente e verso
Pritesh_Diploma de Doutorado .pdf	Diploma de Doutorado - frente e verso
grupo de pesquisa CNPq.pdf	Comprovante de Cadastro no Diretório de Grupo de Pesquisa do CNPq
Pritesh_Currículo do Sistema de Currículos Lattes (Pritesh Jaychand Lalwani).pdf	Currículo Lattes do CNPQ Atualizado
Pritesh_comprovande de residencia.pdf	Edital N.003_2021_POP, C,T&I_Comprovante de Residência atualizado com modelo de declaração.
Anuência da Instituição Executora (vínculo formal.pdf	Anuência da Instituição Executora (vínculo formal)
Pritesh_Proposta Complementar - PCTI EMERGESAÚDE AM 2020.pdf	Proposta Complementar - PCTI EMERGESAÚDE AM 2020