UNIVERSIDADE FEDERAL DO RIO DE JANEIRO

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METABOLOMIC PROFILING OF SEVERE COVID-19: INSIGHTS FROM COHORT STUDIES WITH ADULTS AND PREGNANT WOMEN

RIO DE JANEIRO

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Doctoral thesis submitted to the Post-Graduate Program in Nutrition, Josué de Castro Institute of Nutrition, Federal University of Rio de Janeiro, as a requirement for obtaining the title of Doctor in Nutrition.

Academic Supervisor:
Tatiana El-Bacha Porto

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A todos aqueles abandonados pela pós-graduação

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Eu refleti bastante sobre esse trecho, pois sei que não é obrigatório, e mesmo assim nunca tinha visto um trabalho ao qual não havia agradecimentos. Questionei se as pessoas realmente sentiam gratidão por entregar um trabalho acadêmico, ou se elas só estavam escrevendo os "agradecimentos" porque é o tipo de coisa que você faz sem refletir, porque todos os outros antes de você fizeram. Por esse motivo também, eu não escrevi agradecimentos na minha dissertação de mestrado, não estava certo se eu me sentia agradecido.

Agora no doutorado, eu refleti melhor sobre o assunto. E cheguei à conclusão de que eu não me sinto agradecido, eu me sinto aliviado. É um alívio enorme ter chegado ao final da pós-graduação sem maiores sequelas, físicas, psíquicas e financeiras. Escrevo isso porque eu vi muita gente abandonar, pelos mais diferentes motivos, a maioria deles foi por depressão ou ansiedade debilitante. Todos assumiam que não tinham como ficar curados no ambiente que os tinha adoecido. Mas cada um apontava uma ou mais causas específicas pra essa decisão. Uns apontavam a excessiva carga de trabalho, que sempre gera um desequilíbrio entre vida pessoal e profissional, afinal há sempre um resultado pendente para analisar, uma apresentação a ser feita ou um artigo pra ler nas suas horas de descanso e finais de semana. Alguns mencionavam o baixíssimo pagamento recebido pelas bolsas, que não cobre seque o custo de subsistência de qualquer um que se aventure a viver por si no Rio de Janeiro. Eu poderia citar ainda amigos que acusaram a falta de perspectiva com mercado de trabalho e carreira, os que foram incapacitados pela negligência com saúde mental e física em prol da vida academia, e os que se sentiram sobrecarregados por pressões externas e ou autoimpostas. Acredito que parte de tudo isso que testemunhei, eu também pude experienciar.

Como cientista, questionei se minha observação seria apenas um recorte das pessoas que conheci na UFRJ, mas não é. Em abril de 2018, saiu na Nature um estudo em que foram entrevistados mais de 2.200 estudantes de 26 países, sendo 90% de doutorado. A descoberta foi que estudantes de pós-graduação têm seis vezes mais chance de enfrentar depressão e ansiedade, com 41% e 39% dos entrevistados apresentando sinais de ansiedade e depressão, respectivamente, de nível moderado ou grave. Enquanto na população mundial geral, em média, esses índices são ambos de 6% (EVANS et al, 2018). Me pergunto o quanto a situação do Brasil é melhor ou pior do que essa média mundial.

Existe muito material na literatura científica sobre como a pós-graduação é fator de risco pra ansiedade/depressão, e mais do que isso existem centenas de relatos e exemplos perambulando pela UFRJ. Não parece ser um problema invisível, apenas um problema negligenciado pela própria comunidade, pela CAPES, CNPQ, FAPERJ ministério da educação

e de ciência e tecnologia, agências de fomento que escolhem fomentar a precarização da pesquisa e da pós-graduação. Ofertar bolsas que mal batem 1 salário-mínimo, que mesmo após aumento continuam defasadas em relação aos valores de 2013 (G1 GLOBO, 2023), e ao mesmo tempo transfere para os alunos o custo por transporte, alimentação, aquisição de computador para trabalhar, além de não prever direitos de trabalho como férias. As agencias chegam ao absurdo de exigir devolução dos valores de bolsa recebidos em caso de desistência sem a devida concordância de CAPES/CNPQ.

Dito isso, eu gostaria de deixar claro que eu não mais acredito que as pessoas que conheci abandonaram a pós-graduação ou a pesquisa, elas é quem foram abandonadas. Porque de todos os motivos e motivações que ouvi, ninguém chegou a dizer que não gostava mais de ciência. Elas só não conseguiram suportar os maus-tratos dos quais gostar e trabalhar com ciência fazem parte.

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EVANS, T. et al. Evidence for a mental health crisis in graduate education. **Nature Biotechnol** 36, 282–284 (2018).

G1 GLOBO, site G1, 2023, disponível em https://g1.globo.com/educacao/noticia/2023/02/16/governo-divulga-reajuste-em-bolsas-de-pesquisa-nesta-quinta-valor-deve-subir-40percent-em-media.ghtml

ABSTRACT

Brazil has the second-highest COVID-19 death rate worldwide, and Rio de Janeiro is among the states with the highest rate in the country. Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS)-based metabolomics were used to identify metabolites associated with COVID-19 pathophysiology and disease outcome. This study analyzed metabolites in severe COVID-19 cases from two Rio de Janeiro Intensive Care Unit (ICU) reference centers. The cohort included 35 severe COVID-19 patients (18 survivors and 17 non-survivors) and 12 non-infected controls. In severe cases, the findings reveal significant reductions in choline-related metabolites, serine, glycine, and betaine, highlighting dysregulated methyl donor pathways. Non-survivors exhibited elevated creatine/creatinine, 4-hydroxyproline, gluconic acid, and N-acetylserine levels, indicating liver and kidney dysfunctions. Sex-specific differences in metabolic changes were noted, suggesting the need for gender-specific strategies in pandemic surveillance and treatment. Additionally, the study extends its focus to pregnant women, a group particularly vulnerable to severe COVID-19 due to immunologic, cardiovascular, and respiratory changes. Utilizing 1H NMR-based metabolomics, the research identified significant disruptions in insulin sensitivity, lipid metabolism, and inflammation markers in pregnant women with severe COVID-19, resembling metabolic patterns in gestational diabetes. Elevated glucose, lactate, triacylglycerol, LDL, VLDL, betaine, glycine, citrate, and acetoacetate levels were observed, indicating a metabolic shift driven by inflammation. These findings underscore the importance of vigilant monitoring for both maternal and neonatal health, highlighting that maternal COVID-19 infections may pose long-term neurodevelopmental risks to offspring. This research highlights the pivotal role of metabolomics in elucidating the molecular mechanisms underlying severe COVID-19, which is crucial for enhancing patient outcomes through targeted therapeutic interventions.

Keywords: Metabolomics; Severe COVID-19; Pregnancy Pathophysiology; Maternal health.

RESUMO

O Brasil tem a segunda maior taxa de mortalidade por COVID-19 no mundo, e o Rio de Janeiro está entre os estados com a maior taxa no país. Utilizando metabolômica baseada em Ressonância Magnética Nuclear (RMN) e Espectrometria de Massas (EM), foram identificados metabólitos associados à fisiopatologia da COVID-19 e ao desfecho da doença. O estudo analisou metabólitos em casos graves de COVID-19 de dois centros de referência de UTI no Rio de Janeiro. A coorte incluiu 35 pacientes com COVID-19 grave (18 sobreviventes e 17 não sobreviventes) e 12 controles não infectados. Os resultados revelam reduções significativas nos metabólitos relacionados à colina, serina, glicina e betaína em casos graves, destacando vias de doadores de metil desreguladas. Os não sobreviventes exibiram níveis elevados de creatina/creatinina, 4-hidroxiprolina, ácido glucônico e N-acetilserina, indicando disfunções hepáticas e renais. Diferenças metabólicas específicas de sexo foram observadas, sugerindo a necessidade de estratégias específicas de gênero na vigilância e tratamento da pandemia. Além disso, o estudo estende seu foco às gestantes, um grupo particularmente vulnerável à COVID-19 grave devido a alterações imunológicas, cardiovasculares e respiratórias. Utilizando metabolômica baseada em RMN de 1H, a pesquisa identificou interrupções significativas na sensibilidade à insulina, metabolismo lipídico e marcadores de inflamação em gestantes com COVID-19 grave, assemelhando-se aos padrões metabólicos vistos no diabetes gestacional. Foram observados níveis elevados de glicose, lactato, triacilglicerol, LDL, VLDL, betaína, glicina, citrato e acetoacetato, indicando uma mudança metabólica impulsionada pela inflamação. Esses achados enfatizam a necessidade de monitoramento vigilante da saúde materna e neonatal, pois infecções maternas por COVID-19 podem representar riscos de neurodesenvolvimento a longo prazo para os filhos. Esta pesquisa destaca o papel crítico da metabolômica na elucidação dos mecanismos moleculares subjacentes à COVID-19 grave e suas implicações para a melhoria dos resultados dos pacientes através de intervenções terapêuticas direcionadas.

Palavras-chave: Metabolômica; COVID-19 Grave; Gravidez; Fisiopatologia; Saúde Materna

LIST OF ILLUSTRATIONS

Introduction

Chapter 1 - Integrated NMR and MS Analysis of the Plasma Metabolome Reveals Major Changes in One-Carbon, Lipid, and Amino Acid Metabolism in Severe and Fatal Cases of COVID-19

Chapter 2 - Metabolomic Analysis of Severe COVID-19 During Pregnancy Revealed Metabolic Changes Similar to Diabetes

LIST OF TABLES

LIST OF ABBREVIATIONS AND ACRONYMS

ACE2	Angiotensin-converting enzyme 2
ARDS	Acute respiratory distress syndrome
BMI	Body Mass Index
CoVs	Coronaviruses
COVID-19	Coronavirus disease 2019
DAMPs	Damage-associated molecular patterns
DNA	Desoxyribonucleic acid
DMVs	Double-membrane vesicles
E	Envelope
FCS	Furin cleavage site
GC-MS	Gas chromatography-mass spectroscopy
ICU	Intensive care unit
IFN	Interferons
Ig	Immunoglobulins
IL	Interleukin
ISG	IFN-stimulated gene
LC-MS	Liquid chromatography-mass spectroscopy
M	Membrane
MERS	Middle Eastern respiratory syndrome
MS	Mass spectrometry
N	Nucleocapsid
NMR	Nuclear Magnetic Resonance
NSP	Nonstructural proteins
ORFs	Open reading frames
PAMPs	Pathogen-associated molecular patterns
PRR	Pattern recognition receptor
RLR	RIG-I-like receptors
RNA	Ribonucleic acid
S	Spike
SARS	Severe acute respiratory syndrome
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2

SR	Scavenger receptors
ssRNA	Positive-sense ribonucleic acid
TMPRSS2	Transmembrane protease serine 2
TLR	Toll-like receptors
TNF	Tumor necrosis factor
VOC	Variants of concern
VOI	Variants of interest
VUM	Variants under monitoring
WHO	World Health Organization
WT	Wild type

SUMMARY

1 INTRODUCTION

1.1 COVID-19

1.1.1 Epidemiology Background

Over the past two decades, Coronaviruses (CoVs) have been responsible for triggering three significant outbreaks, namely severe acute respiratory syndrome (SARS), Middle Eastern respiratory syndrome (MERS), and most recently, the coronavirus disease 2019 (COVID-19) pandemic. The emergence of the COVID-19 pandemic can be traced back to a cluster of pneumonia cases linked to a seafood market in Wuhan City, within the Hubei Province of China (ZHOU et al., 2020b). Following the probable transfer of a zoonotic disease, subsequent investigations eventually confirmed the causative agent to be a new Betacoronavirus closely associated with the SARS-CoV. The initial onset of human symptoms occurred on December 1, 2019, marking the beginning of instant human-to-human transmission and subsequent global dissemination (HUANG et al., 2020). By March 11, 2020, the World Health Organization (WHO) declared COVID-19 caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) a global pandemic (WHO, 2020).

As a single-stranded ribonucleic acid (RNA) virus, it is genetically similarity to SARS-CoV-1, the virus responsible for the SARS outbreak between 2002 and 2004 (DHAMA et al., 2020). However, SARS-CoV-2 has a higher transmission potential and lower pathogenicity. Even though most affected individuals experience asymptomatic infections, a significant proportion has the potential to develop severe disease, requiring intensive care to prevent fatal outcomes. Severe cases are more prevalent in individuals with preexisting comorbidities, advanced age, or immunodeficiency (HU et al., 2021).

In the four years since the beginning of the COVID-19 pandemic, over 775 million cases have been confirmed, with more than 7 million deaths worldwide (MATHIEU et al., 2024). Until May 2024, Brazil had 38 million notified cases with 712,000 deaths (PAINEL CORONAVÍRUS BRASIL, 2024). These numbers place Brazil in the third-highest number of confirmed cases and second-highest death toll from COVID-19 in the world, with the United States and India in the first and second in the ranking (WHO, 2024).

Besides the effects of SARS-CoV-2 itself, around 10 to 20% of infected subjects may experience long-term symptoms after recovery from the initial illness (WHO, 2021). The condition, also known as post-acute sequelae of COVID-19, or long COVID, is characterized

by multisystem and complex symptoms comprising mainly of neurological, gastrointestinal, pulmonary, and cardiovascular alterations and which can be highly debilitating (FERNÁNDEZ-DE-LAS-PEÑAS et al., 2021). While long COVID seems to be more frequent in subjects that presented severe cases of COVID-19, even those who presented mild symptoms in the acute phase of the disease may later develop post-acute COVID-19 syndrome. The reported incidence of long COVID varies, affecting 10 to 30% of non-hospitalized cases, 50 to 70% of hospitalized cases, and 10 to 12% of vaccinated cases. This condition can affect individuals of all ages and severities of the initial disease, with high prevalence in people ranging from 36 to 50 years old. In absolute numbers, most long COVID cases are found in non-hospitalized patients who experienced a mild acute illness, as they constitute most COVID-19 cases overall (DAVIS et al., 2023).

Although the WHO estimates that COVID-19 mortality could be much higher based on global excess mortality (MSEMBURI et al., 2023), the prevalence of vaccination for SARS-CoV-2 infection resulted in a significant reduction in the number of severe cases and deaths from the disease. However, the transition of COVID-19 into an endemic state has introduced new challenges in public health management. Despite the widespread vaccination efforts, the virus continues circulating, with periodic outbreaks occurring in various regions. These outbreaks are often characterized by the emergence of new variants, which can partially evade immune protection provided by previous infections or vaccinations. Consequently, public health authorities are tasked with the ongoing surveillance of viral mutations and the adaptation of vaccines to ensure their efficacy (COHEN; SPIRO; VIBOUD, 2022). Additionally, the focus has shifted towards booster campaigns and the development of treatments to mitigate the impact of infections. The endemic nature of COVID-19 necessitates a sustained and flexible response to manage the disease effectively, balancing prevention strategies with the need to maintain normal societal functions (WILLIAMS et al., 2023).

1.1.2 SARS-CoV-2: The Virus and The Infection Mechanism

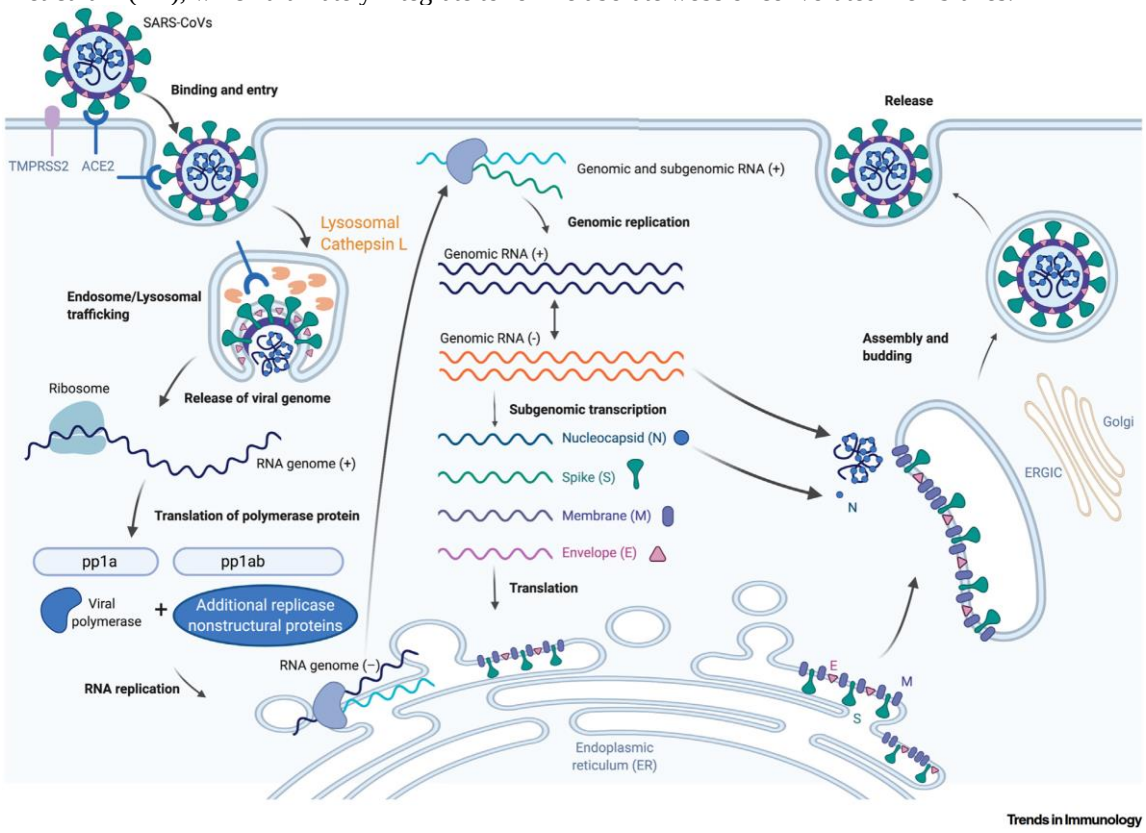
SARS-CoV-2, a member of the Coronaviridae family and the Betacoronavirus genus, is a virus that possess a spherical structure with 80 to 120 nm diameter range. It is distinguished by a lipid bilayer that encases a single strand of positive-sense ribonucleic acid (ssRNA) with a length of 26-32 kilobases (LU et al., 2020). Comparative genome analyzes indicate that the sequence of SARS-CoV-2 shares approximately 80% similarity with SARS-CoV and around 50% with MERS-CoV. Its genome comprises 14 open reading frames (ORFs), with most of

them, at least two-thirds, being responsible for encoding 16 nonstructural proteins (NSP 1–16) that constitute the replicase complex. The remaining one-third of the ORFs encode nine accessory proteins and four key structural proteins, namely spike (S), envelope I, membrane (M), and nucleocapsid (N) (ZHANG; HOLMES, 2020).

Viral tropism is dependent on the susceptibility and permissiveness of particular cellular characteristics, which are essential for understanding the pathogenesis of viral diseases. The SARS outbreak brought attention to patients presenting with respiratory symptoms that progressed to severe pneumonia (PEIRIS et al., 2003), a pattern also observed in COVID-19 cases, suggesting that SARS-CoV-2 primarily targets the lungs. Subsequent research confirmed that SARS-CoV and SARS-CoV-2 both utilize the angiotensin-converting enzyme 2 (ACE2) receptor for entry, prompting further study into the molecular dynamics of viral infection. ACE2 receptors are extensively expressed across various epithelial cells, including those in the lungs, small intestine, and other organs such as the heart, kidney, and esophagus (HAMMING et al., 2004). Significant mutations in the receptor-binding domain of the SARS-CoV-2 S protein have been found to enhance its interaction with ACE2, potentially increasing its binding affinity and, consequently, its infectivity (YAN et al., 2020).

The virus S protein is instrumental in attachment, fusion, entry, and transmission, as it contains a receptor-binding domain (RBD) responsible for establishing direct contact with the cellular receptor ACE2. The S protein undergoes cleavage into two subunits, S1 and S2. The RBD of the S1 subunit engages with the host ACE2 while the S2 subunit secures the S protein to the host membrane (PERLMAN; NETLAND, 2009). The interaction between the RBD and ACE2 triggers significant conformational alterations in both S protein subunits, revealing the S2 site, also known as the priming site. The cleavage of this site is a prerequisite for membrane fusion and the eventual liberation of the viral genome into the cytoplasm of the host cell. SARS-CoV-2 typically relies on the transmembrane protease serine 2 (TMPRSS2) for the cleavage at the’S2' site. In scenarios where TMPRSS2 is absent, the virus employs clathrin-mediated endocytosis for cell entry, and the’S2' site is cleaved by cathepsin L (FIGURE 1). Both TMPRSS2 and cathepsin L, acting in proteolytic cleavage are key factors for viral entry, as TMPRSS2 plays a crucial role in facilitating viral entry at the surface of the plasma membrane, while cathepsin L is involved in activating the SARS-CoV-2 Spike within endosomes, enabling the virus to enter cells lacking TMPRSS2 (JACKSON et al., 2022).

Figure 1 - The SARS-CoV-2 Lifecycle. The SARS-related coronavirus (SARS-CoV and SARS-CoV-2) lifecycle commences by binding of the envelope Spike protein to its cognate receptor, angiotensin-converting enzyme 2 (ACE2). Efficient host cell entry then depends on: (i) cleavage of the S1/S2 site by the surface transmembrane protease serine 2 (TMPRSS2); and/or (ii) endolysosomal cathepsin L, which mediate virus–cell membrane fusion at the cell surface and endosomal compartments, respectively. Through either entry mechanism, the RNA genome is released into the cytosol, where it is translated into the replicase proteins (open reading frame 1a/b: ORF1a/b). The polyproteins (pp1a and pp1b) are cleaved by a virus-encoded protease into individual replicase complex nonstructural proteins (nsps) (including the RNA-dependent RNA polymerase: RdRp). Replication begins in virus-induced double-membrane vesicles (DMVs) derived from the endoplasmic reticulum (ER), which ultimately integrate to form elaborate webs of convoluted membranes.



Source: Reproduction from HARRISON; LIN; WANG, 2020

SARS-CoV-2 also appears to exploit a subset of type II alveolar cells that exhibit elevated levels of ACE2 and additional pro-viral genes, enabling effective replication. The infectivity and transmission efficiency of SARS-CoV-2 is also dependent of a critical factor for membrane fusion between the virus and airways cells, that is facilitated by furin-mediated cleavage at the furin cleavage site (FCS) (PEACOCK et al., 2021b). Furthermore, the role of the lung as the primary site affected by SARS-CoVs could be linked to the modulation of ACE2 expression, both at the gene and protein levels (KUBA et al., 2005). For instance, in human airway epithelial cells, the expression of the ACE2 gene is increased by type I and II interferons (IFNs) in response to viral infections (ZIEGLER et al., 2020).

The initial cellular targets of SARS-CoV-2 during natural infection in humans are probably multi-ciliated cells within the nasopharynx or trachea, as well as sustentacular cells in

the nasal olfactory mucosa (AHN et al., 2020; KHAN et al., 2021). Furthermore SARS-CoVs also infect vascular endothelial cells, and alveolar macrophages (KUBA et al., 2005). These cells are likely initial sites of infection due to their ACE2 receptor expression, which enables the virus to attach and enter (ZIEGLER et al., 2020). Although ACE2 mRNA is present in human and other mammalian lung tissues, its expression is relatively low compared to other tissues, indicating the relevance of other cellular factors that influence susceptibility to SARS-CoV-2 infection. One such factor is TMPRSS2, which is critical for viral entry. Even low levels of ACE2 can facilitate SARS-CoV infection if TMPRSS2 is present, highlighting the complex relationship between host factors and viral infectivity (SHULLA et al., 2011). The importance of TMPRSS2 to viral entry can be confirmed by the evidence that human coronaviruses are known to cause gastrointestinal infections of varying severity (CHOLANKERIL et al., 2020). Notably, the proteins ACE2 and TMPRSS2 are highly expressed in the intestinal lining of humans and several other mammals, specifically within the brush border of enterocytes (WONG; LUI; SUNG, 2020). This correlates with the common occurrence of gastrointestinal symptoms among COVID-19 patients, which aligns with the detection of SARS-CoV-2 in the fecal matter of individuals with SARS (XIAO et al., 2020).

Upon cellular entry, the viral genome is released into the cytosol, and SARS-CoV-2 commandeers the host endogenous cellular machinery to work on the transcription, replication, and translation of its RNA genome (HOFFMANN et al., 2020). This occurs through the ORF1a and ORF1b that are translated into viral replicase proteins (SNIJDER; DECROLY; ZIEBUHR, 2016), thereby initiating the replication and propagation of the virus within the host organism. The translation of these ORFs yields two polyproteins, pp1a and pp1ab, which are pivotal for infection. Proteases encoded by ORF1a initiate the proteolytic cleavage of these polyproteins to release 16 NSPs, which are vital for viral replication (V'KOVSKI et al., 2021).

1.1.3 SARS-CoV-2 Evasion of Host Immune Response

In this initial stage of infection, to ensure survival, viruses have developed sophisticated methods to inhibit the signaling pathways of receptors involved in antiviral immunity. This is achieved by encoding immunomodulatory proteins that antagonize the host immune system,

disrupting the balance between positive and negative regulation of pattern recognition receptor (PRR)-initiated immune responses (CHATHURANGA et al., 2021; THOMPSON et al., 2011).

PRRs, are responsible for recognize conserved molecular structures identified as a pathogen-associated molecular patterns (PAMPs) or those damage-associated molecular patterns (DAMPs). PAMPs are linked with microbial pathogens, whereas DAMPs are associated with the components of the host cell that are released upon cellular injury or death. The primary families of PRRs responsible for identifying viral RNA within endosomes are the Toll-like receptors (TLR), while RIG-I-like receptors (RLR) are responsible for recognizing cytoplasmic viral RNA (OKAMOTO et al., 2017). In the PRR family, we also have the scavenger receptors (SR), that represent a distinct set of receptors capable of interacting with viruses in a non-specific manner. This category of receptors lies at the crossroads of immunity and metabolism, predominantly found on stromal macrophages and dendritic cells (PRABHUDAS et al., 2017). Additionally, SRs can serve as co-factors for PRRs, such as TLRs, in the identification and elimination of viruses by innate immune cells; however, in certain instances, they can also serve as an entry point for viruses, including SARS-CoV-2, to invade cells (WEI et al., 2020).

Upon activation of these receptors that directly or indirectly identifies viral infection, the signaling occurs via recruited adaptor proteins, ubiquitin ligases, and kinases, culminating in transcription factors and ultimate expression of immune genes, including IFNs, cytokines, and chemokines, resulting in the initiation of an antiviral innate immune response, mainly linked to the generation of IFN (DALSKOV; GAD; HARTMANN, 2023). The IFN pathway is often a primary target of evasion due to its rapidity and potency in eliminating viral infection, and that is why CoVs have developed multiple mechanisms to hijack the activation pathway of several PRR-IFN to ensure its viability. In fact, CoVs exhibit a pronounced sensitivity to IFNs, leading them to engage in multifaceted antagonism against mammalian immune detection. This antagonism manifests through the disruption of downstream signaling and the inhibition of specific IFN-stimulated gene (ISG) products (TOTURA; BARIC, 2012). CoVs also employ evasion tactics such as the creation of double-membrane vesicles (DMVs) that conceal viral nucleic acids from PRRs, and the direct impairment of immune signaling molecules by viral proteins (KINDLER; THIEL; WEBER, 2016). The structural and functional parallels of these proteins within the Betacoronavirus genus, and notably between the non-structural proteins of SARS-CoV and SARS-CoV-2, imply the utilization of similar suppressive strategies by SARS-CoV-2. Clinical observations of severe COVID-19 cases reveal a dysregulated immune response characterized by elevated levels of pro-inflammatory cytokines and chemokines, yet

diminished levels of circulating IFN-β or IFN-λ, leading to sustained viremia (HADJADJ et al., 2020). Notably, SARS-CoV-2 has been shown to significantly inhibit the expression of type I and type III IFNs, more so than other respiratory viruses, in both human bronchial epithelial cells and animal models. The circumvention of IFN signaling by SARS-CoV-2, coupled with reduced IFN production in human peripheral blood immune cells, may contribute to efficient viral replication, transmission, and the severe pathogenesis observed in COVID-19 (BLANCO-MELO et al., 2020).

The process of mutation also serves as a critical survival mechanism for viruses. These genetic alterations can enhance viral adaptability, allowing for the evasion of host immune defenses and the exploitation of new ecological niches. The high mutation rates, coupled with short generation times and large population sizes, enable viruses to adapt swiftly to the host environment. Additionally, factors like polymerase fidelity, replication mechanisms, and host-virus interactions play significant roles in modulating viral mutation rates (SANJUÁN; DOMINGO-CALAP, 2016). During the pandemic, mutations that optimized the furin cleavage site (FCS) in the Alpha and Delta variants spike proteins were linked to their increased transmissibility, estimated to be 55 to 65% greater than the original wild-type Wuhan variant (LUBINSKI et al., 2022; PEACOCK et al., 2021a). In contrast, the evolutionary advantage of the Omicron variant is not attributed to FCS optimization but rather to an altered entry mechanism and significant immune evasion, enabling it to infect individuals with prior immunity. The Omicron BA.1 strain, with over 30 spike protein mutations, was initially thought to be less virulent, yet it led to substantial hospitalizations and fatalities (CARABELLI et al., 2023).

1.1.4 COVID-19 Clinical Manifestation and Transmission

In general, CoVs infection tend to have symptoms associated to a common cold, resulting in mild upper respiratory tract symptoms and sometimes affecting the digestive system (KSIAZEK et al., 2003). In contrast, infections from more virulent coronaviruses like SARS-CoV-2 leading to intense symptoms similar to influenza, as described in the early pandemic stages could escalate to severe respiratory distress, pneumonia, kidney failure, and even cause a fatal outcome (WANG et al., 2020a). During the initial phase of the COVID-19 outbreak, in China, clinical characteristics from 1,099 affected subjects showed that the most common clinical characteristics were fever (88.7%,), cough (67.8%) and radiologic findings (59.1%) such as abnormalities on chest radiograph (GUAN et al., 2020). The same study demonstrated

a considerable frequency of symptoms such as sputum production (33.7%) and fatigue (38.1%), but few cases of symptoms like diarrhea (3.8%) and nasal congestion (4.8%). Other studies indicated that the most common associated symptoms of COVID-19 also included dyspnea, myalgia, fatigue, anosmia, and ageusia (EBRAHIMI; MALEHI; RAHIM, 2020). Additionally, some less frequent somatic symptoms like shortness of breath, palpitations, and pain in arms, legs, and joints have also been reported by COVID-19 affected subjects. The onset of COVID-19 is fast, with an average incubation period of approximately 5 to 6 days, compared to the range of 2 to 11 days for SARS-CoV (LI et al., 2020; SU et al., 2016).

Considering the fact that COVID-19 has undergone various changes over time, as the novel infection grew to inflict upon more people in different regions worldwide, other symptoms emerged, while new variants presented new symptoms associations. In a study conducted with 15.626 SARS-CoV-2 positive subjects (alpha, delta and omicron variants), showed that fever was the common symptom among all individuals, while cough was an important predictor of COVID-19 in all variants except for Omicron. Also, the prevalence of shivering, myalgia and ageusia or anosmia was more prevalent in subjects with infected with the Delta and Omicron variants. Artificial intelligence (AI) studies (predictive models and supervised neural networks) using 18 independent variables to determine the importance of predictive symptoms, showed the age, sore throat, myalgia, along cough and diarrhea (81.7% accuracy), were the most important variables that fitted to the model using the initial Wuhan strain. AI also showed that with the subsequent new variants the most important variables were diarrhea, anosmia/ageusia in the Alpha (75.7%); ageusia and anosmia, cough, and diarrhea in the Delta (69.2%) and shivering and diarrhea in the Omicron (62.5%) (TORABI et al., 2023).

Regarding the broad spectrum of the clinical presentations of COVID-19, the prevalence of asymptomatic cases ranged from at least 30% to 45% of infected individuals (KIMBALL et al., 2020; ORAN; TOPOL, 2020). Additionally, most symptomatic subjects experience mild disease, while approximately 5% of infected subjects progressed to severe illness (RAHMAN et al., 2021). Some studies also point that, even though the incubation period of SARS-CoV-2 range between 3 to 7 days, some cases showed a potential extension up to 18 days. Notably, the incubation time varies across different waves of the pandemic and variants of SARS-CoV-2. Specifically, the median incubation period decreases over successive waves, with 5 days for cases associated with the alpha variant and approximately 3.5 days for cases associated to the omicron variant (WU et al., 2022). Importantly, viral transmission can occur even before the onset of symptoms, including individuals who remain asymptomatic throughout the course of infection. Asymptomatic carriers were estimated to contribute to approximately 50% of all virus

transmissions (JOHANSSON et al., 2021). Interestingly, a recent study evaluating patients with long lasting positive RT-PCR for SARS-CoV-2, reported the presence of replication-competent viruses for up to 128 days after symptom onset (LEITÃO et al., 2021). Data that suggest the potential risk for continued transmission for a longer period than previously described by literature.

From the start of the pandemic, COVID-19 cases have been concentrated among adults. Children, in general, have been less affected, exhibiting lower infection rates and milder clinical manifestations (MOLTENI et al., 2021). However, severe disease presentations, characterized by acute respiratory distress syndrome (ARDS), metabolic acidosis, and disseminated intravascular coagulation, have primarily been observed in elderly patients. Interestingly, individuals aged 5 to 9 years present the lowest case fatality rates, with exponential increases in fatality rates as age advances (O'DRISCOLL et al., 2021). Furthermore, numerous studies have highlighted increased mortality risk in individuals with comorbidities, including obesity, diabetes, cardiovascular disease, chronic kidney disease, and chronic pulmonary conditions (TUTY KUSWARDHANI et al., 2020). Additionally, sex-based differences exist in mortality rates for hospitalized patients with severe COVID-19, males appear to be at higher risk of developing severe forms of the disease compared to females (CHATURVEDI et al., 2022). In a global meta-analysis conducted analyzing the impact of sex, age, cardiovascular disease, hypertension and diabetes, indicated that males had 16% higher risk of mortality than females. Overall patients with pre-existing comorbidities such as cardiovascular disease, hypertension, and diabetes have a increased risk of death up to 1.96-fold,1.73-fold, and 1.59-fold, respectively (MOULA et al., 2020).

Severe illness in COVID-19 typically manifests approximately one week after the onset of symptoms. Among the hallmark symptoms of severe disease, dyspnea (shortness of breath) stands out as a consequence of hypoxemia (WANG et al., 2020a; ZHOU et al., 2020a). Subsequently, patients with severe COVID-19 progress to respiratory failure, often meeting the criteria for ARDS. The development of COVID-19 symptoms involves intricate mechanisms but can be theoretically understood through established models of three primary inflammatory processes: typical local inflammation, acute widespread inflammation, and persistent low-grade systemic inflammation. The risk of the latter increases with age and is more prevalent among individuals with metabolic disorders, diabetes, and other chronic conditions (GUSEV et al., 2021). ARDS represents a form of lung injury marked by inflammation, pulmonary vascular leakage, and subsequent loss of aerated lung tissue. In subjects with COVID-19 experiencing hypoxic respiratory failure, evidence points to systemic hyperinflammation. Pro-inflammatory

cytokines, including interleukin-1 (IL-1), IL-6, IL-8, and tumor necrosis factor (TNF), are released, contributing to the pathogenesis. Elevated levels of inflammatory markers such as D-dimer, ferritin, and C-reactive protein further underscore the systemic immune response (CHEN et al., 2020; DEL VALLE et al., 2020).

Initial observations of individuals presenting with cough, pulmonary ground-glass opacities, and the subsequent development of severe pneumonia have indicated that SARS-CoV-2 may be transmitted through respiratory pathways (ZHOU et al., 2020b). These first studies confirmed that the primary mode of transmission of SARS-CoV-2 include person-to-person contagion through airborne droplets and aerosols. Additionally, alternative routes such as transmission via contaminated objects, food, and water have been investigated, with some studies demonstrating the persistence of SARS-CoV-2 RNA on these surfaces, indicating their potential role in contamination and transmission (ARIENZO et al., 2023; SUKHIKH et al., 2021). Understanding these various modes of transmission was crucial for implementing effective control measures to prevent the spread of COVID-19 in different settings and populations, especially considering the evolving nature of the pandemic and the emergence of new variants.

Transmission between asymptomatic and symptomatic individuals with COVID-19 varies significantly. Asymptomatic carriers, despite showing less severe pulmonary damage, have been identified as potential sources of transmission, with a shorter duration from disease onset to peak progression stage compared to symptomatic patients (TAN et al., 2022). Studies have estimated that asymptomatic cases can lead to a lower infection rate compared to symptomatic cases, some studies show that the rate of asymptomatic SARS-CoV-2 infection was 34.9%, which also indicate a reduced transmission risk through asymptomatic exposure (EL-GHITANY et al., 2022). Furthermore, mathematical models analyzing different COVID-19 variants have shown an increasing proportion of asymptomatic infections over time, emphasizing the role of asymptomatic individuals in the spread of the epidemic and the need for collaborative prevention and control measures to curb transmission effectively (XU et al., 2023).

Acute COVID-19 typically persists for up to 4 weeks, while symptomatic COVID-19 can extend for up to 12 weeks. Long COVID has different definitions across different healthcare systems and countries, leading to challenges in standardization and diagnosis (SRIKANTH et al., 2023). The most recent published reviews indicate that long COVID-19 is a condition that follows recovery from acute COVID-19 infection or unresolved illness. It is characterized by persistent symptoms occurring after the acute phase, yet lacks a universal definition, resulting

in variations in symptom duration and clinical manifestations. These symptoms typically emerge approximately 3 months after the initial infection and persist for more than 3 months. Importantly, they cannot be explained by alternative diagnoses. Estimates of the prevalence of long COVID-19 symptoms vary widely, influenced by subjects demographics, inclusion of control groups, and duration of follow-up. Potential mechanisms contributing to long COVID include SARS-CoV-2 persistence, reactivation of other viruses (Epstein-Barr virus), virus-triggered autoimmunity, persistent tissue damage, immune-triggered inflammation, and formation of microthrombi in vascular beds (endothelial cell activation) (LENZ et al., 2024). Endothelial damage and subsequent dysfunction may be a mechanism, as long-term viral infection, chronic hypoxia, and inflammatory responses can lead to persistent vascular endothelial injury, coagulation, microthrombosis, and systemic functional impairments (MONJE; IWASAKI, 2022; NEWELL; WAICKMAN, 2022). Alterations in the host microbiome may also play a role (LIU et al., 2022a).

SARS-CoV-2 primarily targets the respiratory system. However, mounting evidence reveals that its impact extends beyond the lungs, affecting various organs and systems. The virus RNA and proteins have been detected in multiple organs, including the heart, intestines, brain, male genitals, and kidneys. Additionally, infectious virus has been identified in various body fluids such as mucus, saliva, urine, cerebrospinal fluid, semen, and breast milk (TRYPSTEEN et al., 2020). Beyond lung involvement, SARS-CoV-2 may lead to extrapulmonary diseases, encompassing gastrointestinal symptoms, acute cardiac, kidney, and liver injury, cardiac arrhythmias, rhabdomyolysis, coagulopathy, and shock (ASKARI et al., 2023)

Amid the urgency of the COVID-19 pandemic, discovering precise, efficient, and cost-effective diagnostic methods for large-scale use is paramount to mitigating contagion risks and reducing mortality. Accurate identification of patients likely to progress to severe disease or multiple organ failure versus those who will remain stable is essential for personalized treatments and optimized medical resource allocation. High-throughput omics technologies — such as genomics, transcriptomics, proteomics, epigenomics, metabolomics, and microbiomics — have become vital to precision diagnostics. These technologies generate extensive data, enabling the identification of vulnerable populations and the discovery of omics-based biomarkers, which drive advancements in early diagnosis, disease prognosis, individualized treatments, and vaccination (MA et al., 2022).

In particular, metabolomics stands out as a key strategy. By globally analyzing metabolites—small molecules indicative of the metabolic status of organs and tissues—

metabolomics provides a metabolic profile or “metabotype.” This approach facilitates the identification of specific metabolic profiles for diagnosis and prognostication, paving the way for personalized medicine strategies. Beyond patient profiling, metabolic signatures enhance the understanding of disease-associated biological pathways and generate mechanistic hypotheses, making metabolomics a cornerstone in the fight against COVID-19 and other diseases(VLASOVA-ST. LOUIS et al., 2023).

1.1.5 COVID-19 Pathogenesis

The precise pathophysiological mechanisms driving COVID-19 remain unknown considering this is a complex disease with a wide range of clinical presentations and that both host and viral determinants are involved. The pathogenesis of COVID-19 exhibits a spectrum of intensity and even tissue distribution. While the majority of individuals experience symptoms related to the upper respiratory tract and lungs, severe cases may present with extensive thrombosis affecting both small and large blood vessels, damage to the microvasculature, dysrhythmia, neurological impairments, diarrhea, and gastrointestinal hemorrhage, all of which pose significant mortality risks (EBRAHIMI; MALEHI; RAHIM, 2020; RAHMAN et al., 2021).

SARS-CoV-2 is described to utilize at least three of traditional viral pathogenic tactics. The first involves virus recognition by cellular receptors, which are divided into three functional groups: (a) receptors that facilitate virus entry into the cell, aiming to increase binding affinity and broaden the range of these receptors and their co-receptors; (b) receptors that transmit information advantageous to the virus to the cell; and (c) receptors that initiate an antiviral response upon virus detection, and the virus strategy is to inhibit these receptors and their signaling pathways. The second strategy is the inhibition of the host antiviral defenses, affecting both the infected cells and the host immune system. This includes reducing the early antiviral response of type I and type III IFN, disrupting universal cellular stress signals or specific immune responses, and protecting the virus from the direct action of antiviral response factors. The third strategy is to ‘provoke’ the host immune system to attack its own tissues, a unique viral survival tactic that results in autoimmune and autoinflammatory processes (GUSEV et al., 2022). Emerging evidence indicates that severe COVID-19 arises from virus-induced perturbations within the immune system, resulting in tissue damage, alterations in critical immune cell populations, activation of the complement system, and intricate interactions between innate immunity and the coagulation cascade, ultimately promoting inflammatory

thrombotic events (BORCZUK; YANTISS, 2022). These multifaceted changes can be categorized into three distinct groups characterized by a humoral immunodeficiency characterized by B-cell dysfunction, a hyperinflammatory state marked by T-cell subset depletion and elevated cytokine levels driven by IL-6, IL-1β, and TNF-α, and injury mediated by complement activation (DUPONT et al., 2021)

In the context of SARS-CoV-2 pathogenesis IFN responses play a pivotal role in mediating viral defense mechanisms. While IFNs are integral to combating viral infections, an excess can precipitate tissue damage, underscoring the significance of both timing and tissue location in the resultant effects of IFN-mediated immune activity. Variations in interferon levels across different tissues have been observed to correspond with the severity of COVID-19, suggesting a complex interplay between IFN response and disease progression (UNTERMAN et al., 2022). In the early stages of infection, type I IFN responses are critical; however, these responses remain elevated in patients experiencing prolonged viral replication. Conversely, an upsurge in type II IFN has been linked to severe manifestations of the disease across various organ systems. Type III IFN, predominantly found in the upper respiratory tract, appears to be associated with milder cases characterized by high viral loads (KARKI et al., 2021; MONTALVO VILLALBA et al., 2020). The Type III IFN also have a role in SARS-CoV-2 resolution in the upper airways, as it is hypothesized to contribute to the clearance of SARS-CoV-2. However, in the case of antiviral efforts fail, a shift towards a pro-inflammatory state ensues, driven by types I and II IFN within the lower respiratory tract (SPOSITO et al., 2021).

Peripheral blood mononuclear also cells exhibit an increased type I IFN profile correlating with initial viral load, which subsequently diminishes as the viral load decreases. Notably, in severe COVID-19 cases, IFN responses are often attenuated or delayed (BERGAMASCHI et al., 2021). This suboptimal IFN activity may stem from individual biological factors such as genetic factors, the presence of neutralizing autoantibodies, or a reduction in plasmacytoid dendritic cells, which are known for producing TLR7—a toll-like receptor that recognizes single-stranded viral RNA and stimulates IFN responses (SONG et al., 2020). Furthermore, genetic susceptibilities linked to severe COVID-19 have been identified in genes that are instrumental in the induction and amplification of type I interferon via TLR3-dependent and TLR7-dependent pathways, as well as in the detection of type I interferon (ASANO et al., 2021; ZHANG et al., 2020). This underscores the critical role of interferon signaling in the immune response against SARS-CoV-2. Reinforcing this notion are studies indicating an association between neutralizing autoantibodies to IFNα and severe COVID-19 outcomes (BASTARD et al., 2020). Such autoantibodies are found in approximately 4% of

uninfected individuals over the age of 70 and are estimated to account for around 20% of fatalities of patients over 80 years related to COVID-19. As these autoantibodies appear to increase with age, these findings provides one possible explanation for the major increase in the risk of critical COVID-19 in the elderly, (BASTARD et al., 2021).

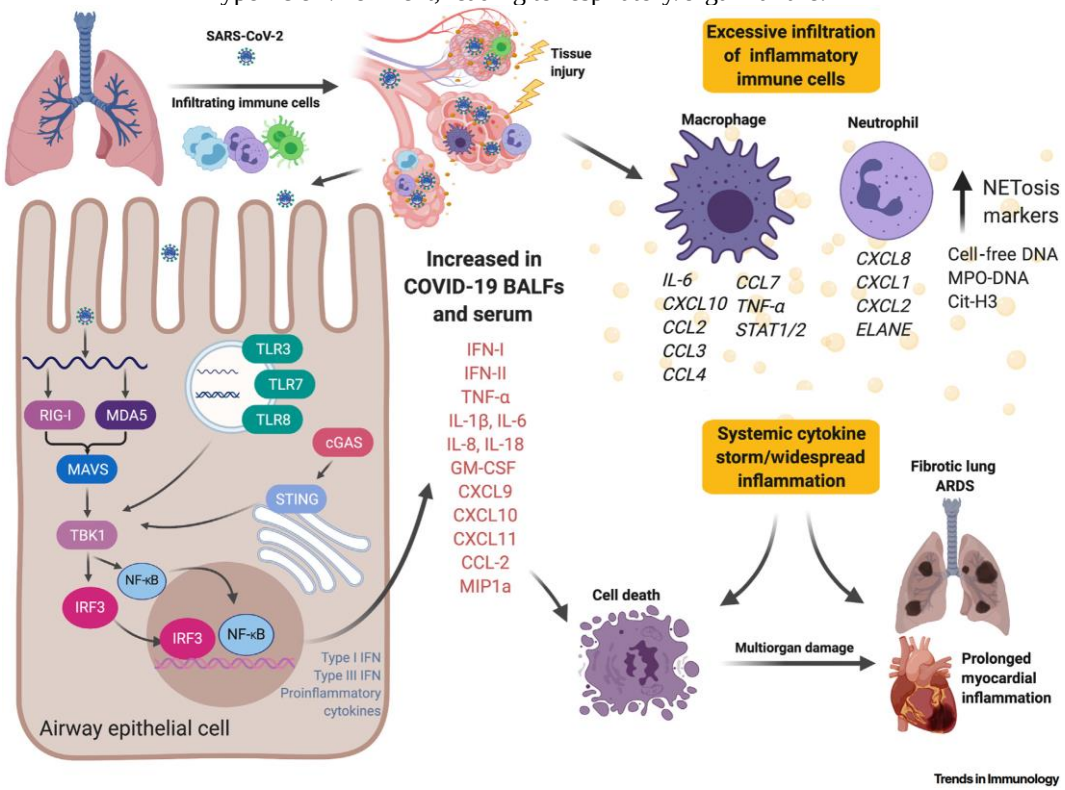
The dichotomy of IFN responses in the pathogenesis of COVID-19 presents a complex puzzle in the landscape of viral infections. While the protective versus detrimental roles of IFNs in COVID-19 remain a subject of debate, emerging literature suggests that the efficacy of IFN responses may be contingent upon the stage of infection and the severity of disease. Recent investigations have illuminated that severe COVID-19 is often accompanied by compromised IFN signaling pathways (BASTARD et al., 2020; COMBES et al., 2021). In contrast, other studies posit that an exacerbated and protracted IFN response correlates with adverse clinical outcomes (LEE et al., 2020; LUCAS et al., 2020). In published study that describes an in-depth analysis of IFNs in COVID-19, elucidates that robust viral replication of SARS-CoV-2 triggers an effective IFN-III response in the upper respiratory tract of younger individuals or those with milder symptoms, whereas patients with severe illness exhibit pronounced IFN levels in the lower respiratory tract, both at the mRNA and protein levels. These findings corroborate to the notion that IFNs exert divergent effects along the respiratory tract, potentially reconciling the conflicting reports regarding IFN dynamics in COVID-19. An efficient early IFN response in the upper airways may facilitate swift viral clearance and impede progression to the lower respiratory tract. However, if the virus elude initial immune defenses, the intensified IFN response in the lungs may exacerbate the inflammatory milieu, contributing to the cytokine storm and subsequent tissue damage observed in severe-to-critical cases of COVID-19, which are marked by diminished cellular proliferation and enhanced pro-apoptotic p53 transcriptional activity (SPOSITO et al., 2021).

There is accumulating evidence suggesting that dysregulated inflammation and cytokine alterations plays a significant role in the mortality and morbidity of the disease(LUCAS et al., 2020). When compared with influenza, the ARDS associated with SARS-CoV-2 infection features a increased cytokine levels. Patients with COVID-19 with hypoxic respiratory failure have evidence of systemic hyperinflammation, including release of pro-inflammatory cytokines, such as interleukin-1 (IL-1), IL-6, IL-8 and TNF, while also presenting elevated concentrations of inflammatory markers, including D-dimer, ferritin and C-reactive protein. Serum levels of IL-6, IL-8, and TNF at the time of hospitalization are strong and independent predictors of patient survival (DEL VALLE et al., 2020).

This disturbed cytokine profile is also correlated with the recruitment of functionally altered immune cells, as in SARS-CoV-2 infection neutrophils present a higher degree of degranulation, which also increases the cytokine levels (CAMBIER et al., 2022). While macrophages presents precipitate activation syndrome, which is characterized by increased type II IFN-related responses as well as elevated IL-6, IL-1β, TNF-α, and ferritin levels(MCGONAGLE et al., 2020). These two cells also interact with each other in cascade of proinflammatory events that represents an worsening of the patients condition, as monocytes release proinflammatory cytokines being responsible for pneumocyte apoptosis. Macrophages release chemokines and cytokines, which increase capillary permeability and also cause the recruitment of neutrophils. Excessive degranulation of neutrophils causes permanent damage to pneumocytes breaking the alveolar–capillary barrier (FIGURE 1). The end result of all these mechanisms is a transmigration of blood proteins resulting in alveolar and interstitial edema. This acute and diffuse inflammatory damage into the alveolar-capillary barrier is associated with a vascular permeability increase and reduced compliance, compromising gas exchange and causing hypoxemia, occasionally evolving to ARDS (BATAH; FABRO, 2021). Histological examination of human lung tissues indicates that this diffuse alveolar damage is the predominant pattern of injury in patients infected with COVID-19 (CARSANA et al., 2020).

Figure 2 - A Brief Overview of Lung Pathology in Patients with COVID-19. Following inhalation of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) into the respiratory tract, the virus traverses deep into the lower lung, where it infects a range of cells, including alveolar airway epithelial cells, vascular

endothelial cells, and alveolar macrophages. Upon entry, SARS-CoV-2 is likely detected by cytosolic innate immune sensors, as well as endosomal toll-like receptors (TLRs) that signal downstream to produce type-I/III interferons (IFNs) and proinflammatory mediators. The high concentration of inflammatory cytokines/chemokines amplifies the destructive tissue damage via endothelial dysfunction and vasodilation, allowing the recruitment of immune cells, in this case, macrophages and neutrophils. Vascular leakage and compromised barrier function promote endotheliitis and lung edema, limiting gas exchange that then facilitates a hypoxic environment, leading to respiratory/organ failure.



Source: Reproduction from HARRISON; LIN; WANG, 2020

Alveolar cell death or damage leads to a disruption of the alveolar epithelium, which sets the exudative phase of diffuse alveolar damage, which involves hyaline membrane formation due to the polymerization of fibrin present in plasma leaked into interstitial space. The formation of hyaline membranes are dangerous because as a fibrin-rich exudates that seal the alveoli from fluid accumulation, it can also limit oxygen exchange (IBA et al., 2020). This exudative phase is accompanied by alveolar–capillary barrier injury, resulting in red blood cell extravasation and inflammatory cell infiltration. This process leads to a proliferative phase, which involves excessive fibroblast and myofibroblast proliferation resulting in acute fibrinous organizing pneumonia with extracellular matrix deposition and the formation of fibrin thrombi (TANG et al., 2020). This explains why lung radiological examination show bilateral ground-glass opacities along with peripheral distribution. In the most severe cases of COVID-19 fibrin thrombi are found in the small arterial vessels (KLOK et al., 2020). This prothrombotic state seen in patients with COVID-19 is hypothesized to be a reminiscent process known as immunothrombosis, in which immune and coagulation systems would work through activated

neutrophils and monocytes that interact with platelets and the coagulation cascade, leading to intravascular clot formation in small and larger vessels, which block pathogens and limit their spread, creating an intravascular barrier that facilitates the recognition, containment, and destruction of pathogens, thereby protecting host integrity without inducing major collateral damage to the host (BONAVENTURA et al., 2021; ENGELMANN; MASSBERG, 2013).

The precise mechanisms inciting coagulation system imbalances in COVID-19 remain elusive; however, the initial disruption of the alveolar epithelium is a suspected trigger(MEYER; GATTINONI; CALFEE, 2021). A plethora of stimuli, including hypoxia, cytokines, chemokines, and damage-associated molecular patterns, are known to induce permeability in both endothelial and epithelial barriers, disrupting intercellular junctions (MILLAR et al., 2016). Endothelial dysfunction serves as a hallmark of microvascular impairment, precipitating a shift in vascular homeostasis favoring vasoconstriction and a prothrombotic state. Early in the pandemic, case reports suggested that SARS-CoV-2 infection precipitates endotheliosis across multiple organs. This phenomenon may elucidate the observed systemic microcirculatory dysfunction across diverse vascular beds, contributing to the clinical sequelae manifest in patients with COVID-19 (VARGA et al., 2020). A relevant pathophysiological hallmark of COVID-19 is the emergence of a prothrombotic state, characterized by elevated blood levels of fibrinogen, von Willebrand factor (VWF), and D-dimer, the latter being a fibrin degradation product. Despite these indicators, patients typically exhibit minimal alterations in conventional coagulation metrics such as prothrombin time, activated partial thromboplastin time, antithrombin, activated protein C levels, and platelet counts (PANIGADA et al., 2020; TANG et al., 2020).

1.1.6 SARS-CoV-2 Evolution and Genetic Variants

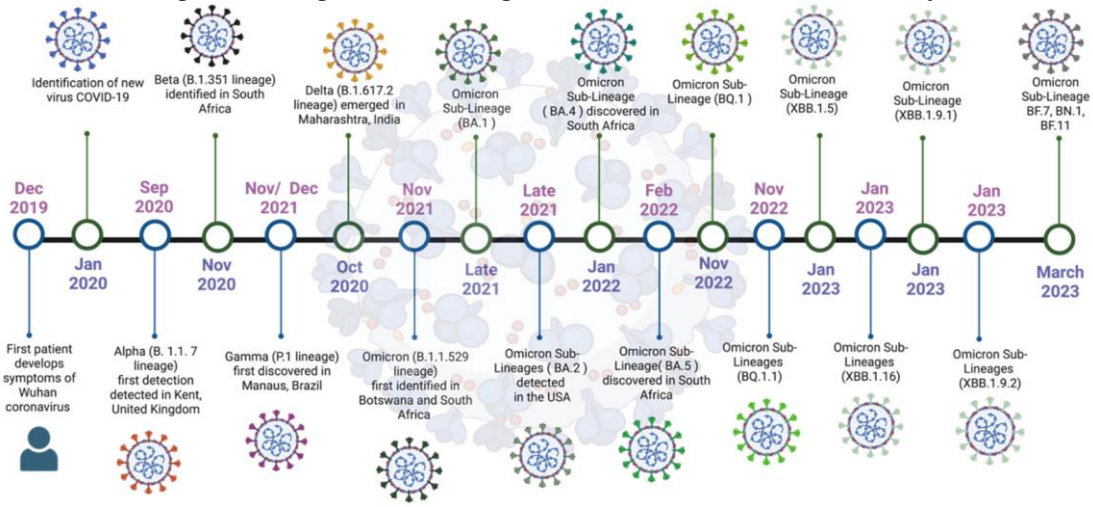
The evolutionary trajectory of SARS-CoV-2 has been marked by the emergence of multiple variants, driven by a diverse array of genetic alterations encompassing recombination events, point mutations, deletions, and amino acid substitutions. Usually, genome size inversely correlates with mutation rate, which influences the genetic diversity of viruses. That is why RNA viruses mutate faster than desoxyribonucleic acid (DNA) viruses, with single-stranded RNA viruses exhibiting higher mutation rates than double-stranded RNA viruses. While

recombination, facilitated by copy choice of the RNA template and polymerase-based mutations, leads to intramolecular recombination in coronaviruses, contributing to the evolution of new strains (SANJUÁN; DOMINGO-CALAP, 2021). Considering this, upon the initial identification of SARS-CoV-2, it was posited that the mutation rate in coronaviruses would be constrained due to the necessity of preserving the integrity of their large genome (RAUSCH et al., 2020), which are among the largest of RNA viruses, encompassing roughly 30,000 nucleotides (HARTENIAN et al., 2020). This presumption of genomic stability misguided pharmaceutical strategies to develop vaccines targeting the spike protein to curb the spread of SARS-CoV-2, not considering the molecular epidemiological dynamics that resulted in the rapid spread and succession of SARS-CoV-2 new strains(YANG et al., 2020).

Unfortunately, Over three years, since the first genome sequence was published, the SARS-CoV-2 genome has accumulated nearly 120 mutations per genome, a stark contrast to the original Wuhan genomic sequence, reflecting the virus's ongoing evolution (COLSON et al., 2024). These changes are particularly pronounced within the RBD of the S protein. The S protein itself is bifurcated into two subunits by host cell proteases: the S1 subunit, which contains the RBD and is responsible for recognizing and binding to the host cell surface receptor, and the S2 subunit, which facilitates the fusion of the viral and host cell membranes. The S1 subunit’s C-terminal domain specifically interacts with the human ACE2, initiating the infection process (WANG et al., 2020b). Both the S1 and S2 subunits are pivotal in the virus’s pathogenesis and serve as primary targets for antiviral-neutralizing antibodies. Genetic variations within the S1/S2 domains and the RBD have led to the genesis of novel SARS-CoV-2 variants, each with potentially distinct pathogenic profiles and implications for vaccine efficacy and public health strategies(ANDERSEN et al., 2020).

To assist with public discussions about significant SARS-CoV-2 variants, the WHO has classified the SARS-CoV-2 variants into three main categories, such as the variants of concern (VOCs), variants of interest (VOIs), and variants under monitoring (VUMs). The classification with most impact worldwide, the VOC, have been identified by their genetic distinctions and epidemiological effects, being differentiated from the original CoV identified in Wuhan by any of the subsequent alterations, such as: increased transmissibility; increased virulence; change in clinical disease presentation; or decrease in the effectiveness of public health and social measures, such as available diagnostics, vaccines, or therapeutics (FIGURE 3). The VOC were named using the Greek alphabet: Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2), and Omicron (B.1.1.529) (WHO, 2023).

Figure 3 - Timeline of different VOCs of SARS-CoV2. More than 17 different variants have been discovered starting from its origin in 2019 through the most recent VOC discovered early in 2023.



Source: Reproduction from ANDRE et al., 2023

The alpha variant of COVID-19, also known as the B.1.1.7 variant, was first detected in late 2020 and rapidly became the dominant strain in the United Kingdom (BRAYBROOK et al., 2021). This variant contains mutations in the S protein that enhance infectivity and immunity evasion, potentially leading to more severe clinical outcomes and increased resistance to vaccines developed based on the original strain. Such adaptations increased transmittance and virulence, and research concluded that the death risk to this Alfa variant were 39%-72% higher when compared to the Wuhan wild-type strain (MENG et al., 2021). It spread quickly, being the the predominant variant in England, accounting for over 95% of cases by February 2021 (LOCONSOLE et al., 2021). The alpha variant's prevalence during this period was linked to a 3.8-fold rise in the risk for death or intensive care unit (ICU) hospitalization, suggesting a more severe inflammatory disease phenotype (VASSALLO et al., 2021).

The emergence of the Beta variant, B.1.351 variant, initially detected in South Africa, marked a significant development in the COVID-19 pandemic. This variant rapidly became the predominant strain within the region, distinguished by a heightened transmissibility rate. The spike protein of B.1.351 exhibited a multitude of mutations, which are believed to contribute to its increased spread. During the Beta wave of infections in South Africa, a notable 12.6% of infected individuals required hospitalization. Of these hospitalized cases, 63.4% progressed to severe disease states, culminating in a 28.8% mortality rate (JASSAT et al., 2022). Comparative analyses between the Alpha (B.1.1.7) and Beta (B.1.351) variants, focusing on clinical outcomes such as 60-day and 28-day mortality rates, as well as the necessity and duration of mechanical ventilation, revealed no statistically significant disparities (ULRICH et al., 2022).

Despite the global dissemination of the B.1.351 variant, the WHO have not reported any exacerbation in disease severity attributable to this variant, even in the face of its augmented infectivity and capacity for neutralizing antibody evasion. Moreover, amidst the severity associated with the Alpha and Beta variant infections, preliminary data indicates that a single administration of the mRNA-1273 vaccine (also know as bivalent) provided substantial protection. The vaccine efficacy extends to preventing B.1.1.7 and B.1.351 infections, hospitalizations, and fatalities associated with COVID-19, irrespective of symptomatic presentation (CHEMAITELLY et al., 2021).

The Gamma variant (P.1), identified as the third VOC, was initially detected in the timeframe of November to December 2020 within Manaus, Brazil (DA SILVA et al., 2021). Characterized by its rapid proliferation, the Gamma variant extended its reach to over 36 countries. Concurrently, Brazil experienced a second wave of COVID-19, marked by a surge in case numbers and mortality rates. This escalation was fueled by the circulation of several VUMs, notably the Zeta (P.2) and Gamma (P.1) variants, with the latter achieving widespread prevalence by January 2021 (FARIA et al., 2021). Despite the commencement of the national vaccination campaign on January 17, 2021, the death toll attributed to COVID-19 in Brazil continued to escalate, culminating in a daily peak of 4,249 fatalities in April 2021. This peak was succeeded by a decline in daily case numbers and associated deaths. The Zeta variant played a minimal role in the initial epidemic wave, persisting until March 2021, before being swiftly supplanted by the Gamma variant. Between February and June 2021, a significant spike in case numbers was observed, with the Gamma variant accounting for 96% of cases in Brazil. However, its dominance was short-lived, as it was eventually overtaken by the highly transmissible Delta variant (GIOVANETTI et al., 2022). In terms of immunity, prior infection conferred a protection range of 54 to 79% against the P.1 variant. This finding was corroborated by pseudotyped neutralization assays utilizing anti-SARS-CoV-2 RBD monoclonal antibodies, which demonstrated a diminished neutralization capacity in plasma from vaccinated or previously infected individuals (WANG et al., 2021b). Despite the concerns regarding increased reinfection risk or reduced vaccine efficacy posed by the P.1 variant, the threat level does not appear to be as pronounced as that of the B.1.351 variant (WANG et al., 2021a).

The B.1.617.2 variant, the Delta variant, emerged as the fourth and the most significant VOC in October 2020, originating from Maharashtra, India. This variant was characterized by a mutation at the FCS, which led to an increase in pathogenicity and fusogenic activity (SAITO et al., 2022). Such changes were associated with heightened infection severity and the emergence of atypical symptoms. The Delta variant’s increased virulence is partly attributed to

enhanced binding affinity to the ACE2 receptor, which facilitated viral entry into host cells. Similar to the Kappa variant, the Delta variant exhibited a notable capacity for immune evasion, reducing the neutralization effectiveness of both monoclonal and vaccine-induced polyclonal antibodies. The increased RBD-ACE2 interaction also contributed significantly to the variant's ability to circumvent immune defenses (SAVILLE et al., 2022). The Delta variant's global impact was profound, with reports indicating its presence in over 119 countries and its role as a primary driver of the COVID-19 pandemic's spread, despite ongoing vaccination efforts worldwide (MLCOCHOVA et al., 2021). During its peak period from late 2020 to early 2021, the Delta variant was responsible for approximately 99% of all SARS-CoV-2 cases, surpassing the earlier B.1.617.1 (Kappa) variant. Its dominance was attributed to an estimated 40–60% increase in transmissibility compared to the original SARS-CoV-2 strain (CAMPBELL et al., 2021). Vaccination efforts, particularly those utilizing mRNA-based vaccines such as Moderna's mRNA-1273 and Pfizer-BioNTech's BNT162b2, as well as Johnson & Johnson's adenoviral vector vaccine, proved to be 95% effective in reducing hospitalizations and fatalities associated with the Delta variant. While vaccines have shown high efficacy in preventing severe disease and hospitalization for up to six months post-immunization, a decline in vaccine effectiveness against infection has been observed with Beta, Delta, and Omicron variants 20 weeks following the secondary vaccination. It was observed that the protective effect against infection diminishes over time post-primary immunization, highlighting the need for continued vigilance and potential booster vaccinations to combat emerging variants (LOPEZ BERNAL et al., 2021).

The Omicron variant, classified under the B.1.1.529 lineage, emerged as the fifth and final VOC identified during the COVID-19 pandemic. The initial detection of the original Omicron strain occurred in Botswana and South Africa in late November 2021. The variant rapidly disseminated internationally, leading to a significant spike in case numbers. By December 2021, the United States reported daily case figures exceeding one million, and the Omicron variant began to give rise to several subvariants (VITIELLO et al., 2022). Genomic analysis of the S protein sequence revealed the existence of two distinct subclades within the Omicron variant. Subclade 1, characterized by a lower sequence frequency, was predominantly observed in South Africa. In contrast, Subclade 2 demonstrated a higher sequence frequency and a global distribution (WANG; CHENG, 2022).

Data available up to March 2022 indicated that the Omicron variant surpassed the Delta variant in terms of global incidence. Notably, infections with the Omicron variant were associated with the least reduction in body weight and mortality rates among the variants

studied. Comparative analyses utilizing a transgenic mouse model infected with SARS-CoV-2 wild type (WT) from Wuhan and the subsequent Alpha, Beta, Delta, and Omicron variants suggested an attenuating trend in pathogenicity, with the Omicron variant exhibiting the mildest effects (SHUAI et al., 2022). In terms of viral replication dynamics, the Omicron variant exhibited a replication rate in the lungs that was tenfold lower than that of the Delta variant. Conversely, it replicated approximately 70 times more efficiently in the human bronchus compared to both the Delta variant and the original SARS-CoV-2 virus (KHANDIA et al., 2022). The high mutation rate of the Omicron variant has compromised the efficacy of neutralizing antibodies and vaccines, leading to an increased incidence of reinfections in regions such as South Africa (HARVEY et al., 2021).

A mathematical model developed to assess the transmissibility and infection fatality ratio of the Omicron variant in South Africa concluded that, despite its higher transmissibility, the infection fatality was reduced by 78.7% compared to previous variants (LIU et al., 2022b). In both the United States and South Africa, the case fatality ratio associated with the Omicron variant was half that observed with the Delta variant (SIGAL; MILO; JASSAT, 2022). Consistent with these findings, a patient cohort study from Paraná, Brazil, mirrored the observations from South Africa and the United States. The study reported that within a single week, approximately 1.3% of the state's population was infected by the Omicron variant, indicating a high transmission rate. However, the lethality rate was significantly mitigated, likely due to combination of multiple infection waves and the high vaccination coverage (70%) within the population (ADAMOSKI et al., 2022).

1.1.7 COVID-19 Treatment and Prevention

At present, the medical community faces the challenge of lacking a standardized approach for the prevention and treatment of COVID-19. The disease's pathogenesis is primarily propelled by two factors: SARS-CoV-2 infection and subsequent immune system dysfunction. In the initial phase of the disease, pathogenesis is predominantly governed by the virus's life cycle, which encompass its detection, fusion with host cells, entry, and replication, largely regulated by viral proteins. Conversely, in the advanced stages, an overwhelming inflammatory and immune reaction to SARS-CoV-2 precipitates significant tissue damage (PADASAS et al., 2023). Consequently, targeting both viral proteins and host cellular factors

is crucial for mitigating COVID-19 and represents a promising avenue for antiviral intervention. Current therapeutic strategies are categorized based on target specificity and underlying molecular actions. Antiviral medications aim to inhibit various phases of the viral infection cycle, whereas immunomodulatory therapies focus on tempering the host's inflammatory response that exacerbates disease severity (ZHOU et al., 2021a). To date, treatments for COVID-19 have been grouped into three main types: antiviral agents, biological agents, and anti-inflammatory agents. Insights into the structural biology of SARS-CoV-2, its mechanisms of infection, and clinical manifestations in patients have guided the deployment of several therapeutic modalities in clinical trials targeting COVID-19 (PANAHI et al., 2023).

During the early transmission phase of SARS-CoV-2, no antiviral medication had established efficacy against the disease. However, as clinical insights accumulated and research advanced, various antiviral agents emerged as prospective treatments for COVID-19. Numerous pharmaceuticals have been repurposed and evaluated for their potential to prevent the virus's entry into host cells, a critical initial step in the infection process. Among these drugs, Remdesivir has shown considerable promise. Initially conceived as a treatment for hepatitis C, Remdesivir was later repurposed to combat Ebola and Marburg virus diseases before being considered for COVID-19 therapy. Its mechanism involves inhibiting the viral RNA-dependent RNA polymerase, effectively halting viral replication through premature termination of RNA transcription(MARTINEZ, 2020). Chloroquine, an antimalarial medication, and hydroxychloroquine, a derivative used to treat autoimmune conditions such as systemic lupus erythematosus and rheumatoid arthritis, have also been explored for their antientry effects (VINCENT et al., 2005). Hydroxychloroquine is thought to disrupt viral fusion with host cell membranes by increasing endosomal pH. Additionally, it was hypothesized that chloroquine may impede SARS-CoV-2's attachment to cell membranes by inhibiting ACE2 receptor glycosylation. In vitro studies have hinted at an immunomodulatory role for both chloroquine and hydroxychloroquine. Their efficacy and safety in treating COVID-19 have undergone extensive clinical trial evaluation. Regrettably, hydroxychloroquine did not reduce 28-day mortality compared to standard care; instead, it prolonged hospital stays and heightened the risk of requiring invasive mechanical ventilation or resulting in death (HORBY et al., 2020). Therefore, current evidence suggests that hydroxychloroquine does not enhance clinical outcomes for hospitalized patients with mild-to-moderate COVID-19 and may be associated with more adverse events than standard care (CAVALCANTI et al., 2020). Ivermectin, known for its broad-spectrum antiviral and antimicrobial properties, was initially suggested as a potential inhibitor of SARS-CoV-2 based on in vitro studies (CALY et al., 2020). However,

subsequent clinical trials and meta-analyses have consistently demonstrated its ineffectiveness in treating COVID-19 (MARCOLINO et al., 2022). Notably, a significant phase 3 trial and a comprehensive meta-analysis in 2022 concluded that Ivermectin does not reduce mortality or the need for mechanical ventilation, thereby refuting earlier claims of its efficacy in any phase of COVID-19 (BRAMANTE et al., 2022). Notably, repurposing drugs that previously knowledge indicate that it might inhibit the entrance of virus into host cell or block the viral replication have not shown clinical effectiveness.

As no pharmaceutical drug emerged as a definitive treatment for COVID-19, the development and deployment of vaccines have proven to be a pivotal intervention in controlling and mitigating the pandemic's impact. The novelty of SARS-CoV-2 and the limited understanding of protective immune responses have cast uncertainty on vaccine development strategies. Nevertheless, it was crucial to utilize diverse platforms and strategies to develop vaccines, such as DNA, RNA, protein subunits, live attenuated viruses, inactivated viruses, virus-like particles, and non-replicating viral vectors has been crucial in advancing vaccine research (THANH LE et al., 2020). In summary, the vaccines developed were based on four different platforms, inactivated virus vaccines, nucleic acid-based vaccines (specifically mRNA), viral vector vaccines containing recombinant protein, and viral subunit-based vaccines.

It is important to notice that, prior to the emergence of COVID-19, the commercial viability of mRNA vaccines was hindered by concerns over their stability and the uncertainties associated with their formulation (O'CALLAGHAN; BLATZ; OFFIT, 2020). The successful delivery of messenger RNA into the cytoplasm is crucial for vaccine efficacy, leading to the adoption of various methods such as complexes of polymers, nano-emulsions with a positive charge, and lipid-based nanoparticles(KOWALSKI et al., 2019). DNA vaccines utilize plasmids from eukaryotic cells that encode the antigen protein; following uptake by host cells, transcription and translation of the antigen can elicit immune responses that confer protection(PORTER; RAVIPRAKASH, 2017). Viral vector-based vaccines employ modified non-pathogenic viruses like adenovirus to deliver and express target antigens, offering long-term stability and potent immune responses while being amenable to large-scale production(SHARPE et al., 2020). These vaccines combine the high immunogenicity of live attenuated vaccines with the safety profile of subunit vaccines. However, their effectiveness may be compromised if there is pre-existing immunity against the viral vector due to prior exposure to the target virus(URA; OKUDA; SHIMADA, 2014).

The rapid pace of COVID-19 vaccine development marked a historic milestone in immunization technology, with the first vaccine being authorized for use within a year of the disease's emergence (ZHOU et al., 2021b). The global response to SARS-CoV-2 saw an unprecedented variety of vaccines receiving emergency use authorization across different nations. To date, 11 vaccines have received licensing or approval globally, including viral vector-based, RNA-based, inactivated virus, and protein subunit vaccines. Notably, vaccines such as Pfizer/BioNTech, Oxford/AstraZeneca (ChAdOx1-S), Janssen/Ad26.COV 2.S; Moderna; Sinopharm; Sinovac-Coronavac; Bharat Biotech BBV152 COVAXIN; Covovax; and Nuvaxovid have been granted emergency use authorization by the WHO (WHO, 2024). While each country adopted a unique portfolio of vaccines, Russia approved Sputnik V, while China authorized Ad5-nCoV along with CoronaVac and two other inactivated vaccines. In United States, vaccines included BNT162b2 by Pfizer/BioNTech and mRNA-1273 by Moderna; Covishield in England; Covaxin in India (ZHOU et al., 2021b).

To the efficacy and safety of developing vaccines, the Pfizer/BioNTech and the Moderna vaccines were notable for their high efficacy rates of 95% (SKOWRONSKI; DE SERRES, 2021) and 94.1% (CHEMAITELLY et al., 2021), respectively, both utilizing messenger RNA technology. Sputnik V, based on adenovirus types 26 and 5, demonstrated an efficacy of 91.6% despite initial concerns over its rapid production process(LOGUNOV et al., 2021). The vaccine CoronaVac by Sinovac, using the CN02 strain of the virus, underwent phase III clinical trials in multiple countries, with varying efficacy results ranging from 91.25% in Turkey, 67% in Chile and 50.65% in Brazil(PALACIOS et al., 2020). In England, the viral vector-based vaccine Covishield (ChAdOx1 nCoV-19), developed by AstraZeneca and the University of Oxford, was approved to a phase III clinical trial in the UK, Brazil, and South Africa, showing an overall efficacy of 66.7%(VOYSEY et al., 2021). Notably, a dosing regimen involving a low dose followed by a standard dose achieved an efficacy of 80.7%, while two standard doses resulted in 63.1% efficacy. The vaccines before mentioned, typically necessitate a regimen of two or three doses, administered at intervals ranging from 14 to 21 days. In contrast, vaccines requiring only a single dose have been developed to offer greater ease of administration to the population. Janssen Pharmaceutical's Ad26.COV2.S vaccine, offered the convenience of a single-shot regimen with an efficacy of 72% and an overall efficacy of 66% in preventing moderate-to-severe COVID-19 28 days post-injection (SADOFF et al., 2021).

In Brazil, the immunization campaign against COVID-19 commenced on January 17, 2021. Initially, the biological immunizers available for vaccination were CoronaVac and AstraZeneca. Subsequently, vaccines from Janssen and Pfizer were introduced, with Pfizer's

vaccine accounting for the majority of doses distributed across the country (BERNARDEAU-SERRA et al., 2021). The vaccination rollout was structured to prioritize more vulnerable groups within the population. Therefore, elderly individuals, those with comorbidities, and healthcare professionals were among the first to receive vaccinations. In certain federal units, other groups such as indigenous people, residents of quilombo communities, homeless individuals, security personnel, educators, and public transport workers were also classified as priority groups for immunization. Overall, vaccination coverage in Brazil against COVID-19 is higher in the wealthier regions of South and Southeast Brazil. Disparities exist as men, people of color, and those from lower-income backgrounds are more likely to have incomplete vaccination due to missed or delayed second doses. The early initiation of vaccination for individuals under 50 may have impacted the uptake among older populations. Higher vaccination rates correlate with reduced mortality risk, particularly in the elderly, while areas with greater vaccination coverage see increased hospitalization rates, likely due to better access to healthcare and reporting systems. (LI et al., 2024). In the city of Rio de Janeiro, epidemiological observatory data indicate that 91% of the population has received at least two vaccine doses and 62% have received a booster dose. Adherence to both the first and second booster doses is primarily concentrated among older age groups (over 60 years) (PAINEL RIO COVID-19, 2023).

Despite not being included in the initial clinical trials, various authors report the safety of mRNA vaccines in pregnant and breastfeeding women (SHOOK et al., 2022), with a reported lower occurrence of adverse neonatal outcomes and a fivefold lower risk of developing COVID-19 compared to unvaccinated individuals (ROTTENSTREICH et al., 2022). Joubert et al. highlight that 36 pregnant women were inadvertently enrolled in clinical trials, 17 of whom received an mRNA vaccine. None of these vaccinated pregnant women suffered from adverse events, while 2 out of 19 in the placebo group experienced gestational loss. Although limited, these data suggest that vaccination during pregnancy was not associated with an increased risk of spontaneous abortion (JOUBERT; KEKEH; AMIN, 2022). Additionally, in a prospective cohort study with 24 pregnant women and healthcare workers (14 lactating and 10 non-lactating women) enrolled at the time of COVID-19 vaccination, reported no significant difference in the incidence of adverse effects from vaccination, according to surveillance program data from V-safe (a vaccine safety monitoring system from United States) after vaccination health checker, after pregnancy registry, and vaccine adverse event reporting system (CHAREPE et al., 2021).

In general, pregnant and breastfeeding women vaccinated with mRNA formulations exhibited a robust response with the production of high levels of serum antibodies, predominantly IgG. Maternal antibodies are transferred through the highly selective IgG placenta to confer passive immunity to the fetus, and this transfer increases throughout the second and third trimesters, peaking in the last four weeks of intrauterine life (ATYEO et al., 2021). A study by Gray et al. on pregnant and breastfeeding women revealed that antibody titers produced in response to vaccination were significantly higher than those produced by natural infection (GRAY et al., 2021). After the first inoculation, both pregnant and breastfeeding women demonstrated a similar response but lower than non-pregnant women. Upon administration of the second dose, IgG production in breastfeeding women increased substantially, equating to that of non-pregnant women. Pregnant women also showed an increase in antibody production; however, it was lower than breastfeeding and non-pregnant women, and with difficulty in producing functional antibodies (FALSAPERLA et al., 2021). The transfer of immunity to the newborn was observed, being greater the longer the time elapsed from vaccination to delivery and the more doses administered(KASHANI-LIGUMSKY et al., 2021). In addition to the placental route, transmission of IgG and IgA antibodies through breast milk was also observed, with a positive correlation between longer breastfeeding duration and IgG concentrations (SHOOK et al., 2021).

1.2 COVID-19 During Pregnancy

1.2.1 Epidemiology Background

The first cases of pregnant women infected with SARS-CoV-2 were reported in March 2020 and indicated that the clinical manifestations were in general similar to the general population: 86% mild infection, 9.3% severe pneumonia, and 4.7% ARDS (BRESLIN et al., 2020). In May 2020, the United Kingdom Obstetrics Surveillance System published a prospective national cohort of pregnant women admitted with COVID-19, identifying a percentage of severe illness, requiring Intensive Care Unit (ICU) admission and resulting in death, that was comparable to that of the general female population of reproductive age (KNIGHT et al., 2020). The frequency of COVID-19 infection is consistent throughout all gestation. However, symptomatic cases requiring hospital admission are concentrated in the third trimester, around 34 weeks (29-38 weeks) (CROVETTO et al., 2020; KNIGHT et al., 2020). Still during the early onset of pandemics, in August 2020, one of the first systematic

reviews and meta-analysis, utilizing 435 studies December 2019 to July 2020, showed that pregnant women with COVID-19, when compared to pregnant women without COVID-19, were more likely to be admitted in the ICU (odds ratio 3.71), to require mechanical ventilation (odds ratio 2.71), and to present all cause morality (odds ratio 6.09). The same study also indicated that the odds of stillbirth increased from 1.87 to 2.37, and admission to the neonatal ICU were from 2.18 to 3.26, when comparing newborns from COVID-19 versus healthy pregnancies (ALLOTEY et al., 2020). Some studies suggested an increased susceptibility to be infected during pregnancy. In a study comparing the results of universal screening in patients scheduled for surgery and pregnant women in labor, the cases of asymptomatic infection were fifteen times higher in obstetric patients, even after adjusting for age, race, and sex (KELLY et al., 2021).

Findings from the previously mentioned studies reinforced the statement that pregnant women are considered to be a high risk group for SARS-CoV-2 infection. In 2021, pregnancy was listed by OMS as one of the underlying medical conditions that increased the risk of severe illness from COVID-19, when compared with non-pregnant women with a similar age. COVID-19 during pregnancy has also been associated with an increased likelihood of preterm birth (OMS, 2022). The most recent scientific literature identified the primary impacts of the COVID-19 pandemic during pregnancy as follows: an increased risk of severe clinical progression, extended hospitalization periods, and maternal mortality; poor maternal and/or fetal perfusion; thromboembolic disease; hypertensive disorder; elevated rates of cesarean section and preterm births; and the development of depression, anxiety, as well as other emotional issues during and after pregnancy (SMITH et al., 2023). Additionally, although COVID-19 is a recent disease which explains the lack of long-term consequences of SARS-CoV-2 infection during pregnancy, it is know that infections during pregnancy, in particular viral infections, are amongst the environmental factors that can compromise fetal development with an impact on life-long health, both the mother and the offspring (BURTON; FOWDEN; THORNBURG, 2016; GLUCKMAN et al., 2008; KOURTIS; READ; JAMIESON, 2014)

The clinical management of pregnant women with COVID-19 is complicated by the fact that it is not possible to maintain them in a supine position, and the tolerable limit for a decrease in oxygen saturation is lower in these individuals than in non-pregnant individuals, to prevent potential effects of hypoxia to the fetus. The markers of severe COVID-19 illness, in this specific population, are oxygen saturation less than 94% at sea level, (PaO2/FiO2) <300 mm Hg, a respiratory rate faster than 30 breaths/min, or lung infiltrates area bigger than 50%. Critical illness describes patients with respiratory failure, septic shock, or multi-organ

dysfunction (NHI US, 2021).While the risk factors associated with the more severe manifestations of the disease and death are the same in pregnant women as in the general population. Advanced age (≥ 35 years), obesity (Body Mass Index [BMI]>30 kg/m2), arterial hypertension, diabetes mellitus, chronic pulmonary disease, cardiovascular disease, chronic kidney disease are linked to poorer maternal outcomes (GALANG et al., 2021).

The prevalence of SARS-CoV-2 viral infection in the obstetric population is challenging to estimate, as it varies across geographical areas, testing period during the pandemic phases, and different testing methodology strategies, which makes it difficult to obtain a global perspective. Allotey et al. described in a meta-analysis containing 192 cohort studies, that the COVID-19 prevalence in pregnant women during the first wave (between December 2019 and June 2020) was 7% (4-10%) with universal screening, and 18% (10-28%) when based on symptomatology. Approximately three-quarters of the pregnant women were asymptomatic (ALLOTEY et al., 2020). In a comprehensive retrospective cohort analysis conducted by Son et al., encompassing 108,067 pregnant individuals throughout the United States from March to December 2020, it was found that 6.9% of the participants were diagnosed with SARS-CoV-2 infection during their pregnancy (SON et al., 2021).A seroprevalence study conducted in Spain, employing a universal screening strategy for all pregnant women attending first-trimester ultrasound or admitted for delivery between April and May 2020, determined a 14% infection prevalence. Of these, 60% of the pregnant women were asymptomatic, with a variation from 70% in the first trimester to 52% in the third trimester (CROVETTO et al., 2020). Concerning the development of severity, an observational cohort study of pregnant women from the United States from May 2020 until September 2021, pointed that between 5.4% to 12% of pregnant women with SARS-CoV-2 infection developed critical illness (METZ et al., 2021).

In Brazil, in the last report by May 2022, the Brazilian Obstetric Observatory estimates at least 24,000 cases of SARS related to COVID-19 among pregnant and postpartum women, while between the years 2020 and 2021, the maternal mortality ratio increased about five times when compared to 2019. The fatality rate of SARS-CoV-2 among pregnant women in Brazil stands at about 7.2%, markedly higher than the 2.6% average in the broader population (OBSERVATÓRIO OBSTÉTRICO BRASILEIRO, 2022). The same study also revealed that in 2020, there was a 40% increase in maternal deaths compared to previous years. Concerning severe cases, Guimarães et al. published a study utilizing Brazilian nationwide analyses based on data from the Mortality Information System (SIM) for general and maternal deaths and the Influenza Epidemiological Surveillance System (SIVEP-Influenza) for estimates of female and maternal deaths due to COVID-19 and concluded that 59.8% of maternal deaths occurred

during pregnancy compared to postpartum deaths. Even when accounting for the expected rise in overall mortality due to the COVID-19 pandemic, there was a 14% of maternal mortality that exceeded the expected number (GUIMARÃES et al., 2023). The ongoing prevalence of SARS-CoV-2 within the population, coupled with the advent of novel viral variants and the progression of vaccination efforts, persistently impacts the frequency of the disease and its potential ramifications in pregnant women.

1.2.2 Viral Infection During Pregnancy

Infection during pregnancy, particularly viral infection, is among the stressors that can compromise fetal development with long-term health impacts on both the offspring and the mother (GOLDSTEIN; NORRIS; ARONOFF, 2017; KOURTIS; READ; JAMIESON, 2014). Pregnancy is accompanied by a series of physiological changes that may predispose expectant mothers to complications arising from infections, potentially leading to increased maternal and neonatal morbidity and mortality (BRABIN, 1985). These include cardiovascular system alterations, such as an increase in cardiac output due to a higher stroke volume in the first half of pregnancy and a rise in maternal heart rate in the latter half. These changes ensure adequate uterine vascularization and fetal nourishment. Furthermore, the hematological system contributes to the success of pregnancy by maximizing the maternal oxygen transport capacity, achieved through increased plasma volume, erythrocytes, and coagulation factors. However, due to the heightened production of fibrinogen, fibrin products, and coagulation factors VII to X (and a lower concentration of coagulation inhibitors), pregnancy is considered a hypercoagulable state, with an elevated risk of thromboembolism. Concerns also extend to energy metabolism; from a metabolic standpoint, pregnancy induces a state akin to diabetes, characterized by hyperinsulinemia and hyperglycemia. This is attributed to the action of human chorionic somatomammotropin (hPL), which inhibits glucose metabolism in the maternal tissues, ensuring greater availability for the placenta and transfer to the fetus (JAFARI et al., 2021). This metabolic traits also acts as a risk factor, as a higher prevalence of severe outcomes in SARS-CoV-2 has been described in diabetic subjects (BLOOMGARDEN, 2020).

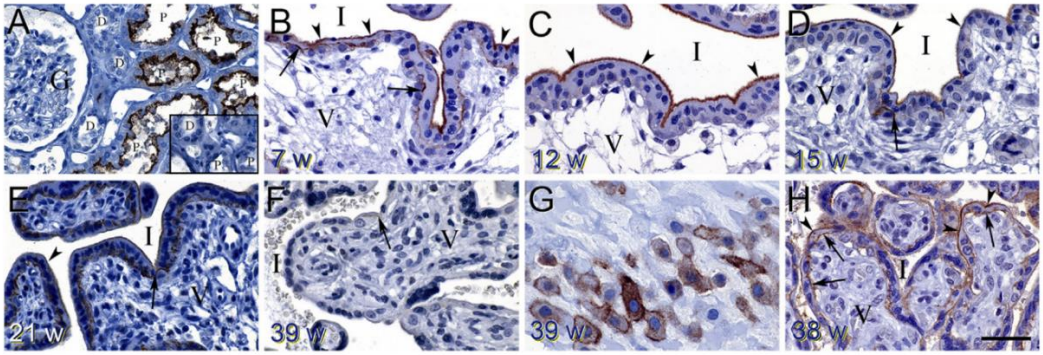
The symptomatology of the SARS-CoV-2 in pregnant women are similar to that of the general population, with the most common symptoms being fever (40%), cough (39%), myalgia (10%), anosmia and ageusia (15%), and diarrhea (7%). When compared to a similar non-pregnant population, pregnant women exhibit less fever and myalgia (ALLOTEY et al., 2020). Existing evidence on other infections such as the influenza A H1N1 virus, SARS, and MERS

suggests that pregnant women are at higher risk of developing more severe forms of the disease than the general population (BABARINSA; OKUNOYE; ODUKOYA, 2021; MOSBY; RASMUSSEN; JAMIESON, 2011). Concerning the different reactions to new variants of SARS-CoV-2, it was reported that infected pregnant women tended to present asymptomatically with pre-Delta and Omicron variants, and symptomatically with Delta variant. While there were fewer cases of severe-critical disease (1.8% Omicron vs 13.3% pre-Delta and 24.1% Delta) and adverse perinatal outcomes during the Omicron wave compared with the pre-Delta and Delta waves (SEASELY et al., 2022).

1.2.3 Vertical Transmission and Maternal-Fetal Responses to COVID-19

The concern about vertical transmission comes from the information that, in addition to the aforementioned lungs, heart, gastrointestinal tract, kidneys, liver, and vascular endothelium, tissue tropism of SARS-CoV-2 extends to the placenta (LAMERS; HAAGMANS, 2022). For vertical transmission to occur the pathogen must cross the placenta, the critical maternal-fetal interface. SARS-CoV-2 exhibits tissue tropism that includes the placenta, where ACE2 expression is noted in syncytiotrophoblast, cytotrophoblasts, endothelium, and vascular smooth muscle of both primary and secondary villi (VALDÉS et al., 2006). It is hypothesized that ACE2 modulates vascular dynamics through the renin-angiotensin system within the chorionic and extravillous trophoblasts, facilitating trophoblast invasion (ANTON et al., 2008). The functional parallelism of the ACE2 and TMPRSS2 in the placenta, similarly to its role in pulmonary tissue, remains to be elucidated (PIQUE-REGI et al., 2020). Notably, several studies pointed that ACE2 protein expression peaks during the first trimester and diminishes as pregnancy progresses, reaching its lowest in the final stages of gestation (FIGURE 4). Thus, the data reflect a momentary infectious state and cannot be extrapolated to a consistent vulnerability profile of the placenta to SARS-CoV-2 throughout gestation, suggesting an increased susceptibility to infection in the early stages of pregnancy (BLOISE et al., 2021; LU-CULLIGAN et al., 2021).

Figure 4: ACE2 protein expression in the placenta varies with gestational age. (A) Human kidney used as a positive control revealed strong apical staining of the proximal tubules (P). The distal tubules (D) and glomerulus (G) were negative. The inset shows a serial section of the same kidney stained with non-immune rabbit serum, resulting in no staining; (B–D) Placentas derived from normal pregnancies between 7 and 15 weeks of gestation demonstrated strong, uniform, apical microvillus syncytiotrophoblast staining (arrowheads) and patchy strong basolateral staining at the cytotrophoblast-syncytiotrophoblast contact zone (arrows). V, villous core; (E) A normal 21-week placenta still exhibited syncytiotrophoblast surface staining (arrowhead) but to a lesser extent than the earlier samples. Cytotrophoblast-syncytiotrophoblast contact zone staining was still prominent (arrow); (F) A representative normal placenta at 39 weeks revealed almost no ACE2 staining. Occasionally, staining at the cytotrophoblast-syncytiotrophoblast contact zone was noted (arrow); (G) Normal extravillous invasive trophoblasts from a 39-week placenta demonstrated strong surface expression of ACE2 with variable cytoplasmic staining; (H) Representative image of ACE2 expression in a 38-week placenta derived from an individual with symptomatic maternal COVID-19. (A–H) Scale bars represent 50 mM



Source: Reproduction from LU-CULLIGAN et al., 2021

Vertical transmission is shown to be rare, estimated to be about 2-3% (KOTLYAR et al., 2021), and COVID-19 pregnancies do not seem to affect the rates of miscarriage and congenital anomalies (SUKHIKH et al., 2021). The few studies that report documented cases of feto-placental infection with and without fetal sequelae have been criticized about wide range of accuracy, lack of reproducibility, and varying methods of virus detection that create doubt about cross-contamination between maternal blood, placental tissue, and the neonate (PATANÈ et al., 2020; VIVANTI et al., 2020). Usually published data support the hypothesis that in utero SARS-CoV-2 vertical transmission, while low, is possible, and usually dependent on the strong maternal inflammatory response (FENIZIA et al., 2020). A 2021 systematic review of the literature on early RNA detection of SARS-CoV-2 postpartum posits that vertical transmission, while feasible, is infrequent and predominantly associated with infections in the third trimester. Notably, the lack of data from the first trimester hinders a comprehensive evaluation of vertical transmission rates during early gestation and the potential implications for fetal morbidity and mortality (KOTLYAR et al., 2021).

Breastfeeding was once one of the proposed routes of mother-to-child transmission of SARS-CoV-2. Despite some evidence of the presence of SARS-CoV-2 in colostrum, the overwhelming majority of documented breast milk samples in the literature do not contain viral

RNA particles (HERNÁNDEZ-CARAVACA et al., 2023). Breast milk contains a multitude of nutrients, hormones, digestive enzymes, and immune cells such as macrophages and antibodies. Breastfeeding has been proven to be a protective factor against various infectious, cardiovascular, gastrointestinal, behavioral, neoplastic, and atopic pathologies, and it also has a positive impact on neurodevelopment and reduces the risk of sudden infant death (ALOTIBY, 2023). The passive immunity that is obtained by the neonate with the transfer of immunoglobulins (Ig) from breast milk may also act as a protective factor against COVID-19. A cross-sectional study was developed with 165 participants with COVID-19 infection during pregnancy and their newborns reported that colostrum presenting anti-SARS-CoV-2 IgA was found in 117 (70.9%) women. The presence of anti-SARS-CoV-2 IgA in colostrum was independently associated with fewer clinical symptoms in their newborns. SARS-CoV-2 seems to spare human breast milk, and horizontal transmission from mother to neonate might occur through respiratory droplets rather than through breast milk (DUTRA et al., 2023)

Irrespective of vertical transmission and placental infection, SARS-CoV-2 infection during pregnancy elicits an immune response that affects both the maternal-fetal interface and systemic health. The balance of this immune reaction is crucial; while it can combat the infection, an excessive response may induce pregnancy complications, affecting maternal and fetal health (NADEAU-VALLÉE et al., 2016). A 2021 study utilizing bulk and single-cell transcriptomics analysis revealed that in most cases, placental cells were not infected by SARS-CoV-2, and no significant difference in S protein IgG and IgM antibodies was observed in the plasma of infected women. Nonetheless, large-scale RNA sequencing of placenta villi and assessment of differentially expressed genes revealed upregulated pathogen-response pathways and downregulated physiological pathways, crucial for the success of pregnancy (LU-CULLIGAN et al., 2021). This suggests that even without direct infection, the placenta immune gene expression is significantly altered, particularly IFN-regulated genes, underscoring the profound influence of maternal infection on placental function. The same study highlighted distinctive interactions between NK cells and T cells within the placenta of infected women, a phenomenon absent in uninfected individuals. It was inferred that the full-term placenta may present an inflammatory response even in the absence of an active local infection, particularly in cases involving maternal upper respiratory tract infections.

The potential adverse effects of maternal SARS-CoV-2-induced inflammation on offspring are of concern, particularly in asymptomatic infection. A 2022 study that aimed to characterize the maternal-fetal immune responses triggered by SARS-CoV-2 during pregnancy, stated that this infection is linked to inflammatory reactions both in maternal circulation and at

the maternal-fetal interface (GARCIA-FLORES et al., 2022). The authors reported elevated IgM and IgG levels in maternal blood, with only IgG present in neonatal cord blood, implying that direct fetal infection is unlikely. The increased levels of IgG in the cord blood are explained by the fact that this immunoglobulin crosses the placenta via the neonatal Fc receptor, which is highly expressed in the syncytiotrophoblast layer (LEACH et al., 1996), which by contrast is not the same to IgM due to large molecular weight (HAIDER, 1972). Additionally, infected mothers and their neonates exhibit higher pro-inflammatory cytokine levels. In particular, infected women showed increased systemic levels of IL-8 (5.9-fold change), IL-10 (2.3-fold change), and IL-15 (1.5-fold change), while neonates born from women infected with SARS-CoV-2 had increased concentrations of IL-8 (2 fold change) compared to those born from control mothers. Correlation analyses between maternal blood and placental single-cell RNA sequencing data suggest that infection alters the transcriptome of both the mother and the neonate, with some changes reflected in placental tissues. These findings indicate that while SARS-CoV-2 does not elicit fetal hematopoietic immune responses in the placenta, it does influence the neonatal immune system (GARCIA-FLORES et al., 2022). Taken together, these data indicate that SARS-CoV-2 infection not only causes a maternal cytokine response but also induces neonatal inflammation, which can lead to long-term morbidities.

1.2.4 Obstetric and Neonatal Outcomes

One of the first systematic review published found that COVID-19 may be accompanied by some obstetrics outcomes such as decreased fetal movement, intrauterine fetal distress, developed anemia, and preterm labor (BANAEI et al., 2020). Later on, during the COVID-19 pandemic, several authors observed a potential increased association between SARS-CoV-2 infection during pregnancy and an elevated relative risk of pre-eclampsia (NASCIMENTO et al., 2023). Pre-eclampsia is a complex syndrome characterized by the onset of hypertension, systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg measured on two separate occasions at least 4 hours apart, after 20 weeks of gestation in a previously normotensive woman, associated with proteinuria, hematological issues such as thrombocytopenia, organ dysfunction as renal insufficiency, hepatic function impairment, pulmonary edema, and/or neurological symptoms (ACOG, 2020). Although traditionally defined by hypertension and proteinuria, recent understandings have highlighted the need for more precise diagnostic criteria, potentially incorporating biochemical markers like angiogenic or anti-angiogenic factors. A most precise pathogenesis of pre-eclampsia remains largely

unknown, but, it is widely believed that multiple factors, involving genetic, environmental as well as other complicated facets, may contribute (BURTON et al., 2019).

Observational studies that examined the relationship between obstetric and neonatal outcomes and the timing of infection concluded the odds of developing pre-eclampsia were significantly higher among pregnant women with SARS-CoV-2 infection than among those without SARS-CoV-2 infection (7.0% vs 4.8%) (CONDE-AGUDELO; ROMERO, 2022). A meta-analysis that combined data from 10 studies of preeclampsia in pregnant women infected with SARS-CoV-2 estimated a prevalence of 8.2% (KARIMI-ZARCHI et al., 2021). In Brazil, a meta-analysis that included 10 studies and 2988 women estimated the prevalence of pre-eclampsia in 6.7% of pregnancies (GUIDA et al., 2022). The overall frequency of pre-eclampsia in Brazil was similar to that reported worldwide (SAY et al., 2014).

The condition affects both the mother and fetus, as pre-eclampsia is part of a spectrum of pregnancy complications linked to disordered placentation, which includes late spontaneous miscarriage, abruptio placentae, fetal growth restriction, pre-term rupture of membranes, and premature delivery. Currently, there are no treatments that reverse the underlying pathophysiology of pre-eclampsia, and prolonged pregnancy can increase the risk of fetal death due to uterine artery obstruction (BURTON et al., 2019). Pre-eclampsia is one of the leading causes of maternal morbidity and mortality, as well as the primary cause of iatrogenic prematurity (FOX et al., 2019).

The international INTERCOVID study, which included data from 18 countries with varying socioeconomic levels, demonstrated a significantly higher risk of severe neonatal complications in pregnant women with COVID-19 (17% vs. 7.9% in uninfected pregnant women). This disparity may also be explained by the global inequality in access to healthcare, reinforcing the notion that these complications could be partially prevented if pregnant women had access to appropriate obstetric care (VILLAR et al., 2021).

The challenge of accurately determining global maternal mortality rates among pregnant women with COVID-19 is due, at least in part, to pre-existing disparities in maternal healthcare. Before the COVID-19 pandemic, lower-income countries already experienced significantly higher rates of maternal mortality and morbidity compared to wealthier nations (KHAFAIE; RAHIM, 2020). While studies have identified geographical disparities in SARS-CoV-2 infection rates and related health outcomes in the general population — attributed to factors such as population density, median age, and urbanization — there remains a dearth of high-quality evidence regarding the global variation and extent of these differences among pregnant women (MILLER; BHATTACHARYYA; MILLER, 2020). The limited data available, often

derived from small cohorts or case reports, fail to provide a comprehensive understanding of the impact of SARS-CoV-2 infection on maternal and neonatal health (DUBEY et al., 2021). In a meta-analysis including 117 studies with a total of 11758 pregnant women, Karimi et al reported that maternal mortality was 1.3%, of which mortality rate from high income countries including the United Kingdom, United States, Italy, Switzerland, France, Sweden, Portugal, Netherlands, Ireland, Spain, Canada, and Australia were 0.19%, while women in middle-income countries including China, Iran, Iraq, Jordan, Peru, Turkey, India, Venezuela, Thailand, Brazil, and Honduras has a mortality rate of 8.51% (KARIMI et al., 2021). Unfortunately, the same study pointed that the highest mortality rate was reported in Brazil, in a study using the Brazilian Ministry of Health's ARDS Surveillance System, reporting a pregnancy death rate of 12.7% (TAKEMOTO et al., 2020).

Four years into the COVID-19 pandemic, longitudinal data on the offspring of COVID-19 pregnancies are just emerging. Epidemiological research consistently links maternal infections during pregnancy, including those caused by other viruses like influenza, to a spectrum of adverse neurodevelopmental outcomes in children (AL-HADDAD et al., 2019). These adverse outcomes include autism spectrum disorders, schizophrenia, cerebral palsy, cognitive impairments, bipolar disorder, and mood disorders such as anxiety and depression, and are related to all kinds of viral infections during pregnancy (ADAMS WALDORF; MCADAMS, 2013; AL-HADDAD et al., 2019). The significant immune response observed in SARS-CoV-2 infected individuals indicates that maternal and placental inflammation (SHOOK et al., 2022), and the shift in cytokine profiles induced by infection during critical periods of neurological development (URAKUBO et al., 2001), may harm maturation of the fetal brain.

Neonates from mothers infected with SARS-CoV-2 exhibit disrupted immune functions, notably neutrophil activation, a phenomenon also observed in pediatric COVID-19 cases (SEERY et al., 2021). This response could be linked to increased IL-8 levels in the cord blood, which is known to stimulate neutrophils, indicating a subtle neutrophilic reaction in these newborns (NÉMETH; SPERANDIO; MÓCSAI, 2020). The significance of even a mild immune response cannot be underestimated, as viral infections during gestation, such as influenza and Zika virus, have been connected to negative long-term effects (MWANIKI et al., 2012). In particular, heightened IL-8 levels in neonates have been implicated in the onset of neurological disorders. This underscores the importance of monitoring and understanding the implications of maternal infections on neonatal health (BARTHA et al., 2004). This hypothesis is also supported by data from animal models, that stablished that it is the maternal immune response and increased inflammation, not a specific pathogen, which is a risk factor for

neurodevelopmental disorders (HAGBERG; GRESSENS; MALLARD, 2012). Those studies illustrate that maternal immune activation can alter the normal trajectory of brain development, leading to disorders that have developmental origins including autism and schizophrenia (PLOEGER et al., 2010; RAPOPORT; GIEDD; GOGTAY, 2012).

One of the first retrospective cohort study, that aimed to evaluate whether in utero exposure to SARS-CoV-2 is associated with risk for neurodevelopmental disorders in the first 12 months after birth, found that preterm delivery was more likely among 14.4% of infected mothers versus 8.7% from uninfected, and utilizing the International Statistical Classification of Diseases and Related Health Problems, Tenth Revision (ICD-10), maternal SARS-CoV-2 positivity during pregnancy was associated with greater rate of neurodevelopmental diagnoses in unadjusted models (odds ratio 2.17) as well as those adjusted for race, ethnicity, insurance status, offspring sex, maternal age, and preterm status (odds ratio 1.86). The author also emphasizes that, particularly due to neurodevelopmental effects, some of these disorders may not manifest until adolescence or adulthood, and the true risks of maternal immune activation may not become apparent for decades (EDLOW et al., 2022).

Nonetheless, several studies have described an increased incidence of prematurity, low birth weight, and admission to neonatal ICU, with the latter being more prevalent in premature neonates (GALANG et al., 2021; LOKKEN et al., 2021; NORMAN et al., 2021). It has also been established that neonatal outcomes are correlated with maternal health, with adverse outcomes more likely in symptomatic mothers, particularly in cases of severe or critical illness (KIM, 2021). It is important to note that some authors highlight that the higher occurrence of prematurity may be a confounding factor, as many cases were induced deliveries due to maternal health deterioration, rather than a direct effect of infection (NORMAN et al., 2021).

1.3 Metabolomics

1.3.1 Metabolomic Based Studies

Metabolomics provides knowledge concerning small molecules, usually those with a molecular weight of less than 1,500 Da, linked to human metabolism. This field has gained prominence within systems biology, offering a new way to explore metabolic dynamics in different physiological and pathological situations. Unlike DNA, RNA, or proteins, metabolites

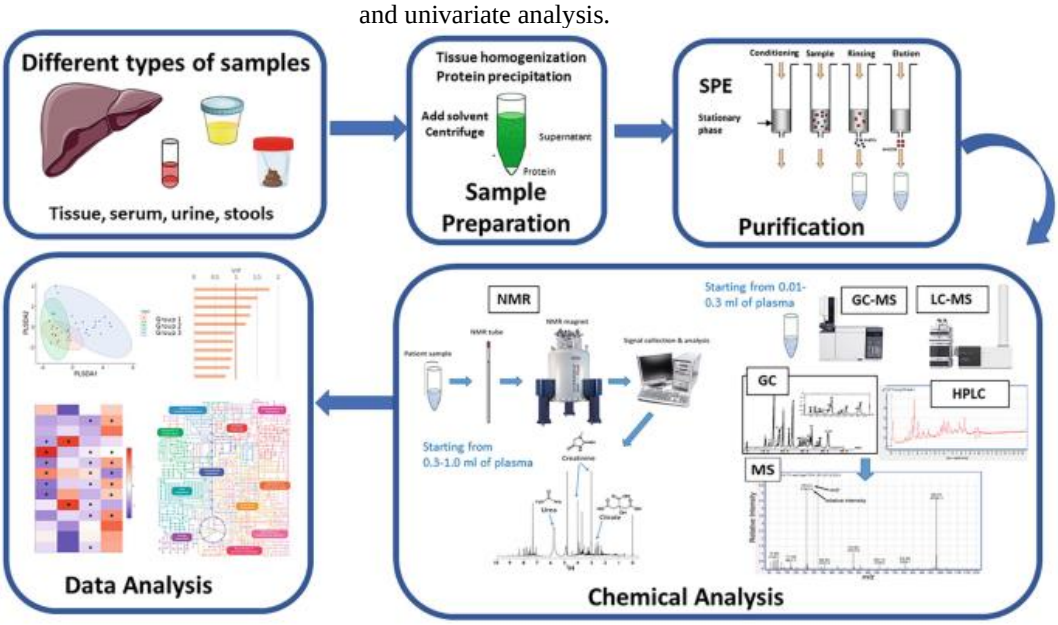
provide real-time snapshots of biochemical activity, directly reflecting the underlying physiological state of cells and tissues (DS, 2005).

Blood, as an integral component of the circulatory system, is intimately connected with all organs and tissues in the human body. In the pathological state, changes in metabolites in the bloodstream may reflect changes in the diseased tissues. Additionally, blood samples are easily obtainable and less invasive than tissue samples, making them ideal for metabolomics-based research.

The human serum metabolome, which encompasses all metabolites present in serum, is increasingly used to identify disease biomarkers (PSYCHOGIOS et al., 2011). These metabolites, being the end products of various metabolic pathways, closely link genotype and phenotype, making them valuable indicators of disease (HOLMES; WILSON; NICHOLSON, 2008). Furthermore, measuring the entire serum metabolome rather than individual metabolites simplifies the biomarker discovery process and enhances the reliability of results (HOLMES et al., 2008). Comparative metabolomics studies reveal potential diagnostic and prognostic biomarkers, inform therapeutic strategies, and unveil the impact of external chemical exposures (WANG et al., 2022). These investigations shed light on altered metabolic pathways in diseases, assess treatment efficacy, predict drug responses, and even stratify the risk of multiple common ailments simultaneously (T et al., 2022).

The two most successful analytical platforms used in metabolomics are mass spectrometry (MS, including liquid chromatography-mass spectroscopy [LC-MS] and gas chromatography-mass spectroscopy [GC-MS]) and nuclear magnetic resonance spectrometry (NMR). While MS and NMR are pivotal techniques in metabolomics, each one presents distinct advantages and limitations, such as different systematic workflow, that may encompass sample collection, viral inactivation, metabolite extraction, statistical analysis, identification, and data interpretation (GOWDA; RAFTERY, 2023).

Figure 5: Schematic overview of typical workflow metabolomic investigation. The usual metabolomics workflow is divided into: (a) pre-analytical including sample collection and sample preparation, (b) chemical analysis and data generation which involves spectra acquisition, spectral processing, and the generation of spectral bins and/or metabolite concentrations. (c) Data analysis was performed for both data types multivariate and univariate analysis.



Source: Reproduction of FENIZIA; SCODITTI; GASTALDELLI, 2023

MS is renowned for its exceptional sensitivity, allowing for the detection and analysis of a significantly greater number of metabolites compared to NMR in a smaller amount of sample. This high sensitivity is largely facilitated by the widespread use of LC in approximately 80% of MS studies, which separates metabolites prior to detection (EDISON et al., 2021). Despite this, LC-MS faces considerable challenges, such as ion suppression and adduct formation, leading to variations in peak intensities and increased spectral complexity. Conversely, NMR, while less sensitive and possessing limited spectral resolution for complex biological samples, offers unique and advantageous features. Unlike MS, NMR typically does not employ chromatography, as it would reduce sensitivity and increase variability. NMR is lauded for its high reproducibility and quantitative accuracy, enabling absolute quantitation of metabolites either with a single internal standard or without any. It serves as the gold standard for unambiguous identification of unknown metabolites and allows for minimal sample preparation, preserving the sample for repeated measurements or other analyses due to its non-destructive nature. Additionally, the ability of NMR to differentiate between various isotopes makes it valuable for tracing and quantifying metabolite fluxes across multiple pathways from a single measurement. The technique also supports the detection of the same metabolites through different atomic nuclei, such as ^{1}H, ^{13}C, ^{31}P, or ^{15}N, providing diverse options for metabolite analysis and metabolic pathway exploration. These distinct characteristics and

capabilities of NMR, including its reproducibility, quantitative nature, minimal sample processing requirements, non-destructiveness, and versatility in isotope detection, significantly outweigh its limitations in sensitivity and resolution, presenting a robust complement to the high sensitivity of MS in the field of metabolomics (GOWDA; RAFTERY, 2023; WISHART, 2019).

1.3.2 Application of Metabolomics in Human Disease

For research purposes, understanding specific metabolic changes during disease progression is crucial, as blood-based metabolite markers can enhance disease diagnosis. By tracking changes in metabolites and correlating them to specific pathways, researchers can integrate metabolomics, proteomics, and genomics to gain mechanistic insights into disease pathogenesis. Risk stratification is essential for disease prevention, and over the past decade, increasingly complex phenotypic information has become available, extending beyond traditional demographic and laboratory data (LIN et al., 2018). Established clinical predictors such as blood cholesterol levels are well-known indicators of cardiovascular disease risk (GOFF et al., 2014), yet numerous other blood metabolites have been associated with common disease phenotypes. Recent studies have advanced beyond examining individual markers, linking comprehensive metabolomic profiles to aging (AHADI et al., 2020), disease onset, and mortality, thereby recognizing the human blood metabolome as a direct representation of physiological states (SCHÜSSLER-FIORENZA ROSE et al., 2019). Furthermore, identifying metabolites that significantly alter in the early stages of certain diseases can serve as predictive indicators, potentially allowing for early risk assessment and intervention (LI et al., 2015). The onset and development of many diseases can be tracked to disturbances in specific metabolites, such as glucose (WÜRTZ et al., 2012) and lipid (HOLMES et al., 2018) to diabetes, and protein metabolism to Alzheimer's disease (TYNKKYNEN et al., 2018).

Importantly, in recent years, assays such as ^{1}H-NMR metabolomics have become more frequent in epidemiology studies. This method evolved to allow the assessment of representative screening changes in metabolites within patient samples, in a robust and low-cost way (SOININEN et al., 2015). The most up-to-date reviews report that predictive information of patients metabolic profiles matched established clinical variables, such as type 2 diabetes, dementia, and cardiovascular diseases, while specific metabolites related to amino acids metabolism and creatinine have been linked to all-cause and disease specific mortality (T et al., 2022). In 2016, Louis et al., aiming to create a discriminating model based on ^{1}H-NMR

metabolic profiles of plasma between 233 patients with lung cancer and 226 controls, found that higher levels of glucose and lower levels of lactate and phospholipid were found in lung cancer patients. The same study stated that the model makes it possible to correctly classify 78% of patients with lung cancer and 92% of controls (LOUIS et al., 2016). A study using ^{1}H-NMR analyzed serum from patients with chronic obstructive pulmonary disease, a prevalent chronic condition characterized by airflow obstruction, discovering lower levels of phenylalanine, tyrosine, alanine, valine, leucine, isoleucine, and high-density lipoprotein, along with higher levels of glycerophosphocholine, compared to healthy controls (WANG et al., 2013). These findings suggest that NMR-based metabolomic profiling holds significant potential for clinical use, both as an alternative to extensive laboratory tests and as an additional source of detailed information to enhance risk assessments for various diseases concurrently.

1.3.3 Metabolomics in COVID-19 Patients

The multisystemic nature of SARS-CoV-2 infection necessitates an integrated approach to biological systems to understand the molecular mechanisms associated with COVID-19 pathogenesis. In this context, metabolomics, serving as a central omics field in the translation of information (COSTA DOS SANTOS; RENOVATO-MARTINS; DE BRITO, 2021), provides the metabolic signature of organs and biological fluids under different conditions, making it an excellent tool for elucidating metabolic pathways associated with the infectious process. Host energy and lipid metabolism alterations, as well as metabolites related to the immune system, are frequently observed as the virus disrupts and exploits host metabolic pathways to enable its replication (EL-BACHA; DA POIAN, 2013; GIRDHAR et al., 2021) . These metabolic changes play a critical role in modulating the host's immune response and determining the disease outcome in both the short and long term.

The limited understanding of the biological mechanisms related to COVID-19 hampers the development of evidence-based therapeutic strategies. While hyperinflammation is known to play a significant role in patients with severe COVID-19, the extent to which direct cellular and tissue damage, activation of coagulation pathways, immune responses, and metabolic changes contribute to the clinical picture remains unclear. Various clinical markers, such as sex, age, and pre-existing chronic disease, are associated with more severe COVID-19 outcomes (MOULA et al., 2020). Conditions linked to metabolic disorders, like obesity or diabetes, show a higher correlation with severe outcomes, suggesting that metabolic disturbances play an important role in the progression of COVID-19 (BLOOMGARDEN, 2020; DIETZ; SANTOS-

BURGOA, 2020). In this context, metabolomics, as a method of identifying an individual's metabolic profile, acts as a relevant tool for studying the metabolic status of different organs, tissues, or fluids associated with diseases or altered metabolic states.

Among studies aiming to identify metabolic changes during coronavirus infection, it has been demonstrated that free fatty acids are mobilized to form the viral envelope membrane (DIAS et al., 2020), double-membrane vesicles, and lipid compartments essential for replication (WOLFF et al., 2020; YAN et al., 2019). Other studies on patients with severe COVID-19 indicate that more than 100 lipid species, including glycerophospholipids, sphingolipids, and fatty acids, were downregulated in serum (RICCIARDI et al., 2022; SHEN et al., 2020). Additionally, an increase in serum triglycerides and dysregulation in apolipoproteins were positively correlated with the severity of SARS-CoV-2 infection and fatal outcomes (WU et al., 2020). Taken together, these alterations in host lipid metabolism appear to be related to the disease's pathogenesis and may also suggest the presence of multiple dysfunctions in tissues such as the liver, intestine, and kidneys.

As a result of metabolic disruption, COVID-19 frequently leads to widespread thrombotic disorders. It has been reported that 15 of the 17 proteins involved in platelet degranulation were downregulated in individuals infected with SARS-CoV-2, which correlated positively with the most severe and fatal cases of COVID-19 (GIRDHAR et al., 2021). Although inflammation plays a significant role in thrombotic events in COVID-19 patients, it may also be involved in alterations in one-carbon metabolism (STARK; MASSBERG, 2021). A study indicated that homocysteine, a metabolite often associated with hypercoagulability (EDIRISINGHE, 2004), was decreased, while S-adenosyl-homocysteine was increased in hospitalized COVID-19 patients, independent of their inflammatory status measured by IL-6 concentration (THOMAS et al., 2020). These changes suggest a dysregulation in methyl donors, which may be related to SARS-CoV-2's ability to modulate host folate metabolism and the one-carbon metabolism of infected cells that are involved in viral genome replication (ZHANG et al., 2021). Disruptions in one-carbon metabolism affect a multitude of cellular functions, including epigenetic regulation, redox homeostasis, and the metabolism of glucose, amino acids, nucleotides, and lipids (DUCKER; RABINOWITZ, 2017). Therefore, a comprehensive investigation of the metabolites (de)regulated by SARS-CoV-2, could help identify common metabolic pathways involved in multi-tissue dysfunction, and biomarkers of disease severity, and potentially lead to the development of new therapeutic strategies.

2 OBJECTIVES

2.1 Main Objectives

The purpose of this study was to characterize in depth the metabolic alterations associated with the severe cases of COVID-19 and to investigate potential markers of disease progression in (a) a cohort of adult female and male subjects and (b) in a cohort of pregnant women.

2.2 Specific Objectives

Chapter I - Metabolomics of Severe COVID-19 Patients

Evaluate the metabolic changes associated with the severity of COVID-19 in a well-characterized cohort of adult individuals presenting with severe COVID-19, divided into survivors, non-survivors, and healthy individuals. The study aims to provide a possible explanation for the metabolic biases observed in COVID-19, establishing an important foundation for a better understanding of the metabolic alterations in severe COVID-19 and disease progression.

Chapter II – Severe COVID-19 Induces Metabolic Alterations in Pregnancy

Using serum samples from a cohort of pregnant women infected with SARS-CoV-2, we aim to identify metabolic alterations and pregnancy outcomes related to disease severity. These analyses will contribute to the understanding of the effects of infection in this group and help identify predictors for clinical outcomes, placental responses and long-term health effects of the infection to the mother and the offspring.

3 RESULTS

3.1 Chapter I - Metabolomics of Severe COVID-19 Patients

In this section, we present the results corresponding to the specific objectives outlined in Chapter 1. This analysis aims to evaluate the metabolic changes associated with the severity of COVID-19 in a well-characterized cohort of adult individuals presenting with severe COVID-19, divided into survivors, non-survivors, and healthy individuals. The study aims to provide a possible explanation for the metabolic biases observed in COVID-19, establishing an important foundation for a better understanding of the metabolic alterations in severe COVID-19 and disease progression.

Those results were already published as: GAMA-ALMEIDA, M. C. et al. Integrated NMR and MS Analysis of the Plasma Metabolome Reveals Major Changes in One-Carbon, Lipid, and Amino Acid Metabolism in Severe and Fatal Cases of COVID-19. Metabolites, v. 13, n. 7, p. 879, 24 jul. 2023. DOI: 10.3390/metabo13070879

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Integrated NMR and MS Analysis of the Plasma Metabolome Reveals Major Changes in One-Carbon, Lipid, and Amino Acid Metabolism in Severe and Fatal Cases of COVID-19

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Abstract: Brazil has the second-highest COVID-19 death rate worldwide, and Rio de Janeiro is among the states with the highest rate in the country. Although vaccine coverage has been achieved, it is anticipated that COVID-19 will transition into an endemic disease. It is concerning that the molecular mechanisms underlying clinical evolution from mild to severe disease, as well as the mechanisms leading to long COVID-19, are not yet fully understood. NMR and MS-based metabolomics were used to identify metabolites associated with COVID-19 pathophysiology and disease outcome. Severe COVID-19 cases (n = 35) were enrolled in two reference centers in Rio de Janeiro within 72 h of ICU admission, alongside 12 non-infected control subjects. COVID-19 patients were grouped into survivors (n = 18) and non-survivors (n = 17). Choline-related metabolites, serine, glycine, and betaine, were reduced in severe COVID-19, indicating dysregulation in methyl donors. Non-survivors had higher levels of creatine/creatinine, 4-hydroxyproline, gluconic acid, and *N*-acetylserine, indicating liver and kidney dysfunction. Several changes were greater in women; thus, patients' sex should be considered in pandemic surveillance to achieve better disease stratification and improve outcomes. These metabolic alterations may be useful to monitor organ (dys) function and to understand the pathophysiology of acute and possibly post-acute COVID-19 syndromes.

Keywords: SARS-CoV-2; metabolomics; ^{1}H-NMR; high-resolution mass spectrometry; fatal COVID-19; virus-host interactions; metabolic alterations; sex differences

1. Introduction

The existing vaccines for SARS-CoV-2 infection resulted in a significant reduction in the number of severe cases of the disease. However, it is anticipated that the coronavirus disease 2019 (COVID-19) will transition into an endemic state [1]. By the time this article is being written, COVID-19 will have exceeded 767 million cases with more than 6.9 million deaths worldwide [2]. Brazil has recorded more than 700,000 deaths, making it the country with the second-highest number of deaths worldwide [2]. Rio de Janeiro, where this study took place, had 2.8 million confirmed cases with more than 77,000 deaths. The state is among the ones with the highest mortality rate [3].

The replication of SARS-CoV-2 triggers a systemic immune response that leads to tissue damage and the reprogramming of whole-body metabolism [4,5]. Additionally, around 20% of infected subjects may experience long-term symptoms after recovery from the initial illness [6], a condition that is associated with neurological, gastrointestinal, pulmonary, and cardiovascular alterations and which can be highly debilitating [7]. Even patients who present mild symptoms in the acute phase of the disease may later develop post-acute COVID-19 syndrome [8].

These observations reveal the complexity of COVID-19 pathophysiology and its profound impact on different tissues and cells. However, the underlying molecular mechanisms leading to clinical evolution from mild to severe disease, as well as the mechanisms associated with post-acute COVID-19 symptoms, are not yet known. The complex multisystemic nature of SARS-CoV-2 infection calls for a system-level approach that provides a better understanding of the molecular mechanisms underlying COVID-19 pathophysiology.

Metabolomics is the central omics in information translation [9], providing the metabolic signature of organs and biological fluids in different conditions. We and others have shown that metabolomics was essential in the development of new approaches that improved the understanding, therapeutics, and clinical management of emerging viral diseases such as Dengue [10,11], Chikungunya [10], SARS [12], and Zika [13,14], and may be helpful in elucidating the metabolic pathways associated with COVID-19. Alterations in host energy, amino acid, and lipid metabolism are frequently observed in viral infections as the virus disturbs and exploits host metabolic pathways for its own benefit [15,16]. These metabolic alterations have a critical role in disease outcome and in modulating the host immune response.

The modulation of host lipid metabolism is a feature shared by coronaviruses and is essential for viral RNA replication [17], as it enables the synthesis of the viral envelope membrane as well as double-membrane vesicles and lipid compartments. Indeed, our group has shown that lipid droplets accumulate in monocytes isolated from COVID-19 subjects and serve as an assembly platform for SARS-CoV-2 particles [18]. The orchestration of lipid flow within different cell compartments by the SARS-CoV-2 non-structural protein 6 ensures the proper organization of double-membrane vesicles as well as their effective communication with lipid droplets [19]. All these events are essential for SARS-CoV-2 replication.

One of the first studies to use a multi-omics approach to gain insight into the pathophysiology of COVID-19 was performed by Shen et al. with a small cohort of patients. In that study, proteomics and metabolomics approaches revealed that mild and severe COVID-19 patients presented metabolic and immune dysregulation [20]. More than 100 lipid species, including glycerophospholipids, fatty acids, lipoproteins, and several amino acids, were downregulated in sera from subjects infected with SARS-CoV-2 if compared to controls [20]. Alterations in lipoproteins using NMR metabolomics were also confirmed in larger cohorts [21], and single-cell metabolomics of monocytes reinforced the idea that modulation of intermediary metabolism, in particular organic acids, plays crucial

roles in COVID-19 severity [22]. Regarding the modulation of amino acid metabolism, several studies have reported that patients have low levels of plasma tryptophan, which is now considered a marker for the extent of inflammation and COVID-19 severity [20,23–25]. Additionally, it has been reported that COVID-19 inpatients show dysregulation in the metabolism of methyl donors, including higher levels of S-adenosyl-homocysteine and lower levels of homocysteine, regardless of IL-6 levels [26]. Indeed, SARS-CoV-2 genome replication seems to depend on folate and methionine cycle modulation [27]. Therefore, SARS-CoV-2 infection is thought to affect various aspects of host metabolism, and the extent of these changes is believed to be linked to the severity of the disease. On the other hand, the specific metabolic differences that may distinguish the severe cases of COVID-19 from those that are fatal have not yet been fully addressed.

The purpose of this study was to characterize in depth the metabolic alterations associated with the severe cases of COVID-19 and to investigate potential markers of fatal outcome. ^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy and Liquid Chromatography-High-Resolution Mass spectrometry (LC-HRMS)-based metabolomics were used in a well characterized prospective cohort of subjects with severe COVID-19, including survivors and non-survivors, and healthy subjects. Patients' samples were collected in Rio de Janeiro, Brazil, between April and July 2020. We were particularly interested in investigating metabolites associated with one-carbon metabolism and with lipid and amino acid metabolism. Considering that the incidence of post-acute severe outcomes after hospital discharge is very high after severe COVID-19, also in Brazil [28], these metabolic alterations may be useful to monitor patients' organs and tissues (dys)function and to understand acute pathophysiological mechanisms that may lead to post-acute COVID-19 syndrome [29–32].

2. Materials and Methods

2.1. Study Design and Participants

We prospectively enrolled a cohort of 35 RT-PCR-confirmed severe COVID-19 cases within 72 h of intensive care unit (ICU) admission in two reference centers in Rio de Janeiro, Brazil (Instituto Estadual do Cérebro Paulo Niemeyer and Hospital Copa Star), between April and July 2020. Enrichment-dependent SARS-CoV-2 sequencing of a subsample of this cohort showed that over 70% of SARS-CoV-2 samples were phylogenetically related to the emerging clade 20B. Clades 19A and 20A were also detected [33].

All patients were adults (≥18 years of age) classified as having severe COVID-19 (n = 35) according to the WHO working group on the clinical characterization and management of COVID-19 [34]. Severe COVID-19 was defined as critically ill patients presenting with viral pneumonia confirmed by the presence of chest infiltrates on a computed tomography scan and by the need for respiratory support with either non-invasive oxygen supplementation or mechanical ventilation. The complete clinical information was collected prospectively using a standardized form: International Severe Acute Respiratory and Emerging Infection Consortium (ISARIC)/World Health Organization (WHO) Clinical Characterization Protocol for Severe Emerging Infections (CCP-BR). Upon admission, clinical and laboratory data were recorded for all severe patients included in the study. The primary outcome analyzed was 28-day mortality, and patients were classified as survivors (n = 18) or non-survivors (n = 17).

All ICU-admitted patients received the usual supportive care for severe COVID-19. Patients with acute respiratory distress syndrome (ARDS) were managed with neuromuscular blockade and a protective ventilation strategy that included low tidal volume (6 mL/kg predicted body weight) and limited driving pressure (<16 cm H_2O) as well as optimal positive end-expiratory pressure calculated based on the best lung compliance and PaO_2/fraction of inspired oxygen (FiO_2) ratio. A prone position was adopted in those patients with severe ARDS and a PaO_2/FiO_2 ratio < 150. Antithrombotic prophylaxis was performed with 40 to 60 mg of enoxaparin per day. Patients did not receive antivirals, steroids, or other

anti-inflammatory or antiplatelet drugs in accordance with clinical practice at the time of inclusion.

Peripheral blood was also collected from SARS-CoV-2-negative participants (control group; n = 12), confirmed by RT-PCR of nasal swabs on the day of blood sampling. The control group included subjects of matching age and sex distribution compared to infected subjects. These participants had not been on anti-inflammatory or antiplatelet drugs for at least 2 weeks prior to the study.

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the National Review Board of Brazil (Comissão Nacional de Ética em Pesquisa [CONEP] 30650420.4.1001.0008), and informed consent was obtained from all subjects or their caregivers.

2.2. Chemicals and Solvents

All solvents used were of HPLC analytical grade. Acetonitrile and methanol were obtained from TEDIA® (Fairfield, OH, USA), and isopropanol from Sigma Aldrich (São Paulo, Brazil). Water was purified in the Milli-Q device, the Millipore Purification System (Billerica, MA, USA). Mobile phase additives formic acid and ammonium hydroxide were purchased from TEDIA®, and ammonium acetate was obtained from J. T. Baker® (Aparecida de Goiânia, Brazil). Isotopically labeled internal standards, U ^{13}C D-glucose and U ^{13}C L-glutamine, and deuterium oxide were purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA). All other standards were obtained from Sigma Aldrich.

2.3. Sample Processing

Blood samples were drawn into acid-citrate-dextrose and centrifuged (200× *g*, 20 min, room temperature). Plasma was collected and stored at −80 °C until analysis. A citrate-dextrose buffer was chosen to preserve platelets. This choice of buffer prevented us from comparing citrate and sugars among groups and limited the identification of metabolites that present chemical shifts in the ^{1}H NMR spectrum, which is in the vicinity of citrate.

2.4. Nuclear Magnetic Resonance-Based Metabolomics

2.4.1. Sample Preparation

Frozen plasma samples were quickly thawed and diluted 3-fold in sodium phosphate buffer and deuterium oxide (final concentration: 50 mM phosphate buffer and 10% deuterium oxide, pH 7.4). A total of 600 µL of diluted samples were transferred to a 5 mm NMR tube.

2.4.2. NMR Acquisition, Spectra Pre-Processing, and Metabolite Assignment

NMR spectra were acquired on a Bruker Advance III at 500.13 MHz at 300 K, coupled with a cooled automatic sample case at 280 K. 1D-^{1}H NMR spectra were acquired using excitation sculpting to suppress the solvent signal [35] as well as a CPMG (Carr-Purcell-Meiboom-Gill) T2 filter [36] with 32 loop counters and a delay of 0.001 s. 32,768 complex data points were acquired per transient, for a total of 1024 transients. The spectral width was set to 19.99 ppm, resulting in an acquisition time of 3.27 s per FID. The relaxation delay was set to 1.74 s.

Spectra data were pre-processed in the MetaboLab [37] software v. 2022.0726.1733. Prior to the Fourier transform, the FIDs were apodized using an exponential window function with 0.3 Hz line-broadening and then zero-filled to 65,536 data points. After Fourier transform, each spectrum was manually phase corrected, followed by a spline-baseline correction. Finally, all spectra were referenced to the signal of the ^{1}H linked to the anomeric carbon of glucose. Baseline noise and regions corresponding to water and citrate signals were deleted. Spectra data were binned with a 0.005 ppm interval, and the resulting table presented 81,498 data points, corresponding to metabolites' intensities. A generalized log transformation [38] was applied prior to multivariate statistical analysis.

Following 2D spectra, HSQC ^{1}H-^{13}C and TOCSY ^{1}H-^{1}H acquisition, data was uploaded on the COLMAR [39,40] for the assignments. The peak report of all assigned compounds can be seen at https://spin.ccic.osu.edu/index.php/colmarm, session ID 3121-pZ5ZukwXBh, (accessed on 14 July 2023) (COLMAR https://spin.ccic.osu.edu/index.php/colmarm/index2, accessed on 14 July 2023). Supplementary Tables S1 and S2 present the ^{1}H NMR assignment information for the metabolites and broad signals of lipids and proteins that distinguished the groups.

We also overlaid spectra from the BMRB [41] and HMDB 4.0 [42] databanks. The software ICON NMR (Bruker) was used for automatic acquisition.

2.5. Mass Spectrometry-Based Metabolomics

2.5.1. Standards

A stock of internal standard (IS) solution was prepared with a final concentration of 0.15 mg mL^{-1} for U-^{13}C D-glucose and 0.13 mg mL^{-1} for U-^{13}C L-glutamine in acetonitrile/isopropanol/water (3:3:2, % $v/v/v$).

Stock solutions of targeted analytes were prepared at 1.0 mg mL^{-1} in methanol or in different proportions of acetonitrile/water. A standard working solution was prepared by mixing appropriate volumes of each stock solution to reach the final concentration of 2.0–50.0 µg mL^{-1} in acetonitrile/water (1:1, %/v).

2.5.2. Sample Preparation

A total of 30 µL of plasma (in duplicate) were mixed with the same volume of the IS mixture and 500 µL of a degassed mixture of pre-chilled acetonitrile/isopropanol/water (3:3:2, $v/v/v$). After vortexing for 20 s and incubating in ice in an ultrasonic water bath for 5 min, samples were centrifuged at 12,000× g at 4 °C for 5 min, and the supernatant (480 µL) was dried under Nitrogen gas. Samples were reconstituted with 60 µL of acetonitrile/water (1:1, v/v) containing 2 µg mL^{-1} of the IS p-fluoro-L-phenylalanine, vortexed for 15 s, and centrifuged as above. The resulting supernatant was used for LC-MS analysis.

A pooled quality control (QC) sample was prepared by combining 5 µL of each plasma before extraction and processing it in the same way as the specimen samples. QC samples were injected with every tenth biological sample to monitor the stability of the analytical system as well as the reproducibility of the procedure for sample treatment [43].

Subgroups of pooled QC samples (control, survivors, or non-survivors) were created to collect fragmentation spectra via data-dependent acquisition (DDA) mode on the mass spectrometer. The analysts running MS-based experiments were blinded to the sample grouping until the end of data analysis to limit biased peak annotation.

2.6. LC-MS Conditions

Liquid chromatography (LC) analysis was performed on a Dionex UltiMate 3000 UHPLC (Thermo Fisher Scientific, Bremen, Germany) system using a Waters® ACQUITY UPLC® BEH amide column (150 × 2.1 mm × 1.7 µm) by gradient elution at a constant flow rate of 350 µL min^{-1}. The column oven temperature and injection volume were set to 40 °C and 5.0 µL, respectively. Two different mobile phase compositions with different pH values were used. One consisted of (A) water:acetonitrile (95:5, v/v) and (B) acetonitrile:water (95:5, v/v) both with 0.1% formic acid (pH 3), and the other consisted of (A) water:acetonitrile (95:5, v/v) and (B) acetonitrile:water (95:5, v/v) both with 0.05% ammonium hydroxide and 10 mM ammonium acetate (pH 8). The gradient elution was 0–0.5 min 100% B; 0.5–5.0 min 45% B; 5.0–9.0 min 45% B; 9.0–10.0 min 100% B; 10.0–15.0 min 100% B.

The LC was coupled to a hybrid Quadrupole-Orbitrap high-resolution and accurate mass spectrometer (QExactive Plus, Thermo Scientific, Waltham, MA, USA) equipped with a heated electrospray ion source operating in both negative (ESI−) and positive (ESI+) ionization modes. Source ionization parameters were: spray voltage −3.6 kV/+3.9 kV; capillary temperature 270 °C; probe heater temperature 380 °C; S-Lens RF level 50; sheath and auxiliary gases 50 and 10 (arbitrary units), respectively. Samples were analyzed in

Full MS mode in the scan range of m/z 50–710 at a resolution of 70,000 FWHM (full width at half maximum). The automatic gain control (AGC) target was set at 1×10^{-6} with a maximum injection time (IT) of 150 ms.

The solution of target analytes and the subgroup pooled QC samples were analyzed in Full MS followed by data-dependent acquisition (dd-MS2 top5 experiment) in the same scan range as above. For the full MS scan, the mass resolution was set to 17,500 FWHM with the following settings: AGC target of 1×10^{-6} and maximum IT of 80 ms. For the dd-MS2 scan, the mass resolution was set to 17,500 FWHM with the following settings: AGC target at 1×10^{-5}, maximum IT of 50 ms, isolation window at m/z 1.2, normalized collision energy (NCE) of 15, 35 (ESI+), and 10, 30 (ESI−), intensity threshold at 1×10^{-6}, exclude isotopes "on", and dynamic exclusion of 10.0 s.

2.7. Non-Targeted and Targeted LC-HRMS-Based Metabolomics

For the non-targeted analysis, the LC-HRMS data files were submitted to a metabolomics workflow using MS-DIAL software (RIKEN, version 4.80) [44] for data processing, including peak matching against an MS/MS library. The parameters used for both pH 8 and pH 3 analyses are described in Supplementary Table S3. Features were selected assuming coefficient variation (CV) % values less than 30% in QC samples and a Gaussian-like peak shape according to the protocols for quality control used in untargeted metabolomics [43,45]. Prior to multivariate statistics, MS data were normalized by the Total Ion Chromatogram and scaled using Pareto.

Compound annotation was carried out: (i) based on the MS/MS fragment comparison with the standard compounds; (ii) by comparing the aligned m/z ions with a mass error below 6 ppm to those available at the HMDB [42] and METLIN website; and (iii) by comparing the investigated MS/MS spectra with a similarity score $\geq$ 80% to those in the NIST 20 Tandem Mass Spectral Library and MassBank of North America using a customized MSP file in MS-DIAL. Furthermore, molecular formulas were determined using MS-FINDER (RIKEN, version 3.52) [46].

Targeted data analysis was performed as a confirmation step for the non-targeted approach in TraceFinder software v3.1 (ThermoFisher Scientific, Waltham, MA, USA). An in-house library that includes retention time, exact mass, and fragments of the target compounds was used. As identification criteria, mass errors less than 5 ppm and retention time variations of <1% compared to the defined retention time were accepted [47]. Supplementary Tables S3 and S4 present the target compounds monitored via the method at pH 8 and pH 3, respectively.

2.8. Lipoprotein Analysis

Total cholesterol (TC), high-density lipoprotein (HDL), and triglycerides (TGs) were measured by the oxidase-peroxidase method [48,49]. Low-density lipoprotein (LDL) was calculated based on Friedewald's equation (LDL-c = TC − HDL-c − TG/5).

2.9. Statistical Analysis

The sample size was determined by the feasibility of recruitment and eligibility criteria.

Data distribution was analyzed using the Shapiro-Wilk test and median and interquartile intervals, and the Mann-Whitney or Kruskal-Wallis tests were used for data with an asymmetrical distribution. Categorical variables were compared using the Fisher's exact test with absolute (n) and relative (%) frequencies.

Data derived from processed NMR spectra was subjected to multivariate Principal Component Analysis using the MetaboAnalyst 4.0 [50] platform. For the univariate statistics, Kruskal-Wallis and Dunn's post-hoc tests were used for variables' comparison of non-transformed NMR or MS data. The interaction between sex and disease was analyzed using a two-way ANOVA and Tukey's multiple comparison tests. GraphPad Prism version 8.4.3 was used for all analyses. $p < 0.05$ was considered for rejection of the null hypothesis.

Classification and regression tree (CART) models [51] were fitted to assess which metabolites best predicted the observed morbidity class of study participants. The model-fitting algorithms have been implemented in the library "rpart" [52] for the R programming language. We set "method" = "class", and the remaining parameters were kept at their default values for all models. CART models were built using the significant variables identified in the NMR and MS-based metabolomics (considering separate and combined datasets), in addition to the subjects' sex and age. For the combination of NMR and MS results, a unified matrix was built by normalizing the data as their z-score.

3. Results

3.1. Subjects' Demographics and Clinical Parameters

A total of 47 individuals were included in this study: 12 non-infected control subjects and 35 severe COVID-19 cases, grouped into survivors (n = 18) and non-survivors (n = 17) according to the 28-day mortality outcome. The demographics and clinical characteristics of all subjects included in the study are shown in Table 1. Briefly, age, sex distribution, and co-morbidities were similar among groups. Non-invasive oxygen supplementation was used in 44% of subjects in the survivors group, whereas 100% of the subjects in the non-survivors group received mechanical ventilation.

Table 1. Demographics and clinical characteristics of control, COVID-19 survivors, and non-survivors.

	Control (n = 12)	Survivors (n = 18)	Non-Survivors (n = 17)	*p* Value
Age, years	50 (36–59)	56 (39–63)	58 (51–73)	0.344
Sex, male; n (%)	5 (41)	7 (38)	10 (58)	0.727
Respiratory support; n (%)				
Noninvasive O_2 supplementation	0 (0)	8 (44)	0 (0)	**0.003**
Mechanical ventilation	0 (0)	10 (66)	17 (100)	
SAPS II	n.a.	55 (37–64)	68 (59–78.5)	**0.001**
PaO_2/FiO_2 ratio	n.a.	196 (154–429)	139 (177–178)	0.099
Time from symptom onset to blood sample (days)	n.a.	10 (7–14)	10 (3–14)	0.975
Comorbidities; n (%)				
Obesity	1 (8.3)	5 (27.7)	2 (11.7)	0.650
Hypertension	2 (16)	4 (22)	5 (29)	0.855
Diabetes	0 (0)	6 (33)	6 (35)	0.990
Cancer	0 (0)	2 (11)	2 (11)	0.990
Heart disease [1]	0 (0)	2 (11)	2 (11)	0.990
Laboratory findings at admission				
Leukocytes, ×1000/μL	n.a.	12.4 (9.1–14.5)	14.8 (11.5–21.7)	0.097
Lymphocytes, cells/μL	n.a.	1288 (939–1.579)	1035 (284–1.706)	0.521
Monocytes, cells/μL	n.a.	495 (448–742)	738 (599–1.005)	**0.009**
Platelet count, ×1000/μL	n.a.	198 (154–324)	187 (131–240)	0.125

Continuous variables are represented as the median and interquartile range. Categorical variables are represented as *n* (frequency %). n.a.—not applicable [1] Coronary artery disease or congestive heart failure. Categorical variables were compared using the two-tailed Fisher exact test, and continuous variables were compared using student's *t* or ANOVA tests for parametric and Mann-Whitney U or Kruskal-Wallis tests for nonparametric distributions. Significant *p* values are in bold.

Laboratory findings revealed that patients in the survivors group presented ~50% more monocyte counts ($p = 0.009$) if compared to non-survivors. At admission to the ICU, leukocyte and platelet counts were similar between the two groups of infected patients.

3.2. ^{1}H NMR- and MS-Based Metabolomics

Representative spectra of aliphatic (Figure 1A,B), amidic, and aromatic (Figure 1C) regions indicate important differences in the metabolite profile among the three groups. Discriminating metabolites such as $(CH_3)_3$ choline-related metabolites, creatine/creatinine, amino acids, organic acids, and broad residual signals of lipids are depicted, followed

by arrows indicating higher/lower contents in severe COVID-19 cases. A discriminating metabolite profile was confirmed with the Principal Component Analysis (PCA) scores plot (Figure 1D), where principal components 1, 2, and 3 accounted for approximately 70% of the variation among groups. PCA loading factors plot highlights $(CH_3)_3$ choline, creatine/creatinine, lactate, acetate, and broad signals of CH_3 and CH_2 lipoproteins as discriminating variables (Supplementary Figure S1).

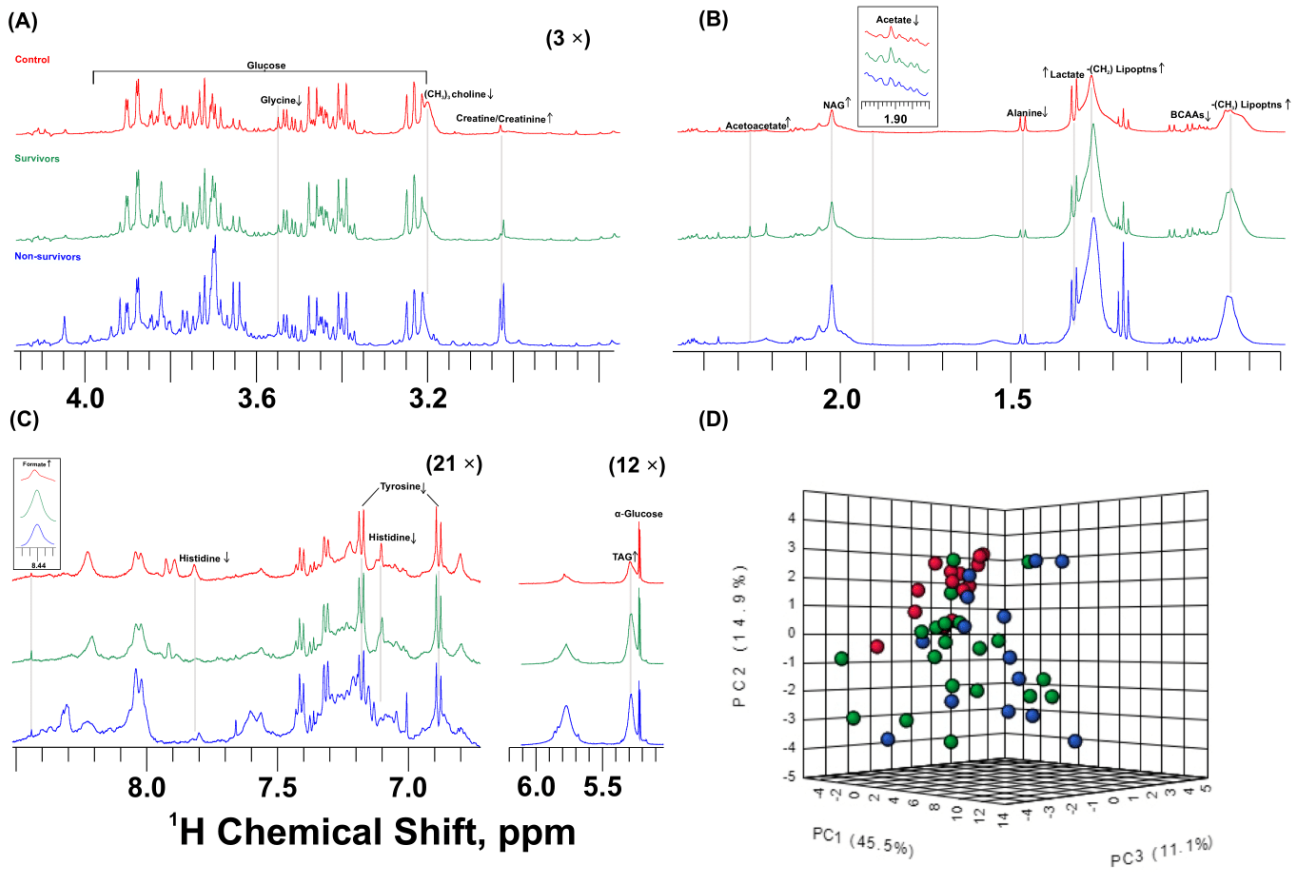

Figure 1. ^{1}H NMR-based metabolomics shows different plasma metabolite profiling in severe COVID-19 patients compared to control subjects. ^{1}H NMR representative spectra of control (red), COVID-19 survivors (green), and non-survivors (blue). Metabolites that differ significantly among groups are indicated as having higher (↑) or lower (↓) contents compared to controls; (**A**,**B**) aliphatic region (3× magnified); (**C**) amidic and aromatic regions (21× magnified); (**D**) Principal Component Analysis 3D score plot shows discriminating profiling among groups; control (red), COVID-19 survivors (green), and non-survivors (blue).

To gain meaningful insights into the changes associated with disease severity and outcome, metabolites that exhibited significant differences in content among groups according to the PCA results were selected. NMR-based metabolomics revealed that the levels of $(CH_3)_3$-choline metabolites, including glycerophosphocholine, phosphocholine, and choline, as well as glycine, which is associated with one-carbon metabolism, were significantly lower in both survivors and non-survivors if compared to controls (Figure 2A,B). Additionally, ^{1}H NMR-based metabolomics identified several metabolites related to glucose, insulin sensitivity, and inflammation that were significantly higher in infected subjects (Figure 2C–G), including creatine/creatinine (considered together due to signal overlap), *N*-acetyl ^{1}H of glycoproteins, and lactate. Importantly, creatine/creatinine levels at admission set non-survivors apart from survivors and control subjects, as the levels were higher in patients that expired after up to 28 days of ICU stay. Lower levels of acetate and

higher levels of formate, a byproduct of bacterial metabolism in the gut, were observed in infected subjects but not in controls (Figure 2F,G). Additionally, residual signals of (CH_2) of VLDL-lipoproteins, lipids (CH=CH olefinic protons of triacylglycerols), and acetoacetate, a ketone body, were significantly higher in both groups of infected subjects if compared to controls (Figure 2H–J).

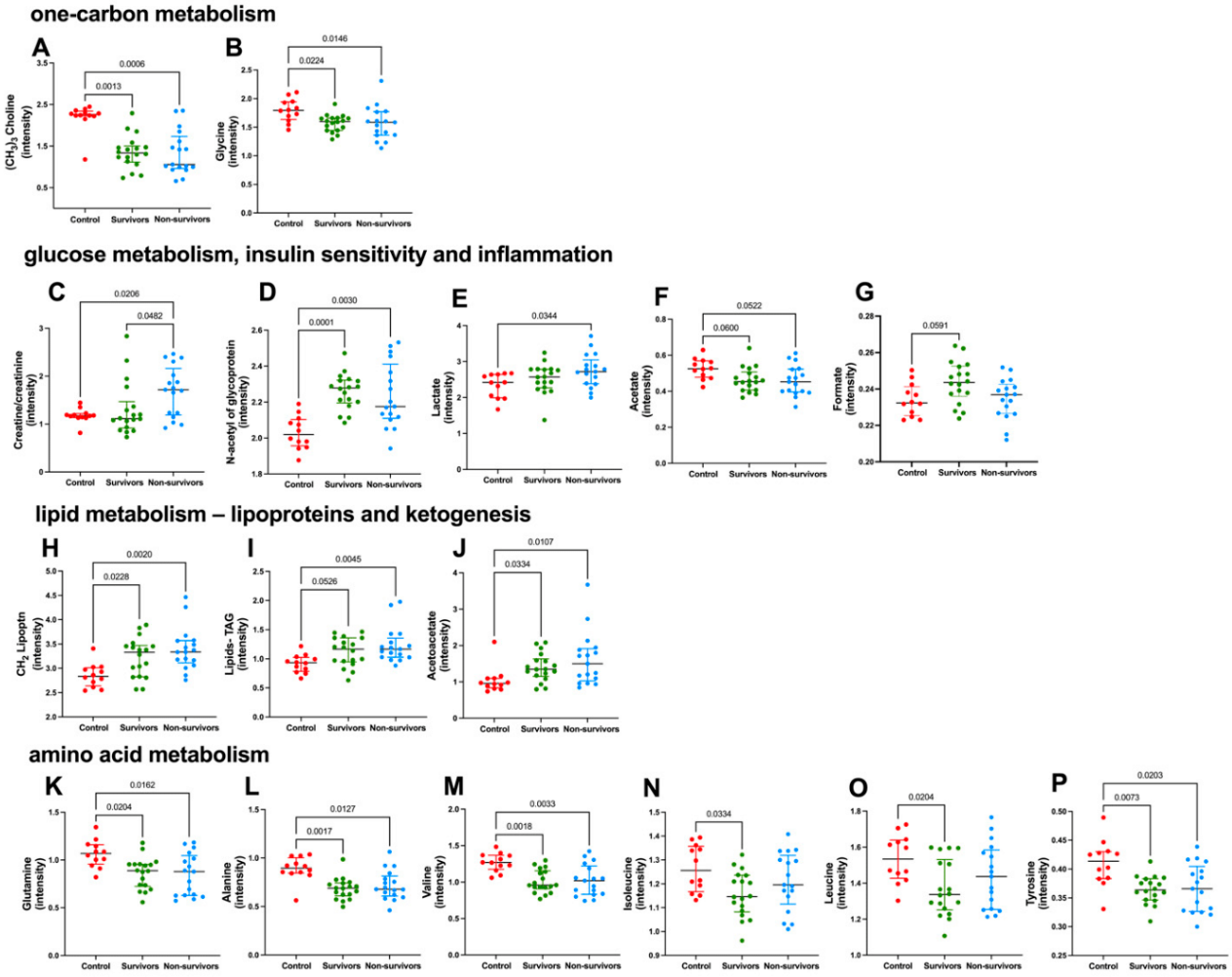

Figure 2. Metabolites that were significantly altered in severe COVID-19, according to ^{1}H NMR-based metabolomics. Most discriminating metabolites according to PCA loading factors, presenting significant differences among groups. Control, n = 12 (red circles); survivors, n = 18 (green circles); non-survivors, n = 17 (blue circles). Metabolites related to one-carbon metabolism (**A**,**B**): $(CH_3)_3$ choline-related metabolites and glycine; metabolites related to glucose metabolism, insulin sensitivity, and inflammation (**C**–**G**): creatine/creatinine, *N*-Acetyl of glycoproteins, lactate, acetate, and formate; metabolites related to lipid metabolism (**H**–**J**): CH_2 lipoproteins (mainly VLDL), lipids-TAG (CH=CH olefinic protons of triacylglycerols) and acetoacetate; metabolites related to amino acids and protein metabolism (**K**–**P**): glutamine, alanine, valine, isoleucine, leucine, and tyrosine; metabolites' contents were determined according to their respective peak intensity. Data were presented as medians with an interquartile range, and only significant *P* values are shown, according to Kruskal-Wallis and Dunn's post-hoc tests.

^{1}H NMR-metabolomics findings also suggest that the dysregulation in amino acid metabolism is a function of severe COVID-19, as survivors and non-survivors had plasma levels of glutamine, alanine, branched-chain amino acids (valine, leucine, and isoleucine), and tyrosine lower than those observed in controls (Figure 2K–P). Supplementary Tables S1 and S2 present the ^{1}H NMR assignment information for the metabolites and broad signals of lipids and proteins that distinguished the groups in both PCA and univariate analysis.

To further investigate the changes in the plasma metabolome associated with severe COVID-19, LC-high-resolution mass spectrometry (LC-HRMS)-based metabolomics was used. In the current study, LC-HRMS allowed us to confirm the alterations detected by ^{1}H NMR-based metabolomics in amino acid and protein metabolism and in ketogenesis. Importantly, it allowed us to discriminate the metabolic abnormalities associated with fatal COVID-19 and to complement the NMR data, providing insightful information regarding the alterations in one-carbon metabolism and in metabolites associated with insulin sensitivity and inflammation. The metabolites related to one-carbon metabolism, glycerophosphocholine, serine, betaine, and histidine, were lower (Figure 3A–D), whereas xanthine and hypoxanthine were higher in infected subjects if compared to controls (Figure 3E,F). The lower levels of glycerophosphocholine in the survivors and non-survivors groups, when compared to control subjects, support the ^{1}H NMR-based metabolomics results, which detected lower $(CH_3)_3$ choline-related metabolites in the two groups of infected patients. Metabolites associated with inflammation were significantly higher in infected subjects compared to controls. Importantly, creatinine, 4-hydroxyproline, gluconic acid, and *N*-acetylserine in admission were predictive of ICU-related mortality, being significantly higher in the non-survivors compared to both survivors and control groups (Figure 3G–J). The higher levels of creatinine in non-survivors confirmed the results of the NMR analysis (Figure 2C). Moreover, asymmetric dimethylarginine and methylmalonic acid were significantly higher in survivors and non-survivors when compared to control subjects (Figure 3K,L). MS-based metabolomics revealed significantly higher content of β-hydroxybutyrate—a ketone body (Figure 3M) and lower content of the amino acid tryptophan (Figure 3N) in infected subjects if compared to controls. Supplementary Figure S2 shows the essential stability of the QC samples throughout the run, which was within the SD limit. Additionally, the IS p-fluoro-DL-phenylalanine and U-^{13}C D-glucose, which were added to all samples, showed that the CV was less than 10%, indicating that the differences observed in metabolites among groups could be attributed to biological variation and not to the variability of the analytical system. Supplementary Tables S6 and S7 show the parameters of the assigned metabolites with significant differences among groups according to the non-targeted MS-metabolomics in the negative and positive modes, respectively.

CART models were built to assess the predictive power of metabolite levels in classifying study participants into control, survivors, and non-survivors. These models were built using the data from the NMR and MS metabolomics and considered only the metabolites that showed discriminatory power among the groups (metabolites shown in Figures 2 and 3). Additionally, the subjects' sex and age were included in the models. Supplementary Figures S3 (NMR dataset) and S4 (NMR + MS dataset) show the CART models fitted to data indicating that choline-related metabolites at ICU admission had the highest predictive power with a first partition point at $\geq$0.8813 (normalized data). The classification trees show that over 90% of control subjects followed the classification of $\geq$0.8813, and most survivors (95%) and non-survivors (89%) were classified as <0.8813. A second partition criteria was observed at creatine/creatinine < 0.1156 (supplementary Figure S3) and at *N*-acetyl serine < 0.00137 (supplementary Figure S4). For this second partition, over 70% of survivors were included. Conversely, for the non-survivors, the majority (64%) were classified at $\geq$0.116 for creatine/creatinine and $\geq$0.00137 for N-acetyl serine. Considering the models using the MS variables, hypoxanthine had the highest predictive power with a first partition point at <-0.2694, where 100% of control subjects followed this classification and over 80% of survivors and non-survivors were classified as ≥-0.2694 (Supplementary Figure S5). A second partition criteria was observed

at *N*-acetylserine < 0.2264, where most survivors were included, whereas over 50% of non-survivors were classified at ≥0.2264. Lastly, a CART model was built with creatinine, 4-hydroxyproline, gluconic acid, and *N*-acetylserine, metabolites that were higher in the non-survivors compared to survivors and controls (Supplementary Figure S6). This model shows that *N*-acetylserine had the sole predictive power with a first partition point of <0.2264 (100% of control and 94% of survivors). Most non-survivors (65%) were classified at ≥0.2264, confirming the results of the models presented in Supplementary Figures S4 and S5. The second partition point included the majority of control subjects at *N*-acetylserine < −0.5714 and survivors at ≥−0.5714.

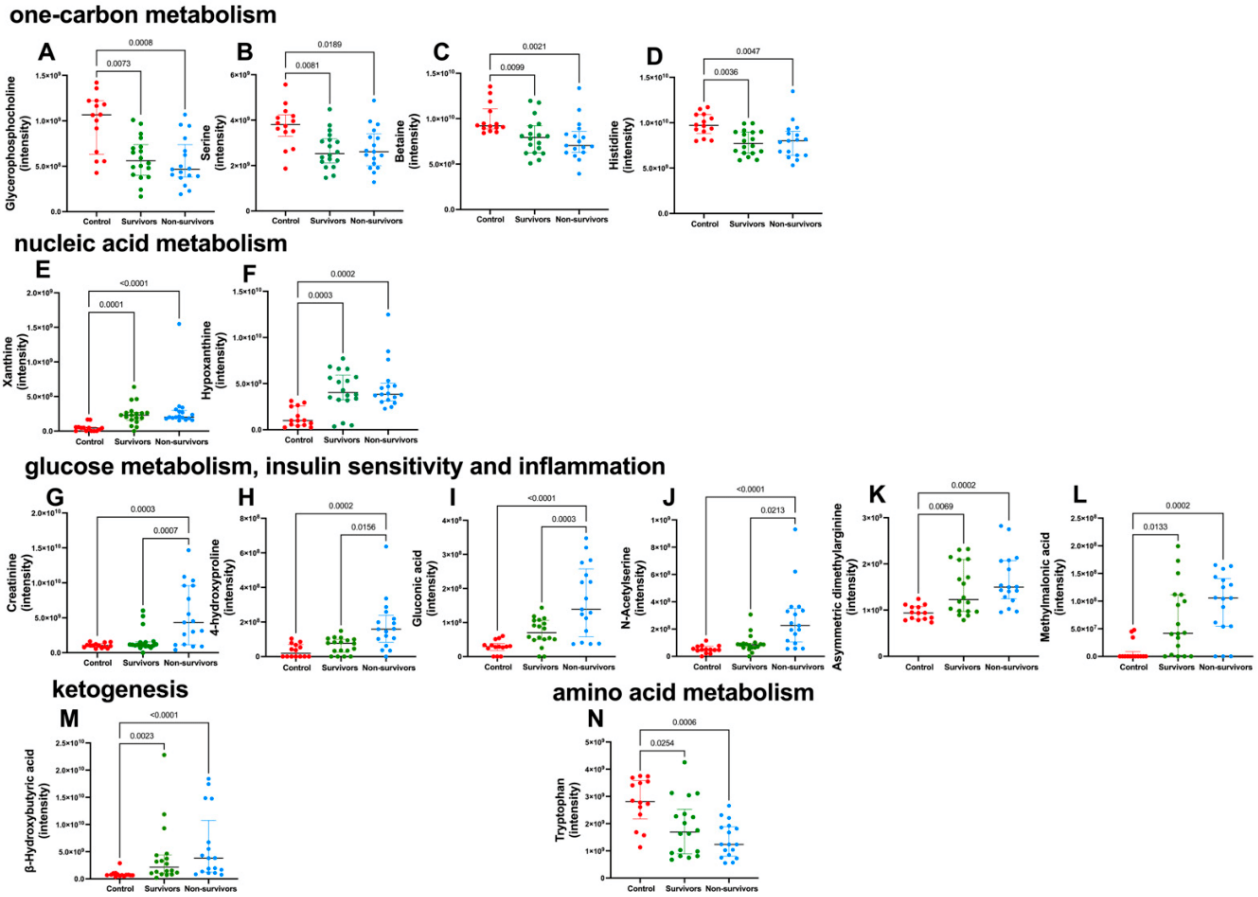

Figure 3. High-resolution mass spectrometry-based metabolomics shows an altered plasma metabolic profile in severe COVID-19. Assigned metabolites in the untargeted approach and confirmed in the targeted approach, presenting significant differences among groups. Control, n = 12 (red circles); survivors, n = 18 (green circles); non-survivors, n = 17 (blue circles). Metabolites related to one-carbon metabolism (**A**–**D**): glycerophosphocholine, serine, betaine, histidine, and nucleic acid metabolism (**E**,**F**): xanthine, hypoxanthine; metabolites related to glucose metabolism, insulin sensitivity, and inflammation (**G**–**L**): creatinine, 4-hydroxyproline, asymmetric dimethylarginine, gluconic acid, *N*-acetyl serine, and methylmalonic acid. Metabolite related to lipid metabolism (**M**): *β*-hydroxybutyrate; metabolite related to amino acid metabolism (**N**): tryptophan. Metabolites' contents were determined according to their respective peak intensities. Data were presented as medians with an interquartile range, and only significant *p* values are shown, according to Kruskal-Wallis and Dunn's post-hoc tests.

Supplementary Figures S3–S6 present the final allocation contingent among the morbidity classes by applying the classification criteria described above. These results clearly suggest that choline-related metabolites at ICU admission have a protective effect on 28-day outcomes. As opposed to *N*-acetylserine, creatine/creatinine, and hypoxanthine, where higher contents at admission predicted fatal outcomes.

3.3. Lipoprotein Dynamics

A complete characterization of lipoproteins was performed (Figure 4), and significant changes were observed as a function of severe COVID-19. The results show that survivors and non-survivors presented lower concentrations of total cholesterol, LDL, and HDL if compared to controls (Figure 4A–C). VLDL and triacylglycerol concentrations were significantly higher (Figure 4D,E), and non-HDL cholesterol (Figure 4E) was significantly lower in the non-survivors if compared to controls.

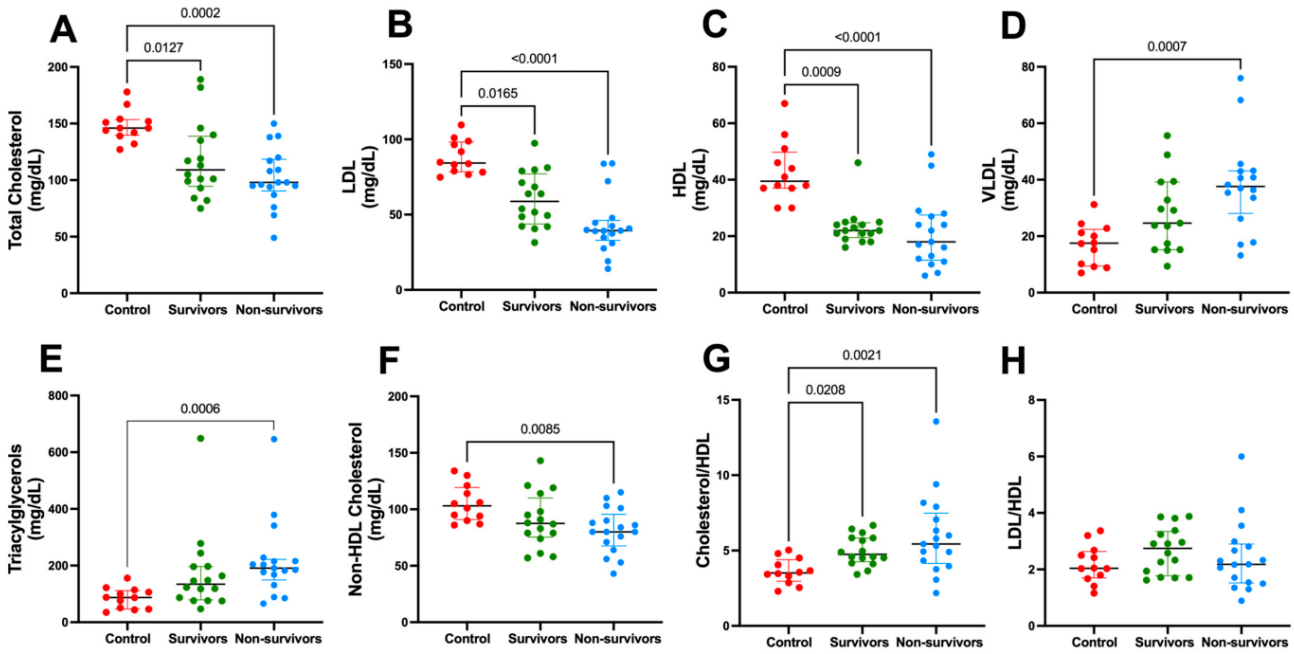

Figure 4. Lipoprotein dynamics changed significantly in severe COVID-19. (**A**) Total Cholesterol; (**B**) Low-Density Lipoprotein—LDL; (**C**) High-Density Lipoprotein—HDL; (**D**) Very Low-Density Protein—VLDL; (**E**) Triacylglycerol; (**F**) Non-HDL Cholesterol; (**G**) Cholesterol-to-HDL ratio; and (**H**) LDL-to-HDL ratio. Control, n = 12 (red circle); survivors, n = 18 (green circle); non-survivors, n = 17 (blue circle). Data were presented as medians with an interquartile range, and only significant *p* values are shown, according to Kruskal-Wallis and Dunn's post-hoc tests.

3.4. Sex-Based Differences in Lipoproteins and Metabolites

Lastly, we investigated whether changes in lipoproteins and in metabolites associated with severe COVID-19 differed according to sex by two-factor analysis (Figure 5). In general, the changes observed in female subjects mirrored the changes described above when considering all subjects. Our findings indicate that the strongest effect was seen in severe COVID-19 cases, and no differences between men and women within the group were observed. However, for HDL, total cholesterol, non-HDL cholesterol, LDL to HDL, and HDL to cholesterol ratios, significant interactions between disease and sex were observed. Additionally, lower contents of HDL (Figure 5C) and higher contents of VLDL (Figure 5D) and triacylglycerols (Figure 5E) were observed in female but not male subjects in the non-survivors group when compared to controls.

The same sex-related pattern changes were observed for selected metabolites analyzed by ^{1}H NMR-based metabolomics, where the strongest effect was seen in severe COVID-19

cases (Figure 5I–L). Lower content of $(CH_3)_3$ choline-related metabolites (Figure 5I) and higher content of *N*-acetyl glycoproteins (Figure 5K) were observed among females in both survivors and non-survivors if compared to controls, whereas among men these differences were observed only between survivors and controls. Additionally, higher contents of acetoacetate (Figure 5J) and creatine/creatinine (Figure 5L), as a function of severe COVID-19, were only observed in women but not in men. Indeed, for acetoacetate and creatine/creatinine, significant interactions between disease and sex were observed. There were no significant differences in amino acids and one-carbon metabolism-related compounds when sex was considered a variable. The metabolic alterations observed in severe COVID-19 are shown in Figure 6.

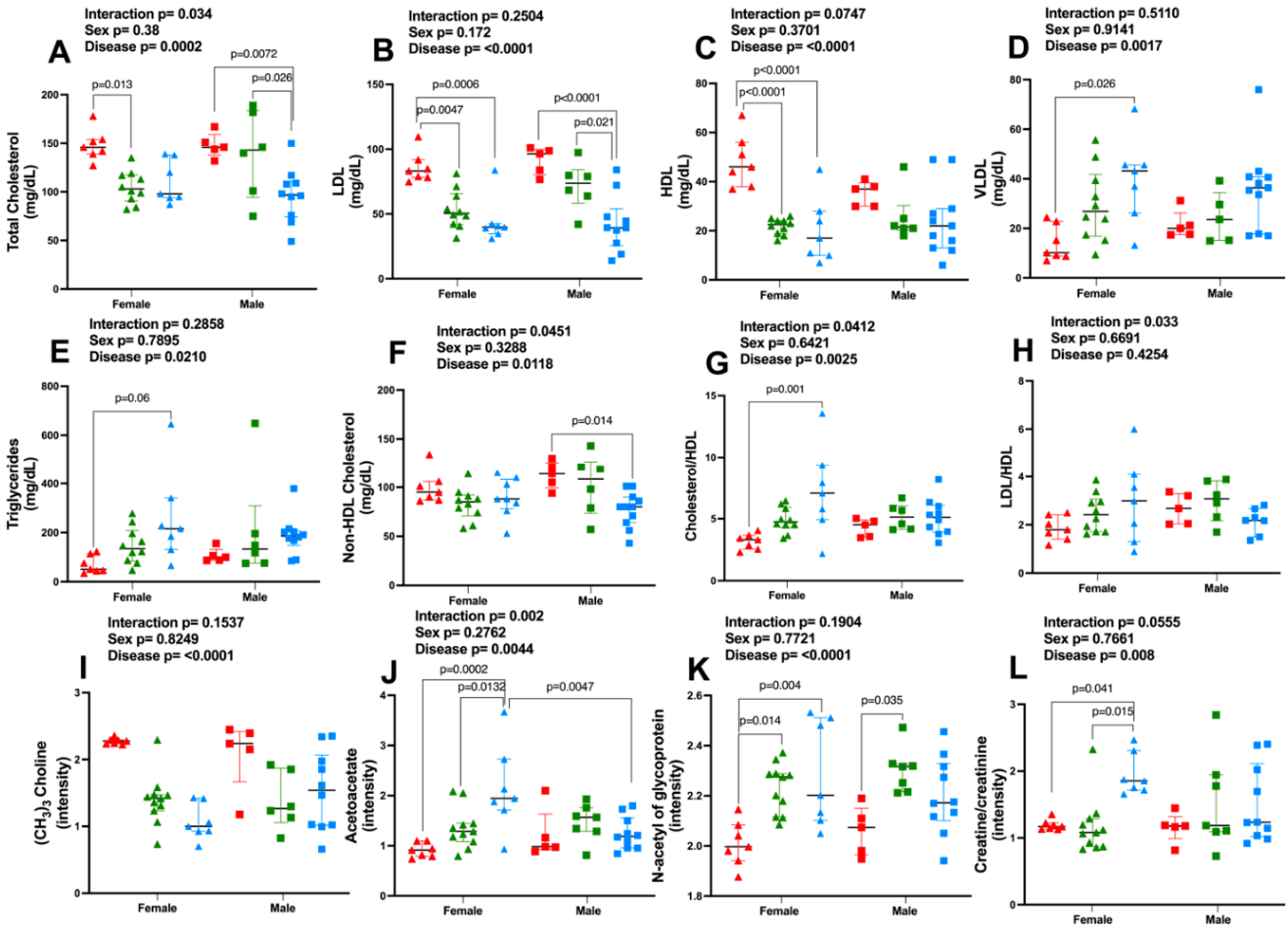

Figure 5. Severe COVID-19-induced changes in lipoproteins and metabolites are greater in women than in men. (**A**) Total Cholesterol; (**B**) Low-Density Lipoprotein—LDL; (**C**) High-Density Lipoprotein—HDL; (**D**) Very Low-Density Protein—VLDL; (**E**) Triacylglycerol; (**F**) Non-HDL Cholesterol; (**G**) Cholesterol-to-HDL ratio; (**H**) LDL-to-HDL ratio; (**I**) $(CH_3)_3$ Choline; (**J**) Acetoacetate; (**K**) *N*-acetyl of glycoprotein; and (**L**) Creatine/creatinine. Female subjects: control, n = 7 (red triangles); survivors, n = 11 (green triangles); non-survivors, n = 7 (blue triangles). Male subjects: control, n = 5 (red squares); survivors, n = 7 (green squares); non-survivors, n = 10 (blue squares). Data were presented as medians with an interquartile range, and *p* values are shown according to a two-factor ANOVA and Tukey's multiple comparison tests.

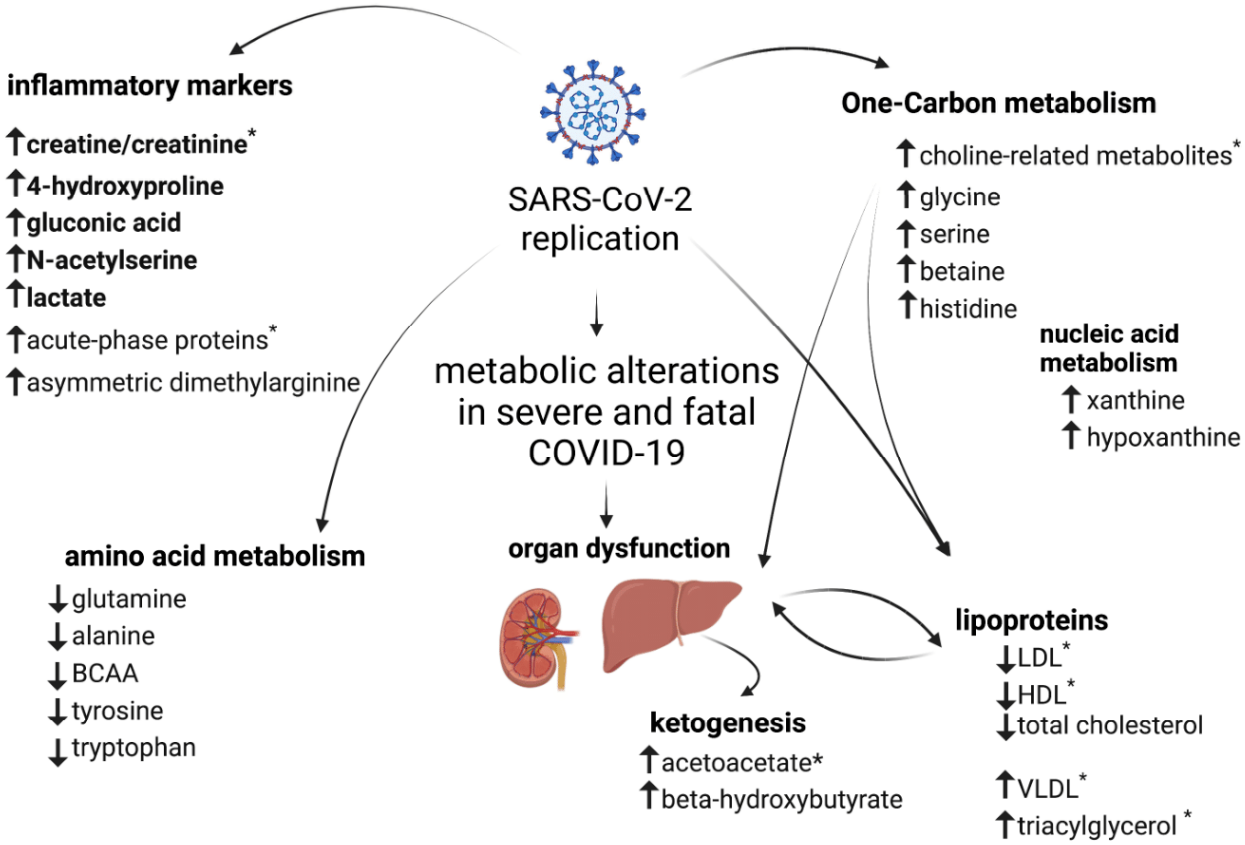

Figure 6. Metabolic alterations in severe COVID-19. Summary of changes in plasma metabolites in survivors and non-survivors. Metabolites that differ significantly among groups are indicated as having higher (↑) or lower (↓) contents compared to controls. Metabolites in bold were higher in the fatal cases of the disease compared to survivors and controls. * indicates metabolites with changes greater in women than in men. This figure was created with BioRender.com.

4. Discussion

The present study aimed to investigate plasma metabolic changes in severe COVID-19 patients at admission that are associated with ICU-related mortality. Our goal was to search for potential metabolic pathways that could be involved in severe COVID-19 pathophysiology and disease outcome. We identified significant changes in a plethora of metabolites, indicating that severe COVID-19 dysregulates one-carbon, lipid, and amino acid metabolism and lipoprotein dynamics.

Higher contents of creatine/creatinine, 4-hydroxyproline, gluconic acid, and *N*-acetylserine in non-survivors if compared with survivors and control groups indicate that these metabolites are associated with uncontrolled inflammation, multi-organ dysfunction, particularly liver and kidneys, and some degree of insulin resistance associated with fatal COVID-19 outcomes. They may be considered biomarkers for prognostic purposes and to monitor how different organs and tissues respond to severe infection. For instance, gluconic acid has been previously linked to hyperglycemia and brain injury in ischemic stroke [53], and it may be considered a marker of oxidative stress. Additionally, *N*-acetylation of amino acids, including the formation of *N*-acetylserine, has been associated with SARS-CoV-2 infection and COVID-19 pathogenesis [54], whereas higher levels of *N*-acetylserine have been recently considered a marker of the progression of chronic kidney disease [55]. Our data, built on these previous observations, highlights an association between these metabolic alterations and poor outcomes in a cohort of ICU-admitted patients.

Higher plasma contents of creatine/creatinine can be an indication of lower sensitivity to insulin, as we observed in the fatal cases of COVID-19. Although higher creatine levels in severe COVID-19 have been associated with kidney dysfunction [26] and are important for viral replication [56], recent findings place creatine as a key metabolite involved in the regulation of adipocyte thermogenesis, whole-body energy metabolism, and immunity [57,58]. Therefore, higher levels of creatine/creatinine in infected subjects can be regarded as a biomarker of detrimental effects on metabolic health and immune responses imposed by SARS-CoV-2 infection.

Higher levels of 4-hydroxyproline found in non-survivors are a strong indication of disturbed amino acid metabolism induced by SARS-CoV-2 infection, as already suggested [20,23–25]. In humans, 4-hydroxyproline in the blood is a product of protein degradation, mainly collagen. Most 4-hydroxyproline is recycled back to the liver and kidneys to synthesize glycine, and this seems to be an important source of glycine for cells and tissues [59]. Therefore, the significantly higher levels of 4-hydroxyproline in non-survivors can be regarded as a feature of severe COVID-19, suggesting liver dysfunction and a lower availability of glycine. This result reveals a potential disruption in the one-carbon metabolism pathway, which is significant as glycine serves as a crucial methyl donor in this pathway. This disruption has already been described previously in cells infected with SARS-CoV-2 in vitro [27] and confirmed in patients with severe COVID-19 in the present study and mild to moderate COVID-19 in another [26].

In addition to glycine, significant lower contents of serine, betaine, and histidine, which are metabolites also involved in one-carbon metabolism, were found in infected subjects. Importantly, the significant drop in choline-related metabolites (as observed in the NMR-based metabolomics), which agrees with a drop in glycerophosphocholine (by MS-based metabolomics), strengthens the idea that severe COVID-19 alters one-carbon metabolism and affects the availability of methyl donors. Indeed, a comprehensive characterization of the alterations in one-carbon metabolism would help integrate the metabolic changes in glucose, amino acid, nucleotide, and lipid metabolism [60], as well as provide a better understanding of the alterations in epigenetic regulation and redox homeostasis associated with severe COVID-19.

Phosphatidylcholine is the most abundant phospholipid, and in humans, its synthesis is probably the main point of deviation for one-carbon donors [61]. Our results show that severe COVID-19 alters lipoprotein dynamics, as indicated by lower contents of total cholesterol and HDL- and LDL-cholesterol and higher contents of VLDL and triacylglycerols, particularly in fatal COVID-19 (Figure 4). Therefore, one may speculate that the alterations in one-carbon metabolism observed in infected subjects caused a drop in choline-related metabolites, which significantly disrupted lipoprotein dynamics, as indicated by other studies [61,62]. Conversely, lower choline availability may have contributed to reduced phospholipid synthesis by the liver and disturbed lipoprotein dynamics, as choline deficiency is known to alter lipid metabolism and induce liver fibrosis [62]. Another indication of the effect of SARS-CoV-2 infection on host lipid metabolism and lipoprotein dynamics was recently shown by our group, where simvastatin, by disrupting lipid rafts in human epithelial lung cells, prevented SARS-CoV-2 entry and replication [63]. Importantly, NMR-based metabolomics has also shown that alterations in lipoprotein dynamics may be associated with the systemic effects of COVID-19, even in subjects in the recovery phase of the disease. Particularly, the HDL subfraction negatively correlates with inflammatory cytokines [64]. Additionally, apolipoprotein alterations are more pronounced in the fatal cases of COVID-19 [65].

This scenario is compatible with the CART models fitted to data where choline-related metabolites had a protective effect at ICU admission (Supplementary Figures S3 and S4). Recent evidence links choline metabolism in the liver and the gut microbiota to endothelial function and thrombosis [66,67], which is one of the clinical manifestations associated with disease progression and severe COVID-19 [68]. Indeed, choline seems to be essential to sustain mitochondrial energetics and maximal platelet activation, and, therefore, to regulate

thrombosis [69]. Additionally, CART models indicated that higher levels of *N*-acetylserine, creatine/creatinine, and hypoxanthine at admission are strong predictors of COVID-19 fatality (Supplementary Figures S4–S6). These results add to the discussion about the detrimental effects of severe COVID-19 on liver and kidney function and metabolic health.

We also found increased levels of asymmetric dimethylarginine in infected subjects, which is produced by arginine methylation. Therefore, asymmetric dimethylarginine synthesis is directly related to one-carbon metabolism for methyl donor availability. Additionally, asymmetric dimethylarginine has been strongly associated with fibrosis in the liver, kidney, and heart [70,71], tissues that are compromised in COVID-19. Therefore, these data strongly support that alterations in one-carbon and amino acid metabolism are linked to liver damage in the most severe outcomes of COVID-19.

Our study confirmed that severe COVID-19 induced important changes in amino acid metabolism, where significantly lower contents of alanine, BCAA (valine, isoleucine, and leucine), glutamine, tryptophan, glycine, and tyrosine were found in both survivors and non-survivors if compared to control subjects. Lower amino acid levels may indicate an increase in amino acid catabolism to provide the necessary supply of carbon and ATP to support viral replication [16,72,73]. For instance, isoleucine [74] and glutamine [75] seem to be essential for SARS-CoV-2 replication. In the case of glutamine, it has been shown that inhibition of glutaminolysis halted SARS-CoV-2 in primary astrocytes in a rodent model; the authors suggested that lower availability of glutamine is a contributing factor for the neurological impairments observed in long post-acute COVID-19. As observed in the plasma of infected subjects, lower contents of salivary amino acids are associated with SARS-CoV-2 infection [76]. Additionally, a decrease in plasma glutamine [77] and lower salivary levels of tyrosine and BCAA [78] seem to be associated with COVID-19 severity, which is consistent with our findings. Despite the fact that the literature shows an association between levels of circulating amino acids and COVID-19 severity, amino acid levels seem not to be good predictors of ICU admission or disease fatality [79].

Lower levels of glutamine in the plasma may indicate increased use of this amino acid, especially by the host's liver and immune cells. This increased use may be needed to support the synthesis of proteins and inflammatory mediators during the acute phase of infection. Indeed, the liver is actively involved in the synthesis of acute-phase proteins. In this context, the assigned broad signals, δ^1H = 2.04–2.08 ppm, of *N*-Acetyl glycoproteins, which were higher in severe COVID-19 subjects (Figure 2D), may include signals from the acute-phase proteins, such as α1-acid glycoprotein, α1-antitrypsin, and haptoglobin, and to the ^{1}H from sidechains of *N*-acetyl-glucosamine and *N*-acetylneuraminic acid [80]. ^{1}H signals of *N*-acetyl glycoproteins have been suggested to be markers of SARS-CoV-2 infection and inflammation and to be implicated in long post-COVID symptoms as well [81]. Indeed, enrichment analysis of genes associated with cases of severe COVID-19 indicates acute phase response and inflammation among the most significant biological functions altered, according to a recent multi-omics analysis of COVID-19 datasets [82].

Other authors have shown that liver infection by SARS-CoV-2 directly contributes to hepatic dysfunction and that deceased subjects often presented abnormal liver enzymes, microvesicular steatosis, and mild inflammatory infiltrates in the hepatic lobule and portal tract [83,84]. The increase in purine metabolites (xanthine and hypoxanthine) may also be regarded as a direct effect of SARS-CoV-2 infection in the liver that enables virus replication. Higher levels of deoxycytidine, associated with increased viral load, have already been associated with severe and fatal outcomes [85]. Accordingly, the liver of patients with severe COVID-19 shares many similarities with that of non-alcoholic fatty liver disease (NAFLD), a common manifestation of the metabolic syndrome that can progress to hepatocyte injury, inflammation, and fibrosis [86,87].

Lastly, the higher levels of acetoacetate and ß-hydroxybutyric acid observed in infected subjects also reflect the impact of severe COVID-19 on liver function. Dysregulation in ketogenesis is also associated with the pathogenesis of NAFLD and decreased insulin sensitivity, an important manifestation of the metabolic syndrome. In this scenario, en-

hanced ketogenesis seems to be a consequence of increased influx and accumulation of lipids in the liver (e.g., triacylglycerols), which in turn results in an increased flux of Acetyl-CoA [62]. Metabolites' profiles in the urine of COVID-19 subjects are also compatible with enhanced ketogenesis, as shown by higher excretion of carnitine and acetone in the acute phase of the disease compared to the recovery period and control subjects [88]. In addition, our group has recently pointed out the central role of the acetyl-CoA pathway in the immunometabolism response associated with the Sinovac vaccine [89].

The metabolic disturbances observed in COVID-19 subjects seem to be influenced by the SARS-CoV-2 variant and clinical presentation [90], which highlights the novelty of the results presented in this study. Interestingly, there have been some suggestions that the metabolic disturbances observed in hospitalized subjects with COVID-19 are dependent on the wave of the infection [91]. In this regard, arginine and threonine were altered in the early wave (between May and July 2020) but not in the latter wave (September 2020 to June 2021) of COVID-19 in that particular study. On the other hand, a machine learning approach identified the same pattern of change in glutamate, aspartate, glycolithocholic acid, and methionine sulfoxide across both waves of COVID-19, corroborating our results.

Our results show that alterations in key metabolites, such as choline metabolites, creatinine/creatinine, ^{1}H signals of *N*-acetyl of glycoproteins, and acetoacetate, as well as changes in lipoproteins, are greater in women. Current evidence indicates that men are more vulnerable to severe COVID-19, and higher mortality rates have been observed in this category [92]. Male subjects presented significant alterations in the tryptophan-kynurenine pathway and plasmalogen, which were associated with increased inflammation and stress biomarkers [93,94]. On the other hand, women seem to be more susceptible to developing post-acute COVID syndrome [95]. Therefore, our study highlights the importance of considering the sex-based differences here described when monitoring and treating COVID-19 patients.

5. Conclusions

This is the first study to demonstrate host metabolic disturbances associated with severe COVID-19 and to investigate the responses in the fatal cases of the disease in hospitalized subjects in Rio de Janeiro during the early months of the COVID-19 pandemic in Brazil. While our study provides new insights into the metabolic disturbances associated with fatal outcomes of COVID-19, one limitation is its reduced sample size. We identified that severe COVID-19 dysregulates one-carbon, lipid, and amino acid metabolism and lipoprotein dynamics. The higher contents of creatine/creatinine, 4-hydroxyproline, gluconic acid, and *N*-acetylserine observed in the fatal COVID-19 outcome may be associated with uncontrolled inflammation, multi-organ dysfunction, and some degree of insulin resistance. Since the incidence of severe outcomes after hospital discharge can be very high in Brazil [28], these metabolic alterations may be considered to improve our understanding of the pathophysiology of post-acute COVID-19 syndrome, as already suggested for the metabolic alterations associated with post-acute cardiovascular events [96]. Additionally, the sex differences observed in our study should also be considered when designing strategies for pandemic surveillance. Doing so may lead to better disease stratification and improved patient outcomes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/metabo13070879/s1. Table S1: Assignment table of metabolites that discriminated in the PCA and univariate analyses; Table S2: Assignment table of broad signals of compounds that discriminated in the PCA and univariate analysis; Table S3: Parameters used for untargeted analysis in MS-DIAL software; Table S4: List of the target compounds monitored in the method at pH 8 [97]; Table S5: List of the target compounds monitored in the method at pH 3; Table S6: Assigned metabolites with significant differences among groups according to the non-targeted MS-metabolomics in the negative mode; Table S7: Assigned metabolites according to the non-targeted MS-metabolomics in the positive mode; Figure S1: PCA loading factors plot highlights the discriminant metabolites for the separation of groups according to ^{1}H NMR-based metabolomics; Figure S2: PC1

score plot versus sample in run order at pH 8 and pH 3 analyses, indicating the stability of the system throughout the analytical run; Figure S3: Classification and Regression Tree (CART) model indicates that $(CH_3)_3$-choline related metabolites and creatine/creatinine present high predictive power in assigning subjects to their morbidity class; Figure S4: Classification and Regression Tree (CART) model indicates that $(CH_3)_3$-choline related metabolites and *N*-acetylserine present high predictive power in assigning subjects to their morbidity class; Figure S5: Classification and Regression Tree (CART) model indicates that hypoxanthine and *N*-acetylserine present high predictive power in assigning subjects to their morbidity class; Figure S6: Classification and Regression Tree (CART) model indicates that *N*-acetylserine presents high predictive power in assigning subjects to their morbidity class.

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Informed Consent Statement: Written informed consent has been obtained from the patients or their caregivers to publish this paper.

Data Availability Statement: The NMR data presented in this study was assigned using COLMARm, https://spin.ccic.osu.edu/index.php/colmarm, session ID 3121-pZ5ZukwXBh, (accessed on 14 July 2023). The raw NMR and MS data presented in this study are available on request from the corresponding author. The data are not publicly available as multivariate analyses are being performed.

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3.2 Chapter II – Severe COVID-19 Induces Metabolic Alterations in Pregnancy

In this section, we present the results corresponding to the specific objectives outlined in Chapter 2. Using serum samples from a cohort of pregnant women infected with SARS-CoV-2, we aim to identify metabolic alterations and pregnancy outcomes related to disease severity. These analyses will contribute to the understanding of the effects of infection in this group and help identify predictors for clinical outcomes, placental responses and long-term health effects of the infection to the mother and the offspring.

The following results, from second chapter, was written in the format for publication in the journal Frontiers of Physiology. The manuscript will be sent for publication in due course.

frontiers

1H NMR Serum Metabolomics of Severe COVID-19 During Pregnancy Reveals Metabolic Changes Similar to Diabetes and indicative of placental dysfunction

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Abstract

Pregnancies complicated by Coronavirus Disease 2019 (COVID-19) are at an increased risk for severe morbidity due to physiological changes in the immune, cardiovascular, and respiratory functions. Despite that, there are very few studies on the metabolic effects of severe COVID-19 during pregnancy. Maternal infections, including COVID-19, pose long-term developmental risks for offspring. This study investigates the metabolic changes in pregnant women with COVID-19 and their correlation with disease severity. Using ^{1}H NMR-based metabolomics, we identified significant alterations in insulin sensitivity, lipid metabolism, and inflammation markers in the severe cases. Pregnant women with severe COVID-19 showed higher levels of glucose, lactate, triacylglycerol, LDL, VLDL, and 1-carbon metabolites like betaine and glycine. Additionally, severe cases exhibited increased levels of citrate and acetoacetate, indicating a metabolic shift due to inflammation. This study highlights the metabolic reprogramming in severe COVID-19 during pregnancy, similar to gestational diabetes, and underscores the importance of monitoring maternal and neonatal health. These findings contribute to understanding the metabolic impact of COVID-19 on pregnant women and the potential long-term effects on their offspring's neurodevelopment.

Introduction

COVID-19 during pregnancy has been associated with various obstetric complications, including decreased fetal movement, intrauterine fetal distress, anemia, and preterm labor (1). Emerging data suggest a higher risk for preeclampsia, characterized by hypertension and potential organ damage after 20 weeks of gestation, among pregnant women with SARS-CoV-2 infection (2,3). Observational

studies indicate that pregnant women with COVID-19 have significantly higher odds of developing preeclampsia compared to those without the infection (4,5) Additionally, strong evidence indicate increased severe neonatal complications and disparities in the access to healthcare among different locations, exacerbating these risks (6). The impact of COVID-19 Pandemic was more pronounced in low-income countries, which already faced higher maternal morbidity and mortality rates (7). Neonates from infected mothers may exhibit disrupted immune functions, indicating the need for careful monitoring of maternal and neonatal health (8,9). Studies have reported increased prematurity, low birth weight, and neonatal intensive care unit (ICU) admissions, particularly in cases of severe maternal illness (10,11).

The initial case series of pregnant women with COVID-19, reported in March 2020, indicated clinical manifestations similar to the general population: 86% mild infection, 9.3% severe pneumonia, and 4.7% acute respiratory distress syndrome (ARDS)(12). Indeed, a prospective national cohort study from the UK published in June 2020 revealed similar severe illness rates requiring ICU admission and resulting in similar rates of mortality for pregnant women and the general female population of reproductive age (13). Symptomatic COVID-19 cases requiring hospital admission in pregnant women are primarily concentrated in the third trimester (13,14). On the other hand, later in September 2020, a meta-analysis showed that pregnant women with COVID-19 were more likely to be admitted to the ICU, to require mechanical ventilation, and to present higher all-cause mortality when compared to non-infected pregnant women. Regarding unfavorable outcomes to the newborn, the odds of stillbirth and ICU admission also increased significantly (15).

The primary impacts of COVID-19 on pregnancy include increased risk of severe clinical progression, extended hospital stays, maternal mortality, poor maternal/fetal perfusion, thromboembolic disease, hypertensive disorders, higher cesarean section rates, preterm births, and mental health issues (16).

Risk factors for severe disease manifestations and death in pregnant women mirror those of the general population, including advanced age, obesity, hypertension, diabetes, chronic pulmonary and cardiovascular diseases, and chronic kidney disease (10). In Brazil, the maternal mortality rate increased fivefold during 2020-2021, with a 7.2% COVID-19 fatality rate among pregnant women (17). The ongoing prevalence of SARS-CoV-2 and the emergence of new variants continue to impact pregnant women significantly (18).

Viral infections during pregnancy can compromise fetal development and long-term health for both the mother and offspring (19,20). Maternal infections, such as encephalitis, pneumonia, influenza, pyelonephritis, during pregnancy, have long-term neurodevelopmental implications for offspring, potentially leading to disorders such as autism and schizophrenia (21,22). Pregnancy induces physiological changes, such as increased cardiac output and hypercoagulability, raising the risk of complications (23). Additionally, pregnancy-related metabolic shifts, like hyperinsulinemia and hyperglycemia, heighten susceptibility to severe outcomes from infections, including SARS-CoV-2 (24,25). Pregnant women are at higher risk of severe illness from infections like H1N1, SARS, and MERS (26,27).

The multisystemic nature of SARS-CoV-2 necessitates an integrated biological approach to understand COVID-19 pathogenesis, with metabolomics offering insights into metabolic disruptions in organs and fluids, essential for viral replication and immune modulation (28–30). Metabolic disorders like obesity and diabetes are associated with severe COVID-19 outcomes. Therefore,

understanding the pathways involved might help to underpin the molecular mechanisms associated with the pathophysiology of COVID-19

Previous studies on metabolomic analysis of plasma from pregnant women with COVID-19 reported dramatic lipidomic and proteomic changes, with an increase in the acute phase response proteins, serine protease inhibitors, complement factors, and dysregulated lipid metabolism e.g. apolipoproteins, phosphatidylcholines, and acylglycerols (31). While pregnant women exhibiting elevated anti-SARS-CoV-2 antibody titers, reduced plasma concentrations of acetate and urea (32). Investigation on COVID-19 in effects on maternal-placental-fetal interaction discovered increased levels of lysophospholipids, triglycerides, sphingomyelins, and oxidized lipids were in maternal and cord plasma after COVID-19 infection, with the increased being more noticeable when the infection occurred in the third trimester (33).

Despite the extensive literature on the effects of COVID-19 in the general population, the metabolic changes that occur in women during pregnancy and the potential risks of COVID-19 during pregnancy on maternal and neonatal outcomes are still poorly understood, in particular changes related to placental responses during SARS-CoV-2 infection. Therefore, this study aim to comprehensively investigate the metabolic profile and alterations in pregnant women affected by COVID-19 and to assess the associated risks posed by the disease severity during pregnancy.

Materials and Methods:

Study Design and Participants

We prospectively enrolled a cohort of adult (≥19 years of age) pregnant women (n=85) at two referral centers in Rio de Janeiro: (a) A total of 72 subjects who presented for COVID-19 testing at the Center for the Study and Management of Emerging and Re-emerging Infectious Diseases (NEEDIER) located at Federal University of Rio de Janeiro (UFRJ), from March 2020 to May 2022. The NEEDIER-UFRJ was established as a screening and diagnostic center for COVID-19, offering diagnostic testing for COVID-19 to public health service workers, public safety professionals, and students and staff of UFRJ; (b) A total of 13 subjects presenting severe COVID-19 at the intensive care unit (ICU) of the São Sebastião State Institute of Infectious Diseases (IEISS) located at São Sebastião Hospital in Rio de Janeiro, between July and December 2021. Severe COVID-19 was defined as critically ill patients presenting with viral pneumonia confirmed by the presence of chest infiltrates and by the need for respiratory support with either non-invasive oxygen supplementation or mechanical ventilation, classified according to the WHO working group on the clinical characterization and management of COVID-19 (84).

For all individuals, RT-PCR was performed on nasopharyngeal swab samples, and serological evaluation was conducted on blood samples only for the severe group. Nasopharyngeal samples were collected from both nostrils using a rayon-tipped swab in each nostril. These samples were stored in Modified Eagle's Medium (DMEM; ThermoFisher Scientific) at 4°C until transport to the laboratory, where they were then stored at -80°C (average time of 2 hours until storage). Blood samples for serology were collected by venipuncture from a peripheral vein, placed in gel collection tubes with a clot activator, centrifuged, and frozen until testing.

Pregnant women were divided into three groups: symptomatic negative for SARS-CoV-2 (n=55), characterized by nonspecific acute respiratory illness; symptomatic non-severe positive for SARS-CoV-2 (n=17); and severe positive for SARS-CoV-2 (n=13). Blood samples were obtained from the individuals at the time of testing or upon admission to the intensive care unit (ICU). Clinical,

laboratory, and sociodemographic data were recorded upon admission for all patients included in the study. This study was approved by the Ethics Committee of Clementino Fraga Filho University Hospital (CAAE: 30161620.0.1001.5257), and informed consent was signed by all participants or their guardians at the time of inclusion in the study.

Information regarding maternal and neonatal outcomes and overall health status of the participant of this study were obtained from the medical charts (for in-patients) and also from active search through structured questionnaires using the Kobo toolbox (supplementary material 1).

Blood and sample processing

Blood samples were drawn into gel collection tubes with a clot activator and centrifuged at 200 × g, 20 min, room temperature. Serum samples were collected and stored at −80 °C until analysis.

Nuclear Magnetic Resonance-Based Metabolomics

Sample Preparation

Frozen serum samples were quickly thawed and diluted 3-fold in sodium phosphate buffer and deuterium oxide (final concentration: 50 mM phosphate buffer and 10% deuterium oxide, pH 7.4). A total of 600 µL of diluted samples were transferred into a 5 mm NMR tube.

NMR Acquisition, Spectra Pre-Processing, and Metabolite Assignment

NMR spectra were acquired on a Bruker Advance III at 500.13 MHz at 300 K, coupled with a cooled automatic sample case at 280 K. 1D-1H NMR spectra were acquired using excitation sculpting to suppress the solvent signal as well as a CPMG (Carr-Purcell-Meiboom-Gill) T2 filter with 32 loop counters and a delay of 0.001 s. 32,768 complex data points were acquired per transient, for a total of 1024 transients. The spectral width was set to 19.99 ppm, resulting in an acquisition time of 3.27 s per FID. The relaxation delay was set to 1.74 s.

Spectra data were pre-processed in the MetaboLab software v. 2022.0726.1733 (34). Prior to the Fourier transform, the FIDs were apodized using an exponential window function with 0.3 Hz line-broadening and then zero-filled to 65,536 data points. After Fourier transform, each spectrum was manually phase corrected, followed by a spline-baseline correction. Finally, all spectra were referenced to the signal of the 1H linked to the anomeric carbon of glucose. Baseline noise and regions corresponding to water and ethanol signals were deleted. Spectra data were binned with a 0.005 ppm interval, and the resulting table presented 81,498 data points, corresponding to metabolites' intensities. Pareto scaling was applied prior to multivariate statistical analysis.

Following 2D spectra, HSQC 1H-13C and TOCSY 1H-1H acquisition, data was uploaded on the COLMAR (35,36) for the assignments. Supplementary Tables S1 and S2 present the 1H NMR assignment information for the metabolites and broad signals of lipids and proteins that distinguished the groups. For manual profiling, samples were analyzed using the Chenomx NMR Suite version 8 (Chenomx, Inc. Alberta, Canada) using the software-provided 500 MHz compound library. We also overlaid spectra from the HMDB 6.0 databanks (37). The software ICON NMR (Bruker) was used for automatic acquisition.

overlaid spectra from the HMDB 6.0 databanks (37). The software ICON NMR (Bruker) was used for automatic acquisition.

Statistical Analysis

The sample size was determined by the feasibility of recruitment and eligibility criteria.

Data distribution was analyzed using the Shapiro-Wilk test and median and inter-quartile intervals, and the Mann-Whitney or Kruskal-Wallis tests were used for data with an asymmetrical distribution. Categorical variables were compared using the Fisher's exact test with absolute (n) and relative (%) frequencies.

Data derived from processed NMR spectra was subjected to multivariate Principal Component Analysis using the MetaboAnalyst 6.0 platform. For the univariate statistics, Kruskal-Wallis and Dunn's post-hoc tests were used for variables' comparison of non-transformed NMR or MS data. GraphPad Prism version 8.4.3 was used for all analyses. $p < 0.05$ was considered for rejection of the null hypothesis.

Results:

Subjects Demographics and Clinical Parameters

A total of 85 individuals were included in this study: 55 negative for SARS-CoV-2 subjects, presenting symptoms of acute respiratory illness (negative group), and 30 symptomatic positive for SARS-CoV-2, grouped into non-severe (positive group; n = 17) and severe cases (severe group; n = 13) according to the WHO working group on the clinical characterization and management of COVID-19. Age, gestational trimester and symptoms of the subjects included in the study are shown in Table 1. Notably, there were two deaths in the severe group, while no deaths occurred in the other groups. Body Mass Index (BMI) was similar between severe and positive, while in severe group the weight was not recorded in the ICU admission. The prevalence of symptoms is shown for the negative and positive groups only as the severe group was already in a critical state. Age, prevalence of comorbidities, and gestational trimester did not differ among groups. However, regarding the gestational trimester, it is worth noting that in the negative and positive groups, about 35% of the pregnant women were in the first trimester, while in the severe group only 8% were in the early stages of pregnancy. This may indicate an increased risk of severe outcomes when the disease occurs later in pregnancy. Regarding the presence and frequency of symptoms, there was also no clear difference between groups, except for cough, which is a well-described symptom of SARS-CoV-2 infection and was more prevalent in subjects with COVID-19, at 76%, compared to the symptomatic negative group, at 53%. These data reinforce that pregnant women with a negative result for SARS-CoV-2 represent a group with symptoms of nonspecific acute respiratory syndrome.

Table 1: Characteristics and symptoms prevalence of pregnant women participating in the study

	Negative (n=55)	Positive (n=17)	Severe (n=13)	P value
Age, years	30 (17-41)	30 (20-42)	26 (17-39)	0.189
BMI [n]	26.98 (19.75-38.54) [23]	26.16 (21.16-31.24) [6]	-	0.813
Gestational Trimester				
1°	39% (21)	35% (6)	8% (1)	0.199
2°	37% (21)	41% (7)	54% (7)	0.533
3°	24% (13)	24% (4)	39% (5)	0.740
Deceased	0% (0)	0% (0)	15% (2)	-
Vaccine	25% (14)	35% (6)	46% (6)	0.379
GDM or Hypertension	7.3% (5)	0% (0)	15% (2)	0.532
Fever	15% (8)	24% (4)	-	0.459
Cough	53% (29)	77% (13)	-	0.098
Dyspnea	25% (14)	18% (3)	-	0.545
Diarrhea	20% (11)	35% (6)	-	0.326
Headache	67% (37)	71% (12)	-	0.965
Asthenia	58% (32)	59% (10)	-	0.969

Continuous variables are represented as median and interquartile range, and categorical variables are represented as frequency % (n). Selected the most prevalent symptoms in the study participants, relevant to acute respiratory syndrome. Comparisons were made using ANOVA for parametric distributions and Mann-Whitney U or Kruskal-Wallis for non-parametric distributions.

In Table 2 we show the COVID-19 persistent symptoms, pregnancy complication and newborn characteristics, based on the answer given by the participants who responded to our contact for interview. Persistent COVID-19 symptoms were present in both the positive and severe groups, with all severe cases reporting fatigue, memory loss, and headache. COVID-19 vaccination rates were high in all groups, with 100% vaccination in the negative and positive groups, and 60% in the severe group. Pregnancy complications were reported in all groups, with hypertension being consistently present in two cases per group. Preeclampsia was observed in two cases in the negative group and one in the positive group but was absent in the severe group. Gestational diabetes mellitus was reported in each group, with the highest occurrence in the positive group (n=2). Labor complications were highest in the positive group (n=3). Newborn outcomes indicated that the severe group had heavier (4.01 kg) and taller (52 cm) babies on average. Breastfeeding duration was longest in the severe group, averaging 13 months.

Table 2: Maternal and neonatal characteristics and pregnancy outcomes of the participants of the study.

	Negative (n=16)	Positive (n=11)	Severe (n=3)
COVID-19 Persistent Symptoms (n)	0	3	3
Fatigue	0	3	3
Memory Loss	0	1	3
Muscle Pain	0	1	0
Headache	0	1	3
Depression or Anxiety	0	0	0
Pregnancy Complication (n)	7	7	3
Hypertension	2	2	2
Preeclampsia	2	1	0
Gestational Diabetes Mellitus	1	2	1
Labor Complication	2	3	0
Newborn (n)	16	11	2
Weight (kg)	3.36 (2.58-4.31)	3.32 (2.75-3.85)	4.01 (3.95-4.10)
Height (cm)	50 (47-52)	50 (38-52)	52 (51-54)
Sex Female:Male	9:7	6:5	1:1
Breastfeeding (months)	9.5	9	13

Information gathered from interviews of the participants who responded to our contact.

Descriptive analysis of laboratory findings at the admission to the ICU revealed that pregnant women with severe COVID-19 showed notable abnormalities (Table 3). Within the blood count, red blood cell count was within the normal range with no inadequacies. Hemoglobin and hematocrit levels, although largely normal, 20% women showed inadequacy. Leukocytes and platelets were also mostly within reference values, though 30% of the cases were inadequate. Coagulation parameters such as INR, prothrombin activity time, and prothrombin activity were within normal values, but the partial thromboplastin time was inadequate in 33% of the cases. The D-dimer levels were elevated in all women where this information was collected, indicating possible risk for thrombosis, which is a common trait of COVID-19. Blood glucose, total serum protein, and urea levels were normal, while albumin concentration was inadequate in 25% of women. Notably, fibrinogen levels were elevated in all cases, indicating an acute phase inflammatory response. C-reactive protein (CRP) was significantly elevated in 86% of cases, reflecting inflammation. Liver function tests indicated elevated levels of direct bilirubin, aspartate aminotransferase (AST), alanine transaminase (ALT), and gamma-glutamyl transferase (GGT) in 43% of cases. Alkaline phosphatase was elevated in 80% of cases. Enzymes such as amylase and lipase were significantly elevated in 1 case, suggesting possible pancreatic involvement. Bicarbonate levels were inadequate in 54% of cases, and lactate dehydrogenase (LDH) was elevated in all tested cases, further indicating tissue damage or inflammation. The anion gap was elevated in 66% of cases, suggesting metabolic acidosis. Electrolytes such as sodium and potassium were within normal ranges, but calcium was inadequate in 33% of cases. Magnesium and phosphorus levels were normal. Overall, these findings highlight significant inflammation, possible liver and pancreatic involvement, and a potential for metabolic acidosis in severe COVID-19 cases among pregnant women.

Table 3: Laboratory parameters associated with severe group

Severe (n)	Mean	Reference Value	Inadequancy
Hemoglobin (10)	11.5 g/dL	11-14.9 g/dL	20%
Hematocrit (10)	34.20%	33 - 44.1%	20%
Leukocytes (10)	7292 mm3	5.000-12.000/mm^3	30%
Platelets (10)	208,340 mm3	150,000-450,000/mm^3	30%
INR (4)	1.058	0.8-1.5	0%
Prothrombin Activity Time (3)	10.87 s	10-14 s	0%
Prothrombin Activity (2)	91.50%	>70%	0%
Partial Thromboplastin Time (3)	33.1 s	25-33 s	33%
D-dimer (2)	1716 ng/mL [FEU]	< 500ng/mL {FEU]	100%
Blood Glucose (3)	113 mg/dL	<140 mg/dL	0%
Total Serum Protein (4)	6.688 g/dL	6-8 g/dL	0%
Albumin (4)	3.373 g/dL	3-5 g/dL	25%
PCR (7)	12.39 mg/dL	0,3-1 mg/dL	86%
Urea (10)	14.2 mg/dL	10-45 mg/dL	0%
Creatinine (10)	0.53 mg/dL	0.5-1.2 mg/dL	0%
Uric Acid (4)	4.165 mg/dL	2.4-6 mg/dL	25%
Total Bilirrubin (7)	0.5586 mg/dL	<1.2 mg/dL	0%
Direct Bilirrubin (5)	0.346 mg/dL	<0.3 mg/dL	0%
Aspartate Aminotransferase (9)	31.33 U/L	5-40 U/L	22%
Alanine Transaminase (9)	21.22 U/L	5-30 U/L	11%
Gamma-glutamyl Transferase (7)	38.43 U/L	9-36 U/L	43%
Alkalyne Phosphatase (5)	229 U/L	40-120 U/L	80%
Creatine Phosphokinase (3)	47.67 U/L	30-170 U/L	0%
MB Creatine Phosphokinase (2)	8.5 U/L	< 24 U/L	0%
Bicarbonate (11)	20.7	22-26 mEq/L	54%
Lactate Dehydrogenase (3)	358 U/L	135-214 U/L	100%
Lactate (8)	0.72 mmol/L	0,63-2,44 mmol/L	38%
Anion Gap (9)	11.4 mEq/L	8-12 mEq/L	66%
Sodium (8)	136.4 mEq/L	135-145 mEq/L	0%
Potassium (8)	3.775 mEq/L	3,5-5,5 mEq/L	0%
Calcium (3)	8.333 mg/dL	8.6-10 mg/dL	33%
Magnesium (5)	1.86 mg/dL	1.6-2.6 mg/dL	0%
Phosphoforus (5)	3.52 mg/dL	2.3-4.3 mg/dL	0%
Chloride (2)	104 mEq/L	20-295 mEq/L	0%

Laboratory test performed on a blood sample collected at the time of admission of severe patients to the intensive care unit. Reference value relative to pregnant women, or to women in eutrophic conditions. Inadequacy refers to the percentual of individuals of a particular parameter falling outside the established reference range or normal values.

^{1}H NMR -Based Metabolomics

Representative spectra of aliphatic (Figure 1A,B), amidic, and aromatic (Figure 1C) regions indicate important differences in the metabolite profile among the severe, positive and negative groups. Discriminating metabolites such as glucose, -(CH=CH) olefinic protons of triacylglycerol, broad residual signals of –(CH2) and –(CH3) of lipoproteins, N-acetyl 1H of glycoproteins, acetoacetate, and aromatic amino acids such as tryptophan are depicted on Figure 1 A-C, followed by a confirmation of discriminating metabolite profile in the Principal Component Analysis (PCA) score plot in Figure 1 D, where principal components 1, 2, and 3 accounted for approximately 85%. Figure E shows a heatmap of all identified metabolites clustered using Hierarchical Clustering, indicating a significant different clustering profile for the severe group. The PCA loading factor indicated lactate, residual signals of lipids, 3-hydroxykynurenine, N-acetyl of glycoprotein and betaine as discriminating variables (Fig. 1F).

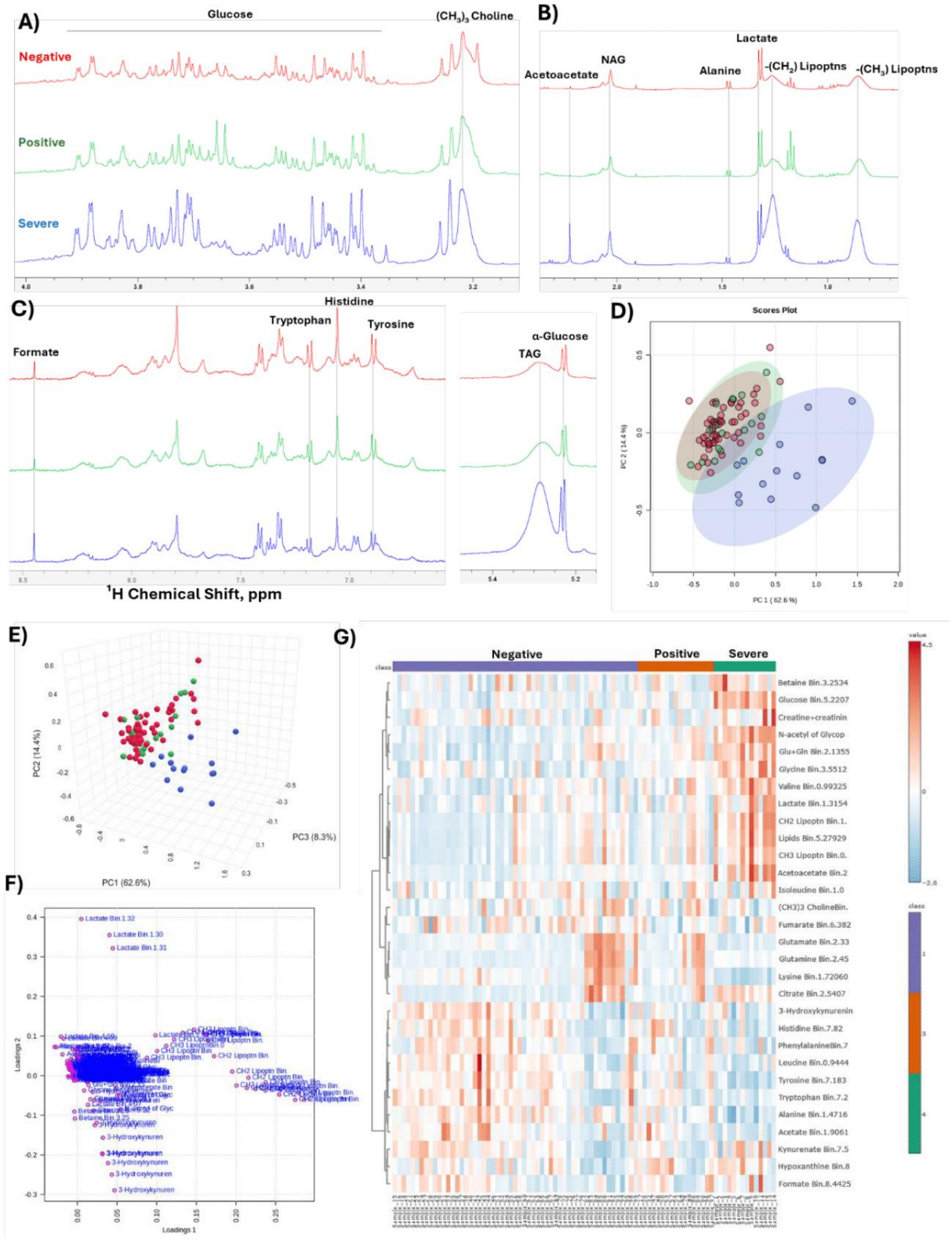


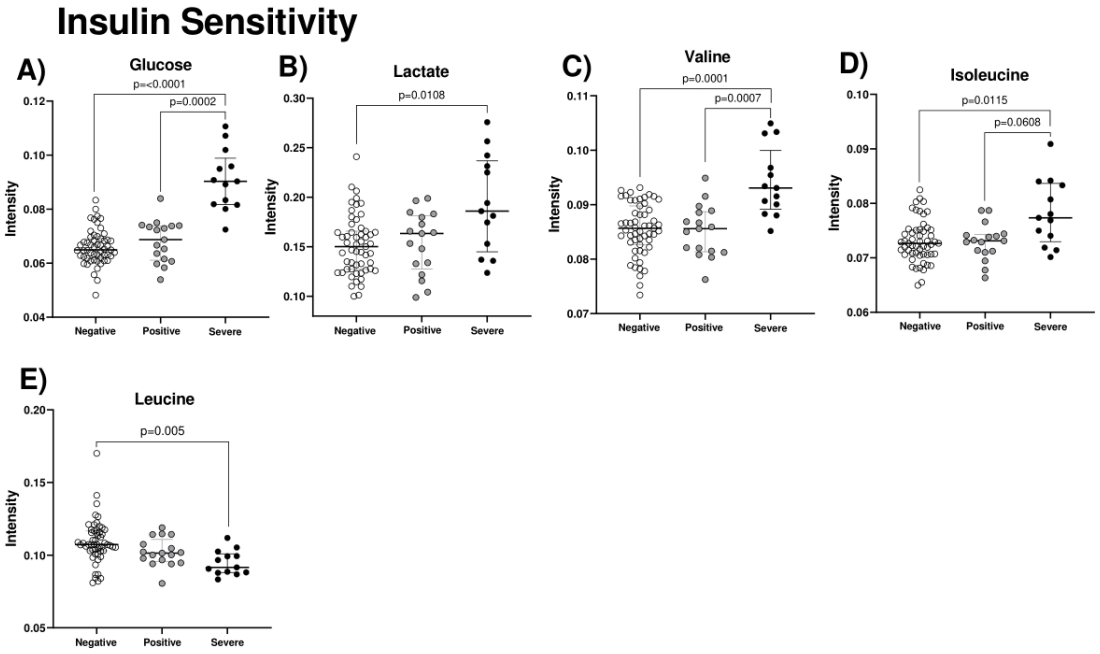
Figure 1. [1]H NMR-based metabolomics shows different plasma metabolite profiling in severe COVID-19 pregnant women compared to negative and positive subjects. 1H NMR representative spectra of negative (red), positive (green), and severe (blue). Metabolites that differ significantly among groups are indicated in representative spectra; **(A,B)** aliphatic region; **C)** amidic and aromatic regions; **D)** Principal Component

Analysis 3D score plot shows 85% discriminating profile for principal components 1, 2, and 3 among groups; negative (red), positive (green), and severe (blue); **E)** Heatmap of all identified metabolites, each column represents a sample and each row represents the variation in the specific metabolite; **F)** PCA loading factors plot highlights the discriminant metabolites for the separation of groups according to 1H NMR-based metabolomics; The fold change from the overall mean concentration are shown in a color-coded manner, with blue representing a decrease and red an increase.

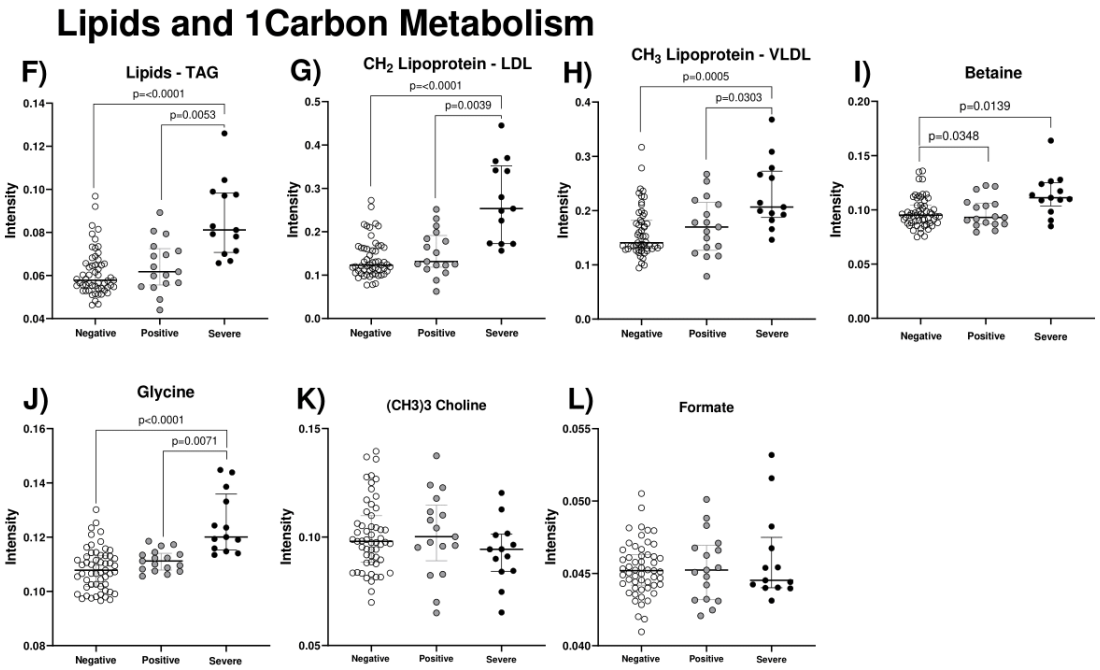
To elucidate the changes associated with disease severity and outcome, we next performed univariate analysis, to identify the molecular pathways that are disrupted during the progression of the disease. For the majority of metabolites identified, the biggest changes were in the group of pregnant women compared to the negative and positive groups, indicating that severe COVID-19 during pregnancy imposes a particular metabolite profile and also that the metabolic alterations of non-severe COVID-19 are, in, in many aspects, similar to a non-specific acute respiratory infection.

Data from NMR-based metabolomics indicate that the severe group exhibits significantly higher levels of metabolites related to insulin sensitivity glucose, lactate, valine and isoleucine compared to the negative group, while only leucine presented lower levels (Figure 2 A-E. Considering the changes in lipoproteins and metabolites related to lipids and 1 carbon metabolism, the severe group shows significantly higher levels of triacylglycerol, LDL, and VLDL and glycine compared to the negative and positive groups, indicating disruptions in lipid metabolism with severe COVID-19 (Figure 2 F-H). Interestingly, betaine levels were higher in severe COVID-19 and lower in the non-severe positive compared to the negative group (Figure 2I). While (CH3)3 choline-related metabolites and formate were similar among the groups (Figure 2 K,L).

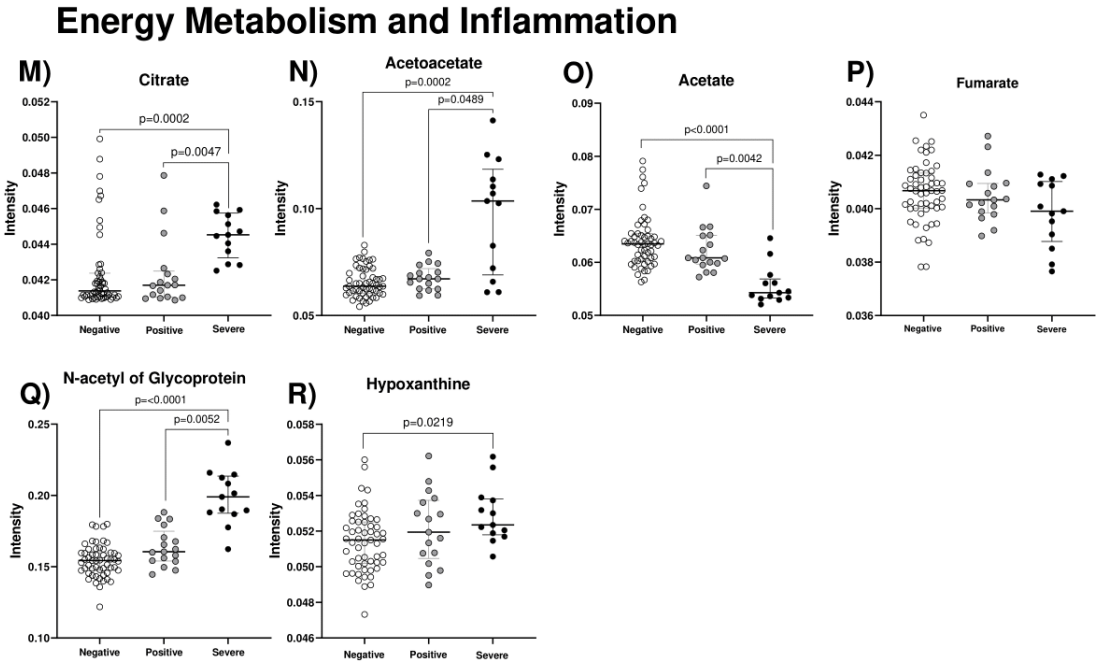
Additionally, metabolites related to energy metabolism and inflammation seem to be significantly altered in pregnant women from severe group. The severe group have higher levels of citrate, acetoacetate, N-acetyl of glycoprotein compared to the negative and positive groups (Figure 2 M,N and R). Conversely, they also present lower levels of acetate (Figure 2 O and P). Hypoxanthine levels were higher in severe COVID-19 compared only to the negative group (Figure 2R). 1H NMR-metabolomics findings also suggest that the dysregulation in amino acid metabolism indicating a potential damage to some tissue in severe COVID-19, as severe patients had plasma levels of lysine, alanine, histidine, tyrosine, tryptophan and derivates, phenylalanine and creatine+creatinine lower than the observed in positive and negative group, only glutamine+glutamate were higher in severe group than the other two groups (Figure 2 S-AB).



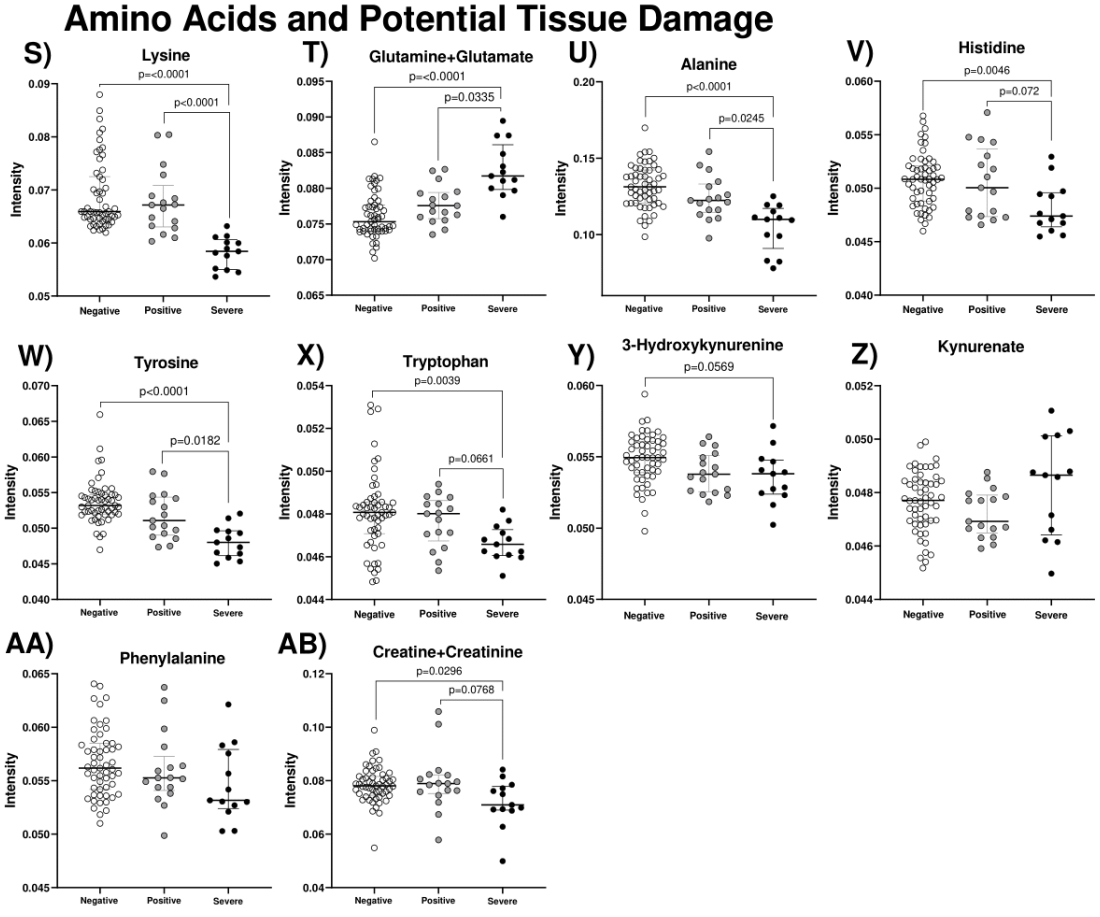
299



300


Energy Metabolism and Inflammation
M)
Citrate
p=0.0002
p=0.0047
N)
Acetoacetate
p=0.0002
p=0.0489
O)
Acetate
p<0.0001
p=0.0042
P)
Fumarate
Q)
N-acetyl of Glycoprotein
p=<0.0001
p=0.0052
R)
Hypoxanthine
p=0.0219
Intensity
Negative
Positive
Severe

301


Amino Acids and Potential Tissue Damage
S)
Lysine
p=<0.0001
p<0.0001
T)
Glutamine+Glutamate
p=<0.0001
p=0.0335
U)
Alanine
p<0.0001
p=0.0245
V)
Histidine
p=0.0046
p=0.072
W)
Tyrosine
p<0.0001
p=0.0182
X)
Tryptophan
p=0.0039
p=0.0661
Y)
3-Hydroxykynurenine
p=0.0569
Z)
Kynurenate
AA)
Phenylalanine
AB)
Creatine+Creatinine
p=0.0296
p=0.0768
Intensity
Negative
Positive
Severe

302

303

Figure 2: Metabolites that were identified in the three groups, according to 1H NMR-based metabolomics. Most discriminating metabolites according to PCA loading factors, presenting significant differences in the severe group. Negative, n = 55 (white circles); Positive, n = 17 (grey circles); Severe, n = 13 (black circles). **A-G** Metabolites related to insulin sensitivity: glucose, lactate, valine, isoleucine, leucine, citrate and fumarate; **H-M** metabolites related to lipids and 1 carbon metabolites: triacylglycerol, LDL, VLDL, betaine, glycine and choline; **N-U** Energy metabolism and inflammation related metabolites: acetate, n-acetyl of glycoproteins, acetoacetate, formate, hypoxanthine, tryptophan, 3-hydroxykynurenine, kynurenate; **V-AB** Metabolites identified as amino acids and potential markers of tissue damage: lysine, glutamine+glutamate, alanine, histidine, tyrosine, phenylalanine, creatine+creatinine. Data were presented as medians with an interquartile range, and only significant and trend P values are shown, according to Kruskal-Wallis and Dunn's post-hoc tests.

Discussion:

Infection during pregnancy, in particular infection caused by viruses, make up the list of stressors that can compromise the development of the fetus with impacts on the long-term health of the offspring and also the mother. Therefore, the investigation of maternal determinants and specific changes caused by the infection during pregnancy is extremely important for the management of the disease through individualized assistance to pregnant women.

As pregnancy is a period marked by physiological and immunological adaptations essential for adequate fetal development, SARS-CoV-2 infection increases the risk of complications for maternal and fetal/neonatal health. Complications that can potentially have long-term health consequences. However, little is known about how the severity of COVID-19 affects the metabolic profile of pregnant women and how this differs from disease progression in the non-gravid state. This study aims to identify metabolic differences in pregnancy with and without COVID-19 and to evaluate metabolic variations corresponding to the severity of COVID-19 infection, clinical parameter and demographical characteristics from the pregnant cohort. In summary our findings demonstrated that pregnant women with severe COVID-19 infection had significant changes in metabolites related to insulin sensitivity, to serum lipids metabolism and to inflammation markers. While non-severe COVID-19 infection presented similar metabolic profile as a non-specific acute respiratory infection symptoms.

Notably, in the demographic characteristics of our cohort, only 8% of the severe COVID-19 group were in the first trimester of pregnancy, whereas the positive COVID-19 group exhibited an even distribution across all gestational trimesters. Our findings align with current evidence suggesting that the frequency of COVID-19 infection is generally consistent throughout gestation. While regarding symptomatic cases requiring hospital admission, literature points that they are predominantly concentrated in the third trimester, approximately around 34 weeks (29-38 weeks) (13,14).

During the ICU hospitalization, 2 deaths among the 13 severe cases were registered, resulting in a mortality rate of 15% in this group. There were no registered deaths in the other two groups, at least according to the information obtained from the active search. To notify global maternal mortality rates accurately among pregnant women with COVID-19 is challenging due to pre-existing disparities in maternal healthcare worldwide. The limited available data, often derived from small cohorts or case reports, fails to provide a comprehensive understanding of the true impact of SARS-CoV-2 infection on maternal and neonatal health (38). In a meta-analysis of 117 studies encompassing 11,758 pregnant women, Karimi et al. reported a maternal mortality rate of 1.3%. Notably, the mortality rate reported in high-income countries was 0.19%, whereas in middle-income countries, including Brazil, mortality

rate was 8.51% (39). Sadly, the highest mortality rate that has been reported was 12.7%, in a study using the ARDS Surveillance System from the Brazilian Ministry of Health published at the end of July 2020, i.e., before the vaccine was available (40).

The laboratory parameters indicated that in severe COVID-19, fibrinogen levels were elevated in all cases, indicating an acute phase inflammatory response. C-reactive protein (CRP) was significantly elevated in 86% of cases, reflecting the inflammation impact in severe cases of COVID-19. Bicarbonate levels were inadequate in 54% of cases, and lactate dehydrogenase (LDH) was elevated in all tested cases, further indicating tissue damage or inflammation with possible impact of an acidoses. The anion gap was found elevated in 66% of cases, suggesting metabolic acidosis. Previous studies that focused on high anion gap and metabolic acidosis to evaluate outcomes in patients admitted to ICU due to COVID-19, found that of the 465 patients, 14% had metabolic acidosis and patients with acidosis and increased anion gap had 88% mortality rate (41).

The ketoacidosis is the most common consequence of hyperglycemia, the classical diabetic ketoacidosis occurs in the setting of relative or absolute insulin deficiency, which leads to reduced glucose utilization and unchecked lipolysis, causing excessive formation of ketone bodies and finally metabolic acidosis (42).). Diabetic ketoacidosis is itself a pro-inflammatory state, with positive correlation with increased circulating levels of TNF-a, IL-6, IL1-beta and PCR (43). While often related to decompensated diabetes, ketoacidosis is also related as common precipitating factor for underlying illness (44) including COVID-19 severe cases (45). A systemic review of literature from 2020 revealed that diabetic hospitalized patients with COVID-19 had mortality rate as high as 50% (46). Besides, only one women from the severe group developed gestational diabetes, and none of them were previously diagnosed with diabetes, which indicates that severe COVID-19 during pregnancy may induce similar responses to gestational diabetes.

Acetoacetate, a ketone body, was found markedly increased in pregnant women from the severe group. SARS-CoV-2 infection can cause ketosis and ketoacidosis, and subjects with high blood ketone bodies levels have longer hospitalization and increased mortality rates (45). Multiple factors may be contributing to acidosis in COVID-19 patients. Respiratory acidosis occurs due to a buildup of carbon dioxide in the body, while lactic acidosis occurs due to mitochondrial electron transport chain dysfunction or pyruvate dehydrogenase complex inhibition. The metabolism of ketone bodies, unlike glucose metabolism, does not raise the levels of lactic acid and may even decrease acidosis by lowering the rate of glycolysis and lactic acid synthesis (47).

Another evidence that points to the fact that severe COVID-19 during pregnancy may induce responses similar to diabetes is the increase in citrate levels. Citrate and other tricarboxylic acid cycle metabolites were previously described to be decreased in non-pregnant severe COVID-19 patients (48) which indicates that severe COVID-19 in pregnancy unveils different metabolic responses than in the non-gravidic state. Citrate is produced in mitochondrial via reaction of Acetyl-CoA and oxalaoacetate, and it is a marker of de novo fatty acid synthesis in the liver (49). As de novo lipogenesis is usually associated with increased insulin resistance, the increased citrate levels found in our study may be associated with the observed higher levels of glucose and also increased serum lipids.

We found significantly higher levels of glucose and lactate in the severe group. Lactate increase has also been reported in inflammatory environments, ranging from 1.5–3 mM in healthy individuals to 10–40 mM in a inflammatory state (50) and this accumulation of lactate can lead to lactic acidosis. Indeed, in gestational diabetes, excessive maternal blood glucose could facilitate overproduction of lactate in the placenta, resulting in metabolic acidosis. The amount of lactate produced by the placenta

is directly proportional to the maternal glucose concentration, as elevation of the glucose concentration from 4.2 to 10.9 mmol/l during placental perfusion in vitro induced a fourfold increase in lactate production (51). And unlike many organs, the placenta synthesizes considerable amounts of lactate under aerobic conditions(52). Also, placental-derived lactate is an essential fuel for the fetus. Therefore, higher levels of lactate is another indication of severe COVID-19 inducing a diabetic-like state, with effects on placental function that might compromise fetal development.

The significant increase in lipid signals in the severe group were corroborating the findings of other studies but specifically within the pregnant population. We found that the severe group had higher triacylglycerol levels, which are the same observation in a prior study on COVID-19 severity. High triglyceride levels are associated with a worse prognosis of COVID 19 (49). In the last trimester of pregnancy, there is a physiological rise in maternal circulating lipids – both lipoproteins and non-esterified fatty acids, rendering a supply of lipids available for active transfer across the placenta for the developing fetus. On the other hand, the literature has suggested associations between maternal lipids, specifically triglycerides, and birthweight in normal-weight and a wide range of maternal BMI (53).

Higher birthweight is usually associated with higher adiposity at birth, with increased risks of obesity throughout the life course and its associated co-morbidities. Therefore, increased in maternal lipids in the severe group not only suggests disease severity and a diabetic state but also may impact lifelong health of the offspring. And this needs to be determined. (54–56)

In our study we found a significant alteration in betaine and glycine, both markedly higher in severe group. This is of particular interest as glycine is considered a conditionally essential amino acid for fetal development, in particular for preterm infants (57). Glycine is involved in many metabolic processes, as collagen and elastin synthesis, conjugation of bile acids and as an important intermediate in the one carbon metabolism. Additionally, glycine is essential for gluthatione synthesis, the most abundant intracellular antioxidant (58). There are evidences that maternal-fetal transfer of glycine is limited, and placental supply via serine, betaine and choline contributes to glycine availability to the fetus (59,60)

Therefore, one might speculate that the significant higher levels of glycine and betaine is suggestive of impaired placental uptake, probably compromising fetal supply in severe COVID-19. Another important observation is that choline levels were similar among groups. We recently showed that plasma choline levels were significantly lower in non-gravidic subjects presenting severe COVID-19, and the magnitude of its decrease associated with disease fatality. Therefore, plasma choline might present a protective effect in the severe cases of COVID-19 (61).

Choline in pregnancy is not only is important as a source of glycine for fetus but also it is an important methyl donor to fulfil the needs for placental functions and fetal growth (62). Indeed, inadequate dietary supply of choline, and also betaine, have been associated with increased risk of neural defects in humans (63). In the present study, choline levels were similar among groups. Therefore, this could represent a protective mechanism, assuring choline supply to the placenta and the fetus.

Our findings demonstrated significant lower level of acetate in severe COVID-19 compared to the negative and positive groups. Acetate, a short-chain fatty acid, is primarily produced by gut microbiota through the fermentation of fibers and can also be derived from acetyl-CoA via glycolysis (64). Recent studies indicate that alterations in gut microbiota composition can influence immune

435 reactions, thereby potentially affecting COVID-19 outcomes, as acetate is suggested to have antiviral
436 properties, by increasing interferon-β production and expression of interferon-stimulated genes (65).
437 This observation aligns with findings that memory CD8+ T cells take up acetate and expand their
438 acetyl-CoA pool and its function, reciprocally regulating energy metabolism and immune function
439 alertness (66). Additionally, it has been recently demonstrated that the maternal microbiome promotes
440 placental development in mice (67). Therefore, lower levels of acetate is another metabolic alterations
441 that links COVID-19 severity and placental dysfunction.

442 The interplay among immunity, inflammation, and energy metabolism may be seem when
443 immune cells undergo significant metabolic changes that switch immune cells to resting state to an
444 activated state, and the resulting changes in energy supply and demand can cause metabolic acidosis
445 and reduced oxygen delivery or availability, leading to significant hypoxia that is sufficient to induce
446 transcriptional and translational alterations in tissue phenotype (68). This metabolic programming in
447 M1 macrophages likely evolved to increase proinflammatory cytokine production. M2 macrophages
448 are programmed to oxidize pyruvate and fatty acids into acetyl-CoA for normal citric acid cycle
449 function and oxidative phosphorylation (69), which has been shown to facilitate anti-inflammatory IL-
450 10 secretion (70). Lung-resident macrophage polarization to either a proinflammatory M1 state or an
451 anti-inflammatory M2 state is largely controlled by the cytokines secreted by the other cells present in
452 the environment (71). These cytokines also influence the catabolic pathways that are activated to fuel
453 cellular energy needs. M1 macrophages have increased glycolytic activity and lactate production due
454 to the presence of a dysfunctional citric acid cycle (72). The dysfunctional citric acid cycle resulted in
455 the accumulation of citrate that increased fatty acid synthesis (73).

456 Increased N-acetylation of amino acids was found higher in the severe group, and it has been
457 previously associated with SARS-CoV-2 infection and illness progression (74), and in women with
458 gestational diabetes (75). N-acetyl of glycoprotein were also correlated with chronic kidney disease
459 progression (76), systemic inflammation and all-cause mortality (77).

460 Our study also confirmed that severe COVID-19 induced important changes in amino acids
461 metabolism, where significantly lower contents of lysine, alanine, histidine, tyrosine, and tryptophan,
462 were found in women from severe group. These data corroborate with literature reviews that revealed
463 that 16 out of 20 amino acids were altered in severe and mild COVID-19 (78). The lower content of
464 amino acids in the severe cases of COVID-19 is associated with increased amino acid catabolism and
465 worse prognosis. The underlying mechanism is believed to involve a hyperproliferative response of
466 lung parenchymal cells during the early stages of infection (79). Impaired placental perfusion are
467 associated with an inability to regulate the tryptophan/kynurenine pathway during pregnancy
468 Individual differences in maternal tryptophan and/or kynurenine during pregnancy could have
469 substantial long-term effects on fetal brain development (80,81).

470 The findings of this study may be linked to an increased prevalence of adverse
471 neurodevelopmental outcomes in neonates from COVID-19 positive pregnancies, as epidemiological
472 research consistently links maternal infections during pregnancy, including those caused by other
473 viruses like influenza, to a negative impact on neurodevelopmental outcomes in children (21). Four
474 years into the COVID-19 pandemic, longitudinal data on the offspring of SARS-CoV-2-infected
475 pregnancies is just emerging. Neonates from mothers infected with SARS-CoV-2 exhibit disrupted
476 immune functions, notably neutrophil activation, a phenomenon also observed in pediatric COVID-19
477 cases (8). This response could be linked to increased IL-8 levels in the cord blood, which is known to
478 stimulate neutrophils, indicating a subtle neutrophilic reaction in these newborns (82). One of the first
479 retrospective cohorts that aimed to evaluate whether in utero exposure to SARS-CoV-2 is associated

with risk for neurodevelopmental disorders in the first 12 months after birth, found that preterm delivery was more likely among 14.4% of infected mothers versus 8.7% from uninfected. Additionally, according to the International Statistical Classification of Diseases and Related Health Problems, Tenth Revision (ICD-10), maternal SARS-CoV-2 positivity during pregnancy was associated with greater rate of impaired neurodevelopment in unadjusted models (odds ratio 2.17) as well as those adjusted for race, ethnicity, insurance status, offspring sex, maternal age, and preterm status (odds ratio 1.86). This study also emphasis that particularly to neurodevelopmental effects, some of these disorders may not manifest until adolescence or adulthood, and the true risks of maternal immune activation may not become apparent for decades (83).

Conclusion:

Serum metabolomics of pregnant women proved to be useful in distinguishing severe COVID-19 from non-severe infection and revealing greater inflammation, and dysregulation of serum lipids, like the general population. Additionally, specific alterations in metabolites might be indicative of placental dysfunction which may compromise fetal development and also lifelong health of the mother and the infant. As such, pregnant people with COVID-19 should be managed similarly to their non-pregnant counterparts, while observed to progress to a similar state observed in gestational diabetes. And, if necessary, consider special support regarding methyl donors and ketone bodies to minimize placental function.

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4 FINAL CONSIDERATIONS

Integrating metabolomics into the study of severe COVID-19 has uncovered critical insights into the complex metabolic disturbances associated with the disease, affecting both the general population and pregnant individuals. The plasma and serum metabolome analysis revealed significant alterations in one-carbon, lipid, and amino acid metabolism, reflecting systemic dysregulation and correlated with disease severity and outcomes. In non-pregnant individuals, particularly those with fatal outcomes, increased levels of creatine/creatinine, 4-hydroxyproline, gluconic acid, and N-acetylserine suggest uncontrolled inflammation and multi-organ dysfunction, highlighting the urgent need for enhanced understanding of post-acute COVID-19 syndrome. Similarly, in pregnant women, metabolic shifts similar to those observed in gestational diabetes underscore an increased risk of complications, including potential placental dysfunction that may affect both maternal and neonatal health. These findings emphasize the importance of considering sex-specific and pregnancy-related metabolic changes in the management and surveillance strategies for COVID-19. By advancing our understanding of the metabolic underpinnings of severe COVID-19, these studies provide a foundation for improving patient stratification, therapeutic approaches, and long-term care for affected individuals.

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6 APPENDICES

Anexx 1 - Questionnaire Administered To Pregnant Women

COVID - gestação

ACEITARIA PARTICIPAR DE UMA NOVA COLETA DE SANGUE PARA CONTINUAÇÃO DAS ANÁLISES DO PROJETO?

- ◯ Sim
- ◯ Não
- ◯ Não sabe

Dados Pessoais

NOME:	DATA DE NASCIMENTO: yyyy-mm-dd

PESO (KG):	ALTURA (M):	PESO ANTES DA GESTAÇÃO (KG):	GANHO DE PESO NA GESTAÇÃO (KG):

Dados da gestação

CONCLUIU A GESTAÇÃO: ◯ Sim ◯ Não ◯ Não sabe	DATA DO PARTO: yyyy-mm-dd	IDADE GESTACIONAL:

IDADE GESTACIONAL

TIPO DE PARTO:

- ◯ Cesariana
- ◯ Normal

TEVE ALGUMA INTERCORRÊNCIA DURANTE A GESTAÇÃO?	TIPO DE INTERCORRÊNCIA	OUTRA INTERCORRÊNCIA :	HOUVE ALGUMA INTERCORRÊNCIA NO MOMENTO DO PARTO:	QUAL INTERCORRÊNCIA :
◯ Sim ◯ Não ◯ Não sabe	☐ Diabetes Mellitus Gestacional ☐ Hipertensão ☐ Pré-eclâmpsia ☐ Anemia ☐ Outros		◯ Sim ◯ Não ◯ Não sabe	

Dados do recém nascido

PESO DO BEBÊ AO NASCER:	COMPRIMENTO DO BEBÊ AO NASCER:	PERÍMETRO CEFÁLICO DO BEBÊ

APGAR

SEXO DO BEBÊ

◯ Femino

◯ Masculino

A SRA. AMAMENTOU SEU BEBÊ:	SE SIM, POR QUANTO TEMPO:
◯ Sim ◯ Não ◯ Não sabe	

Dados COVID

DURANTE O PERÍODO DE GESTAÇÃO A SRA TESTOU POSITIVO PARA COVID-19?

- ◯ Sim
- ◯ Não
- ◯ Não sabe

ALGUM SINTOMA PERSISTIU APÓS A RESOLUÇÃO DA INFECÇÃO POR COVID-19?

- ☐ Nenhum
- ☐ Cansaço
- ☐ Dor de cabeça
- ☐ Falta de ar
- ☐ Dor muscular
- ☐ Dor nas articulações
- ☐ Hipertensão
- ☐ Diabetes Mellitus Gestacional
- ☐ Dislipidemia
- ☐ Trombose
- ☐ Alopécia
- ☐ Ansiedade
- ☐ Depressão
- ☐ Insônia
- ☐ Dificuldade de memória e ou concentração
- ☐ Alteração no paladar
- ☐ Outros

OUTROS:

Dados de vacinação

A SRA. FOI VACINADA PARA COVID-19?

- ◯ Sim
- ◯ Não
- ◯ Não sabe

QUANTAS DOSES?

- ☐ 1
- ☐ 2
- ☐ 3
- ☐ 4
- ☐ 5 ou mais

QUAL LABORATÓRIO?

- ☐ Pfizer
- ☐ Astrazeneca
- ☐ Jansen
- ☐ CoronaVac
- ☐ Outro

OUTRO LABORATÓRIO:

APÓS O PERÍODO DE EXAME NO CTD, HOUVE OUTRA GESTAÇÃO?

- ◯ Sim
- ◯ Não
- ◯ Não sabe

A GESTAÇÃO FOI CONCLUÍDA?

- ◯ Sim
- ◯ Não
- ◯ Não sabe

OBSERVAÇÕES

Anexx 2 – Ethics Committee Approval for Chapter 1

COMISSÃO NACIONAL DE ÉTICA EM PESQUISA

PARECER CONSUBSTANCIADO DA CONEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Caracterização da resposta imune de pacientes hospitalizados com COVID-19: Um estudo prospectivo

Pesquisador: Fernando Augusto Bozza

Área Temática:

Versão: 1

CAAE: 30650420.4.1001.0008

Instituição Proponente: INSTITUTO D'OR DE PESQUISA E ENSINO

Patrocinador Principal: INSTITUTO D'OR DE PESQUISA E ENSINO

DADOS DO PARECER

Número do Parecer: 3.979.658

Apresentação do Projeto:

As informações contidas nos campos "Apresentação do Projeto", "Objetivo da Pesquisa" e "Avaliação dos Riscos e Benefícios" foram obtidas dos documentos contendo as Informações Básicas da Pesquisa (PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1535044.pdf de 08/04/2020) e do Projeto Detalhado.

INTRODUÇÃO

As doenças infecciosas estão entre as principais causas de óbito em todo mundo. Além das doenças infecto -contagiosas já conhecidas, vírus emergentes têm sido reconhecidos como potencial causa de epidemias e pandemias. A evidência sugere que a possibilidade de uma pandemia aumentou no último século devido ao aumento das viagens internacionais e da integração global, urbanização, mudanças do uso da terra e aumento da exploração do meio ambiente. Pandemias, além de poder aumentar significativamente a morbi-mortalidade em uma ampla área geográfica, tem o potencial de causar rupturas políticas, sociais e econômicas significativas. Novos agentes infecciosos, como SARS, MERS e outros novos coronavírus, novos vírus da influenza, vírus que causam febre hemorrágica viral (por exemplo, Ebola) e vírus que afetam o sistema nervoso central (SNC), como TBEV e Nipah, exigem investigação para compreender a biologia e a patogênese do patógeno no hospedeiro. Mesmo para infecções conhecidas, a resistência às terapias antimicrobianas é difundida e faltam tratamentos para

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controlar as respostas potencialmente deletérias do hospedeiro. Recentemente, o novo coronavirus SARS-CoV-2 (doença do coronavírus 19 – COVID-19) tem causado uma epidemia cujo epicentro foi a província de Wuhan na República Popular da China e se espalhou para diversos outros países. No dia 11 de março de 2020, a Organização Mundial de Saúde (OMS) declarou a epidemia de COVID-19 oficialmente uma pandemia. A emergência do SARS-CoV-2, depois da síndrome respiratória aguda grave (SARS-CoV) em 2002 e da síndrome respiratória do Oriente Médio (MERS-CoV) em 2012, marca a terceira introdução de uma epidemia em grande escala causada por um coronavírus altamente patogênico no século 21. Até o dia 24/03/2020, havia 414.277 casos confirmados de COVID-19 ao redor do mundo, com 18.557 mortes. Em 26 de fevereiro de 2020 foi confirmado no Estado de São Paulo o primeiro caso da COVID-19 no Brasil, doença transmitida pelo vírus SARS-CoV-2. Desde então, o número de casos no Brasil começou a crescer, acrescido da recente constatação de transmissão comunitária em algumas das maiores capitais do país. Até o dia 29 de março mais de 4.309 casos de covid- 19 foram registrados em todos os estados e 139 óbitos forma notificados. Para desenvolver uma compreensão mecanística dos processos da doença, de modo que os fatores de risco para a doença grave possam ser identificados e os tratamentos possam ser desenvolvidos, é necessário compreender as características do patógeno associadas à virulência, a dinâmica da replicação e a evolução do patógeno no hospedeiro, a dinâmica da resposta imune, a farmacologia das terapias antimicrobianas ou direcionadas a imunomodulação, a dinâmica da transmissão e os fatores subjacentes da suscetibilidade individual.

HIPÓTESE

A compreensão das características do patógeno associadas à virulência, a dinâmica da replicação e a evolução do patógeno no hospedeiro, a dinâmica da resposta imune, a farmacologia das terapias antimicrobianas ou direcionadas a imunomodulação, a dinâmica da transmissão e os fatores subjacentes da suscetibilidade individual pode auxiliar na identificação dos fatores de risco para a doença grave e no desenvolvimento de tratamentos.

METODOLOGIA

Trata-se de um estudo de coorte prospectivo observacional. Este protocolo foi desenhado para permitir que dados e amostras biológicas sejam coletados prospectivamente e rapidamente em um cronograma de amostragem harmonizado. O estudo será realizado em vários centros no estado do Rio de Janeiro, incluído centros públicos e privados como o Instituto Estadual do Cérebro Paulo

Niemeyer (IEC) e os hospitais Copa D'Or, Copa Star, Caxias D'Or, Glória D'Or e Quinta D'Or. Pacientes que apresentam doença aguda suspeita ou confirmada por este patógeno poderão ser incluídos. Em todos os casos, uma ficha clínica (CRF em papel ou "eCRF" eletrônico baseada na web) será preenchida. As amostras serão coletadas no dia da inclusão (Dia 1; idealmente na apresentação inicial a uma unidade de atenção à saúde), depois nos dias 3, entre os dias 5 a 7 e entre os dias 11 a 14 após a inclusão, então semanalmente até a alta hospitalar e novamente aos 3 e 6 meses após a inclusão.

CRITÉRIOS DE INCLUSÃO
Nova infecção COVID-19 suspeita ou comprovada como o principal motivo de hospitalização em pacientes adultos (idade 18 anos).

CRITÉRIOS DE EXCLUSÃO
Diagnóstico confirmado de um patógeno não relacionado aos objetivos deste estudo e ausência de indicação ou de probabilidade de infecção concomitante com um patógeno relevante.
Recusa pelo participante ou representante apropriado.

Objetivo da Pesquisa:
OBJETIVOS PRIMÁRIOS
Descrever as características clínicas da doença COVID-19 no nosso meio.
Caracterizar as respostas do hospedeiro à infecção e à terapia, incluindo respostas imunes inatas e adquiridas, tromboplaquetária, níveis circulantes de moléculas de sinalização imuneinflamatórias e o perfil de expressão gênica no sangue periférico.
Avaliar o papel das células imunes nas respostas inflamatória e anti-viral de pacientes com COVID-19.
Descrever a resposta ao tratamento, incluindo o tratamento de suporte e as novas terapêuticas.
Observar a replicação, a excreção e a evolução do patógeno no hospedeiro e identificar determinantes de severidade e transmissão utilizando o sequenciamento de alto rendimento dos genomas de patógenos obtidos a partir das vias aéreas, sangue, e outras amostras.
Identificar biomarcadores e variantes genéticas do hospedeiro associadas à progressão da doença ou severidade.
Compreender a transmissibilidade e as probabilidades de diferentes desfechos clínicos após a exposição e a infecção.

Avaliação dos Riscos e Benefícios:

RISCOS

Flebotomia.

Os participantes podem ter sangue coletado com mais frequência do que o exigido para o padrão de tratamento. A flebotomia pode estar associada à dor no local da coleta e raramente à infecção. Os volumes de coleta de sangue diários foram restringidos, para que a amostragem clínica e de pesquisa combinada esteja dentro dos limites recomendados. O desconforto será minimizado com a equipe especializada obtendo as amostras de sangue e combinando a amostragem da pesquisa com a amostragem clínica de rotina, quando possível, o que normalmente ocorre diariamente em pacientes doentes no hospital.

Desconforto das amostras respiratórias.

A coleta de esfregaços respiratórios pode causar desconforto transitório. O desconforto e o risco serão minimizados pelo uso de equipe clínica experiente em cada centro e as amostras serão coletadas ao mesmo tempo que as amostras clínicas, a fim de minimizar esses riscos.

BENEFÍCIOS

Não haverá benefício direto para os participantes da pesquisa. O estudo pode incluir amostragem biológica, além da amostragem exigida para o tratamento médico. Os resultados dos testes realizados nessas amostras podem não contribuir para melhorar a saúde do participante. Os resultados deste estudo podem não estar disponíveis a tempo de contribuir para o tratamento do participante. Quando possível, os resultados dos testes relevantes para o tratamento do paciente serão informados ao participante e/ou ao médico responsável pelo tratamento. A viabilidade disso dependerá dos recursos locais. Alguns ensaios não podem beneficiar imediatamente o paciente porque os dados precisarão ser agrupados com outros ou porque os ensaios levam tempo.

Comentários e Considerações sobre a Pesquisa:

Estudo de coorte prospectivo observacional. Este protocolo foi desenhado para permitir que dados e amostras biológicas sejam coletados prospectivamente e rapidamente em um cronograma de amostragem harmonizado. O estudo será realizado em vários centros no estado do Rio de Janeiro, incluído centros públicos e privados como o Instituto Estadual do Cérebro Paulo Niemeyer (IEC) e os hospitais Copa D'Or, Copa Star, Caxias D'Or, Glória D'Or e Quinta D'Or. Potenciais participantes de pesquisa que apresentam doença aguda suspeita ou confirmada por este patógeno poderão ser incluídos. Em todos os casos, uma ficha clínica (CRF em papel ou "eCRF" eletrônico baseada na web) será preenchida. As amostras serão coletadas no dia da inclusão (Dia 1; idealmente na

apresentação inicial a uma unidade de atenção à saúde), depois nos dias 3, entre os dias 5 a 7 e entre os dias 11 a 14 após a inclusão, então semanalmente até a alta hospitalar e novamente aos 3 e 6 meses após a inclusão.

Considerações sobre os Termos de apresentação obrigatória:

Vide campo "Conclusões ou Pendências e Lista de Inadequações".

Conclusões ou Pendências e Lista de Inadequações:

Não foram observados óbices éticos na documentação do protocolo de pesquisa.

Considerações Finais a critério da CONEP:

Diante do exposto, a Comissão Nacional de Ética em Pesquisa - Conep, de acordo com as atribuições definidas na Resolução CNS nº 466 de 2012 e na Norma Operacional nº 001 de 2013 do CNS, manifesta-se pela aprovação do projeto de pesquisa proposto.

Situação: Protocolo aprovado.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1535044.pdf	08/04/2020 06:42:13		Aceito
Projeto Detalhado / Brochura Investigador	Protocolo_COVID_03_04_2020.pdf	08/04/2020 06:40:30	Fernando Augusto Bozza	Aceito
Outros	COVID19_Ficha_voluntario.pdf	08/04/2020 06:18:09	Fernando Augusto Bozza	Aceito
Outros	COVID19_Ficha_clinica.pdf	08/04/2020 06:17:45	Fernando Augusto Bozza	Aceito
Declaração de Manuseio Material Biológico / Biorepositório / Biobanco	Regimento_biorrepositorio_covid19.pdf	08/04/2020 06:17:06	Fernando Augusto Bozza	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	COVID19_amostras_TCLE28032020_representante.docx	08/04/2020 06:15:50	Fernando Augusto Bozza	Aceito
TCLE / Termos de Assentimento /	COVI19_Amostras_TCLE28032020_voluntario.docx	08/04/2020 06:15:39	Fernando Augusto Bozza	Aceito

COMISSÃO NACIONAL DE ÉTICA EM PESQUISA

Continuação do Parecer: 3.979.658

Justificativa de Ausência	COVI19_Amostras_TCLE28032020_voluntario.docx	08/04/2020 06:15:39	Fernando Augusto Bozza	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	COVI19_Amostras_TCLE28032020_paciente.docx	08/04/2020 06:15:20	Fernando Augusto Bozza	Aceito
Declaração de Pesquisadores	termo_compromisso.pdf	08/04/2020 06:14:52	Fernando Augusto Bozza	Aceito
Declaração de Instituição e Infraestrutura	anuencia_ses_iec.jpeg	08/04/2020 06:14:38	Fernando Augusto Bozza	Aceito
Declaração de Instituição e Infraestrutura	anuencia_ioc.jpeg	08/04/2020 06:14:28	Fernando Augusto Bozza	Aceito
Declaração de Instituição e Infraestrutura	anuencia_idor_covid19.pdf	08/04/2020 06:14:18	Fernando Augusto Bozza	Aceito
Folha de Rosto	folhaDeRosto_covid19.pdf	08/04/2020 06:13:57	Fernando Augusto Bozza	Aceito

Situação do Parecer:
Aprovado

BRASILIA, 19 de Abril de 2020

Assinado por:
Jorge Alves de Almeida Venancio
(Coordenador(a))

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Anexx 3 – Ethics Committee Approval for Chapter 3

COMISSÃO NACIONAL DE ÉTICA EM PESQUISA

PARECER CONSUBSTANCIADO DA CONEP

DADOS DA EMENDA

Título da Pesquisa: CARACTERIZAÇÃO DE FATORES DE RISCO E DESENVOLVIMENTO DE NOVOS TESTES SOROLOGICOS PARA A INFECÇÃO POR SARS-CoV-2

Pesquisador: Amilcar Tanuri

Área Temática: Genética Humana:
(Trata-se de pesquisa envolvendo Genética Humana que não necessita de análise ética por parte da CONEP;);
A critério do CEP

Versão: 4

CAAE: 30161620.0.1001.5257

Instituição Proponente: UNIVERSIDADE FEDERAL DO RIO DE JANEIRO

Patrocinador Principal: UNIVERSIDADE FEDERAL DO RIO DE JANEIRO

DADOS DO PARECER

Número do Parecer: 4.245.490

Apresentação do Projeto:

As informações elencadas nos campos "Apresentação do Projeto", "Objetivo da Pesquisa" e "Avaliação dos Riscos e Benefícios" foram retiradas do arquivo Informações Básicas da Pesquisa (PB_INFORMAÇÕES_BÁSICAS_1599143_E2.pdf, de 21/07/2020).

INTRODUÇÃO

Em 31 de dezembro de 2019, a Organização Mundial de Saúde (OMS) foi notificada sobre os primeiros casos de doença respiratória grave de origem inicialmente desconhecida na cidade de Wuhan, na China. Uma semana depois, foi identificado como agente causador um novo coronavírus (nCoV) que foi oficialmente denominado SARS-nCoV-2. Trata-se de vírus de RNA vírus da ordem Nidovirales, família Coronaviridae. Desde então, o vírus se espalhou pelo mundo e diversos países além da China já apresentam transmissão local (Ministério da Saúde. Plataforma IVS, 2020). A doença causada pelo SARSCoV2 foi oficialmente denominada COVID-19. O espectro clínico tem-se mostrado amplo, podendo variar de um simples resfriado até pneumonia severa. Os sintomas são principalmente respiratórios, tais como febre, tosse e dificuldade para respirar. As complicações clínicas mais comuns são Síndrome Respiratória Aguda Grave (SRAG), lesão cardíaca

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aguda e infecção secundária. Os dados disponíveis até o momento indicam uma maior taxa de hospitalização em indivíduos maiores de 50 anos (Ministério da Saúde. SAES, 2020). No Brasil, o primeiro caso suspeito foi notificado no dia 22 de janeiro de 2020. Em 01 de março de 2020, o Brasil contabilizava dois casos importados confirmados, ambos com histórico de viagem para a região da Lombardia na Itália, na qual o índice de infeção encontra-se alto. Cinco dias depois, um total de 13 casos já havia sido confirmado. O estado de São Paulo concentra 10 casos, enquanto Rio de Janeiro, Bahia e Espírito Santo apresentam apenas 1 caso confirmado até o dia 06 de março. Ao todo, 768 casos suspeitos já foram notificados no Brasil, dos quais 480 já foram descartados (Ministério da Saúde. Plataforma IVS, 2020). As definições operacionais de casos seguem sofrendo atualizações, acompanhando a detecção de novos casos no país. No Boletim Epidemiológico do dia 04 de março de 2020 (Ministério da Saúde. SVS, 2020), os casos suspeitos passaram a ser definidos como pessoas com febre acompanhada de pelo menos um dos sintomas respiratórios (ex: tosse, congestão nasal, dificuldade para respirar, entre outros), e que tenha viajado nos últimos 14 dias para país com transmissão local ou sustentada. Indivíduos com os mesmos sintomas e que tenham tido contato com um caso confirmado nos últimos 14 dias também são considerados casos suspeitos. Contatos domiciliares de casos confirmados e que apresentem os sintomas gripais são considerados casos prováveis. Até o presente momento, os casos confirmados de COVID-19 incluem os casos suspeitos ou prováveis que tenham diagnóstico laboratorial positivo ou aqueles que, além dos sintomas, tiveram contato próximo com algum indivíduo com diagnóstico laboratorial positivo (Ministério da Saúde. SVS, 2020). Até o momento, o diagnóstico é confirmado pela identificação do material genético viral em amostras do paciente (swab nasofaríngeo, swab orofaríngeo, sangue) por qRT-PCR, visto que se trata de um vírus com genoma de RNA fita positiva. Existe também a necessidade do melhor entendimento da resposta imune e da história natural do SARS-CoV-2 para podermos fazer o desenvolvimento de kits diagnósticos simples e rápidos para auxiliar os clínicos na condução clinica dos pacientes e possibilitar estudos epidemiológicos mais acurados. O desenvolvimento de ações coordenadas para a identificação de novos casos e sequenciamento viral para identificação de “clusters” de transmissão viral a serem utilizados em estudos de vigilância epidemiológica é urgente para que ações de saúde pública possam interferir e diminuir as taxas de transmissão, reduzindo o risco de disseminação do vírus e/ou a gravidade de uma possível epidemia no nosso país. No final do mês de fevereiro (28/02/2020), o primeiro genoma do SARSCoV-2 no Brasil foi sequenciado em tempo recorde (48 horas após confirmação do primeiro caso), utilizando a plataforma Nanopore de sequenciamento portátil, no âmbito de uma colaboração entre a Universidade de São Paulo (USP), o

Instituto Adolfo Lutz, o Laboratório de Virologia Molecular da UFRJ e a Universidade de Oxford, no Reino Unido. No dia 03 de março, o mesmo grupo já havia sequenciado também o genoma do segundo caso confirmado no país (Jesus, 2020). Trata-se dos primeiros genomas do SARS-CoV-2 reportados na América do Sul. A geração destes dados em tempo real durante a epidemia será de extrema importância não apenas para o estudo de vigilância genômica e dispersão do vírus pelo mundo, mas também para o mapeamento de regiões e epítopos antigênicos específicos para o desenvolvimento de testes sorológicos ainda não disponíveis.Com o objetivo de dar continuidade ao estudo da COVID-19, pesquisadores da UFRJ com expertise em clínica, virologia, controle de epidemias, diagnóstico, genômica e fatores de risco, propõem um projeto de pesquisa de natureza multidisciplinar, de modo a contribuir para evitar o avanço do novo coronavírus em território nacional.

HIPÓTESE

A reduzida letalidade da infecção frente ao alto potencial de transmissão do SARS-CoV2, bem como a concentração dos casos graves entre indivíduos a partir dos 50 anos de idade sugere que sejam necessários fatores de risco adicionais para o desenvolvimento de complicações clínicas. O presente estudo tem como base a hipótese de que mutações que aumentem a patogenicidade do vírus, confecções ou ainda fatores genéticos do hospedeiro possam estar associados ao desenvolvimento de complicações clínicas tais como a pneumonia, Síndrome do desconforto respiratório agudo (SDRA) e sepse, que requerem a internação e podem evoluir para óbito.

O desenvolvimento de testes que avaliem a presença de anticorpos IgG e IgM específicos para SARS-CoV2 poderá contribuir para o diagnóstico da infecção de forma rápida e eficiente. Além de dispensar estrutura laboratorial complexa, o diagnóstico sorológico permitirá também identificar os casos em que a amostra clínica não tenha sido coletada no momento em que a viremia está mais alta, o que poderia gerar resultado falso negativo em testes moleculares.

METODOLOGIA

Coleta e processamento das amostras. Para este estudo, serão utilizadas amostras clínicas coletadas para o diagnóstico e laboratorial e rotina de acompanhamento clínico dos pacientes. Poderão ser utilizadas sobras de sangue total, swab, líquor ou amostras de sangue já processadas (soro, plasma e buffy coat) para o acompanhamento clínico dos pacientes. Para os casos brandos, poderá ser solicitada a coleta de amostra de 2 mL de sangue, caso não tenha sido realizada coleta no atendimento ambulatorial. Caso seja necessária a coleta sequencial de amostras para reportar a

resolução da infecção pelo SARS-CoV-2, todas as amostras coletadas durante o seguimento dos pacientes serão incluídas no estudo, de modo a obter maior rendimento para a extração do material genético. As amostras de soro e plasma serão utilizadas para a avaliação dos testes diagnósticos por sorologia e para a extração de ácidos nucleicos para sequenciamento do genoma viral e/ou análise metagenômica. A fase celular (buffy coat) será utilizada para extração de DNA e RNA dos pacientes, para a identificação de variantes genéticas e análise de RNAseq. As amostras serão armazenadas em biorrepositório provisório no Laboratório de Virologia Molecular por um período de até 2 anos após o término do estudo, caso sejam necessárias a repetição de um dos testes. Após este período as amostras serão descartadas. Detecção do SARS-CoV-2 por RT-PCR O diagnóstico molecular será realizado através da metodologia de transcrição reversa seguida pela reação em cadeia da polimerase (RT-PCR) para detecção de fragmento específico do material genético do vírus. A detecção será realizada por PCR em tempo real, seguindo recomendações do Ministério da Saúde (Ministério da Saúde. SVS, 2020; Corman et al., 2020). Validação de testes sorológicos e antigênicos. As amostras de soro e plasma obtidas serão utilizadas para a validação de testes sorológicos para a detecção de anticorpos IgG e IgM anti SARSCoV-2. Também será avaliada a utilização de testes para a detecção do antígeno S do SARS-CoV-2 em exsudatos respiratórios para uma maior sensibilidade dos testes rápidos. Os testes ELISA encontram-se em fase de desenvolvimento. Sequenciamento dos Genomas virais O sequenciamento do genoma completo dos vírus será realizado através da metodologia de sequenciamento paralelo massivo, através da tecnologia Ion PGM (ThermoFisher Scientific). Inicialmente, serão sintetizados oligonucleotídeos específicos para a amplificação do material genético viral e construção de bibliotecas genômicas. O conjunto de primers será desenvolvido de modo a fornecer uma cobertura total do genoma do vírus. Os genomas sequenciados serão analisados com o auxílio do software Ion Report (ThermoFisher Scientific). Análise metagenômica. As amostras dos pacientes que apresentarem complicações clínicas serão submetidas à análise metagenômica, a fim de identificar a presença de material genético de outros vírus que possam estar presentes na amostra do paciente. Para este fim, será utilizada a metodologia de sequenciamento Illumina, conforme descrito (Calvet et al., 2016). As bibliotecas de cDNA serão preparadas com o auxílio do sistema TruSeq Stranded Total RNA LT Sample Preparation Kit. O sequenciamento será realizado em sistema MiSeq sequencing system (Illumina). Os dados poderão também ser analisados com o objetivo de caracterizar os perfis de expressão gênica em casos graves. Sequenciamento do exoma e transcriptoma dos pacientes O sequenciamento dos exomas e transcriptomas será realizado a partir de amostras clínicas de sangue total através de metodologia Illumina. Para este fim, serão

selecionados 10 casos com complicações clínicas e 10 indivíduos controle, que apresentarem sintomas brandos da doença. O preparo das bibliotecas para cada uma das análises será realizado conforme descrito (Aguiar et al., 2019; Carvalho et al., 2019). O sequenciamento será realizado na plataforma NextSeq500 sequencer (Illumina Inc., San Diego, Califórnia).

CRITÉRIOS DE INCLUSÃO
Serão considerados elegíveis para o estudo indivíduos maiores de 18 anos de ambos os sexos, independente de idade, que atendam aos critérios clínicos para investigação de infecção por SARS-CoV-2 (casos suspeitos).

CRITÉRIOS DE EXCLUSÃO
Serão excluídos do estudo os indivíduos que não tiverem diagnóstico confirmado através de análise laboratorial.

Objetivo da Pesquisa:
OBJETIVO PRIMÁRIO
O objetivo central do estudo consiste em contribuir para a compreensão dos fatores genéticos associados ao curso clínico da infecção por SARSCoV2 e também para o desenvolvimento e validação de metodologias para o diagnóstico sorológico da infecção.

OBJETIVOS SECUNDÁRIOS
1. Avaliação de testes sorológicos/antigênicos para o diagnóstico da infecção pelo SARS-CoV2;
2. Análise combinada de testes imunológicos e moleculares para o diagnóstico da infecção;
3. Sequenciamento do genoma completo do SARS-CoV-2;
4. Análise metagenômica para identificação de confecções associadas a formas severas e complicações clínicas da COVID-19;
5. Identificação de fatores genéticos do hospedeiro associados ao desenvolvimento de formas severas da COVID-19, através da análise do exoma dos pacientes;
6. Identificação de padrões de expressão gênica em casos de COVID-19 que apresentem complicações clínicas.

Avaliação dos Riscos e Benefícios:

RISCOS

O presente estudo será realizado utilizando o material restante daquele que já seria coletado para os laboratoriais de rotina, incluindo o diagnóstico laboratorial da infecção. Desta forma, o estudo não apresenta risco direto aos participantes além daqueles relativos à coleta de swab de orofaringe e/ou coleta de sangue por punção venosa. Esta última, pode acarretar o aparecimento de manchas arroxeadas no braço. O risco de perda de confidencialidade será evitado através da codificação de todas as amostras e registros em bancos de dados.

BENEFÍCIOS

O presente estudo não visa gerar benefícios diretos aos participantes. O objetivo principal da proposta reside na avaliação de novas metodologias de diagnóstico da infecção por SARS-CoV-2 e identificação de fatores de risco genéticos e confecções associados ao desenvolvimento de complicações clínicas nos pacientes. O resultado dos testes genéticos poderá ser encaminhado ao médico responsável, caso este venha a fornecer algum benefício para o manejo clínico. Os resultados poderão também ser disponibilizados ao paciente, caso seja solicitado. No que diz respeito aos dados genéticos humanos, o conhecimento do resultado não trará qualquer prejuízo ao paciente (estresse psicológico ou estigmatização). Embora o projeto vise o sequenciamento de todo o exoma dos pacientes, apenas os genes relacionados com a resposta a infecção serão analisados.

Comentários e Considerações sobre a Pesquisa:

Trata-se da Emenda 2 ao Protocolo de Pesquisa aprovado pela CONEP em 03/04/2020 (PB_PARECER_CONSUBSTANCIADO_CONEP_3953368.pdf)

Justificativa: A presente emenda é referente à atualização do Projeto Detalhado (versão 04 de 21/07/2020) e do Termo de Consentimento Livre e Esclarecido (versão 03 de 21/07/2020).

1. Alterações no Projeto Detalhado (versão 04 de 21/07/2020), referente ao documento "ProjetoCOVID19_v4.docx", postado em 21/07/2020:

• Atualizado a lista de "Colaboradores do Instituto de Microbiologia da UFRJ": Marcelo Torres Bozza; Luciana Arruda, Juliana Echevarria e Renata M. Pereira.

• Adicionado que serão feitos testes para detectar IgA e IgE. (item Hipóteses);

• Adicionado objetivo específico “Determinação da prevalência de coinfecções na coorte de estudo e associação das mesmas aos desfechos clínicos da COVID-19”. (item objetivos específicos);
• Adicionado que “A coleta será realizada no centro de triagem da COVID-19, vinculado ao Hospital Universitário Clementino Fraga Filho (HUCFF) e localizado no Centro de Ciências da Saúde, da UFRJ” (item desenho do estudo);
• Adicionado que “Poderá ser solicitada a coleta de 20 mL de sangue, bem como amostras de saliva e fezes” (item Coleta e processamento das amostras).
• Adicionado o item “Identificação de coinfecções”: A identificação de outros microorganismos que possam estar presentes em coinfecção com SARS-CoV-2 será realizada em diferentes amostras clínicas (swab, sangue, saliva, fezes) utilizando diferentes metodologias de diagnóstico, incluindo testes sorológicos, PCR e sequenciamento do metagenoma. Para o metagenoma, será utilizada a metodologia de sequenciamento Illumina, conforme descrito.

2. Alterações no Termo de Consentimento Livre e Esclarecido (versão 03 de 21/07/2020), referente ao documento “TCLE_v3.docx”, postado em 21/07/2020:
• Adicionado o trecho “avaliação de marcadores da sua resposta imune que podem estar relacionados com a capacidade de resistir à infecção”.
• Atualizado o “Se você tiver alguma consideração ou dúvida sobre a ética da pesquisa, entre em contato com o Comitê de Ética em Pesquisa (CEP) do Hospital Universitário Clementino Fraga Filho/HUCFF/UFRJ, Rua Prof. Rodolpho Paulo Rocco, n.° 255, Cidade Universitária/Ilha do Fundão, 7º andar, Ala E - de segunda a sexta-feira, das 8 às 16 horas”.

LISTA ATUALIZADA DE CENTROS PARTICIPANTES (de acordo com o documento “PB_INFORMAÇÕES_BÁSICAS_1599143_E2.pdf” de 21/07/2020):
1. Instituto de Pesquisas Biomédicas do Hospital Naval Marcílio Dias - HNMD (Pesquisador Responsável: Shana Priscila Coutinho Barroso)
2. Hospital Universitário Clementino Fraga Filho (Pesquisador Responsável: Roberto de Andrade Medronho)

Considerações sobre os Termos de apresentação obrigatória:
Vide campo "Conclusões ou Pendências e Lista de Inadequações".

COMISSÃO NACIONAL DE
ÉTICA EM PESQUISA

Continuação do Parecer: 4.245.490

Recomendações:

Conclusões ou Pendências e Lista de Inadequações:

Não foram observados óbices éticos nos documentos da presente Emenda.

Considerações Finais a critério da CONEP:

Diante do exposto, a Comissão Nacional de Ética em Pesquisa - Conep, de acordo com as atribuições definidas na Resolução CNS nº 466 de 2012 e na Norma Operacional nº 001 de 2013 do CNS, manifesta-se pela aprovação da emenda proposta ao projeto de pesquisa.

Situação: Emenda aprovada.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_1599143_E2.pdf	21/07/2020 12:23:48		Aceito
Outros	ProjetoCOVID19_v4_realce.docx	21/07/2020 12:23:07	Amilcar Tanuri	Aceito
Projeto Detalhado / Brochura Investigador	ProjetoCOVID19_v4.docx	21/07/2020 12:22:47	Amilcar Tanuri	Aceito
Outros	TCLE_v3_realce.docx	21/07/2020 12:20:33	Amilcar Tanuri	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_v3.docx	21/07/2020 12:20:13	Amilcar Tanuri	Aceito
Outros	folhaDeRosto_v2_editavel.pdf	28/05/2020 14:40:34	Amilcar Tanuri	Aceito
Outros	TermoUtilizacaoDados.doc	28/05/2020 14:40:18	Amilcar Tanuri	Aceito
Outros	TermoUtilizacaoDados_assinado.pdf	28/05/2020 14:40:03	Amilcar Tanuri	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	SolicitacaoDispensaTCLE_assinada.pdf	28/05/2020 14:36:02	Amilcar Tanuri	Aceito
Folha de Rosto	folhaDeRosto_v2_assinada.pdf	28/05/2020	Amilcar Tanuri	Aceito

COMISSÃO NACIONAL DE ÉTICA EM PESQUISA

Continuação do Parecer: 4.245.490

Folha de Rosto	folhaDeRosto_v2_assinada.pdf	14:35:23	Amilcar Tanuri	Aceito
Declaração de concordância	CartaAnuenciaHNMD.pdf	20/03/2020 16:15:16	Amilcar Tanuri	Aceito
Outros	LinksLattes.docx	20/03/2020 09:54:48	CYNTHIA CHESTER CARDOSO	Aceito
Outros	CartaApresentacao.docx	20/03/2020 09:49:11	CYNTHIA CHESTER CARDOSO	Aceito
Outros	CartaApresentacaoAssinada.pdf	20/03/2020 09:48:42	CYNTHIA CHESTER CARDOSO	Aceito
Outros	FolhaDeRostoEditavel.pdf	18/03/2020 16:20:03	CYNTHIA CHESTER CARDOSO	Aceito
Declaração de Instituição e Infraestrutura	TAI_Editavel.docx	18/03/2020 16:19:45	CYNTHIA CHESTER CARDOSO	Aceito
Declaração de Instituição e Infraestrutura	DeclaracaoInfraestruturaEditavel.docx	18/03/2020 16:19:36	CYNTHIA CHESTER CARDOSO	Aceito
Declaração de Instituição e Infraestrutura	TAI_Assinado.pdf	18/03/2020 16:18:19	CYNTHIA CHESTER CARDOSO	Aceito
Declaração de Instituição e Infraestrutura	DeclaracaoInfraestruturaAssinada.pdf	18/03/2020 16:17:45	CYNTHIA CHESTER CARDOSO	Aceito

Situação do Parecer:
Aprovado

BRASILIA, 28 de Agosto de 2020

Assinado por:
Jorge Alves de Almeida Venancio
(Coordenador(a))

CARACTERIZAÇÃO DE FATORES DE RISCO E DESENVOLVIMENTO DE NOVOS TESTES SOROLOGICOS PARA A INFECÇÃO POR SARS-CoV-2

Pesquisador Responsável:

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Professor Titular da Universidade Federal do Rio de Janeiro,

Chefe do Laboratório de Virologia Molecular UFRJ

Instituição proponente: Instituto de Biologia da UFRJ

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Resumo

Em dezembro de 2019, a Organização Mundial de Saúde teve conhecimento de casos de uma doença respiratório de origem desconhecida em Wuhan, na China. Posteriormente, umnovo coronavírus foi identificado como o agente causador. Este foi posteriormente denominado SARS-CoV-2, enquanto a doença em si passou a ser denominada COVID-19. Desde então, a doença tem-se disseminado pelo mundo, com transmissão local já presente em diversos países. Embora a maior parte dos indivíduos infectados desenvolva doença branda, semelhante a um resfriado, alguns indivíduos desenvolvem complicações que podem levar ao óbito. Os primeiros casos de infecção pelo novo coronavírus no Brasil foram confirmados no final do mês de fevereiro, e o sequenciamento do genoma viral foi divulgado apenas 48h depois. A fim de contribuir para o entendimento da infecção e controle da disseminação do vírus em nosso país, o presente estudo visa analisar amostras clínicas de indivíduos infectados pelo novo coronavírus utilizando metodologias de sequenciamento em larga escala. As amostras serão coletadas no Hospital Universitário Clementino Fraga Filho e no Hospital Naval Marcílio Dias, e enviadas ao Instituto de Biologia, onde serão realizadas as análises laboratoriais. As amostras de soro e plasmaserão utilizadas na avaliação da eficácia de sistemas de diagnóstico sorológico, além da análise do genoma completo do vírus. As amostras obtidas de casos graves serão submetidas à análise de RNAseq, com o objetivo de identificar o material genético deoutros agentes infecciosos que possam estar contribuindo para o quadro clínico observado, além de determinar perfis de expressão globais nestes casos. O exoma dos pacientes será também analisado de modo a identificar variantes genéticas associadas a casos graves. Esta análise poderá também contribuir para conhecer o repertório de células B destes pacientes,o que pode auxiliar no desenvolvimento de vacinas. Os genes candidatos identificados a partir das abordagens em larga escala (exoma e RNAseq), além de outros descritos na literatura, serão incluídos em estudo caso-controle incluindo todos os participantes dapesquisa.

INTRODUÇÃO

Em 31 de dezembro de 2019, a Organização Mundial de Saúde (OMS) foi notificada sobre os primeiros casos de doença respiratória grave de origem inicialmente desconhecida na cidade de Wuhan, na China. Uma semana depois, foi identificado como agente causador um novo coronavírus (nCoV) que foi oficialmente denominado SARS-nCoV-2. Trata-se de vírus de RNA vírus da ordem Nidovirales, família Coronaviridae. Desde então, o vírus se espalhou pelo mundo e diversos países além da China já apresentam transmissão local (Ministério da Saúde. Plataforma IVS, 2020).

A doença causada pelo SARS-CoV2 foi oficialmente denominada COVID-19. O espectro clínico tem-se mostrado amplo, podendo variar de um simples resfriado até pneumonia severa. Os sintomas são principalmente respiratórios, tais como febre, tosse e dificuldade para respirar. As complicações clínicas mais comuns são Síndrome Respiratória Aguda Grave (SRAG), lesão cardíaca aguda e infecção secundária. Os dados disponíveis até o momento indicam uma maior taxa de hospitalização em indivíduos maiores de 50 anos (Ministério da Saúde. SAES, 2020).

No Brasil, o primeiro caso suspeito foi notificado no dia 22 de janeiro de 2020. Em 01 de março de 2020, o Brasil contabilizava dois casos importados confirmados, ambos com histórico de viagem para a região da Lombardia na Itália, na qual o índice de infeção encontra-se alto. Cinco dias depois, um total de 13 casos já havia sido confirmado. O estado de São Paulo concentra 10 casos, enquanto Rio de Janeiro, Bahia e Espírito Santo apresentam apenas 1 caso confirmado até o dia 06 de março. Ao todo, 768 casos suspeitosjá foram notificados no Brasil, dos quais 480 já foram descartados (Ministério da Saúde. Plataforma IVS, 2020).

As definições operacionais de casos seguem sofrendo atualizações, acompanhando a detecção de novos casos no país. No Boletim Epidemiológico do dia 04 de março de 2020

(Ministério da Saúde. SVS, 2020), os casos suspeitos passaram a ser definidos comopessoas com febre acompanhada de pelo menos um dos sintomas respiratórios (ex: tosse, congestão nasal, dificuldade para respirar, entre outros), e que tenha viajado nos últimos 14 dias para país com transmissão local ou sustentada. Indivíduos com os mesmos sintomas e que tenham tido contato com um caso confirmado nos últimos 14 dias também são considerados casos suspeitos. Contatos domiciliares de casos confirmados e que apresentem os sintomas gripais são considerados casos prováveis. Até o presente momento,os casos confirmados de COVID-19 incluem os casos suspeitos ou prováveis que tenham diagnóstico laboratorial positivo ou aqueles que, além dos sintomas, tiveram contato próximo com algum indivíduo com diagnóstico laboratorial positivo (Ministério da Saúde. SVS, 2020).

Até o momento, o diagnóstico é confirmado pela identificação do material genético viral em amostras do paciente (swab nasofaríngeo, swab orofaríngeo, sangue) por qRT-PCR, visto que se trata de um vírus com genoma de RNA fita positiva. Existe também a necessidade do melhor entendimento da resposta imune e da história natural do SARS- CoV-2 para podermos fazer o desenvolvimento de kits diagnósticos simples e rápidos para auxiliar os clínicos na condução clinica dos pacientes e possibilitar estudos epidemiológicos mais acurados. O desenvolvimento de ações coordenadas para a identificação de novos casos e sequenciamento viral para identificação de “clusters” de transmissão viral a serem utilizados em estudos de vigilância epidemiológica é urgente paraque ações de saúde pública possam interferir e diminuir as taxas de transmissão, reduzindoo risco de disseminação do vírus e/ou a gravidade de uma possível epidemia no nosso país.

No final do mês de fevereiro (28/02/2020), o primeiro genoma do SARS-CoV-2 no Brasil foi sequenciado em tempo recorde (48 horas após confirmação do primeiro caso), utilizando a plataforma Nanopore de sequenciamento portátil, no âmbito de uma colaboração entre a Universidade de São Paulo (USP), o Instituto Adolfo Lutz, o Laboratório de Virologia Molecular da UFRJ e a Universidade de Oxford, no Reino Unido.

No dia 03 de março, o mesmo grupo já havia sequenciado também o genoma do segundo caso confirmado no país (Jesus, 2020). Trata-se dos primeiros genomas do SARS-CoV-2 reportados na América do Sul. A geração destes dados em tempo real durante a epidemia será de extrema importância não apenas para o estudo de vigilância genômica e dispersão do vírus pelo mundo, mas também para o mapeamento de regiões e epítopos antigênicos específicos para o desenvolvimento de testes sorológicos ainda não disponíveis.

Com o objetivo de dar continuidade ao estudo da COVID-19, pesquisadores da UFRJ com expertise em clínica, virologia, controle de epidemias, diagnóstico, genômica e fatores de risco, propõem um projeto de pesquisa de natureza multidisciplinar, de modo a contribuir para evitar o avanço do novo coronavírus em território nacional.

Hipóteses

A reduzida letalidade da infecção frente ao alto potencial de transmissão do SARS-CoV2, bem como a concentração dos casos graves entre indivíduos a partir dos 50 anos de idade sugere que sejam necessários fatores de risco adicionais para o desenvolvimento de complicações clínicas. O presente estudo tem como base a hipótese de que mutações que aumentem a patogenicidade do vírus, coinfecções ou ainda fatores genéticos do hospedeiro possam estar associados ao desenvolvimento de complicações clínicas tais como a peneumonia, Síndrome do desconforto respiratório agudo (SDRA) e sepse, que requerem a internação e podem evoluir para óbito.

O desenvolvimento de testes que avaliem a presença de anticorpos IgG, IgM, IgA e IgE específicos para SARS-CoV2 poderá contribuir para o diagnóstico da infecção de forma rápida e eficiente. Além de dispensar estrutura laboratorial complexa, o diagnóstico sorológico permitirá também identificar os casos em que a amostra clínica não tenha sido

coletada no momento em que a viremia está mais alta, o que poderia gerar resultado falso negativo em testes moleculares.

OBJETIVOS

O objetivo central do estudo consiste em contribuir para a compreensão dos fatores genéticos associados ao curso clínico da infecção por SARS-CoV2 e também para odesenvolvimento e validação de metodologias para o diagnóstico sorológico da infecção.

Os objetivos específicos incluem:

1. Avaliação de testes sorológicos/antigênicos para o diagnóstico da infecção pelo SARS-CoV2;
2. Análise combinada de testes imunológicos e moleculares para o diagnóstico da infecção;
3. Sequenciamento do genoma completo do SARS-CoV-2;
4. Determinação da prevalência de coinfecções na coorte de estudo e associação das mesmas aos desfechos clínicos da COVID-19;
5. Identificação de fatores genéticos do hospedeiro associados ao desenvolvimento de formas severas da COVID-19, através da análise do exoma dos pacientes;
6. Identificação de padrões de expressão gênica em casos de COVID-19 que apresentem complicações clínicas.

MATERIAIS E MÉTODOS

Desenho do estudo

Trata-se de estudo observacional para a identificação de fatores de risco associados a complicações da doença causada pelo novo coronavírus (COVID-19). A inclusão dos

participantes será realizada por um período de 12 meses, a partir da data da aprovação do projeto. A coleta será realizada no centro de triagem da COVID-19, vinculado ao Hospital Universitário Clementino Fraga Filho (HUCFF) e localizado no Centro de Ciências da Saúde, da UFRJ, e no Hospital Naval Marcílio Dias (HNMD). O projeto prevê a inclusãode 500 participantes. Serão considerados elegíveis para o estudo indivíduos maiores de 18 anos de ambos os sexos, que atendam aos critérios clínicos para investigação de infecção por SARS-CoV-2 (casos suspeitos). O diagnóstico será realizado através de avaliação clínico epidemiológica, conforme recomendações do Ministério da Saúde. A identificação laboratorial do SARS-CoV-2 será realizada através da metodologia de RT-PCR (Ministério da Saúde, SVS, 2020). Serão excluídos do estudo os indivíduos que não tiverem diagnóstico confirmado através de análise laboratorial. Os casos com complicações serão também definidos conforme recomendações do Ministério da Saúde (Ministério da Saúde. SVS, 2020; Ministério da Saúde. SAES, 2020). As amostras clínicas serão utilizadas para a avaliação de metodologias de diagnóstico sorológico, sequenciamento dos genomas virais, análise do metagenoma dos pacientes que apresentarem complicações clínicas e análises de transcriptoma e exoma de até 10 participantes, entre casos brandos (controles) e pacientes que apresentarem complicações clínicas (casos). A análise do exoma será importante para auxiliar para identificar variantes genéticas humanas associadas a complicações clínicas, além de contribuir para o conhecimento do repertório de células B, o que poderá auxiliar o desenvolvimento de estratégias de imunização. Os genes que forem identificados através do exoma e transcritoma como potencialmente associados ao desenvolvimento de complicações clínicas serão utilizados como candidatos para análise de associação incluindo todos os participantes da pesquisa.

Coleta e processamento das amostras

Para este estudo, serão utilizadas as amostras clínicas coletadas para o diagnóstico e laboratorial e rotina de acompanhamento clínico dos pacientes. Poderão ser utilizadassobras de sangue total, swab, líquor ou amostras de sangue já processadas (soro, plasma e buffy coat) para o acompanhamento clínico dos pacientes. Poderá ser solicitada a coleta de

20 mL de sangue, bem como amostras de saliva e fezes. Caso seja necessária a coleta sequencial de amostras para reportar a resolução da infecção pelo SARS-CoV-2, todas as amostras coletadas durante o seguimento dos pacientes serão incluídas no estudo, de modo a obter maior rendimento para a extração do material genético.

As amostras de soro, plasma, saliva e fezes serão utilizadas para a avaliação dos testes diagnósticos por sorologia e para a extração de ácidos nucleicos para sequenciamento do genoma viral e/ou análise metagenômica. A fase celular (buffy coat) será utilizada para extração de DNA e RNA dos pacientes, para a identificação de variantes genéticas e análise de RNAseq. As amostras serão armazenadas em biorepositório provisório no Laboratório de Virologia Molecular por um período de até 2 anos após o término do estudo, caso sejam necessária a repetição de um dos testes. Após este período as amostras serão descartadas.

Detecção do SARS-CoV-2 por RT-PCR

O diagnóstico molecular será realizado através da metodologia de transcrição reversaseguida pela reação em cadeia da polimerase (RT-PCR) para detecção de fragmento específico do material genético do vírus. A detecção será realizada por PCR em tempo real,seguindo recomendações do Ministério da Saúde (Ministério da Saúde. SVS, 2020; Cormanet al., 2020).

Validação de testes sorológicos e antigênicos

As amostras de soro, plasma, saliva e fezes obtidas serão utilizadas para a validação de testes sorológicos para a detecção de anticorpos IgG, IgM, IgA e IgE anti SARS-CoV-2. Também será avaliada a utilização de testes para a detecção do antígeno S do SARS-CoV-2em exsudatos respiratórios para uma maior sensibilidade dos testes rápidos. Os testesELISA encontram-se em fase de desenvolvimento.

Sequenciamento dos Genomas virais

O sequenciamento do genoma completo dos vírus será realizado através da metodologia de sequenciamento paralelo massivo, através da tecnologia Ion PGM (ThermoFisher

Scientific). Inicialmente, serão sintetizados oligonucleotídeos específicos para a amplificação do material genético viral e construção de bibliotecas genômicas. O conjunto de primers será desenvolvido de modo a fornecer uma cobertura total do genoma do vírus. Os genomas sequenciados serão analisados com o auxílio do software Ion Report (ThermoFisher Scientific).

Identificação de coinfecções

A identificação de outros microorganismos que possam estar presentes em coinfecção com SARS-CoV-2 será realizada em diferentes amostras clínicas (swab, sangue, saliva, fezes) utilizando diferentes metodologias de diagnóstico, incluindo testes sorológicos, PCR e sequenciamento do metagenoma. Para o metagenoma, será utilizada a metodologia de sequenciamento Illumina, conforme descrito (Calvet et al., 2016).

Sequenciamento do exoma e transcriptoma dos pacientes

O sequenciamento dos exomas e transcriptomas será realizado a partir de amostras clínicas desangue total através de metodologia Illumina. Para este fim, serão selecionados 10 casos com complicações clínicas e 10 indivíduos controle, que apresentarem sintomas brandos da doença. O preparo das bibliotecas para cada uma das análises será realizado conforme descrito (Aguiar et al., 2019; Carvalho et al., 2019). O sequenciamento será realizado na plataforma NextSeq500 sequencer (Illumina Inc., San Diego, California, USA).

METODOLOGIA PARA ANÁLISE DOS DADOS

As análises filogenéticas serão realizadas com base na sequência do genoma completo do novo coronavírus conforme descrito (Jesus, 2020). A análise dos metagenomas será realizada conforme descrito (Calvet et al., 2016). Após análise de qualidade, inicialmente serão removidas as sequências oriundas do genoma humano. As sequências restantes serão analisadas através de alinhamento utilizando o repositório de genomas virais do GenBank,

a fim de identificar outros agentes infecciosos que possam estar presentes na amostra. As análises do perfil transcricional dos pacientes serão realizadas conforme descrito (Aguiar et al., 2019).

As análises dos exomas e RNAseq serão realizadas conforme descrito (Aguiar et al., 2019). A qualidade das bibliotecas será avaliada utilizando a ferramenta FASTQC (http:// www.bioinformatics.babraham.ac.uk/projects/fastqc/). As sequências que atenderem os critérios de qualidade serão alinhadas ao genoma humano de referência (GRCh38). A anotação das variantes será realizada através da ferramenta "Genome Analysis Toolkit" (GATK). A anotação das variantes funcionais será realizada com o auxílio da ferramenta ANNOVAR. Para as análises de RNAseq, os genes diferencialmente expressos em casos graves serão identificados com o auxílio da biblioteca DESeq2, do software R. Asanálises de enriquecimento de vias biológicas serão realizadas com as ferramentas Reactome pathways (https://reactome.org/) e EnrichR.

Estudo de associação (caso-controle genético): as análises de associação serão realizadas conforme descrito em estudos anteriores do grupo (Arruda et al., 2016; Almeida et al., 2018). As frequências dos genótipos de cada polimorfismo serão determinadas por contagem direta e testadas quanto à sua conformidade ao esperado em condições de Equilíbrio de Hardy-Weinberg (EHW) com o auxílio de um teste Qui-quadrado. As frequências genotípicas e alélicas de cada polimorfismo em casos e controles serão comparadas com o auxílio de modelos de regressão logística com ajuste para covariáveis tais como gênero, idade, presença de comorbidades (ex: hipertensão, diabetes, doenças autoimunes). As frequências dos haplótipos serão estimadas separadamente para casos e controles através do método de máxima verossimilhança e comparadas através de modelos de regressão logística.

ASPECTOS ÉTICOS DO ESTUDO

RISCOS

O presente estudo será realizado utilizando o material restante daquele que já seria coletado para os laboratoriais de rotina, incluindo o diagnóstico laboratorial da infecção. Desta forma, o estudo não apresenta risco direto aos participantes além daqueles relativos à coletade swab de orofaringe e/ou coleta de sangue por punção venosa. Esta última pode acarretar o aparecimento de manchas arroxeadas no braço. O risco de perda de confidencialidade será evitado através da codificação de todas as amostras e registros em bancos de dados.

BENEFÍCIOS

O presente estudo não visa gerar benefícios diretos aos participantes. O objetivo principal da proposta reside na avaliação de novas metodologias de diagnóstico da infecção por SARS-CoV-2 e identificação de fatores de risco genéticos e coinfecções associados ao desenvolvimento de complicações clínicas nos pacientes. O resultado dos testes genéticos poderá ser encaminhado ao médico responsável, caso este venha a fornecer algum benefício para o manejo clínico. Os resultados poderão também ser disponibilizados ao paciente, caso seja solicitado. No que diz respeito aos dados genéticos humanos, o conhecimento do resultado não trará qualquer prejuízo ao paciente (estresse psicológico ou estigmatização). Embora o projeto vise o sequenciamento de todo o exoma dos pacientes, apenas os genes relacionados com a resposta a infecção serão analisados.

ORÇAMENTO

O presente estudo não apresenta patrocínio específico, e será conduzido através de colaborações e/ou verba restante de projetos conduzidos atualmente nos locais da pesquisa. Considerando que o proponente e parte da equipe são servidores de Instituto de Biologia,

este foi incluído na Plataforma Brasil como o Patrocinador da pesquisa. A previsão de custo das diferentes etapas do projeto está descrita no quadro abaixo.

Quadro 1: Orçamento previsto para o projeto.

Material	**Valor estimado**
Kits para extração de ácidos nucléicos	R$ 15.000,00
Kits para sequenciamento de genoma completo do vírus	R$ 32.000,00
Kits para análise do exoma dos pacientes	R$ 30.000,00
Kits para metagenômica	R$ 40.000,00
Kits para análise por RNAseq	R$ 50.000,00
Material para análise de testes sorológicos	R$ 30.000,00
Material para genotipagem de polimorfismos em genes candidatos	R$ 25.000,00
Total	**R$ 222.000,00**

CRONOGRAMA

O desenvolvimento do projeto está previsto para um período de 12 meses, a partir de sua aprovação pelo CEP e CONEP.

Atividade	**Semestre de execução**			
	1	**2**	**3**	**4**
Inclusão dos pacientes	X	X		
Diagnóstico por RT-PCR	X	X		

Sequenciamento do genoma total de SARS-CoV-2	X			
Avaliação de testes sorológicos e antigênicos	X	X		
Análise de RNAseq		X	X	
Análise de exoma de casos graves		X	X	
Análise de associação utilizando genes candidatos			X	X
Elaboração de manuscritos para a divulgação dos dados			X	X
Elaboração de relatórios para prestação de contas ao CEP				X

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