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IGOR SALERNO FILGUEIRAS

Análise compreensiva da resposta imune às infecções por ZIKV e SARS-CoV-2 em diferentes contextos patológicos

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IGOR SALERNO FILGUEIRAS

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“Omori did not succumb.”

Omocat LLC, **Omori**. 2020.

RESUMO

FILGUEIRAS, I. S. **Análise compreensiva da resposta imune às infecções por ZIKV e SARS-CoV-2 em diferentes contextos patológicos**. 2023. 134p. Dissertação (Mestrado em Imunologia) - Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2023.

Vírus é um assunto de extrema relevância para a saúde pública mundial, o que é facilmente perceptível no Brasil, visto o elevado número de casos anuais de Dengue, o surto de infeções pelo Zika vírus na última década e a atual pandemia da COVID-19, causada pelo SARS-CoV-2. Mais do que quadros de infecções agudas, por vezes as sequelas causadas por esses vírus podem ser devastadoras, além de persistirem por períodos indeterminados. De forma a estudar os aspectos e redes neuroimunológicas em consequência às infecções virais, o presente estudo se dividiu em três etapas. No primeiro capítulo se apresenta uma revisão de literatura que caracteriza os aspectos clínicos e mecanismos imunopatológicos envolvidos nas manifestações neurológicas acarretadas por infecções de Zika vírus. Temas como a Síndrome Congênita associada à infecção pelo vírus Zika (CZS) e a Síndrome de Guillain-Barré são abordados nessa etapa. Com o irromper da pandemia da COVID-19, redirecionamos o foco do trabalho e os dois capítulos que vem a seguir tratam deste tópico que levou à morte milhões de indivíduos e deixou sequelas devastadoras em outra grande parte da população infectada. O estágio pós-agudo dessa doença, a chamada Síndrome Pós-COVID (PCS) é tema do segundo capítulo desta dissertação. Em colaboração com pesquisadores da Alemanha, analisamos a presença de autoanticorpos provenientes de uma coorte de pacientes acometidos por PCS, incluindo pacientes com encefalomielite miálgica, a síndrome da fadiga crônica (ME/CFS). Através de análises estatísticas e modelos de *machine learning*, observamos uma diminuição nos níveis de alguns autoanticorpos contra moléculas neuroimunológicas, sugerindo que eles desempenham papel fundamental na manutenção e regulação da fisiologia humana. O terceiro e último capítulo desta dissertação aborda a eficácia da terapia celular no tratamento da COVID-19. Este estudo, também desenvolvido em colaboração com pesquisadores internacionais, sumariza os achados de testes clínicos publicados até julho de 2022 e mostra uma redução significativa na mortalidade da doença ao utilizar células tronco mesenquimais. Por fim, a abordagem que desenvolvemos permitiu melhor compreensão holística da resposta imune às infecções por Zika e SARS-CoV-2 em diferentes contextos patológicos. Os achados dessa dissertação contribuem para o avanço do conhecimento da imunologia, permitindo o desenvolvimento de abordagens terapêuticas para o tratamento dessas infecções.

Palavras-chave: Infecções virais. Bioinformática. Zika. COVID-19. Terapia Celular.

ABSTRACT

FILGUEIRAS, I. S. **Comprehensive analysis of the immune response to ZIKV and SARS-CoV-2 infections in different pathological contexts**. 2023. 134p. Masters thesis (Immunology) - Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2023.

Viruses are a subject of extreme relevance for worldwide health services, which is easily seen on Brazil, given the high number of yearly cases of Dengue, Zika virus outbreak in the last decade, and the SARS-CoV-2 driven COVID-19 pandemics that is affecting the whole world right now. More than just acute-phase infections, these diseases may inflict long lasting and devastating outcomes in the patients. In order to study the clinical aspects and neuroimunological consequences of these viral infections, this study has been split in three sections. The first chapter presents a literature review that characterizes the clinical aspects and immunopathological mechanisms involved in the neurological manifestations caused by Zika virus infections. Topics such as Congenital Zika Syndrome (CZS) and Guillain-Barré Syndrome are addressed in this stage. Due to the outbreak of the COVID-19 pandemics, the focus of the research was redirected, and the following two chapters discuss this disease responsible for the deaths of millions of individuals and devastating sequelae in a large part of the infected population. The post-acute stage of this infection, known as Post-COVID Syndrome (PCS), is the subject of the second chapter of this dissertation. In collaboration with researchers from Germany, we analyzed the presence of autoantibodies from a cohort of PCS patients, including patients with myalgic encephalomyelitis, also known as chronic fatigue syndrome (ME/CFS). Through statistical analysis and machine learning models, we observed a decrease in the levels of certain autoantibodies against neuroimmunological molecules and indications that they play a fundamental role in the maintenance and regulation of human physiology. The third and final chapter of this dissertation addresses the efficacy of cellular therapy in the treatment of COVID-19. This study, also developed in collaboration with international researchers, summarizes the findings from clinical trials published until July 2022 and demonstrates a significant reduction in disease mortality when using mesenchymal stem cells. Finally, the approach we developed allowed for a more comprehensive understanding of the immune response to Zika and SARS-CoV-2 infections in different pathological contexts. The findings of this dissertation contribute to the advancement of immunology knowledge, enabling the development of therapeutic approaches for the treatment of these infections.

Keywords: Viral infections. Bioinformatics. COVID-19. Zika. Cell therapy.

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LISTA DE ABREVIATURAS E SIGLAS

Ab	Antibody
AAB	Autoantibody
ACE2	Angiotensin-Converting Enzyme 2
ADCC	Antibody-dependent cellular cytotoxicity
ADRA	Adrenergic receptor alpha
ADRB	Adrenergic receptor beta
AGTR	Angiostensin II receptor
Ahr	Aryl Hydrocarbon Receptor
ANS	Autonomic nervous system
Ang	Angiotensin
APC	Antigen-presenting cells
ARDS	Acute respiratory distress syndrome
AT	Adipose tissue
ATMP	Advanced Cell Therapy Medicinal Product
AXL	AXL receptor tyrosine kinase
BDKRB1	Bradykinin receptor B1
BBB	Blood brain barrier
BM	Bone marrow
CCC	Canadian consensus criterio
CFS	Cerebrospinal fluid
CHIKV	Chikungunya virus
CHRM	Muscarinic acetylcholine receptor
CHRN	Nicotinic acetylcholine receptor
CI	Confidence Interval
CMO	Contract manufacturing organization
CNS	Central nervous system
CSPI	Center for science in the public interest
CXCR3	CXC chemokine receptor 3
CZS	Congenital Zika Syndrome
DAMP	Damage-associated molecular patterns
DENV	Dengue virus
ECDC	European Center for Disease Prevention and Control

ED	Endothelial dysfunction
EDNRA/B	Endothelin receptor type A/B
ER	Endoplasmic reticulum
EU	European union
F2R/PAR-1	Coagulation Factor II Thrombin receptor/Proteinase-activated receptor 1
FDA	Food and drug administration
GBS	Guillain-Barré Syndrome
GPCR	G-protein coupled receptor
HC	Healthy control
hi-NPC	Human-induced neural progenitor cells
HLA	Human leukocyte antigen
IBMIR	Instant blood-mediated inflammatory reaction
IFN	Interferon
IFNAR	Interferon receptor
Ig	Immunoglobulin
IL	Interleukin
IQR	Interquartile range
ISCT	International Society for Cell and Gene Therapy
ISG	Interferon stimulated genes
IV	Intravenous
Mas1	MAS receptor
ME/CFS	Myalgic Encephalomyelitis/Chronic Fatigue Syndrome
MH	Mantel-Haenszel
MHC	Major histocompatibility complex
MoA	Mechanisms of action
MNC	Mononuclear cell
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
MSC	Mesenchymal stem cell
MSH-1	Musachi-1
NA	Not assessed
NCAM1	Neural Cell Adhesion Molecule
ND	Not determined
NK	Natural killer cell

NMOSD	Neuromyelitis optica spectrum disorder
NS	Non-structural protein
OAS	Oligoadenylate synthetase
OR	Odds ratio
PAMP	Pathogen-associated molecular patterns
PCHC	Post COVID-19 healthy control
PCS	Post COVID Syndrome
PEM	Post-exertional malaise
PML	Promyelocytic protein leukemia
PNS	Peripheral nervous system
PRR	Pathogen recognition receptors
PT	Perinatal tissue
RNA	Ribonucleic acid
RR	Risk ratio
RT-PCR	Reverse transcription-polymerase chain reaction
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
STAB1	Stabilin-1
STAT	Signal transducer and activator of transcription
STD	Sexually transmitted disease
TACC	Treatment arm continuity correction
TLR	Toll-like receptor
TNF	Tumor necrosis factor
UC	Umbilical cord
UK	United Kingdom
US	United States
VOI	Variant of interest
WHO	World Health Organization
ZIKV	Zika virus

SUMÁRIO

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1 CAPÍTULO 1 - THE CLINICAL SPECTRUM AND IMMUNOPATHOLOGICAL MECHANISMS UNDERLYING ZIKV-INDUCED NEUROLOGICAL MANIFESTATIONS

"It's the questions we can't answer that teach us the most. They teach us how to think. If you give a man an answer, all he gains is a little fact. But give him a question and he'll look for his own answers. [...] That way, when he finds the answers, they'll be precious to him. The harder the question, the harder we hunt. The harder we hunt, the more we learn."

Patrick Rothfuss, **The Wise Man's Fear**. 2011.

The clinical spectrum and immunopathological mechanisms underlying ZIKV-induced neurological manifestations

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Running title: Zika virus infection and neuroimmunological implications

1.1 ABSTRACT

Since the 2015-2016 outbreak in America, Zika virus (ZIKV) infected almost 900 thousand subjects. This international public health emergency was mainly associated with a significant increase in the number of newborns with congenital microcephaly and abnormal neurologic development, known as Congenital Zika Syndrome (CZS). Furthermore, Guillain-Barré Syndrome (GBS), a neuroimmune disorder of adults, has also been associated with ZIKV infection. Currently, the number of ZIKV infected subjects has decreased and most of the cases recently reported present as a mild and self-limiting febrile illness. However, based on its natural history of a typical example of re-emerging pathogen and the lack of specific therapeutic options against ZIKV infection, new outbreaks can occur worldwide, demanding the attention of researchers and government authorities. Here, we discuss the clinical spectrum and immunopathological mechanisms underlying ZIKV-induced neurological manifestations. Several studies have confirmed the tropism of ZIKV for neural progenitor stem cells by demonstrating the presence of ZIKV in the central nervous system during fetal development, eliciting a deleterious inflammatory response that compromises neurogenesis and brain formation. Of note, while the neuropathology of CZS can be due to a direct viral neuropathic effect, adults may develop neuroimmune manifestations such as GBS due to poorly understood mechanisms. Anti-ganglioside autoantibodies have been detected in multiple patients with ZIKV infection-associated GBS, suggesting molecular mimicry. However, further additional immunopathological mechanisms remain to be uncovered, paving the way for new therapeutic strategies.

1.2 INTRODUCTION

Arthropod-borne viruses or arboviruses are responsible for many important infectious diseases worldwide [1]. Due to many aspects of modern society, such as disorganized urbanization, excessive population growth, and increasing international mobility in the past few decades, arboviral diseases currently represent a serious global public health issue [1]. In this context, the outbreak of Zika virus (ZIKV) infection that started in Brazil in 2015, was declared a state of emergency and global concern by the World Health Organization (WHO) on February 1st of the same year [1,2]. This was mainly driven by the exponential increase of newborns with microcephaly and adults with Guillain-Barré Syndrome (GBS). Currently, there are approximately 4000 cases of ZIKV Congenital Syndrome (CZS) in Brazil. The virus has spread to more than 94 countries, infecting as of today almost 900 thousand people, confirming the relevance of arboviruses as a global threat [3].

ZIKV is mostly vectored by *Aedes aegypti* mosquitoes [4], followed by *Aedes albopictus* [5,6]. It is worth mentioning that it can also be found in human sperm up to 6 months after infection. Consequently, in September 2016, the WHO further classified ZIKV infection as a sexually transmitted disease (STD) [7]. Furthermore, vertical transmission (mother-to-fetus and breastfeeding) and transmission by blood transfusions have been described, and the presence of ZIKV in tears was also reported [8–13]. Noteworthy, vertical transmission was also observed in vectors, as infected *A. egipty* laid infected eggs [6]. Of note, scientists discovered that the *Aedes aegypti*, when exposed to ZIKV, Chikungunya virus (CHIKV), and Dengue virus (DENV) may transmit one, two, or even all three viruses simultaneously [14], resulting in viral co-infection and immune hyper-responsiveness [11].

Symptoms associated with acute-phase ZIKV infection are headache, fever, conjunctivitis, myalgia, exanthem and arthralgia [11], which may confound the initial diagnosis from other arboviruses such as DENV and CHIKV infections. Of note, approximately 80% of individuals infected with ZIKV do not develop any clinical manifestation [1] and only 0.3-0.5% of infected pregnant women have given birth to babies with microcephaly [15]. However, during the peak of the outbreak in Rio de Janeiro (2016), adverse neonatal outcomes reached 46% of the infected cases [16]. These facts indicate multifactorial influences in the outcome of ZIKV infection. In this context, Caires-Júnior *et al.* described that only one of dizygotic twins developed CZS [17]. This suggested that host factors (*e.g.* genetic background and epigenetics) also affect the outcome of ZIKV infection. On the other hand, viral mutations have been shown to be involved in pathogenicity and transmission [18]. In this regard, genetic and phylogenetic

investigations indicate that distinct ZIKV lineages (*e.g.,* those of West African, East African, Asian origin) may affect infectivity, virulence, and clinical presentation. Thus, genetic aspects of the virus need to be considered in the etiopathogenesis of ZIKV infections and outcomes, which have been reviewed in detail elsewhere [19,20].

Many studies have confirmed the tropism of ZIKV for neural progenitor stem cells [21,22] and a causal relationship between ZIKV infection during fetal development and the occurrence of CZS [4,23,24]. The Neural Cell Adhesion Molecule (NCAM1) has been recently reported as the possible ZIKV receptor [25]. However, other entry receptors might be involved since AXL receptor tyrosine kinase (AXL) has been shown to mediate ZIKV entry in human glial cells [26]. For instance, it has been shown that the ZIKV genome interacts with Musachi-1 (MSH-1), an RNA-binding protein in the central nervous system (CNS). MSH-1 has an important function in orchestrating mRNA translation for proper neurodevelopment. ZIKV sequesters MSH-1 to promote its replication in the replisomes, impairing the translation of endogenous neurotrophic factors [27].

Notably, an association between ZIKV infection in adults and the development of autoimmune manifestations such as GBS has also been extensively reported [28]. However, the etiopathology remains not fully understood. Thus, making the investigation of ZIKV–host interactions an important research area to be explored. For instance, there is no specific therapy available for ZIKV, demanding the identification of novel immunopathological mechanisms to develop new therapeutic strategies. Here, we review the neuroimmunopathological mechanisms and disease outcomes associated with Zika virus infection.

1.3 THE CLINICAL SPECTRUM OF CONGENITAL ZIKA SYNDROME

The epidemic of CZS was first reported in Brazil in 2015. The many neurologic abnormalities of CZS may consist of abnormal cranial morphology such as overlapping cranial sutures, severe microcephaly, protruding occipital bone, neurologic impairment and scalp ridges. Brain anomalies can also be present and include abnormal gyral patterns, thin cerebral cortices, larger amount of fluid spaces, calcification of subcortical regions, corpus callosum abnormalities, asymmetric and increased ventricles, lesser amount of white matter, and cerebellar hypoplasia. Ocular anomalies, if present, consist of macular pucker formation, retinal inflammation, focal pigmentary retinal mottling, and hypoplasia or atrophy of the optical nerve. Congenital contractures such as arthrogryposis and congenital talipes equinovarus have also been

described. When neurologic sequelae occur they may include early hypertonia, epilepsy, irritability, and symptoms of extrapyramidal involvement [16,29,30] (**Figure 1**).

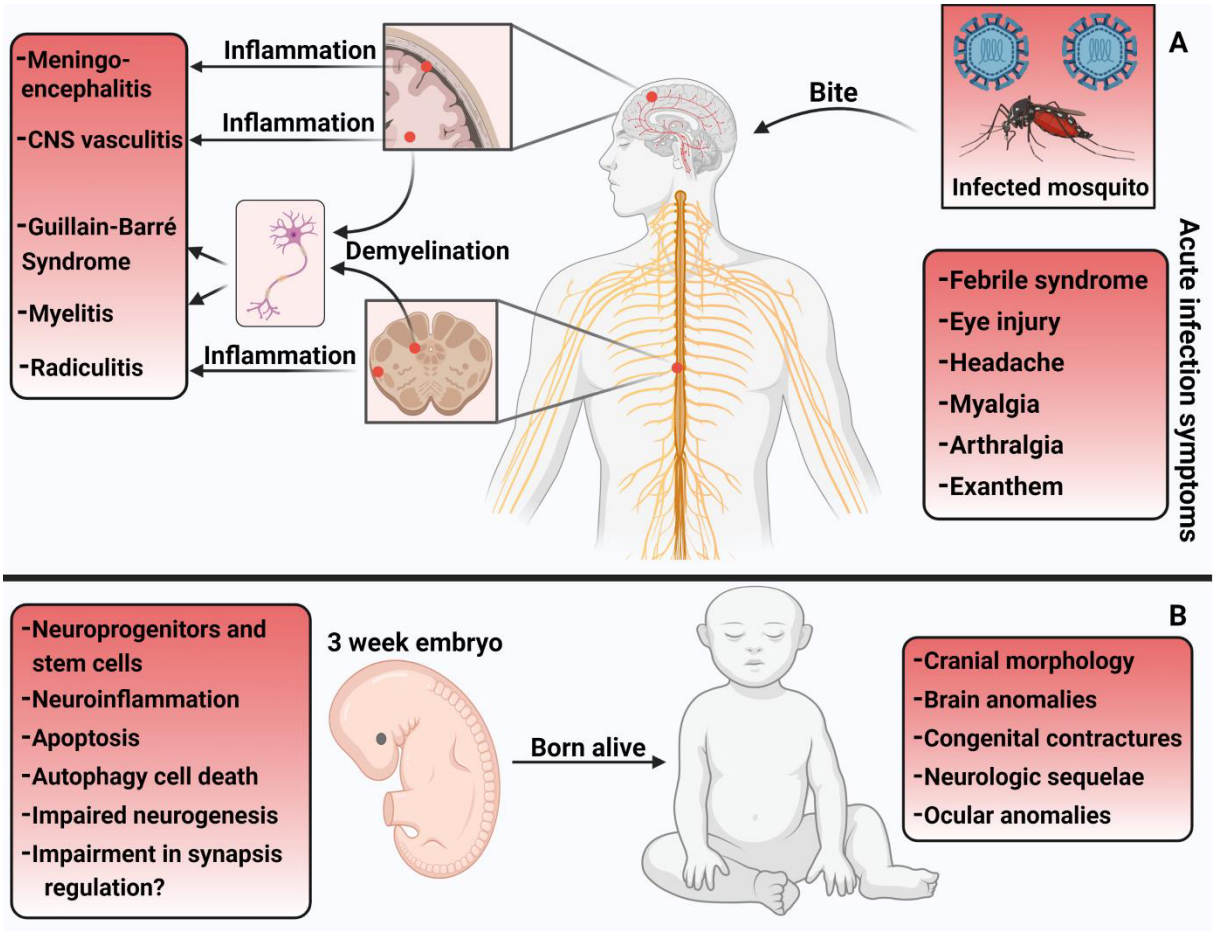

Figure 1. **ZIKV-associated neurological manifestations. (A)** Infected adults may be asymptomatic or present with febrile acute infection symptoms (right panel). The infection may also result in neurological complications, involving the central nervous system and/or peripheral nervous system. **(B)** ZIKV infection during pregnancy can affect the fetus and result in abnormal nervous system development, impairing neurogenesis, and leading to characteristic anomalies. Created with Biorender.com.

1.4 THE IMMUNE RESPONSE DURING PREGNANCY AND THE DEVELOPMENT OF CONGENITAL ZIKV SYNDROME

During pregnancy, the female body employs homeostatic strategies to promote both immune regulation and tolerance while promoting immune surveillance and defense. These immunological processes support embryo development and prevent maternal-fetal infections to avoid placental dysfunction and intrauterine growth restriction [31]. The placenta provides a physical barrier interfacing the maternal and fetal blood circulation, and is also essential for

waste, gas and nutrient exchange. The placenta is an immunologically active barrier to secure implantation and to restrict pathogen invasion [32]. The development of the fetus leads to an expansion of maternal peripheral blood mononuclear leukocytes and their recruitment into the placenta, promoting a tolerogenic environment [33]. In addition, extrinsic and intrinsic factors such as nutritional status, infections, stress, and obesity of a pregnant woman can influence her immune response and promote disorders associated with fetal neurodevelopment [34].

ZIKV can overcome the maternal-fetal immune-physiological barrier by directly inducing cytopathic cell death, and indirectly by tissue damage caused by a local exacerbated inflammatory response [22,35,36]. For instance, a detrimental role of type I interferons (IFNs) in pregnancy has been suggested when mice were inoculated with ZIKV at a gestational age corresponding to the mid and late first trimester in humans. This indicates a gestation stage-dependent ZIKV vertical transmission. By breeding homozygous type I IFN receptor (IFNAR) knockout (-/-) mothers with heterozygous male mice (IFNAR +/-), Yockey *et al.* showed that IFN-β produced in the placenta of heterozygous (IFNAR +/-) litters induced a more pronounced tissue damage and increased viral loads than in homozygous deficient ones (IFNAR-/-)[37]. These findings suggest that the signaling events triggered by IFN-β lead to abortion and growth restriction during ZIKV infection. This observation highlights the complex interplay between host and pathogen during ZIKV infection.

Moreover, new immunopathological mechanisms suspected to be involved in CZS await further investigation. For instance, ZIKV neurotoxicity could impair neurogenesis through a direct cytopathic effect on developing neurons by recruiting leukocytes and activating astrocytes and microglia. This may lead to subsequent congenital abnormalities and/or abortion. Of note, during brain inflammatory responses, astrocytes and microglia express inducible nitric oxide synthase (iNOS) [38]. Diop *et al.* have demonstrated that during initial hours of *in vitro* infection of microglia (CHME-5 cell line) with ZIKV, there is an upregulation of chemokine receptors transcripts involved in leukocyte migration and synapse regulation as well as increase of iNOS and pro-inflammatory molecules such as TNF-α, IL-1β, and IL-6 [39]. Nitric oxide (NO) is a gaseous bioactive compound that exerts protective and regulatory function on different cell types and influences the vascular smooth muscle tone. However, NO can present both anti- and proapoptotic properties, depending on its concentration and source. At low concentrations and when derived from endothelial and neuronal isoforms of NO synthase (eNOS and nNOS), NO normally has protective effects. On the other hand, at higher concentration levels and derived from iNOS, NO is more likely to induce cell death [40].

Therefore, low levels of NO promote the destruction of microorganisms and tumor cells, but at high concentrations and for long term it induces apoptosis of neurons, genotoxic species, and neurodegenerative disorders caused by a S-nitrosylation dependent pathway [41].

1.5 NEUROIMMUNE DISORDERS ASSOCIATED WITH ZIKV INFECTIONS

In addition to CZS, a variety of neurological manifestations affecting both central and peripheral nervous system (PNS) of adults have been reported in patients infected with ZIKV, such as GBS, CNS vasculitis, radiculitis, myelitis, meningoencephalitis, or a combination of these complications [28,42] (**Figure 1**). While CZS is the direct result of the neuropathological effects of the virus [22], these neuroimmune manifestations may occur due to immune dysregulation and autoimmunity triggered after convalescence of ZIKV infection, at least in some subjects [43]. This hypothesis is based on postmortem examination of some infected adults in whom ZIKV viral RNA or antigen were undetectable in the peripheral and central nervous system of patients who had developed GBS.

1.5.1 Guillain-Barré Syndrome

GBS is an autoimmune disease characterized by progressive bilateral weakness and loss of deep tendon reflexes due to peripheral nerve damage [28,43]. The etiopathogenesis of GBS following ZIKV infection may involve molecular mimicry between glycolipids and some ZIKV structural molecules, thus leading to an autoimmune response [44]. A recent systematic review and meta-analysis by Sonja *et al.* characterized the clinical phenotype of ZIKV associated GBS as a general sensorimotor demyelinating syndrome with frequent facial paralysis [45]. The authors observed that the time between the development of infectious symptoms and neurologic manifestations was approximately one week, and ZIKV viral RNA could be detected in the cerebrospinal fluid (CSF) through reverse transcription-polymerase chain reaction (RT-PCR) in only 10 out of 244 cases. While this observation suggested that the outcome of ZIKV infection may involve host self-reactivity, the authors were not able to exclude the possibility that the ZIKV may directly trigger GBS while hiding in CNS or PNS compartments. Of note, some of these individuals showed demyelination associated with inflammation and mononuclear lymphocytic infiltration [28], while others were found to have autoantibodies [46] (**Figure 2**). Beyond, there is evidence that the development of GBS not associated with ZIKV infection involves the activation of the classical complement pathway, disrupting myelin

sheath, nodes of Ranvier, and other membranes of the nervous system [47]. However, the role of the complement system in ZIKV-induced GBS remains to be investigated.

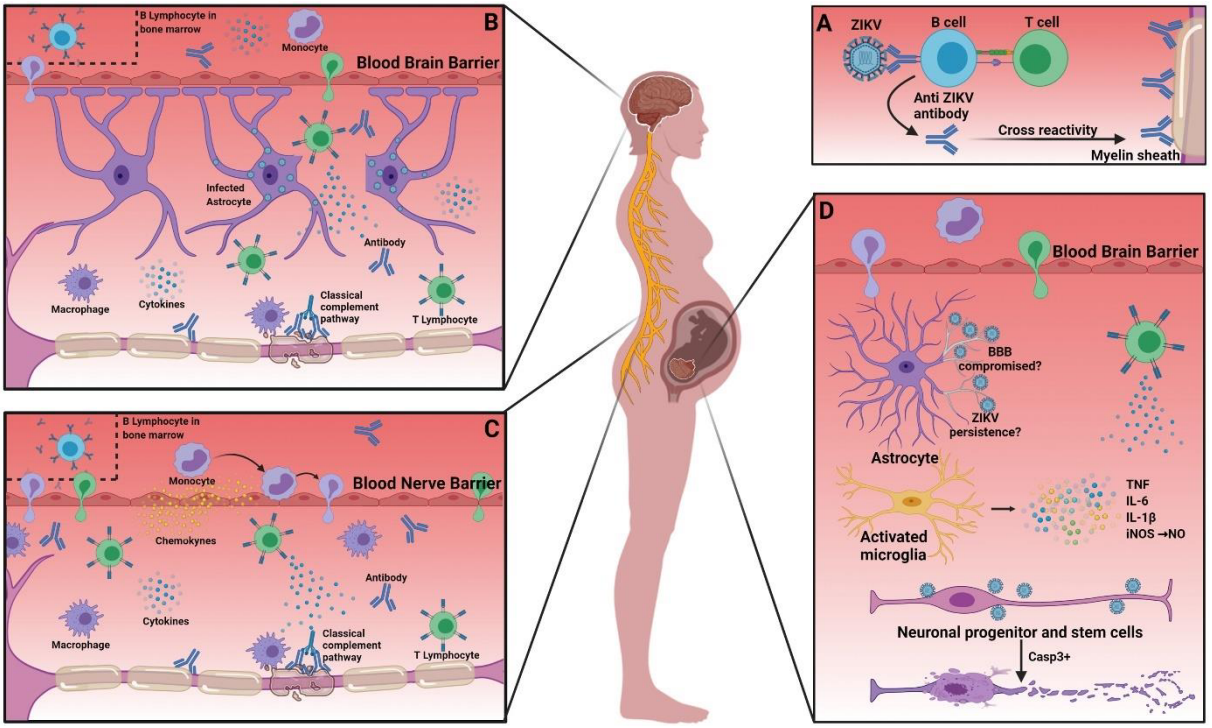

Figure 2. Neuroimmunological mechanisms involved in ZIKV infection. (**A**) Molecular mimicry between ZIKV and gangliosides. (**B**) Infected astrocytes mediate the activation and recruitment of immune cells into the central nervous system (CNS). (**B, C**) Antibodies against ZIKV cross-react with neurons, eliciting an autoimmune response that results in demyelination. (**D**) During fetal development, local infection activates microglia, promoting the production of cytokines. Loss of blood brain barrier (BBB) integrity occurs due to infection of astrocytes, which allows immune cell recruitment, contributing to pathological neuroinflammation. Neural stem cell infection results in autophagy, and possibly other unknown immunopathological mechanisms. The impaired neurogenesis results in congenital abnormalities. Created with Biorender.com.

1.5.2 Encephalitis

A recent study performed in Colombia, where a high number of pediatric ZIKV infections occurred, identified a total of 6 encephalitis cases in children [48]. The symptoms diminished faster than those of encephalitis caused by other infectious agents. Lymphocytosis of the CSF was present in all cases and higher cytokine levels were found in the CSF of one patient when compared with plasma levels, suggesting local inflammation [48].

A single patient with pre-existing multiple sclerosis (MS) who developed acute disseminated encephalomyelitis following ZIKV infection, indicates the possibility that this virus may exacerbate MS symptoms [49]. The patient was found to have ZIKV envelope protein in the brain tissue, indicating the presence of the virus, possibly due to disruption of the blood brain barrier (BBB) directly related to MS. These data suggest an association between neurologic complications due to ZIKV and an existing immune dysregulation background. However,

similar cases are necessary to strengthen this hypothesis. These events raise the question whether ZIKV primary infections in the CNS may lead to severe neuropathological complications in patients with a preexisting MS condition.

1.5.3 Neuromyelitis Optica Spectrum Disorder

Another possible outcome of ZIKV infection is the occurrence of neuromyelitis optica spectrum disorder (NMOSD), recently characterized in a single patient [50]. NMOSD is a severe and debilitating condition that mostly affects the spinal cord. The development of NMOSD not associated with ZIKV infection involves antibodies, mainly IgG, against a water channel called aquaporin-4 (Aqua-4) that is mostly expressed by astrocytes. This leads to complement activation as well as antibody-dependent cellular cytotoxicity (ADCC) resulting in extensive damage, as shown by MRI [51,52]. Initially, the patient with ZIKV infection presented with a positive RT-PCR for the virus in the CSF. Later in the course of the disease, he developed tactile and temperature allodynia of both arms. However, the immunopathological mechanism underlying the development of NMOSD remain to be uncovered. For instance, whether there is a molecular mimicry between ZIKV antigens and Aqua-4 has not been investigated.

1.6 THE IMMUNE RESPONSE TO ZIKV AND ITS TROPISM FOR THE NERVOUS SYSTEM

Blood mononuclear cells such as antigen presenting cells (APCs; *e.g.*, monocytes and dendritic cells) are the most frequently infected leukocyte subpopulations by ZIKV [53]. The recognition of pathogen-associated molecular patterns (PAMP) of ZIKV (*e.g.*, RNA) by host pathogen recognition receptors (PRRs) such as the toll-like receptor 3 (TLR3) is followed by the upregulation of proinflammatory molecules [54]. For example, the recognition of ZIKV by APCs induces the production of proinflammatory mediators and microbicidal mechanisms, such as production of IFNα/β, TNF-α, IL-1β, and NO. These innate immunity events are essential for the activation of T and B lymphocytes, responsible for the adaptive immune response [55], and consequently viral control and elimination [56].

In general, APCs present in human blood and epidermis seem to be the main route of distribution of the virus to other host tissues [56] . However, ZIKV also infects several other cell types such as skin epithelial cells [57], trophoblasts [58], neuronal progenitors, and stem cells [4,22]. During replication, many flaviviruses induce the rearrangement of the endoplasmic reticulum (ER) membrane to support viral production. This process triggers ER stress that

results in an active unfolded protein response and autophagy [59] which is a constitutive process of antigen presentation, but is also potentiated during stress, such as nutrient deprivation. Most cells catabolize proteins to generate energy by carrying and degrading damaged organelles and cytosolic proteins in lysosomes or more complex structures generated by the fusion of these with autophagosomes. If autophagy is upregulated and persists, cells may die [60]. In this context, ZIKV infection of human fetal neural stem cells may impair neurogenesis by aberrant activation of autophagy. I.e., the nonstructural viral proteins NS4A and NS4B synergistically induce cellular dysregulation by suppressing the PI3K-Akt-mTOR pathway [61], which is essential for brain development and autophagy regulation [62,63].

It has been shown that ZIKV co-localizes with autophagosomes [57]. Souza *et al.* developed a biological system of induced neural differentiation obtained by reprogramming human skin fibroblasts. The *in vitro* infection of neural stem and progenitor cells with ZIKV results in the depletion of progenitors and disruption of neural differentiation, as demonstrated by transmission electron microscopy and confocal microscopy [64]. The authors showed impaired cell proliferation and downregulation of caspase-dependent apoptotic cell death. They also confirmed the occurrence of autophagy by the presence of numerous autophagosomes in the perinuclear region of ZIKV-infected cells.

1.6.1 Type I IFNs and signaling pathways involved in the immune response to ZIKV

Among others, type I IFNs trigger the activation of the signal transducer and activator of transcription 1 (STAT1) and STAT2, which play a key role in the antiviral immune response [65]. They induce a state of viral resistance in host cells by activating enzymes such as 2'-5'-oligoadenylate synthetase 1 (OAS1) or ribonuclease L (RNAse L) that catalyze viral RNA degradation [66]. Type I IFNs are also responsible for the upregulation of class I major histocompatibility complex (MHC) and costimulatory molecules (CD80, CD86, CD40), potentializing antigen presentation to T helper (CD4+) and cytotoxic (CD8+) lymphocytes [67]. These IFNs also increase cytolytic action and proliferation of NK cells through the production of cytokines such as IL-15 [68]. Jurado *et al.* [69] demonstrated that transgenic mice lacking type I IFNs have increased viremia in the CNS when infected by ZIKV, mostly due to the antagonistic effect of the ZIKV nonstructural protein 5 (NS5) on STAT1 and STAT2 phosphorylation induced by type I IFNs [70].

In addition, *in vitro* infection of human-induced neural progenitor cells (hiNPCs) by a ZIKV Brazilian strain showed a transcriptional profile related to inflammation, interferon response,

cell death, and growth [71]. Lima *et al.* confirmed this data at the protein level by measuring soluble cytokines and chemokines in hiNPCs supernatants using a multiplex assay [71]. The levels of type I IFNs and of chemokines and cytokines associated with effector leukocyte recruitment and proinflammatory mechanisms were also significantly higher in CSF samples of CZS infants [71]. This raises the possibility that ZIKV affects infants' brains, triggering a local pathological inflammation that compromises neurogenesis and brain development.

1.6.2 ZIKV and immune evasion strategies

ZIKV avoids host immune response by a number of mechanisms, favoring viral replication and vertical transmission. Studies performed both with human cells and animal models clearly demonstrated the importance of type I and III IFNs in the prevention of ZIKV infection [24,72]. Type I IFNs activate cells expressing the IFN-α/β receptor (IFNAR). This event triggers STAT1, STAT2, and IRF-9 to translocate to the nucleus, and induce the transcription of multiple antiviral proteins, called IFN-stimulated genes (ISG), which effectively block viral replication and viral particle assemblage. Interestingly, the ZIKV NS5 protein targets human STAT2 inducing its degradation, abolishing type I IFNs responses [70]. However, this phenomenon in not observed in mice. For this reason, mice are highly resistant to ZIKV virus infection compared to humans, and transgenic or IFN knockout models are required to further investigate the host-pathogen relationship [24,73].

It has also recently been shown that ZIKV triggers the production of kynurenine (Kyn), which activates its receptor called Aryl Hydrocarbon Receptor (Ahr). This receptor is capable of suppressing not only Type I IFNS, but also inhibits the effect of the promyelocytic protein leukemia (PML) protein, which limits ZIKV replication. The use of Ahr antagonists in a murine experimental model of vertical transmission abrogated kyn-induced suppression and led to a better fetal outcome [74].

1.7 A POSSIBLE IMPLICATION OF PYROPTOSIS AND INFLAMMASOME ACTIVATION IN NEUROLOGICAL DISORDERS ASSOCIATED WITH ZIKV INFECTION

Pyroptosis is a type of programmed cell death triggered by the stress of extracellular or intracellular homeostasis [75]. Morphologic alterations associated with pyroptosis are a unique form of chromatin condensation that differs from apoptosis and plasma membrane permeabilization. Pyroptosis utilizes caspase-1-dependent and independent mechanisms.

During Dengue infection, viral RNA is recognized by innate receptors [76]. Cytoplasmic NOD-like receptors activate signaling pathways that subsequently culminate in the activation of a multiprotein complex called inflammasome. Among the components that make up this multi-protein unit, the cellular protease caspase-1 is of relevance [77]. With the activation of inflammasomes, pro-caspase 1 is cleaved into caspase-1 which then cleaves pro-IL-1β and pro-IL-18 as well as gasdermin D (GSDMD), which are released to the extracellular milieu [78]. IL-1β is a pyrogenic cytokine that mediates fever, immune cell migration, BBB disruption, adaptive immune activation and several other functions. IL-18 induces IFN-γ production, which is important to activate effector T-cells and natural killer cells. In turn, GSDMD is known to form pores that are necessary for the rapid release of IL-1β [79,80]. A recent study demonstrated that inflammasome responses are associated with human glioblastoma cell line activation when infected with ZIKV. This infection was linked with increased oxidative stress and pyroptosis, contributing to inflammation and neurological dysfunction [81].

1.8 THE PROTECTIVE ROLE OF MICROGLIA AGAINST NEUROLOGICAL DAMAGE CAUSED BY ZIKV

Microglia are resident macrophages located in the CNS. They are a main component of the local immune response, eliminating apoptotic cells and playing an essential role in brain development, synaptic pruning, memory, and neuronal recycling [35]. Limonta *et al.* demonstrated that primary human fetal astrocytes (HFAs) that promote neuron support, nutrition, and participate in the BBB maintenance, can become a reservoir for ZIKV, shedding virus for at least one month post *in vitr*o infection [82]. ZIKV infects microglia progenitors that derive from the yolk sac (first site of hematopoiesis in both mice and humans), and then, when mature, microglia carrying ZIKV invade the fetal brain [82]. This, provides an explanation on how ZIKV reaches the brain of the fetus.

Moreover, many PAMPs and damage-associated molecular patterns (DAMPs) are associated with brain damage and can trigger microglia activation. Fekete *et al.* hypothesized that microglia sense damage of individual cells before irreversible neuronal injury, recruiting more phagocytic resident cells to the compromised neurons after virus infection via purinergic receptors, promoting phagocytosis and restraining virus dissemination [83]. The authors also demonstrated that ATP delivered by infected neurons exerts chemotactic function, recruiting more precursors of phagocytic cells into the site of brain infection [83].

1.9 CONCLUSIONS

Despite of the advances in understanding the immunopathology of the neurological disorders associated with ZIKV infections, several underlying mechanisms remain poorly undestood. One important aspect is to determine the main routes and biological processes of ZIKV infection in fetuses and adults and whether they overlap. In addition, extensive research is necessary to find therapeutic targets to avoid uncontrolled ZIKV induced neuroinflammation and BBB damage. This is important to prevent the resulting neuropathology due to cell death, dysregulated cell cycle-related pathways, and local immune dysregulation [71,84].

Although the role of type I IFNs and their associated signaling pathways involved in the immune response to ZIKV have been extensively investigated, other protective host factors remain to be uncovered. In addition, immunopathological mechanisms that are poorly understood in humans have been investigated in detail in ZIKV infected mice, including damage of the BBB by astrocytes resulting in a significant infiltration of T lymphocytes into the CNS. This event destroys neurons, causing considerable damage to the brain due to dysregulation of antiviral activity and cytotoxicity resulting in paralysis. This finding supports the role of adaptive immunity in the neurological manifestations that occur in ZIKV infected patients [69,85]. However, the precise mechanisms resulting in immune dysregulation that lead to ZIKV induced autoimmunity remain unclear. Thus, in the absence of approved specific anti-ZIKV therapy or vaccines, a better understanding of the mechanisms that are involved in susceptibility to ZIKV infection and its pathophysiology will be essential for developing effective therapies, reducing morbidity and mortality of newborns and adults due to ZIKV infections.

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2 CAPÍTULO 2 - DYSREGULATED AUTOANTIBODIES TARGETING VASO- AND IMMUNOREGULATORY RECEPTORS IN POST COVID SYNDROME CORRELATE WITH SYMPTOM SEVERITY

"I am no poet. I do not love words for the sake of words. I love words for what they can accomplish. Similarly, I am no arithmetician. Numbers that speak only of numbers are of little interest to me."

Patrick Rothfuss, **The Wise Man's Fear**. 2011.

Dysregulated autoantibodies targeting vaso- and immunoregulatory receptors in Post COVID Syndrome correlate with symptom severity

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2.1 ABSTRACT

Most patients with Post COVID Syndrome (PCS) present with a plethora of symptoms without clear evidence of organ dysfunction. A subset of them fulfills diagnostic criteria of myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS). Symptom severity of ME/CFS has been shown to correlate with natural regulatory autoantibody (AAB) levels targeting several G-protein coupled receptors (GPCR). In this exploratory study we analyzed serum AAB levels against vaso-and immunoregulatory receptors, mostly GPCRs, in 80 PCS patients following mild-to-moderate COVID-19 with 40 of them fulfilling diagnostic criteria of ME/CFS. Healthy seronegative (n=38) and asymptomatic post COVID-19 controls (n=40) were also included in the study as control groups. We found lower levels for various AAB in PCS compared to at least one control group accompanied by alterations in the correlations among AABs. Classification using random forest indicated AABs targeting ADRB2, STAB1 and ADRA2A as the strongest classifiers (AABs stratifying patients according to disease outcomes) of post COVID-19 outcomes. Several AABs correlated with symptom severity in PCS groups. Remarkably, severity of fatigue and vasomotor symptoms were associated with ADRB2 AAB levels in PCS/ME/CFS patients. Our study identified dysregulation of AAB against various receptors involved in autonomous nervous system (ANS), vaso- and immunoregulation and their correlation with symptom severity pointing to their role in the pathogenesis of PCS.

2.2 INTRODUCTION

Post COVID syndrome (PCS) following mild-to-moderate coronavirus disease 2019 (COVID-19) with persistent symptoms for more than 6 months affecting everyday functioning is reported in 10-20% of patients (1-4). PCS symptoms are diverse with debilitating fatigue, post-exertional malaise (PEM), difficulties of breathing, pain and cognitive dysfunction as the most frequently reported (1). We found that half of the PCS patients with fatigue and exertional intolerance fulfill the Canadian consensus criteria (CCC) for myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) (5-7). These patients were referred to as PCS/ME/CFS and the others as PCS/non-ME/CFS, which comprise the COVID-19 outcomes that are of interest to this manuscript (5). Mechanisms of PCS remain poorly understood, but some first evidence point to both immune and vascular dysregulation (8-12). Prothrombotic autoantibodies (AAB) against anti-phospholipid and anti-type I interferon were among the first ones to be described in acute COVID-19 patients (13-15). Wang *et al.* showed elevated functional AAB levels directed against extracellular antigens with a high prevalence of AAB against immunomodulatory proteins like cytokines, chemokines and others (16). In addition, AABs against the vasoregulatory renin-angiotensin-system (RAS)-related proteins Angiotensin-converting enzyme 2 (ACE2) and angiotensin type-1 receptor (AGTR1) were increased in COVID-19 patients and associated with the disease severity (17). In accordance to this, more recently, we showed elevated levels of AAB directed against several vaso- and immunoregulatory G-protein coupled receptors (GPCRs) including RAS-related proteins in moderate and severe acute COVID-19 patients being associated with clinical severity of COVID-19 (18). First studies also found AABs in PCS patients. A microarray analysis detected amongst others AABs against IL2, CD8B, Thyroglobulin and interferons in PCS patients, which were reported before in acute COVID-19 (19). In addition elevated AAB levels against cyclic citrullinated peptide, a predictor for rheumatoid arthritis, against anti-tissue transglutaminase, a predictor of celiac disease (20), and against desmoglein-2, previously described in arrhythmogenic right ventricular cardiomyopathy (21) were described in recovered COVID-19 patients. Moreover, in a first study, functional AABs against vasoregulatory GPCRs were detected in recovered COVID-19 patients with persistent symptoms (22).

AABs against GPCRs are part of normal human physiology. These AABs are dysregulated in various autoimmune and non-autoimmune diseases (23). They can induce or alter signaling and play an important role in regulating autonomic nervous system (ANS), endothelial and immune cell function, which could also be of relevance in COVID-19. As example, AABs directed to

the angiotensin receptor type-1 (AGTR1) induced skin and lung inflammation and were one of the best AABs discriminating mild from severe COVID-19 patients (18, 24). Therefore, GPCR AABs may be useful as biomarkers indicating activation or alteration of respective receptors and pathways (25). In ME/CFS, there is evidence for an altered GPCR AAB network with disease-specific AAB correlations (26-28). AAB levels against β1 and β2 adrenergic receptors (ADRB1/2) as well as muscarinic acetylcholine receptors M3 and M4 (CHRM3/4) measured by ELISA were elevated at least in a subgroup of ME/CFS patients (26, 27). Moreover, AABs against ADRB2 and CHRM4 were significantly declined in clinical responder, but not in non-responder receiving rituximab treatment achieving B-cell depletion (27). Elevated CHRM1 AABs measured by radioligand assay were described in ME/CFS patients in association with muscle weakness and neurocognitive impairment (29). Further, AABs against several vasoregulatory GPCRs measured by ELISA were associated with key symptoms of fatigue and muscle pain in postinfectiouse ME/CFS patients (28). In conclusion, these studies indicate that AAB against the ADRs and CHRMs are associated with ME/CFS (27-29).

Here we investigate levels of IgG AAB directed against vaso- and immunoregulatory receptors including members of the classical RAS (AGTR1/2, BDKRB1) as well as the counter-regulatory ACE2/MAS1 axis, against endothelin receptors (EDNRA/B), against receptors related to the ANS (ADRs, CHRMs, CHRN), and against the protease-activated receptor F2R/PAR-1, the chemokine receptor *CXCR3* and the scavenger receptor stabilin-1 (STAB1). Thus, we aim to get insight into a potential dysregulation of the AAB targets, most of them GPCRs, and linked pathways in PCS. Further, we correlated the AAB levels with symptom severity. Importantly, we found an alteration of various AABs in PCS patients compared to post COVID-19 and seronegative healthy controls (PCHC and HC), as well as associations of AABs with clinical symptom severity.

2.3 METHODS

2.3.1 Patients

Sera of 80 patients with PCS following mild-to-moderate COVID-19 with long persistent fatigue and exertion intolerance and 78 healthy individuals were studied.

PCS patients were enrolled at the Charité Fatigue Center within an observational cohort study between August 2020 and July 2021. PCS patients had a confirmed diagnosis of mild to moderate COVID-19 (PCR or serology), suffer from persistent moderate to severe fatigue and exertion intolerance post severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) infection. Patients which suffer from relevant cardiac, respiratory, neurological, or psychiatric comorbidity, or preexisting fatigue, or evidence of organ dysfunction were excluded (5). Patients were diagnosed at least 6 months following COVID-19. In case of 17 of 80 patients the diagnosis was retrospectively made at most 2 months following blood sampling. Diagnosis of ME/CFS was based on 2003 Canadian Consensus Criteria (CCC), being the recommended diagnostic criteria for ME/CFS by EUROMENE for research purpose (6, 7). PCS/CFS patients fulfilling CCC suffer from persistent fatigue for at least 6 months, PEM, sleep dysfunction, pain, at least two neurological or cognitive manifestations and at least one symptom from two of the following categories: autonomic, neuroendocrine or immune manifestations (7). In contrast to the original CCC classification and in accordance with the studies of Cotler *et al*. 2018 a minimum of 14 hours of PEM duration was required for diagnosis of ME/CFS ((30) and **Table 1**). 40 patients fulfilled criteria for ME/CFS (6) referred to as PCS/ME/CFS and the other patients as PCS/non-ME/CFS. Patient groups were matched for disease duration on time point of blood sampling (**Table 1**).

Table 1: Characteristics of study groups

study group	PCS/ME/CFS (*n*=40)	PCS/non-ME/CFS (*n*=40)	PCHC (*n*=40)	HC (*n*=38)	*p* value
Age, median (range) [years]	46.5 (24-62)	40 (22-67)	35 (21-66)	38 (19-64)	0.0103 ($\mathbf{p}_{PCS/CFS vs. PCHC}$ **=0.0081**)
Female sex, *n*	33	28	23	27	0.1118
COVID-19 severity	moderate: 8 mild: 32	moderate: 8 mild: 32	NA	NA	>0,9999

Months after COVID infection, median (range)	7 (4-14)	7 (4-13)	5.5 (4-10)	NA	0.0049 (**$p_{PCS/CFSvs.PCHC}$ =0.0177; $p_{PCS/non-CFSvs.PCHC}$ =0.0114**)
PEM	40	38	NA	NA	0.1521
PEM >14h	40	11	NA	NA	<0.0001
PEM score median (range)	34 (15-46)	24 (1-40)	NA	NA	<0.0001
Chalder Fatigue Scale median (range)	27 (18-33)	25 (14-32)	NA	NA	0.0234
Bell Disability Scale median (range)	40 (10-80)	50 (30-90)	NA	NA	0.0017
SF36 Physical Functioning median (range)	33 (6-65)	37.5 (10-72)	NA	NA	0.0287
		Symptome severity scores median (range)			
Fatigue	8 (3-10)	7.5 (2-10)	NA	NA	0.2538
Cognitive score	5 (2-10)	4.85 (1-7.3)	NA	NA	0.4073
Headache	6 (1-10)	5 (1-9)	NA	NA	0.2466
Muscle pain	6 (1-10)	6 (1-10)	NA	NA	0.1728
Immune score	3.3 (1-9.3)	2.15 (1-8)	NA	NA	0.0071
COMPASS-31 total, median (range)	36.05 (7-65.16)	29.05 (2.5-62.4)	NA	NA	0.3793
COMPASS-31 orthostatic, median (range)	24 (0-40)	20 (0-40)	NA	NA	0.3958

COMPASS-31 vasomotor, median (range)	0 (0-4.2)	0 (0-4.2)	NA	NA	0.2573
COMPASS-31 secretomotor, median (range)	6.4 (0-15)	2.1 (0-12.86)	NA	NA	0.0857
COMPASS-31 gastrointestinal, median (range)	5.8 (0-15.2)	6.2 (0-17)	NA	NA	0.4670
COMPASS-31 bladder, median (range)	0 (0-5.6)	0 (0-4.4)	NA	NA	0.2678
COMPASS-31 pupillomotor, median (range)	1.483 (0-3.7)	1.3 (0-3)	NA	NA	0.6486
IgG total, median (IQR) [g/l]	10.85 (8.9-14.28)	10.3 (9.45-13.13)	9.7 (8-11.28)	11 (8.65-14.23)	0.1518

Legend Table 1: Kruskal-Wallis test was used when comparing more than two groups and Mann-Whitney-U rank-sum-test when comparing two groups. If the Kruskal-Wallis test results in $p<0.05$, the post hoc Dunn's test was performed and p-values ≤ 0.5 were added to the table in brackets. Chi-square test was used to compare the distribution of gender, COVID-19 severity and of PEM. A two-sided p value ≤ 0.05 was considered statistically significant. [IQR: interquartile range; NA = not assessed]**.**

Healthy individuals include 40 PCHC all after mild-to-moderate COVID-19, and 38 SARS-CoV-2-spike-IgG-negative HC without COVID-19 history. Serum SARS-CoV-2-spike-IgG was determined using Anti-SARS-CoV-2 ELISA (IgG) purchased from Euroimmun (Lübeck, Germany) according to manufactures' protocol. All PCS patients and healthy individuals were required to be SARS-CoV-2 unvaccinated. Samples of PCHC were collected from July 2020 until June 2021 with a similar time period following COVID-19 compared to PCS patients (**Table 1**). As women are more susceptible to PCS and ME/CFS than men (6, 31) study groups were matched for gender, but not for age because of limited number of participants. The study was approved by the Ethics Committee of Charité Universitätsmedizin Berlin in accordance with the 1964 Declaration of Helsinki and its later amendments. All participants signed informed consent.

For comparative statistical analysis of patient characteristics Kruskal-Wallis test with post hoc Dunn's test was performed using GraphPad Prism 6.0. For comparative analysis of distribution of gender, COVID-19 severity and PEM of study groups the Chi-square test was used. A two-sided p value ≤ 0.05 was considered statistically significant.

2.3.2 Determination of AAB

Whole blood samples from each subject were allowed to clot at room temperature and then centrifuged at 2000 x g for 15 min in a refrigerated centrifuge. The serum was purified and stored at −35 °C. IgG AAB against Angiotensin II receptor type 1/2 (AGTR1/2), Angiotensin-converting enzyme 2 (ACE2), MAS1, Bradykinin receptor B1 (BDKRB1), endothelin receptor type A/B (EDNRA/B), ADRA1/2A, ADRB1/2, CHRM1-5, nicotinic acetylcholine receptor subunit alpha 1 (CHRNA1), F2R/PAR-1, STAB1 and CXCR3 were measured using respective sandwich ELISA kits by CellTrend GmbH (Luckenwalde, Germany) as described before (18, 28). In brief, serum samples were diluted at 1:100 ratio for ELISA. The AAB levels were calculated as arbitrary units (U) by extrapolating from the standard curve of five standards ranging from 2.5 to 40 U/ml. The validation of the ELISA kits was carried out in accordance with the Food and Drug Administration's Guidance for Industry: Bioanalytical Method Validation. The concentration of total serum immunoglobulin (Ig)G was analyzed using Human IgG ELISA^{BASIC} Kit purchased from MABTECH AB (Nacka Strand, Sweden) according to manufacturer's protocol.

2.3.3 Symptom assessment by questionnaires

Severity of fatigue and other key symptoms were measured using a Likert Scale (1 = no symptoms up to 10 = most severe symptoms) by the patients. Severity of fatigue was also evaluated using the Chalder Fatigue Scale from 0 (no fatigue) to 33 (heavy fatigue) (32). PEM was assessed by a questionnaire (30), which describes an intolerance to mental and physical exertion triggering an aggravation of symptoms typically lasting for more than 14 hours up to several days (33). PEM score ranges from 0 (no PEM) to 46 (frequent, severe and long PEM) In addition, disability was assessed using the Bell score ranging from 0 (total loss of self-dependence) to 100 (without restrictions) (34) and physical activities of daily life was assessed by Short Form Health Survey 36 (SF-36) ranging from 0 (greatest possible health restrictions) to 100 (no health restrictions) (35). Quantification of the key symptoms ranges from 1 (no symptoms) to 10 (extreme symptoms) (36). Symptoms of autonomic dysfunction were assessed by the Composite Autonomic Symptom Score 31 (COMPASS 31) ranging from 0 (without symptoms) to 100 (strong autonomic dysfunction) (37).

For comparative statistical analysis Mann-Whitney-U rank-sum-test was performed using GraphPad Prism 6.0. A two-sided p value ≤ 0.05 was considered statistically significant.

2.3.4 Visualization of autoantibody targets and pathways interactions

We searched for physical protein interactions (PPIs) between AAB targets using Integrated Interactions Database, IID version 2021-05 (http://ophid.utoronto.ca/iid; (38)), combined with interactions from virus-human interactome (39). The interactions were then used to construct a network figure prepared using NAViGaTOR version 3.0.16 (40). Interactions between the autoantibody targets and their respective Gene Ontology (GO) biological processes (BP) were visualized by NAViGaTOR, as well as their interactions with human and SARS-CoV-2 molecules that are involved in the infection. Comprehensive pathway analysis of the 20 autoantibody targets and their interactors was performed using pathDIP version 4.1 (http://ophid.utoronto.ca/pathDIP, (41)). For the circular plot, emapplots, and enrichment, R version 4.0.5 (42), R studio Version 1.3.959 (43) were utilized, as well as Circos and the R packages ReactomePA, clusterProfiler, ggplot2, and viridis (44-50). After filtering the pathways for the most general level of the ontologies, up to 15 of the most significant pathways were plotted in the emapplots. Given the biological processes ontology, we chose 10 pathways based on their relevance for the discussion out of the most significant ones for each target and performed a circular plot. The R package openxlsx was used through the whole analysis to read and write files (51).

2.3.5 Pairwise comparison and differences in autoantibody concentrations

Differences in autoantibody levels were assessed by Kruskal-Wallis test followed by Dunn test and adjusted for false discovery rate (FDR) via the R package rstatix (52). For each of the AABs, log2-transformed data was used for better visualization. Boxplots were generated using the R packages ggplot2, ggpubr and lemon and plotted based on median and interquartile range (47, 53, 54). Adjusted p-values were represented by: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

2.3.6 Regression Analysis

AABs levels were modeled via the generalized additive models for location, scale and shape (GAMLSS) (55). Lognormal was chosen as the best fitting distribution for the data after analyzing the residual plots. To study potential confounders that may influence the outcomes and the autoantibody levels, i.e., age, sex, and time since infection, which were considered as covariables to model the AABs mean distribution in regard to clinical classification. HC group's

time since infection was considered as 0, and 5 missing values for PCS/ME/CFS patients were substituted by the group's median, 7.

2.3.7 Principal component analysis

Principal Component Analysis (PCA) using a single value decomposition of the data matrix (56, 57) was used to measure the stratification of HC/PCHC and PCS based on the donors' autoantibody levels. Prior to analysis, the raw AAB levels were log2-transformed. PCA was performed using the R package factoextra and the prcomp function, in which data was centered and scaled (58). Number of principal components was chosen according to Kaiser Criterion (59).

2.3.8 Ranking autoantibodies by Random Forest

We used the R package Random Forest (version 4.6.14) (60) to rank the AABs as classifiers of disease outcomes. We trained the random forest model using the 20 AAB levels (for which the number of variables randomly selected for each split, the mtry, was specified as 4), and five thousand trees were used for the classification. Follow-up analysis was conducted with the Gini decrease, number of nodes, and mean minimum depth as criteria to determine variable importance. Receiver Operating Characteristic (ROC) curve (and its area under the curve) and out-of-bags error rate were used to evaluate the stratification of disease groups as previously described (18).

2.3.9 Autoantibody Correlation signatures

Circular networks based on Spearman's rank correlation coefficient were constructed with the R package qgraph (61), using the Log2-transformed AAB levels.

2.3.10 Correlation analysis of AAB with clinical symptoms

Correlation analysis of AAB with clinical symptoms was performed using Spearman's rank correlation using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA). A two-sided p value ≤ 0.05 was considered statistically significant.

2.4 RESULTS

2.4.1 Study population

AAB of 80 PCS patients were measured. The majority of the PCS patients (64 of 80) had mild and 16 had moderate COVID-19 (Table 1) due to pneumonia, according to WHO criteria (62). 40 patients fulfilled the CCC for ME/CFS (6, 7) referred to as PCS/ME/CFS and the other patients as PCS/non-ME/CFS. Convalescent individuals who had COVID-19 (PCHC) during the same period and healthy seronegative individuals without a history of COVID-19 (HC) served as controls. The study design is shown in **Figure 1A**. **Table 1** summarizes demographic characteristics of the study population. The study groups differ in age with PCS/ME/CFS patients being on average 11.5 years older than PCHC. The median time interval since COVID-19 infection was 7 months in patients and 5.5 months in PCHC, respectively. As required as diagnostic criteria for ME/CFS, all PCS/ME/CFS suffered from PEM with a duration of minimum 14 hours ((30) and **Table 1**). 38 of 40 PCS/non-ME/CFS patients fulfilled criteria for PEM according to Cotler J. *et al.*, 2018 (30) with 11 of them showing PEM for more than 14 hours. The PEM score measuring the severity and frequency of PEM was higher in PCS/ME/CFS than in PCS/non-ME/CFS patients. According to the Chalder Fatigue Scale, the level of fatigue was higher in PCS/ME/CFS patients than in PCS/non-ME/CFS ones. Patient's disability assessed by the Bell Disability Scale and physical functioning assessed by SF36 was stronger impaired in PCS/ME/CFS patients than in PCS/non-ME/CFS patients. However, the severity for fatigue, cognitive symptoms, headache and muscle pain measured by symptom score not significantly differ between PCS patient groups. Immune symptoms were severe in PCS/ME/CFS than in PCS/non-ME/CFS. The median total COMPASS-31 score and the subdomains assessing autonomic function do not significantly differ between patient groups.

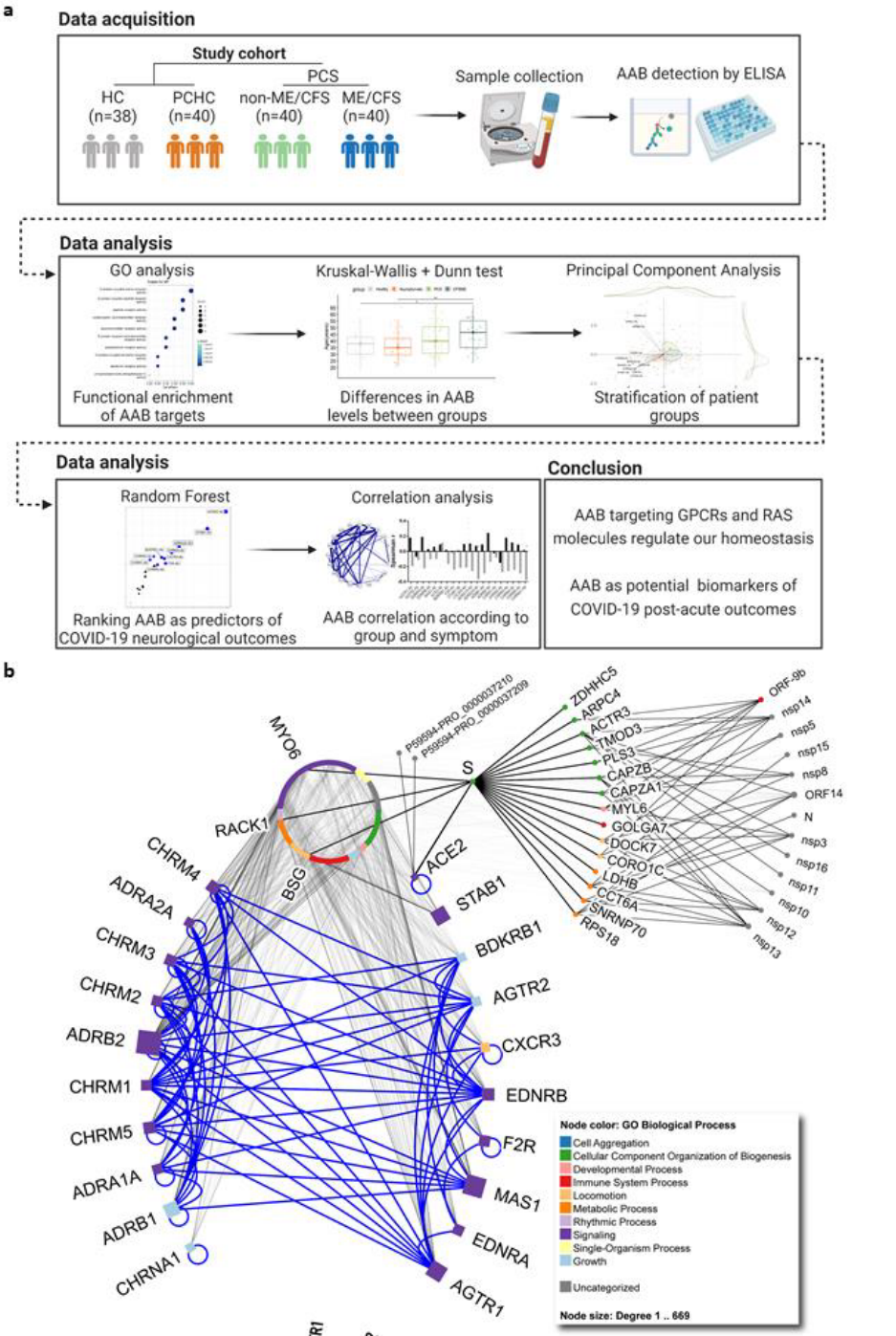
a
Data acquisition
Study cohort
PCS
HC (n=38)
PCHC (n=40)
non-ME/CFS (n=40)
ME/CFS (n=40)
Sample collection
AAB detection by ELISA
Data analysis
GO analysis
Kruskal-Wallis + Dunn test
Principal Component Analysis
Functional enrichment of AAB targets
Differences in AAB levels between groups
Stratification of patient groups
Data analysis
Random Forest
Correlation analysis
Ranking AAB as predictors of COVID-19 neurological outcomes
AAB correlation according to group and symptom
Conclusion
AAB targeting GPCRs and RAS molecules regulate our homeostasis
AAB as potential biomarkers of COVID-19 post-acute outcomes
b
MYO6
RACK1
CHRM4
ADRA2A
CHRM3
CHRM2
ADRB2
CHRM1
CHRM5
ADRA1A
ADRB1
CHRNA1
BSG
P59594-PRO_0000037210
P59594-PRO_0000037209
S
ACE2
STAB1
BDKRB1
AGTR2
CXCR3
EDNRB
F2R
MAS1
EDNRA
AGTR1
ZDHHC5
ARPC4
ACTR3
TMOD3
PLS3
CAPZB
CAPZA1
MYL6
GOLGA7
DOCK7
CORO1C
LDHB
CCT6A
SNRNP70
RPS18
ORF-9b
nsp14
nsp5
nsp15
nsp8
ORF14
N
nsp3
nsp16
nsp11
nsp10
nsp12
nsp13
Node color: GO Biological Process
Cell Aggregation
Cellular Component Organization of Biogenesis
Developmental Process
Immune System Process
Locomotion
Metabolic Process
Rhythmic Process
Signaling
Single-Organism Process
Growth
Uncategorized
Node size: Degree 1 .. 669
c
A
B
C
D
E
F
G
H
I
J
CHRNA1
CHRM5
CHRM4
CHRM3
CHRM2
CHRM1
ADRB2
ADRB1
ADRA2A
ADRA1A
CXCR3
STAB1
F2R
BDKRB1
ACE2
MAS1
EDNRB
EDNRA
AGTR2
AGTR1
A: regulation of angiogenesis
B: vascular process in circulatory system
C: G protein-coupled acetylcholine receptor signaling pathway
D: muscle system process
E: smooth muscle contraction
F: G protein-coupled serotonin receptor signaling pathway
G: positive regulation of cytosolic calcium ion concentration involved in phospholipase C-activating G protein-coupled signaling pathway
H: cell chemotaxis
I: regulation of blood pressure
J: angiotensin-activated signaling pathway

Figure 1. **Study workflow and description of autoantibody targets. (a)** After data acquisition, different statistical analyses (written on the top) were carried out in order to characterize the signature of autoantibodies (AAB) against G protein coupled receptors (GPCRs) and COVID-19-associated molecules (e.g. renin-angiotensin system (RAS)) in Post COVID Syndrome (PCS) when compared with healthy controls (HC) and post COVID-19 healthy controls (PCHC). Created with Biorender. **(b)** The 10 squares on the left represent autonomic nervous system (ANS) related receptors, while the 10 on the right show non-ANS molecules and receptors (e.g. RAS, immune and circulatory systems). Blue edges in the network highlight the interactions among the AAB targets, while gray edges represent other interactions. Node colors map to Gene Ontology (GO) biological processes (BPs) and node size corresponds to number of interacting partners for each target. Circular nodes represent human and SARS-CoV-2 molecules (as well as two Spike (S) proteins with unspecified roles) that are described in the IMEx coronavirus interactome. Circular organization of the proteins on the top middle of the image represent interacting partners of the AAB targets (names are omitted, except for 3 proteins that link ACE2 via S). **(c)** Circular plot with targets and relevant pathways they are associated to. Edge colors differ between each pathway. Edges representing AAB pathways are named from A to J, and the corresponding name is present in the list.

2.4.2 Differences in the AAB levels between PCS/ME/CFS, PCS/non-ME/CFS and controls

The interactions of the AAB targets are represented in **Figure 1B**. The ten adrenergic and muscarinic receptors on the left side are related to the ANS and play a role in regulation of the vascular tone and circulation. The proteins plotted on the right side include members of the RAS System, RAS related receptors and further vaso- and immune-regulatory, non-ANS proteins. Major biological processes, in which this AAB targets are involved are shown in **Figure 1C** and the most significant gene ontologies and their associations can be found in **Supp figure 1**. Majority of the AAB targets are involved in vascular processes of the circulatory system, in the regulation of blood pressure and muscle system processes.

The AABs do not satisfy the criteria for lognormal distribution after log2 data transformation and because of that, non-parametric tests were employed in this analysis. Significantly lower concentrations of 10 AAB that target 8 GPCRs, 1 ionotropic and 1 scavenger receptor were found when comparing either PCS groups with healthy groups, namely: ADRA2A, ADRB2, BDKRB1, CHRM5, CHRNA1, CXCR3, EDNRA, F2R/PAR-1, MAS1 and STAB1 (**Figure 2A; Supp table 1**).

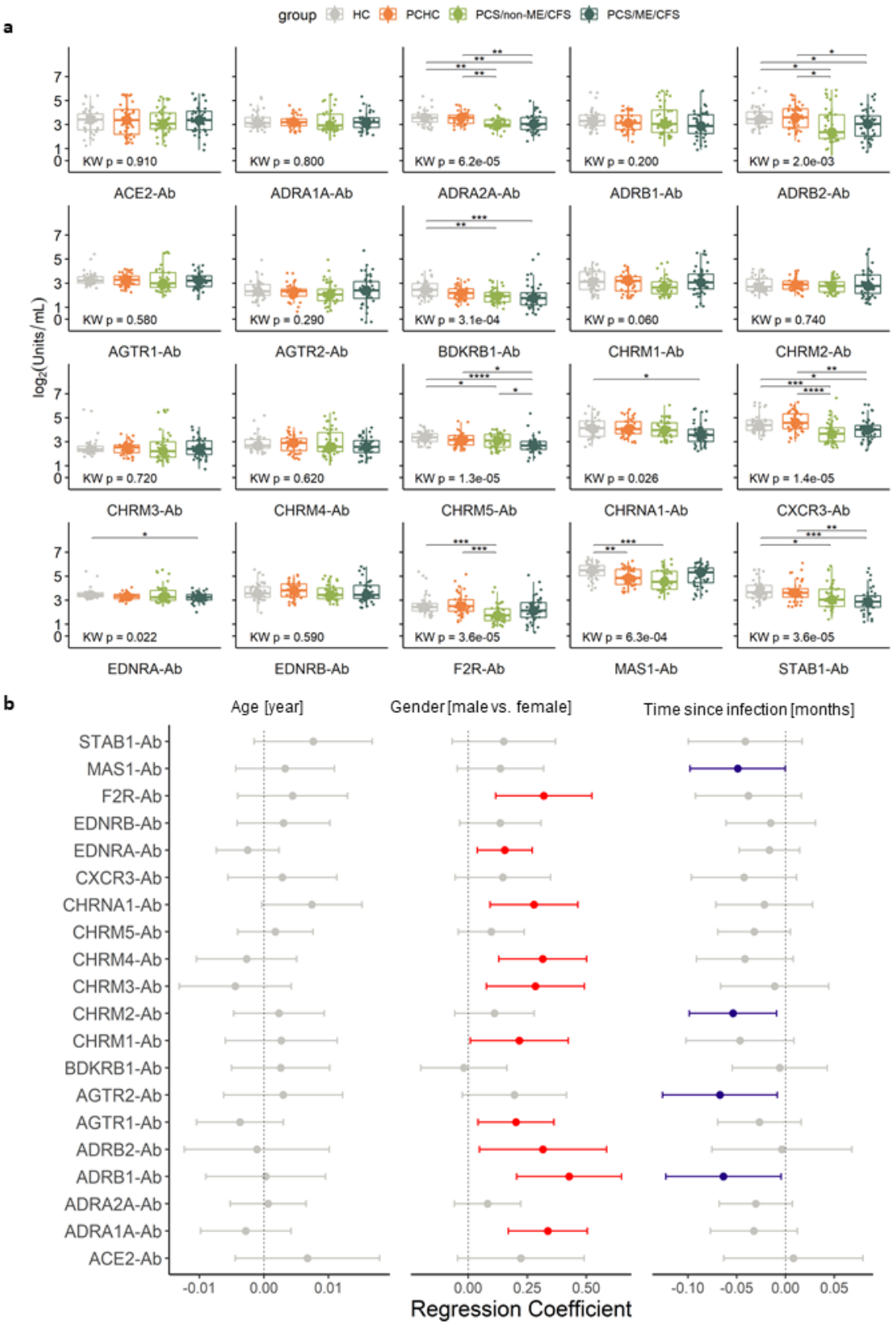

Figure 2. **Autoantibodies against G protein coupled receptors and COVID-19-associated molecules are dysregulated during Post COVID Syndrome. (a)** Box plots of Ab investigated in PCS patients with and without

ME/CFS and healthy controls post or without COVID-19 history (PCHC or HC). Significance determined by Kruskal Wallis test followed by Dunn test as post hoc. Dunn test p values were corrected for FDR. Adjusted p-values are being represented by: * p.adj < 0.05; ** p.adj <0.01; ***p.adj < 0.001; **** p.adj < 0.0001. Boxes represent the median and interquartile range (IQR). **(b)** Forest plot of regression coefficients for the confounding factors age in years, gender (reference being female) and time post COVID-19 in months considering 95% confidence interval (CI). Red dots and CI indicate that variable has a positive influence in the Ab level, blue dots and CI indicate a negative influence and gray ones contain 0 in the confidence interval, therefore are taken as non significant.

Furthermore, we carried out regression analysis to evaluate the influence of age, gender or time post infection on AAB levels. Taken these factors into consideration, age did not significantly affect any of the AAB levels. In turn, while we observed a general tendency for higher AAB levels in males, there is a trend of AAB levels to decrease with time post infection. These confounding effectors, significantly affected the levels of 13 specific AAB with 9 AAB levels being effected by gender, 3 AAB levels by disease duration and ADRB1 AAB level by both (**Figure 2B and Supp table 2**). By adjusting for age, gender and time post infection and in regards to PCS/ME/CFS, most significant regression coefficients were positive, suggesting that lower AAB levels are associated with this phenotype. In contrast, for F2R/PAR-1- and CHRM1-Ab the regression coefficient was negative in PCS/non-ME/CFS, indicating that lower levels of these AABs were associated with the non-ME/CFS phenotype in regards to PCS/ME/CFS, as well as for CHRM2 in HC (**Supp table 3**).

Compared to both HC and PCHC, AAB concentrations against ADRA2A, ADRB2, STAB1, and CXCR3 significantly decreases in both PCS/non-ME/CFS and PCS/ME/CFS (**Figure 2A**). Diminished levels of CHRM5-Ab as well as CHRNA1-Ab were found in PCS/ME/CFS, and reduced F2R/PAR-1 AAB in PCS/non-ME/CFS, exclusively. After adjustment for potential confounders, i.e. sex, age and time since infection, we found in addition to CHRM5-Ab significant differences with higher levels of AABs against CHRM1 and F2R/PAR-1 and lower levels of ADRB1, CHRNA1 and EDNRA in PCS/ME/CFS than in PCS/non-ME/CFS groups (**Figure 2A and Supp table 3**), indicating that PCS patients with and without ME/CFS only barely differ in their AAB homeostasis, at least when taking the concentrations of the 20 AAB into account. Furthermore, only for MAS1-Ab we observed different levels between PCHC and HC (**Figure 2A**). This suggests a similar profile among healthy and asymptomatic post COVID-19 donors. There were no significant differences in levels of total IgG among patient and controls (**Table 1**).

Taken together, we found distinct AAB profiles for each of the studied conditions.

2.4.3 Stratification of study groups based on AAB data

Next, we performed principal component analysis (PCA) to examine the association between AABs (variables) and individuals (observations) while stratifying groups based on the AAB level. According to Kaiser criterion, the first four principal components were considered for the analysis (**Figure 3A**). For dimensions 1 and 2, there was some overlap between the groups, except for HC+PCS/non-ME/CFS and PCHC+PCS/non-ME/CFS (**Figure 3B**). The contribution of each AAB across the PCA dimensions is shown in Figure 3C. Noteworthy, no AABs are negatively related when it comes to the first dimension (F**igure S2A**). PCA plots using different combinations based on dimensions 1 to 4 revealed a similar AAB profile between control groups HC and PCHC as well as between PCS groups (**Figure 3B; Figure S2B-F**).

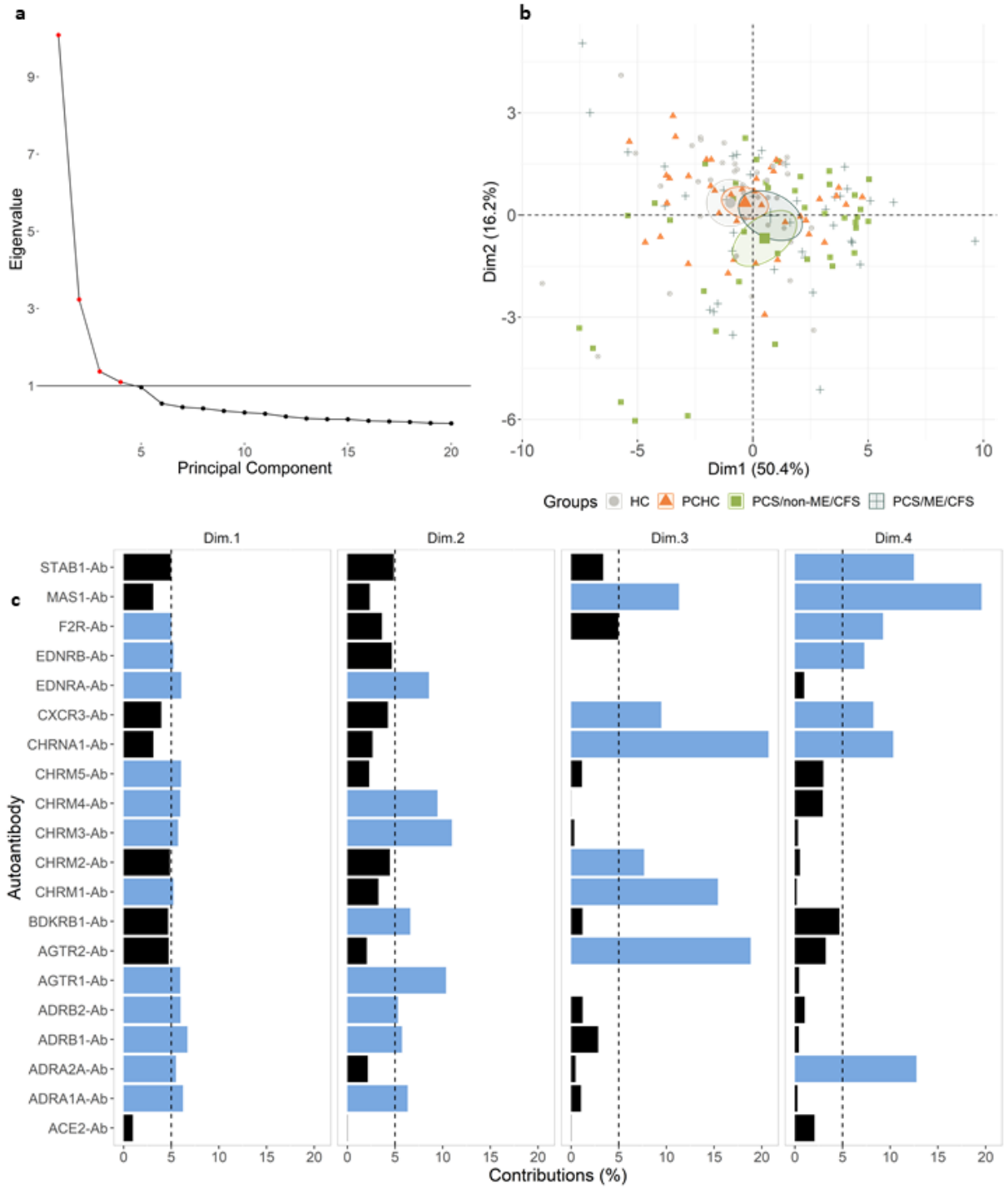

Figure 3. **Autoantibodies stratify patients by post-acute COVID-19 outcomes.** **(a)** Principal component analysis (PCA) with spectral decomposition based on logarithmic values of 20 Abs show the stratification of the four studied groups. Variables pointing to the same sense of the corresponding principal components are positive correlated. Small ellipses are the concentration around the mean points of each group. **(b)** Graphs of variables (Abs) obtained by PCA of all individuals in this study. **(c)** Barplot with the contribution percentages of each variable to each dimension. A black dashed line is plotted on the 5% mark, and blue bars indicate a contribution higher than 5%.

To further investigate the potential of AAB to classify PCS patients we carried out Random Forest analysis, joining HC and PCHC groups (named as Healthy) as well as both PCS groups (referred as PCS patients) due to their similar AAB pattern. This approach indicated an out-of-bag (OOB) error rate of 20.34% (25.86% for Healthy and 15% for PCS patients) and an area under the curve (AUC) of 0.77 for each group (**Figure 4A,B**). In addition, the Random Forest model ranked the AABs based on their ability to discriminate between study groups, identifying ADRB2-Ab, STAB1-Ab and ADRA2A-Ab as the three most important classifiers (**Figure 4C**). In agreement with the PCA results, AABs were able to partially correctly classify the individuals into the Healthy and PCS groups **(Figure 4D).**

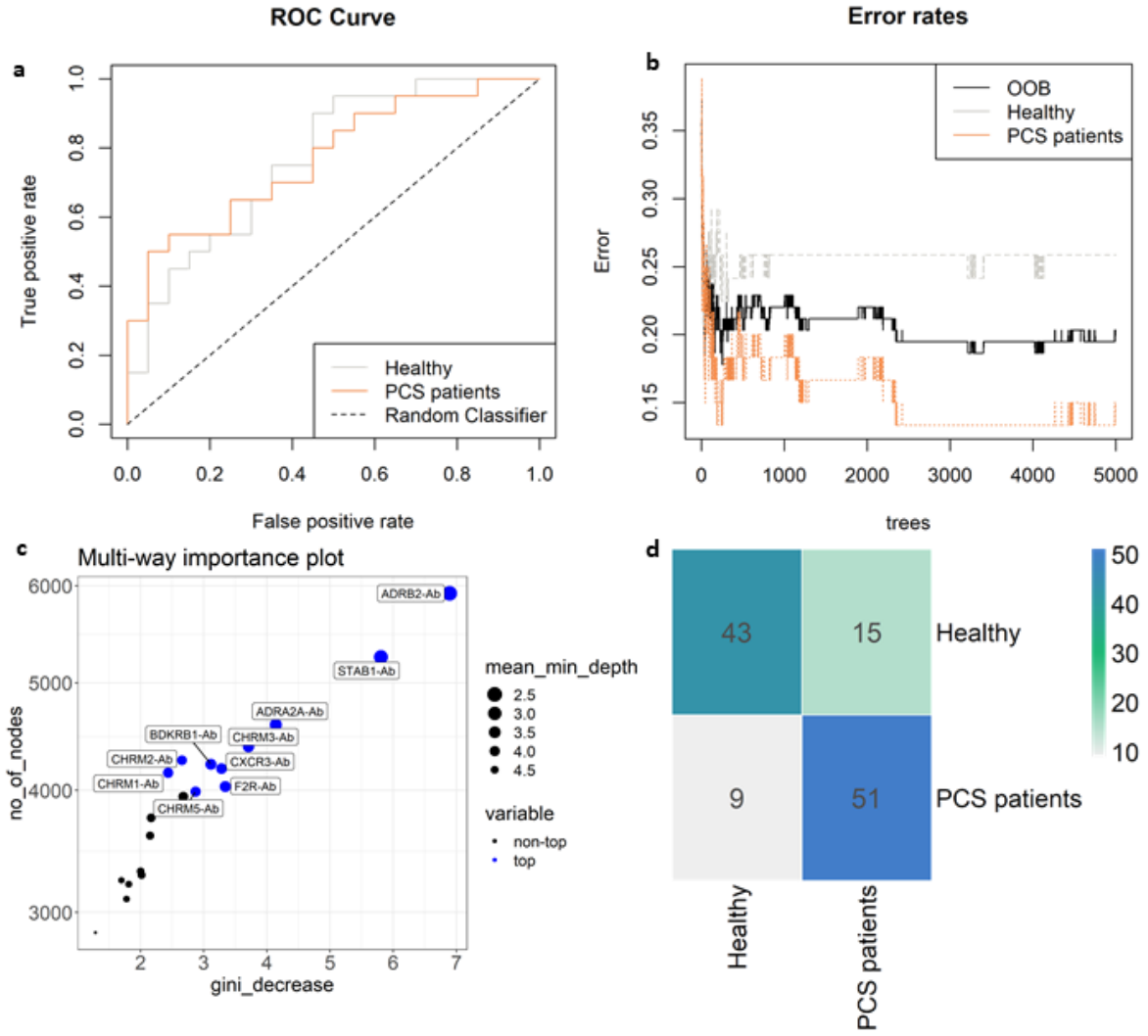

Figure 4. Machine learning classification of study groups based on autoantibodies (a) Receiver operating characteristic (ROC) curves of 20 antibodies (Abs) with an area under the curve (AUC) of 77% for healthy individuals and 77% for PCS patient group. **(b)** Stable curve showing number of trees and out-of-bag (OOB) error rate of 20.34%. **(c)** Variable importance score plot based on Gini decrease and number (no) of nodes, and the mean of minimum depth for each Ab, showing which variable presents a higher score in classifying COVID-19 post-acute infection outcomes. **(d)** Heatmap of the confusion matrix. Numbers represent the amount of occurrences that

happened when training the random forest model in predicted (row) vs actual classification (column), therefore the blueish diagonal identifies the hits, while other cells are mismatches.

2.4.4 AAB correlation signature in study groups

In our previous study, we reported that by clustering AAB correlation, it is possible to associate their signatures with immune homeostasis that leads to either a physiological or to a pathological outcome (23). In this sense, our next step was to investigate how the data we analyzed behave in regard to the AAB levels in each of the four groups. It is possible to identify a pattern characterizing every group, which shows a strong correlation among AAB to ANS receptors. Though there are minor differences noticeable between the groups, namely the strong correlation of CHRM1-Ab with AGTR2-Ab as well as CHRM2-Ab in patients, and between EDNBR-Ab and BDKRB1-Ab mainly in PCS/ME/CFS. Oppositely a weakening in the correlation of ADRB2-Ab with ADRB1-Ab and with CHRM4-Ab in patients in comparison with HC and PCHC was found. In addition, CHRM5-Ab correlated with ADRA2A-Ab in HC and PCHC only (**Figure 5**).

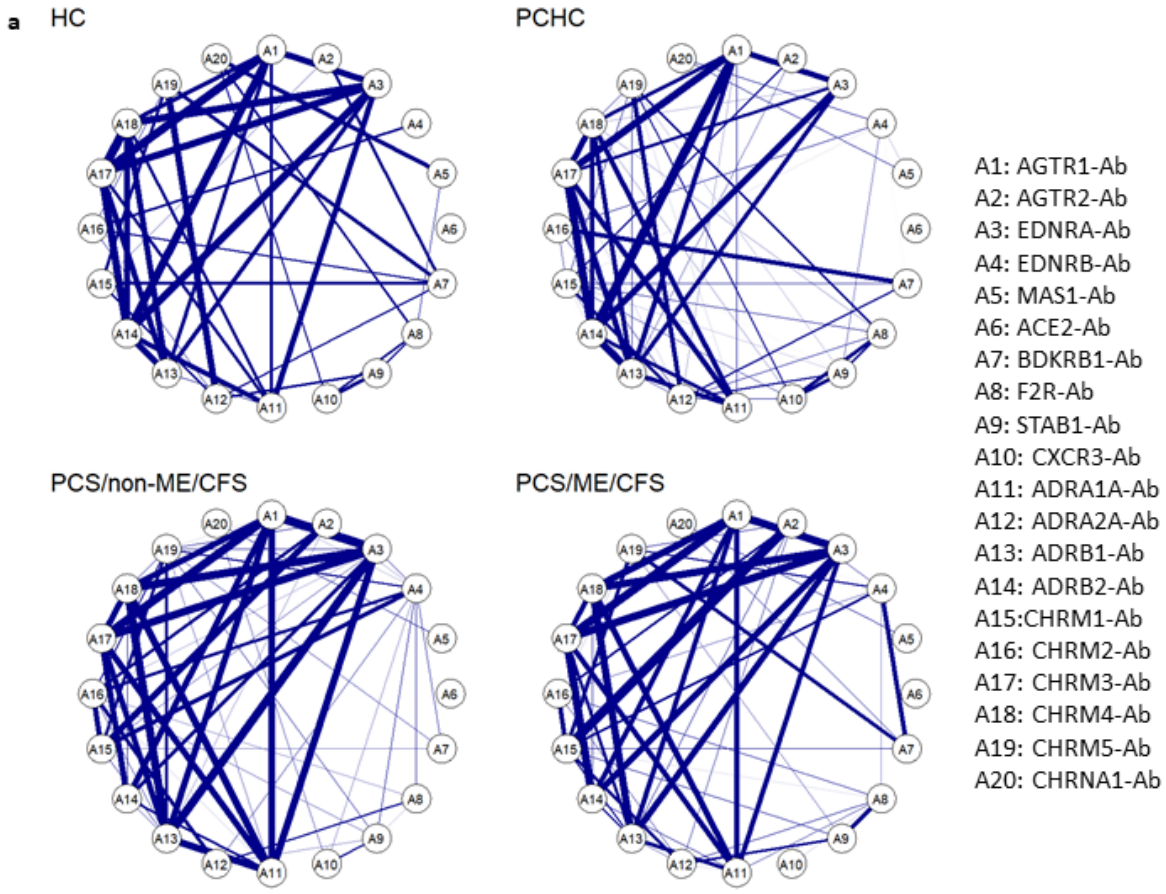

Figure 5. **Autoantibody correlation signatures associate with post-acute infection outcome.** Circular networks based on Spearman's rank correlation for the level of the 20 autoantibodies (-Ab) in post COVID syndrome (PCS) patients with and without ME/CFS and healthy controls post or without COVID-19 history (PCHC or HC). There

is a list with the abbreviations and the Abs names by the right side of the plot. Correlations greater than 0.6 are represented by the blue edges, and thicker edges imply greater correlations.

2.4.5 Correlation of AAB levels with Clinical Symptom Scores

Correlation coefficients of symptom severity with AAB levels in PCS/ME/CFS and PCS patients are shown in **Figure 6A**. In PCS/ME/CFS patients' severity of fatigue correlated positively with levels of several AABs including those against AGTR1, EDNRA, BDKRB1, ADRB1/2, CHRM3/5 (black bars). In contrast, the severity of cognitive symptoms correlated positively with F2R/PAR-1, CXCR3, and STAB1 and immune symptoms correlated with EDNRB, BDKRB1, CHRM5 in PCS/ME/CFS, while severity of muscle pain and headache showed no significant correlations. None of these correlations were significant in PCS/non-ME/CFS (grey bars). In this cohort there were only correlations with CHRM4 with immune symptoms and of ADRB1 and CHRNA1 with headache.

An association was also observed between AABs and severity of autonomic symptoms assessed by COMPASS 31 questionnaire (**Figure 6B**). In PCS/ME/CFS patients, the secretomotor symptoms (dry eyes, dry mouth) correlated negatively with levels of AABs against AGTR1, EDNRA, ADRA1A and ADRB1/2, CHRM3 (black bars). Interestingly, a correlation was found of ADRB2 and of CHRM3 with the vasomotor function (Raynaud symptoms) in PCS/ME/CFS patients. Again none of these correlations were significant in PCS/non-ME/CFS (grey bars). In this cohort we found correlations of gastrointestinal symptoms with MAS1 and of both gastrointestinal and pupillomotor symptoms with F2R/PAR-1, and negative correlation of bladder symptoms with ADRA2A and CHRM2/5.

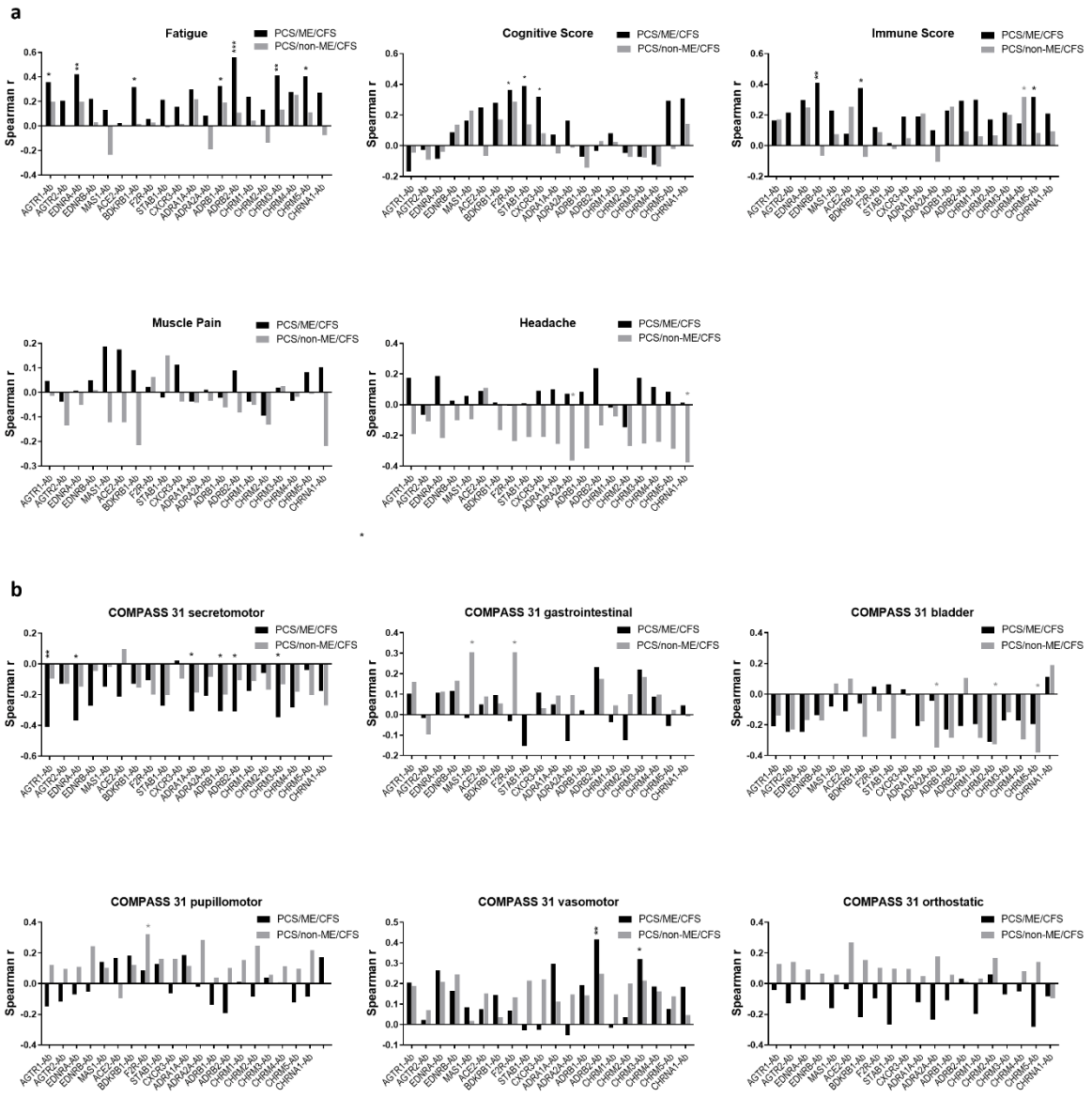

Figure 6. Correlation between autoantibody (-Ab) levels and clinical scores. Plots represent Spearman correlation coefficient (r) of correlation of Abs with **(a)** symptom scores and **(b)** autonomic symptom score assessed by COMPASS-31 questionnaire of PCS/non-ME/CFS (grey) and PCS/ME/CFS (black) patients. p values represented by: *p < 0.05, **p< 0.01 and ***p<0.001.

2.5 DISCUSSION

Here we found in PCS patients decreased levels of AABs directed against various receptors, mainly GPCRs, regulating vascular, muscle and immune processes (**Figure 1C**). Furthermore, while the PCA results of our study revealed a partial stratification overlap between HC and PCHC, as well as both PCS groups, machine learning classification indicated the ANS-related receptors ADRA2A and ADRB2, and angiogenesis regulator STAB1 as the most important classifiers of PCS outcome. All of them are involved in the regulation of vascular processes. Finally, we found strong correlations among the AABs and several associations of AABs with key symptoms of PCS.

In contrast to previous studies in acute COVID-19 with upregulation of several AABs against vaso- and immunoregulatory receptors (16, 18, 63) and the detection of functional GPCR AAB in patients with long COVID-19 (22) a profound downregulation of various AABs was detected in PCS patients accompanied by alterations in the correlations among AABs in this study. Analysed PCS patients suffered from persistent fatigue and exertion intolerance following mostly mild to moderate COVID-19. Elevated levels of AABs against vaso- and immunoregulatory receptors were observed in acute COVID-19 suffering from moderate to severe but not mild disease compared to controls (17, 18). As we did not observe an association of AAB levels, except of MAS1-Ab, with COVID-19 history of healthy controls in our study, we assume that differences to control groups of other studies not explain the different AAB alterations in patient cohorts. More importantly, however, is the gender distribution, as we observed that the male gender was associated with higher AAB levels in our cohort. In contrast to previous studies the majority of PCS patients and gender-matched (PC)HCs were women (17, 18). Therefore, possible effects of gender distribution has to be considered in future studies.

In a first study in 31 recovered COVID-19 patients, with 29 of them suffering from persistent symptoms with 15 suffering from were analysed for functional GPCR AABs using a bioassay (22). Moreover, functional GPCR AABs were found in the both post COVID symptom free controls, although they were rarely detected in healthy controls in previous studies (22). Hence, this study did not show a relationship between the functional vasoregulatory AABs and long COVID. The bioassay used by Wallukat *et al.* 2021 allows the detection of functional AABs which regulate GPCR activation of rat cardiomyocytes by AAB-receptor binding (22). In contrast, in our study the total amount of AAB binding the specific target receptor, independent of their functional potential were measured by ELISA. Although, the current data suggests the involvement of functional AABs, we could not exclude the presence of non-functional AABs.

By acting as ligands to their target receptors, AABs against GPCRs can modulate receptor signaling and in most functional studies, GPCR AABs binding to their corresponding receptors results in agonist stimulation (24, 64-69). Supporting this assumption, functional vasoregulatory GPCR AABs detected in PCS patients showed agonistic effects, with ADRA1-, AGTR1- and ADRB2-Ab stimulating and EDNRA- and MAS1-Ab inhibiting beating rate of cardiomyocytes of neonatal rats *in vitro* (22). AABs against GPCRs appeared to be dysregulated in many diseases and associated with clinical symptoms (23, 25, 28). Dysregulation of GPCR AABs either indicate an altered function of AABs maybe resulting in altered target receptor signaling and/or expression, or indicate a homeostatic response to an upregulation or downregulation of the respective receptors and pathways (25).

AABs reduced in at least PCS/ME/CFS or PCS/non-ME/CFS patients included AABs against adrenergic receptors regulating vascular process in circulatory system (ADRA2A, ADRB2), injury-induced, vasodilative B1 bradykinin receptor (BDKRB1) and the vasodilative MAS1, a potential regulator of cerebral blood flow (CHRM5), the receptors regulating vasoconstriction via vascular and muscle system processes (CHRNA1, EDNRA, F2R/PAR-1), the receptor regulating angiogenesis STAB1 and the chemokine receptor CXCR3. Only for AABs against the blood pressure regulator MAS1 PCHC presented lower levels than HC suggesting a rather similar profile among healthy groups. Expected for vasoregulatory CHRM5-Ab, differences of AAB levels between patient groups where found only after adjustment for age, gender and disease duration (time after infection). In this context, as suggested by our regression analysis, a limitation of our study is the influence of gender and the disease duration as a confounding effect that needs to be considered and investigated in more details by future studies with larger PCS/ME/CFS and PCS/non/ME/CFS cohorts before any conclusions on generalizability and potential diagnostic suitability can be made. . The low number of male patients per group in the cohort (see table 1) preclude a robust correlation analysis in comparison to the female group. Analysis of AABs stratified by sex in PCS patient groups and control groups has to be studied in a larger cohort.

Mechanisms of PCS remain poorly understood; however, some evidence points to both immune and vascular dysregulation (8-10). Both ongoing low-grade inflammation and impaired circulation and oxygen supply could explain many symptoms of PCS including fatigue, cognitive impairment, dyspnea or muscle pain upon exertion. Further marked autonomic dysfunction has been found in PCS (5, 70).

AABs we found diminished in PCS patients, target receptors and pathways playing an important role in ANS and/or vascular regulation and/or inflammation. Among them, AABs against ADRB2 are considered to play a crucial role in endothelial dysfunction in ME/CFS as reviewed in Wirth 2020 (71). Catecholamines binding to ANS receptors ADRA1, ADRA2 and ADRB1 on vascular smooth muscle cells causes vasoconstriction, while ADRB2 mediates vasodilation. Thus, downregulation of both ADRA2A and ADRB2 observed in our study point to a dysregulation in coordination of vasoregulation, which is in accordance with our random forest results. In this context, we found in a previous study an agonistic effect of ADRB2 AAB in HC which was diminished in ME/CFS (69). Further we found a sequence in EBV with high homology with ADRA2 which may induce crossreactive IgG (72). This may be of relevance in PCS, too, as EBV reactivation during COVID was identified as risk factor for PCS (73).

CHRM5 specific AAB were diminished in PCS/ME/CFS patients compared to healthy groups as well as PCS/non-ME/CFS patients. Interestingly, CHRM5 seems to be an important regulator of cerebral blood flow (CBF) (74, 75). In ME/CFS, CBF was impaired at least in a subset of patients (76, 77) and shown to negatively correlate with fatigue severity (78). The scavenger stabilin plays an important role in maintenance of vascular integrity through the clearance of infected apoptotic endothelial cells (79). Further EDNRA, CHRNA, F2R/PAR-1 play a role in vasoconstriction and RAS-related receptors BDKRB1 and MAS1 in vasodilation (80-82). Moreover, F2R/PAR-1, ADRB2 and CHRN play a role in inflammation, with F2R/PAR-1 exerting pro- and CHRN as well as ADRB2 anti-inflammatory responses (83-85). Therefore our study strongly supports a specific dysregulation of the vasculature present in PCS.

Upregulation of CXCL10 the ligand of CXCR3 is associated with COVID-19 severity promoting chemoattraction via CXCR3 for activated lymphocytes and monocytes (86). The CXCL10-CXCR3 axis is also likely to play an important role in COVID-19-induced tissue injury and fibrosis including pulmonary and cardiac fibrosis, endothelitis and endothelial damage. In Sjoegrens Syndrome, an autoimmune disease with a high prevalence of fatigue, anti-CXCR3 AAB levels were also diminished and negatively correlated with circulating lymphocyte counts (87).

In addition to the strong correlations among the AABs targeting vaso- and immunoregulatory receptors, which is in accordance with our recent study (18), our data revealed correlations between the levels of several AABs with severity of fatigue, cognitive and immune symptoms

in PCS/ME/CFS patients thus further pointing to a role of these AAB or of their associated pathways. Severity of fatigue correlated positively with levels of circulating AAB against vasoregulatory EDNRA, ADRB1/2, BDKRB1 and CHRM5 all downregulated in our study and further with AGTR1 which is a strong mediator of vasoconstriction. This finding is similar to previous results in postinfectious non-COVID-19 ME/CFS in which severity of fatigue correlated with AABs against ADRB1/2, EDNRA and AGTR1 (28); BDKRB1 and CHRM5 were not analyzed in this previous study. These correlations suggest that vascular dysregulation plays a role in fatigue in both ME/CFS cohorts. In line with this suggestion, we observed a strong correlation of levels of ADRB2 AAB with Raynaud symptoms in the PCS/ME/CFS cohort in the present study. Similarly, the negative correlation of the secretomotor symptoms (dry eyes, dry mouth) with levels of AAB against vasoregulatory receptors AGTR1, EDNRA, ADRA1A, ADRB1/2 and CHRM3 indicate a vascular mechanism. The severity of cognitive symptoms correlated with AABs against F2R/PAR-1, CXCR3, and STAB1 in PCS/ME/CFS in contrast to our previous results in postinfectious non-COVID-19 ME/CFS in which cognitive impairment correlated with EDNRA and AGTR1 (CXCR3, and Stabilin-1 were not analyzed). As the AAB targets F2R/PAR-1, CXCR3, and STAB1 are involved in inflammatory processes (88, 89), beside their vasoregulatory function (F2R/PAR-1 and STAB1), this finding points to a partly, distinct inflammatory mechanism in PCS/ME/CFS in contrast to vasoconstriction in the non-COVID ME/CFS, which had a much longer median disease duration of 3 years. Thus, one possible explanation may be that early in the disease course or alternatively in PCS/ME/CFS an inflammatory mechanism is more relevant for cognitive impairment, while later in disease course or in non-COVID ME/CFS a vasoregulatory mechanism is more relevant as AGTR1 and EDAR are the two most important vasoconstrictors. None of these correlations observed in the PCS/ME/CFS cohort were found in patients with PCS/non-ME/CFS.

Taken together, levels of several AABs were positively associated with key symptoms of ME/CFS in the PCS/ME/CFS cohort, although serum AAB levels were reduced or unchanged in these patients. One explanation for this observation is, that AABs bind to their targets in the tissue, and consequently circulating antibodies does not reflect the total amount of AABs in the body. The lack of data on the functional properties of the AABs and their localisation in the body of our study cohort is a limitation of our study. Whether, the suggested AAB-target interaction has a functional effect that promotes disease symptoms or is itself a response to pathophysiological changes remains to be elucidated in future studies.

The levels of several AAB were unexpectedly lower in PCS patients compared to control groups, which is in contrast to former studies in ME/CFS (26, 27, 29). While PCS patients analysed here were median 7 months post-infection, ME/CFS patients in these previous studies mostly have been analysed much later in the disease course. Lower GPCR AAB levels were found in vascular diseases such as in acute coronary syndrome or vasculitis and in progressive lung involvement in rheumatic disease, too (25, 87, 90). One explanation could be, that lower levels of circulating AABs are a result of AAB binding to their target molecules upregulated in the post-infection inflammatory endothelium or tissue in PCS patients. During disease progression and regeneration of the inflammatory endothelium, AAB levels may increase again. To address this hypothesis, future AAB studies in samples later during disease progression would be of high interest.

Another explanation for lower serum AAB levels might be anti-idiotype antibodies (anti-IDs) directed against the GPCR AABs. Enhanced GPCR AAB levels during acute COVID-19 may induce enhanced anti-IDs (18). A role of anti-IDs was shown for various autoimmune diseases, like myasthenia gravis and diabetes mellitus, and discussed for PCS as well (91, 92) Consequently, the anti-IDs-Ab –interaction may interfere with the binding of the AABs to their target receptors resulting in reduced serum levels measured by ELISA.

Interestingly the vaso- and immunoregulatory AABs to CXCR3, CHRM5, BDKRB1, MAS1, AGTR1, F2R/PAR-1 and STAB1 were the most significant classifiers of acute COVID-19 severity in our recent study (18). This suggests that dysregulation of these AABs and related pathways during acute COVID-19 may play a role in PCS, too. The separation of the patient from healthy cohorts by PCA and random forest indicates that it may be possible to use the AAB signature as biomarker for PCS, too. However, this needs to be confirmed in further cohorts.

Reduced levels of AAB, which were accompanied by a progressive disruption in their (statistical) relationships in PCS compared to HC/PCHC are in accordance with recent works showing that AAB correlation signatures are associated with both normal physiological and pathological immune homeostasis (18, 23). The dysregulation of any biological process, such as the imbalance (reduction or elevation) of cytokines/chemokines, affects the body equilibrium and homeostasis. Our data support the analogous concept, where an imbalance of the homeostasis of AAB relationships is an underlying pathological mechanism. Thus, the present work reinforces the concept that AAB targeting GPCRs are natural components of the human

physiology that become dysregulated during inflammatory and autoimmune diseases. Considering the importance of GPCRs in human inflammatory and autoimmune diseases, this work expands the comprehension of AAB biology, opening novel avenues for understanding new mechanisms of body homeostasis. In this context, mechanistic studies characterizing the functions of anti-GPCR AAB in patients with PCS hold promise to provide new therapeutic targets. Further, the potential of therapies targeting AABs to restore normal serum levels and deplete AABs with altered binding and function should be explored.

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Authors' contributions

CS and OC-M designed the study. CS, CK, KW, FP, JB-S, RR and UH diagnosed and enrolled the patients. SB performed the sample preparation. HH and KS-F determined the serum autoantibody concentrations. FS and HF managed the collection and maintenance of the clinical and laboratory data. FS, ISF, HF, NS, AHCM, RV, IJ, TL, YS, GR, OC-M and CS provided scientific insights. FS, ISF, DLMF, GCB, MK, OC-M, NS performed the data analysis. FS, ISF, HF, NS, DLMF, GCB, DRP, TL, FL, FMP, FP, GR, OC-M and CS interpreted and discussed the results. FS, ISF, OC-M and CS wrote the manuscript. All authors have read, revised, and approved the final version of the manuscript.

Competing interests

The authors declare that HH and KS-F are managing directors of CellTrend. CellTrend holds together with Charité a patent for the diagnostic use of AABs against ADRB2. CS has a consulting agreement with CellTrend. FP reports grants from the Guthy Jackson Charitable Foundation, during the conduct of the study. The other authors declare no competing interests.

Availability of data and materials

The raw data supporting the conclusions of this article is available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was approved by the Charité ethical committee (EA2_067_20 for assessment of biomarkers, EA2_066_20 for the PA-COVID study). All study participants gave written informed consent.

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2.7 SUPPLEMENTARY FILES

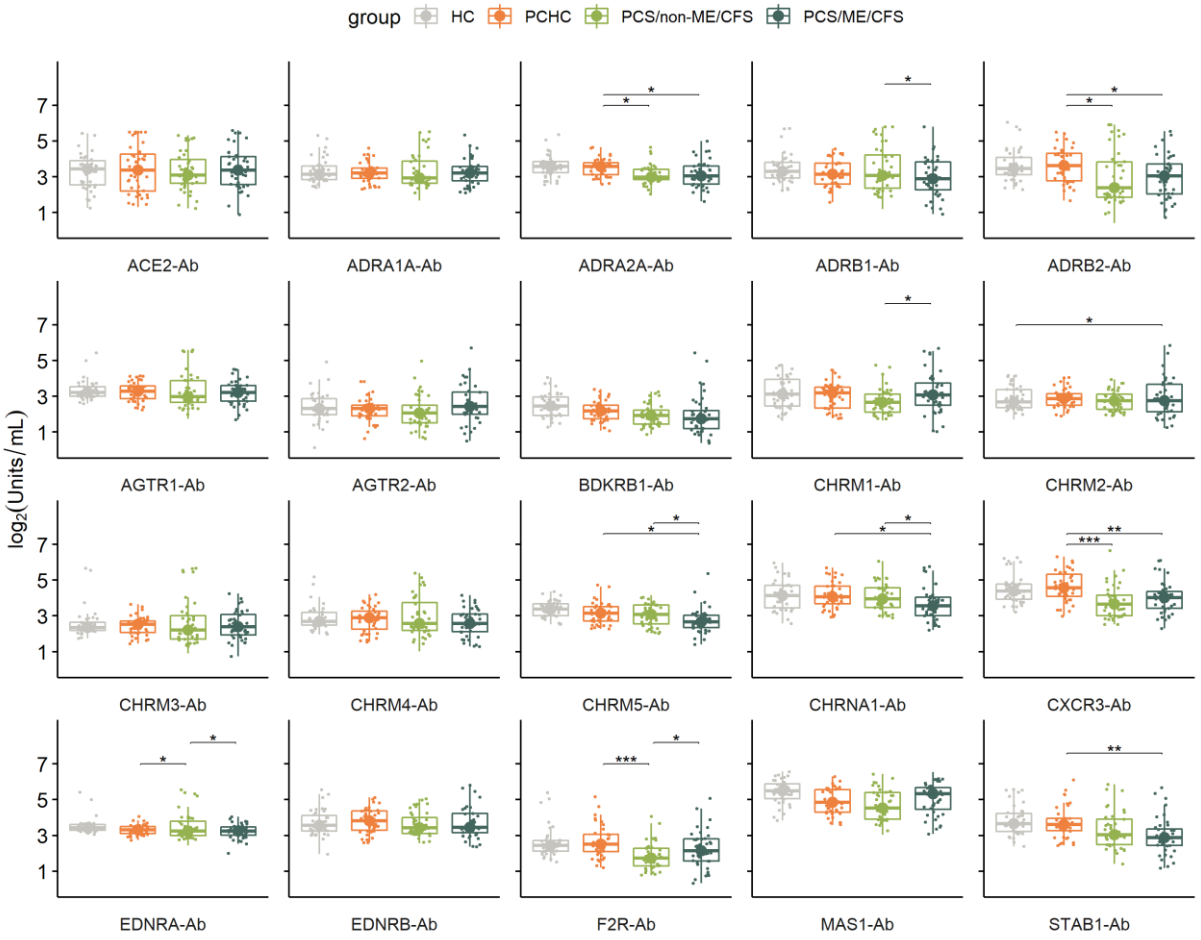

Figure S1. **Regression pvalues for groups.** Box plots of autoantibodies (-Ab) investigated in PCS patients with and without ME/CFS and healthy controls post or without COVID-19 (PCHC or HC). Significance determined by GAMLSS. P values are being represented by: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Boxes represent the median and interquartile range (IQR).

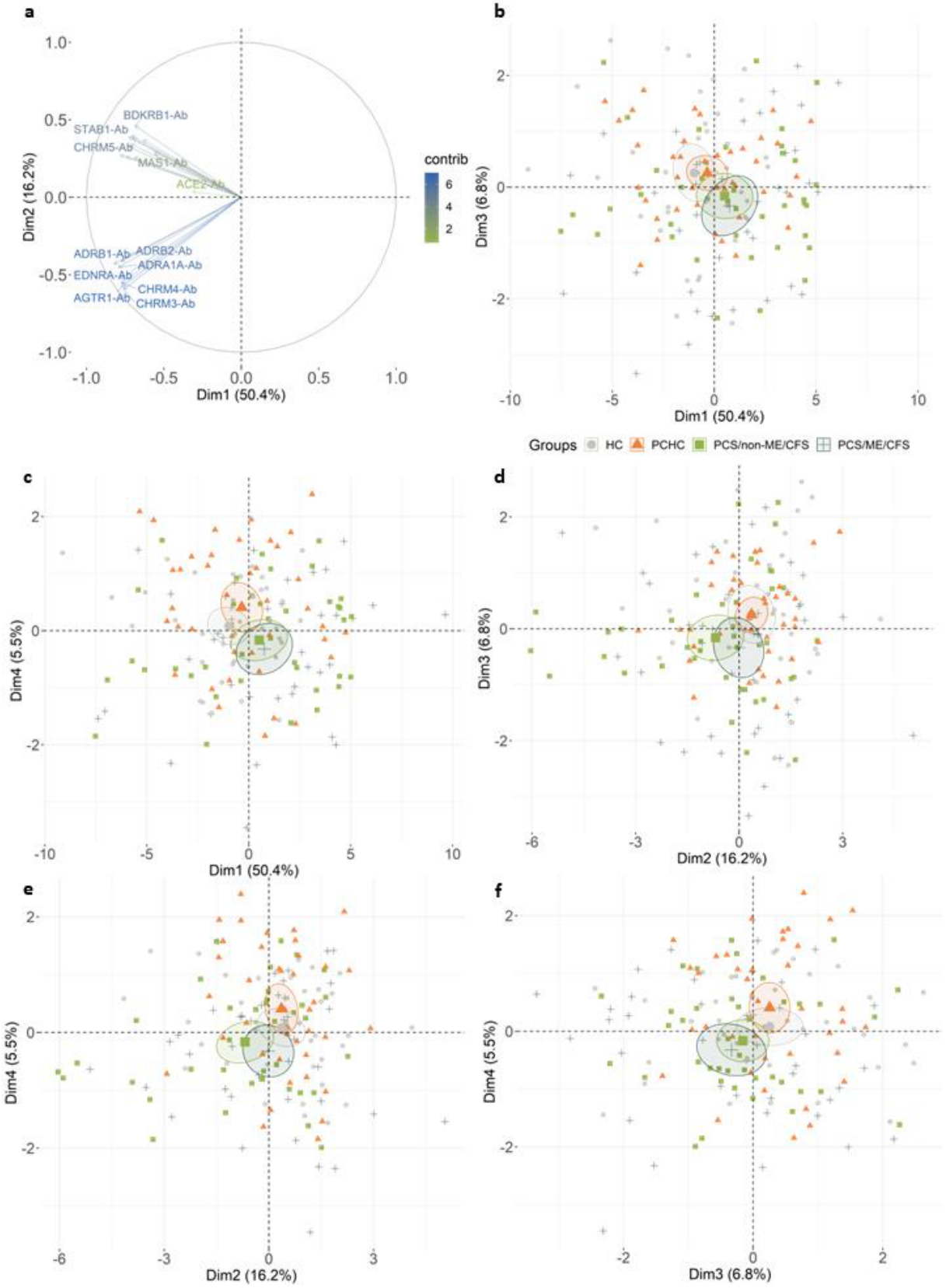

Figure S2. Principal Component Analysis. (a) Variables' contribution to PC1 and PC2. **(b-f)** Plots of the combinations of the first four dimensions.

3 CAPÍTULO 3 - SYSTEMATIC REVIEW AND META-ANALYSIS OF CELL THERAPY FOR COVID-19: GLOBAL CLINICAL TRIAL LANDSCAPE, PUBLISHED SAFETY/EFFICACY OUTCOMES, CELL PRODUCT MANUFACTURING AND CLINICAL DELIVERY

"For without hope, what do any of us have?"

Patrick Rothfuss, **The Wise Man's Fear**. 2011.

Systematic Review and Meta-Analysis of Cell Therapy for COVID-19: Global Clinical Trial Landscape, Published Safety / Efficacy Outcomes, Cell Product Manufacturing and Clinical Delivery

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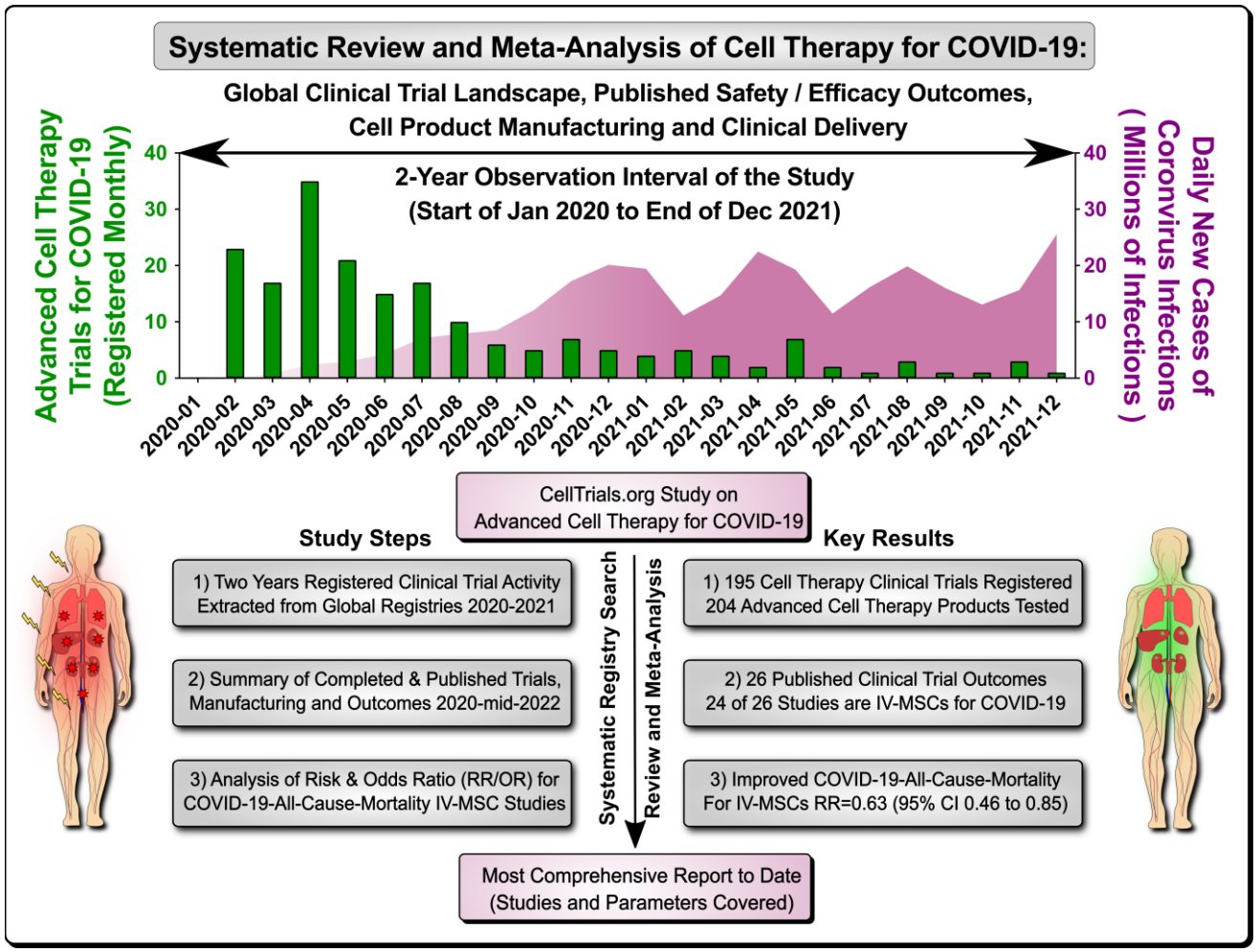
Keywords: Cell and gene therapy (CGT), Advanced therapy medicinal products (ATMPs), Mesenchymal stromal / stem cells (MSCs), Severe respiratory distress syndrome coronavirus 2 (SARS-CoV2), and Coronavirus 2019 induced disease (COVID-19)

3.1 ABSTRACT

During the pandemic of severe respiratory distress syndrome coronavirus 2 (SARS-CoV2), many novel therapeutic modalities to treat Coronavirus 2019 induced disease (COVID-19) were explored. This study summarizes 195 clinical trials of advanced cell therapies targeting COVID-19 that were registered over the two years between January 2020 to December 2021. In addition, this work also analyzed the cell manufacturing and clinical delivery experience of 26 trials that published their outcomes by July 2022. Our demographic analysis found the highest number of cell therapy trials for COVID-19 was in United States, China, and Iran (N=53, 43, and 19, respectively), with the highest number per capita in Israel, Spain, Iran, Australia, and Sweden (N=0.641, 0.232, 0,223, 0.194, and 0.192 trials per million inhabitants). The leading cell types were multipotent mesenchymal stromal / stem cells (MSCs), natural killer (NK) cells, and mononuclear cells (MNCs), accounting for 72%, 9%, and 6% of the studies, respectively. There were 24 published clinical trials that reported on infusions of MSCs. A pooled analysis of these MSC studies found that MSCs provide a relative risk reduction for all-cause COVID-19 mortality of RR=0.63 (95% CI 0.46 to 0.85). This result corroborates previously published smaller meta-analyses, which suggested that MSC therapy demonstrated a clinical benefit for COVID-19 patients. The sources of the MSCs used in these studies and their manufacturing and clinical delivery methods were remarkably heterogeneous, with some predominance of perinatal tissue-derived products. Our results highlight the important role that cell therapy products may play as an adjunct therapy in the management of COVID-19 and its related complications, as well as the importance of controlling key manufacturing parameters to ensure comparability between studies. Thus, we support ongoing calls for a global registry of clinical studies with MSC products that could better link cell product manufacturing and delivery methods to clinical outcomes. Although advanced cell therapies may provide an important adjunct treatment for patients affected by COVID-19 in the near future, preventing pathology through vaccination still remains the best protection to date.

GRAPHICAL ABSTRACT



Title and Legend: Systematic Review and Meta-Analysis of Cell Therapy for COVID-19.

We conducted a systematic review and meta-analysis of advanced cell therapy clinical trials as potential novel treatment for COVID-19 (resulting from SARS-CoV-2 coronavirus infection), including analysis of the global clinical trial landscape, published safety/efficacy outcomes (RR/OR), and details on cell product manufacturing and clinical delivery. This study had a 2-year observation interval from start of January 2020 to end of December 2021, including a follow-up period until end of July to identify published outcomes, which covers the most vivid period of clinical trial activity, and is also the longest observation period studied until today. In total, we identified 195 registered advanced cell therapy studies for COVID-19, employing 204 individual cell products. Leading registered trial activity was attributed to the USA, China, and Iran. Through the end of July 2022, 26 clinical trials were published, with 24 out of 26 articles employing intravenous infusions (IV) of mesenchymal stromal/stem cell (MSC) products. Most of the published trials were attributed to China and Iran. The cumulative results from the 24

published studies employing infusions of MSCs indicated an improved survival (RR=0.63 with 95% Confidence Interval 0.46 to 0.85). Our study is the most comprehensive systematic review and meta-analysis on cell therapy trials for COVID-19 conducted to date, clearly identifying the USA, China, and Iran as leading advanced cell therapy trial countries for COVID-19, with further strong contributions from Israel, Spain, Australia and Sweden. Although advanced cell therapies may provide an important adjunct treatment for patients affected by COVID-19 in the future, preventing pathology through vaccination remains the best protection.

3.2 INTRODUCTION

The outbreak of the novel severe respiratory distress syndrome coronavirus 2 (SARS-CoV2) and its adjunct symptomatic, Coronavirus 2019 induced disease (COVID-19), is one of the most significant world health events in recorded history [1]. Early reports during the initial outbreak in Wuhan, China, found that up to 14% of patients presented with the severe form of COVID-19 and that mortality was as high as 3% [2-4]. Subsequently, the virus became a global pandemic and new variants emerged [5-9]. Major variants responsible for surges of virus infections include "Beta" (South Africa, May 2020), "Delta" (India, October 2020), and "Gamma" (Brazil, November 2020) (**Figure 1A**) [7]. During the summer and fall seasons of 2022, the predominant circulating variants were sub-types of "Omicron", first documented in November 2021 across multiple countries [10]; E.g. the "Omicron" sublineage BQ.1 was designated as a Variant of Interest (VOI) by the European Center for Disease Prevention and Control (ECDC) as of 20th of October 2022 and it was expected that by mid-November to beginning of December 2022 more than 50% of SARS-CoV-2 infections were due to BQ.1/BQ.1.1 [11]. This demonstrates the rapid dynamics in virus changes [9]. By the end of December 2022, the worldwide death toll attributed directly to COVID-19 had surpassed 6.6 million individuals [5-7].

The COVID-19 pandemic created an ideal situation for the convergence of two research quests that had been progressing independently for decades. The first quest came from pulmonology, where researchers have sought to improve mortality from acute respiratory distress syndrome (ARDS) for decades, with mortality levels of 44% in clinical trials since the 1980's [12]. The second quest, dating back from the 1990s, was the scientific effort to demonstrate clinical efficacy for cell therapy products containing multipotent mesenchymal stromal/stem cells (MSCs) [2, 13-18]. Preliminary evidence suggested that MSCs might be beneficial for pulmonary disorders [2, 18]. This is supported by biodistribution studies which demonstrated that MSCs given intravenously (IV) rapidly localize to the lungs, where they may exert their beneficial properties [2, 15, 16, 18]. It is well established that the immunomodulatory and regenerative properties of MSCs entail a plethora of distinct synergistic mechanisms of action (MoAs) that might help ameliorate pulmonary conditions [13, 18].

Between 2011 to 2019, the database CellTrials.org identified 16 clinical trials of MSCs for ARDS, and by April 2020, seven of these trials were completed, and five were published [19]. Unfortunately, none of these publications demonstrated clinical efficacy of MSCs against ARDS. Similarly, a literature search based on published studies of MSCs for ARDS between 1990 to 2020 found nine such studies and confirmed that the improvement in mortality was not

significant [20]. In the past, poor outcomes considering ARDS mortality were often attributed to the high complexity of ARDS etiology and pathology (e.g. many small / difficult to target subgroups) and rapid disease progression (e.g. short time window for interventional treatment). Thus, any studies aiming to prove efficacy in ARDS typically require stringent inclusion/exclusion criteria and large trial cohorts to control for confounders. This has been challenging to achieve, given that patient enrollment only reaches sufficient numbers in larger specialized clinical centers, thus often requiring multi-center studies [12].

This led to the convergence of the quests mentioned above during the COVID-19 pandemic. For the first-time large cohorts were broadly available to effectively study novel therapeutic interventions. Pulmonologists noted very early that COVID-19 differs from classic presentations of ARDS [21], thus calling for an in-depth analysis of clinical trial outcomes. However, current knowledge on the outcome of cell therapy studies for both ARDS and COVID-19 is still fragmented (**Figure 1B**) [2, 18]. Covering the most relevant 2-year interval (Jan 2020 to Dec 2021), we here report the outcomes of the available published clinical trials focusing on cell therapy of COVID-19 today. This also covers specific intricacies of product manufacturing and delivery to patients [2]. Our analysis found that three quarters of cell therapies deployed against COVID-19 relied on MSCs. During 2020 alone more than 100 clinical trials were registered worldwide that employed MSCs to treat COVID-19 pneumonia and acute respiratory distress. Indeed, published outcomes from those trials that focus on MSC therapy for COVID-19 now appear to be sufficient to warrant a first comprehensive examination of the safety and efficacy profiles of MSCs for treating severe COVID-19.

3.3 MATERIALS AND METHODS

This study presents three types of data regarding cell-based therapies for COVID-19 (**Figure 1B**): **(1)** We have collected two years (from Jan 2020 to Dec 2021) of registered clinical trial activity extracted from worldwide registries; **(2)** We have gathered the published clinical outcomes and extracted any available information on manufacturing and clinical delivery of MSC products from the published studies to study the potential impact of cell product manufacturing and mode of delivery on clinical efficacy; **(3)** We have performed a relative risk ratio (RR) and odds ratio (OR) analysis for all-cause COVID-19 mortality for studies employing intravenous use of MSCs (most frequent).

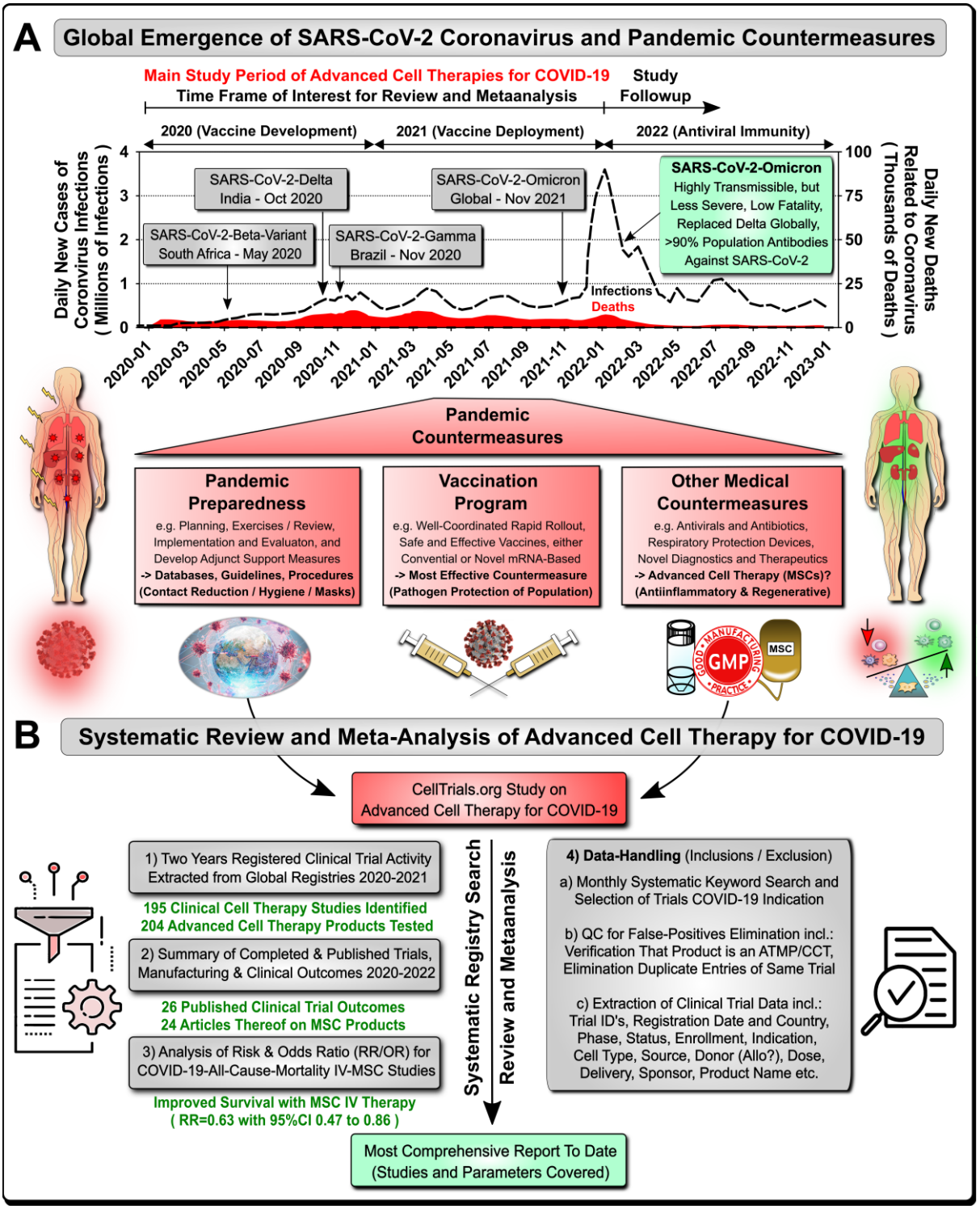


Figure 1. Study Design of Systematic Review & Meta-Analysis of Cell Therapy for COVID-19. (A) Global Emergence of SARS-CoV-2 Coronavirus and Pandemic Countermeasures: Data on daily new registered cases of coronavirus infections (Millions of Infections, dotted black curve) and daily new deaths related to coronavirus infection (Thousands of Deaths, red section/curve at bottom) were obtained from the COVID-Worldometer [6]. Arrows indicate the emergence of major SARS-CoV-2 variants and interventional measures (2020 start of novel vaccine development programs; 2021 start of novel vaccine deployment, some countries already started in the late fall of 2020, e.g. the US started on the 14th of Dec, Israel on the 20th of Dec, and Germany on 26th of Dec 2020; and 2022 emergence of antiviral immunity in population). Our study period of advanced cellular therapy clinical trials for COVID-19 spans from January 2020 to end of December 2021, with follow-up search for publications until the end of July 2022. Pandemic countermeasures are indicated below the graph, depicting 1) Pandemic

Preparedness (e.g. including databases, guidelines, and procedures), 2) Vaccination Programs (most effective countermeasures), and 3) Other Medical Countermeasures (e.g. advanced cell therapies, such as mesenchymal stromal/stem cells, MSCs, of unknown value). **(B)** Systematic Review and Meta-Analysis of Advanced Cell Therapies for COVID-19: Until today most information on advanced cell therapies for COVID-19 is still highly fragmented, in the sense that clinical trials and publications are being compiled separately. This prompted us to conduct this systematic review and meta-analysis to summarize and combine the current knowledge, covering: 1) Two years of registered clinical trial activity, 2) Summary of published clinical trial outcomes, cell product manufacturing and clinical delivery between 2020 and mid-2022, 3) Analysis of risk and odds ratios for COVID-19 all-cause-mortality considering intravenous (IV)-use of MSCs, and 4) A summary of the quality control routines, which were applied for the data-handling (Inclusion / Exclusion) in this study.

3.3.1 Identification of Registered Clinical Trials, Keyword Search, Inclusion/Exclusion Criteria

The methodology for identifying and assembling the database of clinical trials was the same as described earlier by CellTrials.org [22, 23]. Briefly, five main steps were performed monthly: (1.1) Keyword-based search (keywords shown below) for advanced cell therapy clinical trials worldwide, (1.2) Elimination of false positives, (1.3) Elimination of duplicate entries, (1.4) Gathering detailed data from included clinical trials, and (1.5) Extracting trials from the primary data-base where the indication for cell therapy use was COVID-19. The accuracy of the data search relies on the usage of multiple national registries of clinical trials (**Table 1**), including Australia and New Zealand, Brazil, China, Cuba, Germany, India, Iran, Japan, The Netherlands, Singapore, South Korea, Thailand, the United States of America (USA), the European Union (EU), and World Health Organization (WHO). The time frame for identifying advanced cell therapy trials for COVID-19 was between January 2020 until end of December 2021, containing the large majority of so far registered clinical trials (as detailed below). A tabular excel sheet summary of all 195 identified studies including 20 individual parameters can be found in **Table S1**.

3.3.2 Keyword search

The keywords used in the first step, broadly designed to capture all advanced cell therapies, included: “COVID-19”, “cell”, “cell therapy”, “cancer vaccine”, “CAR-T”, “chimeric antigen”, “DC”, “NK”, “TIL”, “tumor infiltrating”, “adoptive”, “regenerative”, “mesenchymal”, “adipose”, “bone marrow”, “cord blood”, and “umbilical”.

3.3.3 Elimination of false positives

A second curation step was needed to screen for studies that were performing advanced cell therapy and were not just a false hit on a keyword. We applied the definitions of Advanced Cell Therapy Medicinal Products (ATMPs) adopted by the European Medicines Agency (EMA), and Human Cellular Tissue Products (HCT/Ps) adopted by the US Food and Drug

Administration (FDA) [24-26]. This step was performed by having at least two scientists review each trial description.

3.3.4 Elimination of duplicate entries

To remove double postings of the same trial in more than one registry, scientific review was applied. If the trial was listed on the US registry ClinicalTrials.gov and another national registry within the same month, then the trial was assigned to ClinicalTrials.gov. If the trial appeared on a second registry months later, it stayed assigned to the month and registry where it first appeared.

3.3.5 Extraction of trial data

The dataset was built by recording the following parameters for each trial: registration date, clinical trial unique ID, secondary ID if any, country of registration, phase, status, cell type, cell source, route of administration, dosage if known, clinical indication, donor type (allogenic or autologous), target enrollment, age ranges of the patient population, type of sponsor (academia or industry), names of the sponsors, and product name if any.

3.3.6 Selection of trials with COVID-19 indication

The final step for this study was to extract the clinical trials of cell-based therapies where the indication for clinical use was COVID-19. On a monthly basis, we posted them online as an open-access community service. Since the early months of the COVID-19 pandemic, our living database of clinical trials has been listed as a resource on the evidence hub of the Center for Science in the Public Interest (CSPI) [27].

3.3.7 Published Clinical Trial Outcomes, Cell Therapy Manufacturing, and Clinical Cell Delivery

The methodology for gathering information on safety and efficacy from clinical trials of cell-based therapies for COVID-19 has been previously described [23]. Briefly, two complementary methods were employed. First, the PubMed registry was searched for publications using the keywords “COVID”, “cell” and “clinical trial”. Second, the search was refined by identifying publications containing the unique ID of each registered trial in our database. We only included publications reporting the outcomes of registered clinical trials but excluded case reports which could not be linked to registered trials. The collected parameters were as follows: connection between trial and publication, country where study was conducted, study design, study endpoints, target enrollment of trial, actual enrollment in paper, cell type(s), cell source(s), cell dose(s), route of administration, adverse events, survival of cell therapy patients and controls.

The cut-off date for including publications from trials registered in 2020 and 2021 was the end of July 2022.

3.3.8 World Map Figures

Global distribution of cell therapy trials for the treatment of COVID-19 per country was displayed as heat map either for the absolute number of trials per country or per capita values. Maps were drawn using the "R" packages 'maps' [28] and 'ggplot2' [29]. The corresponding analysis scripts are available at https://github.com/Starahoush/MSC-COVID19_metaanalysis.

3.3.9 Information on Cell Therapy Manufacturing from Published Studies

Manufacturing and clinical delivery information for each MSC product were obtained upon close inspection of papers to extract information considering: cell sources, donors, cell isolation, cell expansion, cellular passages, medium formulation, storage (fresh or frozen) [30, 31], and quality control steps (e.g. did MSCs fulfill ISCT minimal criteria?) [17, 32, 33]. This search often required checking additional sources when the cell product was supplied by a contract manufacturer or described in an earlier publication.

3.3.10 Risk Ratio and Odds Ratio for COVID-19 All-Cause Mortality for Published Studies

Statistical analysis was carried out in "R" version 4.2.1 [34], the meta package version 6.0.0, and visualized via forest plots from the same package [35]. The Risk Ratio (RR) and the Odds Ratio (OR) were calculated by using the Mantel-Haenszel test [36] and employed to analyze the effect of MSC therapy on the risk/odds of death following COVID-19 infection. The 95% confidence interval for both ratios and the combined statistics are reported for each study. The code used for this analysis was uploaded to https://github.com/Starahoush/MSC-COVID19_metaanalysis.

3.3.11 Handling of Missing Data

The RR and OR calculations require input studies to have two arms, one of the patients undergoing experimental treatment versus a second arm of control/placebo patients. However, during the COVID-19 pandemic, many clinical trials were conducted that did not have a control group. To incorporate their published outcomes, the following methodology was used to integrate single-arm studies, which lacked control data. First, the mean and median data of the controlled studies were obtained. Then, two assumptions were made about the studies without controls: First, it was assumed that the mean behavior of the missing controls was the same as that in the controlled studies, and second, it was assumed that the ratio of MSC patient number to control patient number was the same as the median for the controlled studies. With these two

assumptions it was possible to incorporate the experimental results from single-arm studies to calculate RR and OR for all studies as a group. Of the 24 published MSC studies, 17 had complete information regarding the number of participants and events for both treatment and control groups [37-53]; whereas seven studies did not have a had missing data for the control group [54-60]. Considering that previous studies [43, 46, 49] were double-arm-zero-event, we employed a treatment arm continuity correction (TACC) to incorporate them, since otherwise risk/odds ratios could not have been calculated [61, 62].

3.4 RESULTS

3.4.1 2-Year Registered Global Clinical Trial Landscape of Advanced Cell Therapies for COVID-19

A comprehensive search for advanced cell therapy trials to target the clinical indication COVID-19 and related complications was conducted in 18 national and international registries (**Table 1**). The cell therapy products employed in these trials are referred to as advanced therapy medicinal products (ATMPs) or human cellular and tissue products (HCT/Ps) in the EU and US, respectively [2, 24, 26]. Between January 2020 and December 2021, 195 advanced-cell-therapy-based clinical trials targeting COVID-19 and related complication were registered worldwide (**Table S1**). While we have released first smaller compilations in June 2020 and 2021 [63, 64], the current study covers the most relevant 2-year time window from January 2020 to the end of December 2021 (**Figure 1A and 2A**). The relevance of this time frame is depicted by the initial peak of monthly registered clinical trials in the first months of the pandemic, followed by dramatic decline and a long tail afterwards. The first clinical trial registrations appeared in China and USA in February 2020. Some February trials were registered retrospectively, but subsequent publications revealed that patients began receiving cell therapy for COVID-19 as early as January 2020 in China. The peak of registrations was April 2020. Noteworthy, registrations of cell therapy trials for COVID-19 had only one peak in spring 2020, although global COVID-19 cases went through four major surges during the 2-year timeframe [5-7]. The peak in trial registrations subsided months before the roll out of vaccination programs [65].

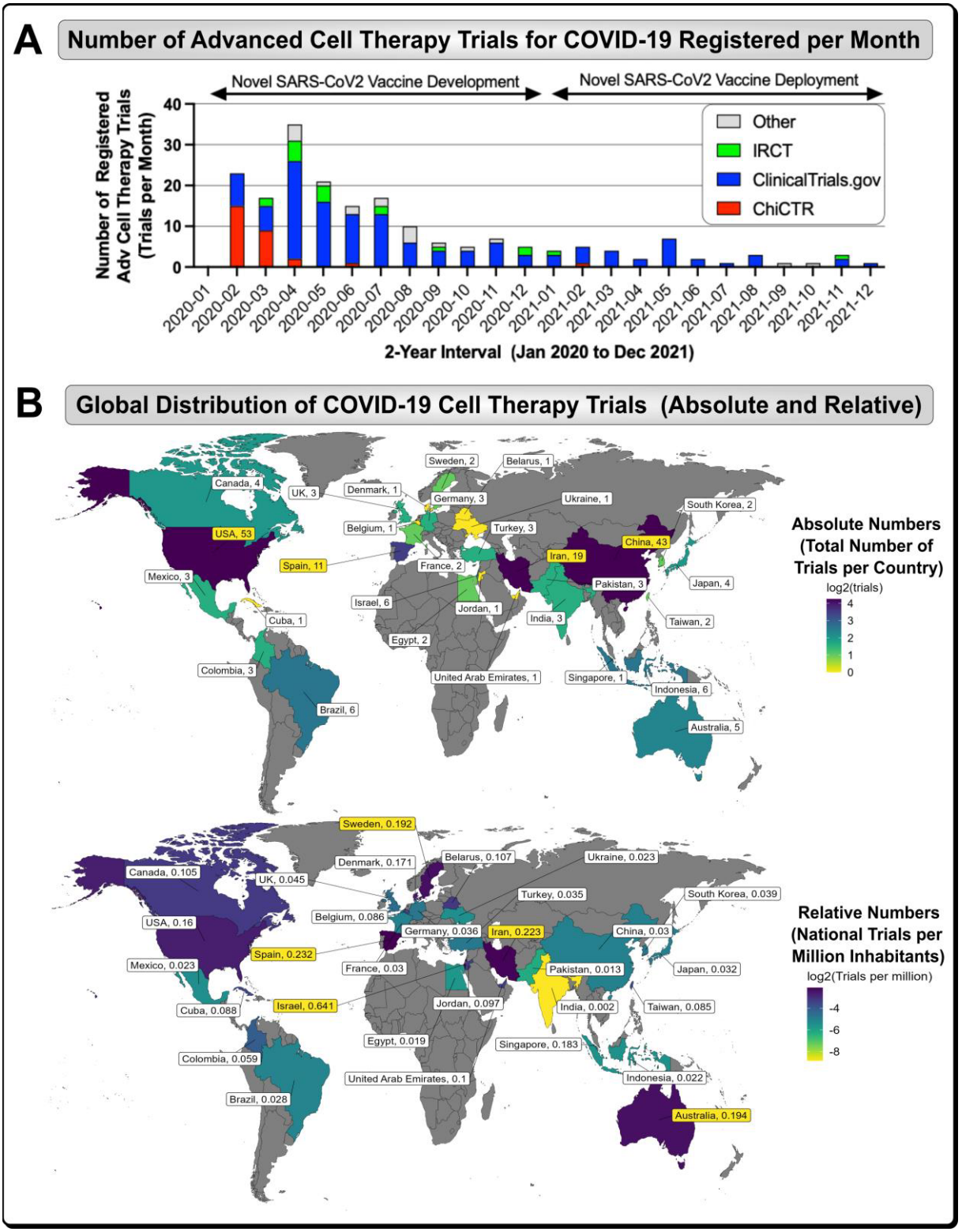


Figure 2. Global Landscape of Cell Therapy Trials for COVID-19. (A) Number of Advanced Cell Therapy Trials for COVID-19 Registered per Month: Data on the number of registered advanced cell therapy trials (Trials per Month; depicted is the 2-year interval of interest from the start of January 2020 to end of December 2021) were collected from available national and international clinical trial registries, e.g. the American registry (ClinicalTrials.gov; shown in blue), Chinese registry (ChiCTR, shown in red), Iranian registry (IRCT, shown in green), and other registries (shown in grey), depicting a peak in advanced cell therapy trial registrations between February to July of 2020, with a subsequent long tail of decline in cell therapy trial activity, which occurred at the

same time the novel SARS-CoV-2 vaccines showed first success in early clinical trials and started to be deployed (e.g. vector or mRNA-based vaccines); and **(B)** Global Distribution of COVID-19 Advanced Cell Therapy Trials (Absolute and Relative No): The top panel depicts the Absolute Numbers (Total Number of Trials per Country) identifying the US (n=53), China (n=43), Iran (n=19), and Spain (n=11), as the most active countries considering the total trial number output, while the bottom panel depicts the Relative Trial Numbers (National Trials per Million Inhabitants) identifying Israel, Spain, Iran, Australia, and Sweden (N=0.641, 0.232, 0,223, 0.194, and 0.192 trials per million inhabitants) as the most prolific countries relative to their (smaller) national population size, again depicting Iran in place three as for the total trial output.

To date, information on advanced cell therapy trials for COVID-19 remains fragmented, although first valuable literature reviews and meta-analyses have been conducted, this is the first study that comprehensively connects trials to subsequent publications. We have listed a summary of prior compilations in descending order of the cut-off date of their conducted search (**Table 2**) [63, 64, 66-86]. Reviews of COVID-19 clinical trials not strictly focused on cell therapy were excluded from the list. Importantly, the 195 trials identified in this article are more than double the number presented by previous authors, which demonstrates the outreach of our data search criteria. Our compilation of cell therapy trials for COVID-19 is so far the only one that offers worldwide trial data versus time for a 2-year time frame (**Figure 2A**). Our review also tracks contributions of different clinical trial registries over time, illustrated by the respective color coding, which indicates a dominance of contributions from the US (blue), Chinese (red), and Iranian (green) registries, while the contributions from other registries (grey) were smaller. This is partly because the most dominant US registry (clinical.trials.gov, 131 of the 195 registered trials) was used as the default template in our search. It must be noted, that in some countries that have a clinical trial registry, researchers are obligated to use their national registry, and cross posting their trial to ClinicalTrials.gov is optional, so that ClinicalTrials.gov should never be relied upon as a complete international record of clinical trials.

A global heatmap of the countries where clinical trials of cell therapy for COVID-19 were conducted, regardless of where they were registered, is shown in **Figure 2B**. Only one trial took place in more than one country. Among 30 participating countries, leaders were the US (n=53, 27%), China (n=43, 22%), Iran (n=19, 10%), and Spain (11, 6%), while other countries hosted <4% (**Figure 2B top**). The highest relative trial numbers per capita came from Israel, Spain, Iran, Australia, and Sweden (N=0.641, 0.232, 0,223, 0.194, 0.192 trials / million inhabitants, respectively) (**Figure 2B bottom**). Our compilation is the only one that identifies Iran as the 3rd absolute and relative leading contributor. Presumably, this is because most trials in Iran are only listed on the Iranian national registry, and not cross-posted to ClinicalTrials.gov. Noteworthy, the list of countries leading in cell therapy for COVID-19 through the end of 2021 (US, China, and Iran) does not match the lists of countries that reported the highest number of

COVID-19 infections (US, India, France, Brazil) or the highest number of COVID-19 related deaths (US, Brazil, India, Russia) during that timeframe [6].

3.4.2 Types of Cell Products in Registered Clinical Trials

Detailed information about the 195 advanced cell therapy trials for COVID-19 registered 2020-2021, including up to 20 individual parameters for each registered trial (listed in the Methods) are listed in **Table S1** with representative plots of important parameters shown in **Figure 3A and the top of 3B**. While the terminology of our database uses "route of administration" and "cell storage", in the discussion these topics are combined as "clinical delivery".

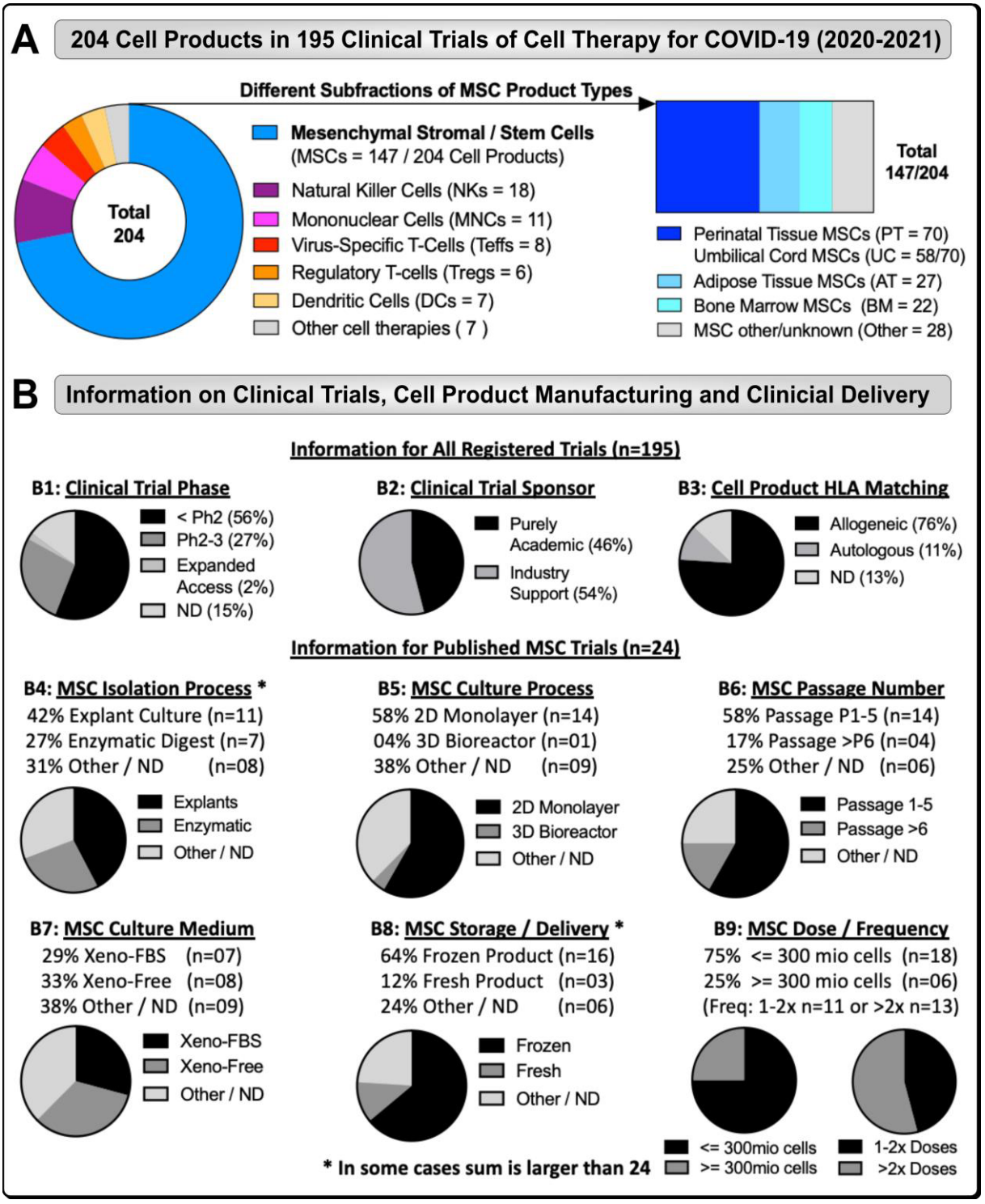


Figure 3. Cell Types and Manufacturing in Cell Therapy Trials for COVID-19. (A) Different Types of Cell Products Tested as Therapy for COVID-19 (2020-2021): The figure depicts the total number of advanced cell therapy products tested as treatment for COVID-19 in clinical trials registered between the start of January 2020 to end of December 2021 (The number of 204 products depicted here is higher than the 195 trials identified in total, due to the testing of multiple products in some studies). The products tested included several hematopoietic cell types (e.g. Natural Killer cells, virus-specific and regulatory T cells, but also mononuclear, dendritic cells, and others types of products), and in particular mesenchymal stromal/stem cells (MSCs), accounted for 147/204 products (left panel), including different subfractions of MSC product types (right panel; e.g. adipose tissue (AT-MSC; 27/147), bone marrow (BM-MSC; 22/147), and perinatal tissue (PT)-derived cells (70/147), with the latter

being most abundant, which was mostly accounted for by the large number of umbilical cord (UC)-derived MSCs (58/147) products tested. **(B)** Clinical Trial Information, Cell Product Manufacturing and Clinical Delivery: **(B1-3)** Depicts information for all registered trials (n=195), including **(B1)** Clinical trial phase, **(B2)** Clinical trial sponsor, and **(B3)** Cell product HLA-matching, while **(B4-9)** Depicts information for the published MSC trials (n=24), including **(B4)** MSC isolation process, **(B5)** MSC culture process, **(B6)** MSC passage number, **(B7)** MSC culture media, **(B8)** MSC storage and clinical delivery, **(B9)** MSC dose and dosing frequency. Some displays in (**B4-9**) are marked with a star (*) to indicate that numbers can be greater than 24, as some published trials employed more than one method or product. Altogether, our analysis depicts a strong dominance in the data set for early stage trials (56%), employing allogeneic MSC products (76%), which were isolated by explant method (42%), cultured in 2D monolayer flasks (58%), expanded up to passage 5 (58%), and delivered intravenously (100%), mainly as cryostorage-derived freeze-thawed product (64%), and dosed below 3 million cells/kg (75%), in either one to two (46%), or even several more doses (54%), most likely to increase the total number of applied therapeutic cells over a given time frame, without increasing the individual therapeutic doses above a limit of 3 million cells/kg. Interestingly, some studies (17%) employed MSCs expanded above passage 5 up to passage 12. Many MSC products were applied thawed shortly upon retrieval from cryostorage as frozen cells (64%), which has previously been shown to compromise the therapeutic properties of clinical MSC products and may even have accounted for earlier trial failures [2, 14, 16-18, 30, 33, 138-142].

Among the 195 registered cell therapy trials for COVID-19, most (n=141, 72%) tested some type of MSC product (**Figure 3A**). The next most common cell types were natural killer (NK) cells and mononuclear cells (MNC), employed in 9% and 6% of the trials, respectively. Interestingly, n=7 of the registered trials used more than one cell type, including more than one MSC type, which is why the total number of cell products is n=204 in the chart (**Figure 3A, left panel**). Even with all cell types counted individually MSC-product-based trials still accounted for 147 / 204 (72%) of the registered cell therapy trials. The most common type of MSC source was perinatal tissue (PT)-derived PT-MSCs, accounting for 70 trials (34% of all cell types, or 48% of MSC products in trials) (**Figure 3A, right panel**). Within this category we included umbilical cord (UC)-derived UC-MSCs in 58 trials, or other perinatal sources in 12 trials. This was followed by adipose tissue (AT)-derived AT-MSCs in 27 studies (13% of cell types in trials, or 18% of the MSC products), and bone marrow (BM)-derived BM-MSCs in 22 studies (11% of all cell types in trials, or 15% of the MSC products), and other types of MSC sources in 28 trials (14% of all cell types, and 19% of the MSC products).

Most of the registered trials were early phase research, with at least 56% below phase 2 (**Figure 3B1 and Table S1**), which is probably an underestimate of early phase trials, since 15% of trials were of unknown phase. There were four phase 3 trials and four trials registered with US FDA under Expanded Access programs. The sponsors of the registered clinical trials were exclusively academic for 46% of the trials (**Figure 3B2**), but the remaining 54% of trials had industry support, typically from the company that manufactured the cell therapy product used in the trial. The large majority, 76% of all registered trials indicated an allogeneic product (**Figure 3B3 and Table S1**), while only 11% were autologous and 13% of trials did not report or define this aspect. The rationale behind the predominant use of (allogeneic) donor cells and

off the shelf products is probably that the enrolled COVID-19 patients were often critically ill, and either unable to provide autologous cells (patient derived), or unable to wait for the autologous product to be manufactured.

3.4.3 Types of Cell Products in Published Outcomes of Clinical Trials

Our search to match clinical trials with reports of their outcomes identified 26 peer reviewed papers accepted for publication by the end of July 2022 (**Table 3)** [37-60, 87, 88]. We also included a clinical trial of MSCs for ARDS that was originally registered in 2017 and published in 2021 [57]. The study was included, since during the pandemic, the researchers pivoted to conduct a study of MSCs for ARDS induced by COVID-19 [38]. Thus, the trial met our inclusion criteria as a published outcome of a registered clinical trial. As stated in the Methods, we excluded publications that could not be associated with registered trials, such as an extensive report on 210 patients that were treated under the approval from the Ministry of Health in Turkey [89], but not registered as a clinical trial. Our database of published trial outcomes includes two pairs of papers from two research groups in China, where in each case the group initially published a safety study [45, 53] and later published their data in a controlled trial [46, 49], respectively. The therapeutic modality reported across the published clinical trials was overwhelmingly allogeneic in all but one of the 26 published trials (96%). This confirms that those allogeneic products favored completion of trials with subsequent publication, while virtually no trials with autologous products were reported within the time frame of our database. Interestingly, many patients received cells from individual (HLA disparate) donors during each infusion in 13 of the 26 published studies (**Table 4**), while two studies used banks of pooled donors, but in 11 studies the donor selection is unknown. This frequent use of allogeneic products raises the issue of potential alloimmune-cross-reactivity from multiple infusions of HLA-mismatched cell products, which should be followed up in more detail in future studies. However, given the widely postulated hypoimmunogenic or immune-privileged status of MSCs, or better said the "immune-evasive nature of MSCs" [90], this critical aspect in clinical cell transplantation appeared to be of less concern in clinical trial design.

By our count, 18 of 26 published trials used cell products from a commercial entity (**Table 4**). Examples are proprietary cell product under development, or cells manufactured by a contract manufacturing organization (CMO), a biotech spin-off, or a cell therapy clinic. By comparison, eight studies used cells manufactured in the lab of an academic center, such as a university lab or a research hospital. This split between commercial facilities *versus* academic labs strongly impacts manufacturing data reporting. In the case of academic labs, manufacturing details are

often available, but frequently buried in a supplement of the COVID-19 study, or in a previous paper. When authors used commercial facilities, they often did not describe cell manufacturing, simply citing that the cell products were approved for clinical use by their government. Some of the commercial entities that provided cells for COVID-19 trials have never described their cell manufacturing in any publication, so it is impossible for a reader to know how the cell product was produced and characterized. Given that MSCs were the dominant cell product in the registered cell therapy trials for COVID-19 (72% of all registered trials), it is not surprising that MSC products were employed in 24/26 (92%) of the published clinical trials, while the remaining two employed memory T-cells from convalescent donor plasma [87], or non-hematopoietic cells from peripheral blood [88]. As indicated above (**Figure 3A, Table 3 and Table S1**) the sources of the MSCs in these studies were remarkably heterogeneous, with many additional variables during their manufacturing. The sources included MSCs from BM, from AT, and from various PT sources, such as from UC-derived Wharton's Jelly, from Wharton's Jelly plus selection for ACE2-negative cells, from the subepithelial layer of the UC after discarding the Wharton's Jelly, from the fetal placenta, from the decidua (maternal side) of the placenta, from menstrual blood, but also MSCs derived from an embryonic cell line, and stromal cells isolated from heart tissue. The closest to a uniform group of cell types is the 11 trials that employed MSCs from Wharton's Jelly alone without further selection.

We have summarized the cell product manufacturing for all 26 published trials in Table 4, while **Figure 3B** summarizes parameters for the 24 published studies on intravenous MSC therapy. Some trials employed more than one MSC product with different processing, such as fresh placenta MSCs and frozen UC MSCs (**Table 3**), so that for some parameters in 3B the total exceeds n=24. The 24 published studies that employed MSCs relied mainly on cell isolation by explants in nine trials (**Figure 3B4 and Table 4**), by enzymatic digestion in five trials, one trial combined MSCs isolated by each method, one trial alternated between MSCs isolated by each method, and the cell isolation method in the other eight MSC trials was unknown. The three studies that started with blood (either peripheral or menstrual blood), used centrifugation as their first step towards cell isolation. In the MSC trials, the cell expansion/culture process was monolayer in 14 trials (**Figure 3B5 and Table 4**), only one MSC trial employed a bioreactor, and not stated in nine studies. The number of passages in MSC products was reported for 18 trials and ranged from P3 to P12 with a median of P4 (**Figure 3B6 and Table 4**), while passages were unknown for six MSC trials. The medium used to grow MSCs for human clinical application contained fetal bovine serum (FBS) in seven of the products (**Figure 3B7 and Table**

4), while xeno-free medium was used in eight MSC products, and the medium formulation was unknown in nine products. Considering their storage and clinical delivery, 15 of the reported MSC trials used a previously frozen product readily derived from prior cryostorage (**Figure 3B8 and Table 4**). Two MSC trials used cells fresh from culture, one trial alternated between fresh or frozen MSC products, and in six trials the storage was not reported. The 24 published MSC trials all delivered MSCs by intravenous (IV) route of administration (**Table 3**). The cell dose was scaled by patient weight in 14 of the published clinical trials but set at a fixed dose in the remaining ten studies (**Figure 3B9**). For a patient weighing 70kg, the average cumulative MSC dose across all the trials was 225 million cells per patient, ranging from a minimum of 70 million to a maximum of 630 million cells per patient, thus typically 1-10 million cells / kg of patient body weight, which is the most commonly reported dose range in clinical trials involving IV delivery of MSCs [2, 16-18]. Considering patient enrollment, despite the difficulty accruing patients for cell therapy trials during a pandemic with moving surges, we found that five of the 26 published trials managed to accrue more patients than the target enrollment listed in their trial registration (**Table 5**). The average target enrollment was 40 patients and the average achieved enrollment was 29 patients.

3.4.4 Published Clinical Trial Outcomes: Safety and Efficacy Based on RR/OR Analysis

Two previous studies that connected advanced cell therapy clinical trials with their reported outcomes both found that only about 20% of these trials get published eventually [23, 91]. To anticipate how many more publications of cell therapy for COVID-19 may be in preparation, we checked the status of all 195 of the 2020 and 2021 clinical trials, as of July 2022. We found that 27 (14%) of the trials had notifications that they had been cancelled, withdrawn, or terminated early. When an explanation was given for these premature endings, typical reasons stated were a lack of funding, or the inability to recruit patients. In addition to the 26 trials (13%) that have been published already, we found another 28 trials (14%) recorded as "completed", which means that additional peer reviewed publications of cell therapy trials for COVID-19 can be anticipated (final publication rate 27%). Most of the 26 trials published so far were controlled studies: 11 were randomized controlled trials, seven trials included a control group without randomization, and eight trials had a single arm (**Table 5**).

We computed a meta-analysis of the survival benefit from IV MSC therapy for COVID-19. As explained above, the Relative Risk (RR) represents the ratio of the treated patients divided by the whole population (**Figure 4A**), while the Odds Ratio (OR) represents the ratio of the treated patients divided by the control group (**Figure 4B**). For the 24 published trials that employed IV

MSCs, we used the survival data tabulated in **Table 5** to assess the clinical efficacy of the treatment relative to controls, according to the statistical procedures described in the Methods. Initially we calculated RR and OR for all 24 studies, employing the missing data compensation described in the Methods. With this approach, there are 305 patients in the MSC treatment groups and 402 in the control groups, with 46 and 90 events (mortality), respectively. In the meta-analysis of these 24 studies, MSC therapy was associated with a diminished risk of all-cause mortality RR=0.63 [95% CI 0.46 to 0.85] ($P < 0.01$) or OR=0.51 [95% CI 0.33; 0.78] ($P < 0.01$). We repeated our RR and OR calculation using only the 17 of 24 IV MSC studies that had a control arm (**Figure S1**). The existence of control arms means it is not necessary to perform any statistical procedures to correct for missing data. This group had a total of 237 patients in the MSC treatment group and 334 in the control group, with 35 and 73 events (mortality) reported, respectively. Here, MSC therapy was associated with a diminished risk of all-cause mortality RR=0.62 [95% CI 0.45 to 0.87], ($P < 0.01$) (**Figure S1A**) or OR=0.48 [95% CI 0.29 to 0.81] ($P < 0.01$) (**Figure S1B**). It is reassuring that the statistical results for this sub-group are almost indistinguishable from the RR and OR results of the full set of 24 studies (**Table 6**).

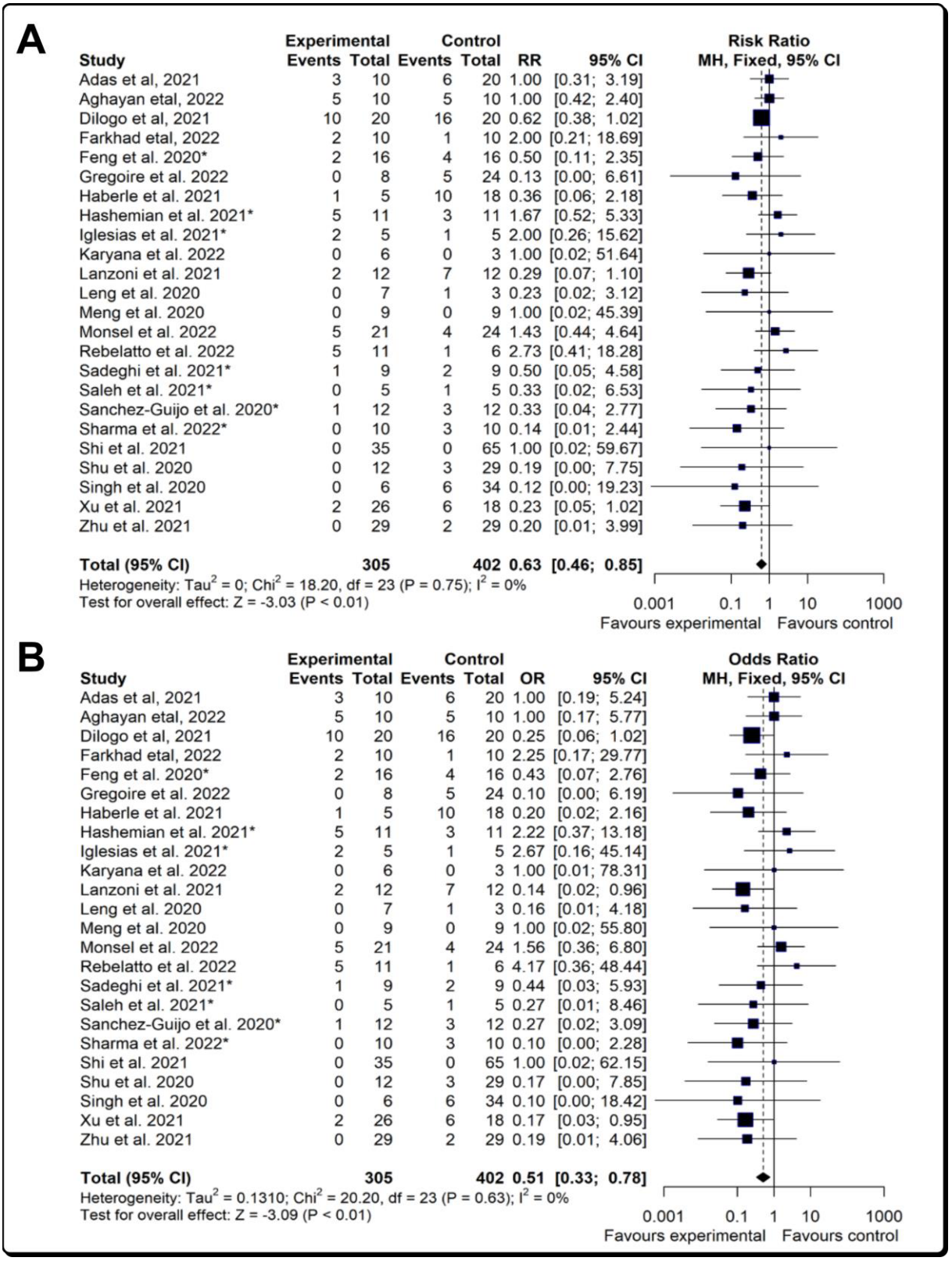


Figure 4. **Treatment Efficacy of MSC Therapy for COVID-19 (RR/OR Analysis).** Comparison of efficacy of mesenchymal stromal/stem cell (MSC) therapy (Experimental) vs. standard of care (Control), depicting calculations of: **(A)** Risk Ratio (RR) or **(B)** Odds Ratio (OR) for published cell therapy trials employing different types of MSC products (n=24 studies). This analysis includes MSC trials registered in the time period Jan-2020 to Dec-2021, with a follow-up period until the end of July 2022, to also detect trials published after the primary time

window. The publications are sorted alphabetically according to the first author. **Abbreviations and Asterisks**: CI, confidence interval; The asterisks (*) indicate the n=7 studies [54-60], where the values for missing controls were computed as indicated in more detail in the methods section. For the double-arm-zero-event studies [43, 46, 49] we employed a treatment arm continuity correction (TACC) to incorporate them, since otherwise RR/OR could not have been calculated [61, 62].

We have summarized the RR and OR meta-analyses for different sub-groups and compared them with previous reports that presented RR / OR survival benefit of cell therapy for COVID-19 (**Table 6)** [92-95]. The first two previous meta-analyses in our table only used studies of IV MSC against controls, finding RR=0.54 for ten studies [92] and RR=0.50 for nine studies [93], respectively. While these two meta-analyses had very similar results, their statistical methods differed slightly. The first one included studies with no mortalities on either arm, whereas the second study excluded them. We have included studies with no mortalities by assigning them RR=1.0. A third previous meta-analysis found OR=0.24 for twelve studies [94], although we caution that their meta-analysis mixed different cell types in the statistics.

3.5 DISCUSSION

To the best of our knowledge, this effort is the first report to date, that comprehensively links clinical trials of advanced cell therapy for COVID-19 with the published outcomes of those trials. This type of linkage requires that the starting database of clinical trials is as complete as possible but avoids/omits any redundancies. Thus, in the process of building the COVID-19 trials database at CellTrials.org, we have incorporated several crucial quality steps, e.g. inclusion of trials from all national registries, exclusion of false positives on keywords, and exclusion of double counting of the same trial. So far, none of the other existing trial compilations that we examined in Table 2 stated that they have employed such steps. We also must point out, that most academic studies of COVID-19 cell therapy trials ran their entire search at a single point in time and selected only for COVID-19 trials. In contrast, CellTrials.org has collected all advanced cell therapy trials monthly and then extracted the COVID-19 trials at the end of each month. In a typical month, CellTrials.org sorts through about 600 clinical trials that hit on keywords and finds that 10% qualify as advanced cell therapy trials.

The advanced cell therapy trials for COVID-19 have been conducted in 30 countries led by the US, China, Iran, and Spain, yet most resulting publications have come from China, Iran, and 11 other countries so far. The initial surge in clinical trials registered to apply cell-based therapy for treating COVID-19 peaked in April 2020 and subsided into an ongoing effort of a few new trials per month. It must be noted that many healthcare policies at both national and local levels influence the ability to launch trials and recruit COVID-19 patients for cell-based therapies.

Despite ongoing outbreaks in the US, we have noted that multiple trials have been suspended because they cannot recruit enough patients at a single hospital. Thus, large research consortia with multi-institutional and multi-national collaboration are needed to tackle this shortcoming and more rapidly develop new treatment approaches for COVID-19. In China, the "Zero COVID" policy was so efficient at suppressing outbreaks for two years, that clinical trials had stopped because they could not accrue patients [54]. Since this policy was changed in late 2022 , new infections and probably also associated severe cases and deaths due COVID-19 are likely to have surged dramatically [96], with a need for effective treatments.

In many Western countries (e.g. Europe being subject to both national and EMA regulation, with considerable variability in regulation between different European nations) [2, 24], strict regulations on human cell therapy mean there were only a limited number of cell therapy products with established safety profiles that could be trialed. While we found that 56% of the trials were below phase 2, we found that 69% of the published outcomes were studies with a control arm. The fraction of the trials employing MSC was 72%, but 92% of the published outcomes are studies that relied on MSCs. The mechanisms of action by which ARDS and COVID-19 patients may benefit from MSC therapy have been exhaustively reviewed by the papers listed in **Tables 2 and 3** [37-53, 63, 64, 66-86, 92-94, 97], relying on multiple synergistic effector mechanisms, such as promoting/triggering beneficial immunomodulatory and regenerative pathways, angiogenesis and antiapoptosis [18]. Hence, we will not repeat that discussion here, but only refer to the most crucial key observations in the discussion further below. Most importantly, in this study, we have also endeavored to quantitatively calculate the safety and efficacy profile of MSC infusions as a novel treatment for COVID-19. However, efforts to treat these topics systematically appear to be fraught with difficulties.

On the issue of MSC safety, we found that all of the 24 published trials claimed that they had no severe adverse events related to the MSC infusion [37-60]. Many of the studies gave anti-coagulant therapy as a prophylaxis [38, 42, 44, 56, 57, 60]. Already in the first reports from the COVID-19 epicenter in Wuhan, and swiftly following global reports, severe coagulopathy was identified as one of the most evident complications arising from SARS-CoV2 infection and critical/severe COVID-19 [98-108]. The increased incidence of thrombotic complications in these patients was verified in large population studies in Sweden [106, 107]. Indeed, MSC-IV therapeutics carry a risk of thrombotic complications, due to their expression of the highly prothrombotic tissue factor (TF/CD142) [2, 16, 17]. Hence, since the first months of the pandemic, we have identified prophylaxis against coagulation as a crucial factor for the safe application of IV MSC

therapeutics in a COVID-19 setting [2, 14, 15]. We were poised to highlight the safety aspect of IV MSC therapy for COVID-19, but we discovered that none of the published trials had conducted monitoring that could address this issue. For example, in one trial that treated patients suffering from severe COVID-19 in a hospital ICU, a patient that received IV MSC therapy died 13 days later following an arterial thrombosis [56]. This event was considered unrelated to MSC therapy because it occurred outside their four-hour window of post-infusion monitoring.

This was common in the 24 publish trials: possible complications arising from MSC infusion were only monitored during or shortly after infusion. Yet, case studies of adverse events associated with MSC infusions found that elevated clotting markers (e.g. TAT and D-Dimer) typically peaked 9-12 hours post-MSC infusion [17], while pulmonary (but not arterial) embolism post MSC therapy could be detected days, weeks, and even months after treatment [17, 109, 110]. This should caution us that the impact of the *instant blood-mediated inflammatory reaction (IBMIR) post IV MSC therapy for COVID-19 may only* become evident at later time points [2, 16, 17]. Given that thromboembolism is a well-known side effect of either COVID-19 infection or MSC infusions, it is currently not possible to retrospectively assign that complication to one or the other group [17], when these studies were not constructed to discriminate the potential causes. Thus, we did not attempt to compute the rate of adverse events in the published trials because the few observed / reported events are largely anecdotal in nature and cannot be quantified at the current stage. Hopefully, future clinical trials will incorporate longer monitoring periods and will have large enough patient groups to statistically identify if any adverse events were increased in response to the experimental (MSC) treatment but not in response to the standard of care.

The primary theme of our analysis was to calculate the efficacy of MSC infusions as a therapy for COVID-19 based on the risk of mortality. The mortality data represent a quantitative set of facts that were extracted from each of the published clinical trials and are listed in **Table 5**. However, we must caution that not all these trials were designed with survival as an endpoint. For example, one study recruited convalescent patients to determine if MSC infusions would speed up the resolution of their lung lesions and consequently none of the patients died on either arm [49]. The previous meta-analyses have also noted that published studies with intravenous use of MSC for COVID-19 have very heterogeneous patient populations, in terms of the severity of their COVID-19 illness as well as the forms of concomitant therapies that they received [92-94, 97]. Our statistical model has included more studies and more patients than previous meta-analyses of MSC efficacy for COVID-19 [92-94, 97]. We corroborate the conclusions of the

previous meta-analyses, that intravenous MSC therapy appears to provide a benefit for the treatment of COVID-19 [66, 92-95]. Recently, earlier timing of MSC infusion has emerged as a new factor that may be associated with improved survival for patients receiving MSC therapy for COVID-19 [111]. This was established in a single center study that gave an IV MSC dose 3 x 3 million cells/kg. We note that their cumulative dose is about three times higher than the average in our 24 studies of IV infused MSCs. We do find that most published studies (17 out of 24) spread out clinical MSC delivery over two to four doses.

Perinatal sources of MSCs (e.g. tissue of UC and/or placenta, collectively called PT-MSCs) [16, 17], were employed in 18 of 24 published trials (**Table 3**). Despite a great deal of preclinical and clinical research, there are still conflicting opinions on the biological characteristics of MSCs isolated from perinatal *versus* adult sources. Multiple earlier studies suggested that PT-MSCs may have superior immunomodulatory properties [112-119], higher proliferation [113, 120], and richer secretome [116, 121-123], compared to adult sources. In turn, some studies also reported that adult sources of MSCs have superior or similar immunomodulatory ability [122, 124, 125], and that adult MSCs have higher production rates of several vital molecular mediators (e.g. VEGF [122, 123], PLGF [122], IL-10 [126], and TGF-β1 [126]) when compared to perinatal cells. Intriguingly, when we performed meta-analysis for subgroups of our data, we found that the RR of the six studies with non-perinatal MSCs was more than factor two better than the RR of the 18 perinatal studies (**Table 6**). All six of the non-perinatal studies, which employed five different MSC products, reported good outcomes. In contrast, among the 18 perinatal studies there were five studies where mortality on the MSC treatment arm was higher than the control baseline [40, 47, 48, 55, 56]. However, this RR comparison was not statistically significant, leading us to argue that more data is needed to confirm or refute this result.

Another focus of our study was to evaluate the diversity of manufacturing methods used to prepare MSC products and to highlight the importance of reporting manufacturing information to enable study comparability (**Figure 3, Tables 3 and 4**). Indeed, diversity in cell product manufacturing parameters, cell dosing, and cell characterization for therapeutic use, but also the completeness of study descriptors, have all been identified as a potential confounder to interpretation of safety and efficacy outcomes in MSC studies and should be monitored / reported more thoroughly in future studies [2, 16, 17, 33, 127-129]. Both, Wiese et al. [127] and Wilson et al. [128] recently highlighted the frequent lack in reporting of study descriptors as a considerable shortcoming to clinical trial reporting and subsequent study interpretation. This aspect is of such importance, that it has been taken up into: “A modified Delphi Study Protocol” for

"Establishment of a Consensus Definition for Mesenchymal Stromal Cells (MSC) and Reporting Guidelines for Clinical Trials of MSC Therapy" [129]. One multi-center randomized control trial (RCT) that employed UC-MSCs to treat COVID-19 found that MSC therapy had no efficacy [47]. However, the MSCs in that study were manufactured in a laboratory that alternated between cell isolation with explants vs. enzymatic digestion (which may impact cell yield and immunophenotype) [130, 131]. Still, the research consortium did not publish any records of which patient received which MSC product [47]. Consequently, the outcome of the entire multi-center RCT may be cast into doubt because it is unknown how manufacturing variability may have compromised the respective results. Another manufacturing issue, which has been largely overlooked in the discussion of cell therapy trials for COVID-19 so far, is the ability to scale up the production of successful therapies [31, 132-135]. Most trials published so far have relied on 2D monolayer cell expansion methods in flasks, with only one study employing a hollow-fiber bioreactor [42]. The monolayer methods are time-consuming, labor intensive, and have limited scalability [131, 136, 137]. Given the potential combinatorial detrimental impact of both freeze-thawing and *in vitro* aging on cell potency [33, 131, 138-142], it is of importance to accurately evaluate the number of population doublings a cell product has accumulated during *ex vivo* culture expansion for COVID-19 therapy and other indications [2, 14, 16]. Of course, it is also relevant to record if the cell product was given either fresh or as a freeze-thawed product derived from cryostorage, since this may impact substantially on the product performance [14, 30, 31, 33, 139-145].

3.6 CONCLUSIONS AND LIMITATIONS

The International Society for Cell and Gene Therapy (ISCT) recently published an editorial calling for a global registry of clinical trials that employ MSCs for COVID-19 to harmonize the data on the limited number of patients and "To collect information on critical process parameters used to manufacture the MSCs" [146]. We support that call to action. Our review of the manufacturing parameters in clinical trials giving cell-based therapy for COVID-19 has revealed a partial disconnect between clinical centers that treat patients versus laboratories that manufacture cell therapy products. Frequently, the clinicians running the trials have acquired cells and delivered them to patients without keeping any records about the cell production. This disconnect could be closed, if the clinical trials participated in a global registry that required completing standardized categories of information. In this study we have compiled two years of worldwide clinical trials testing cell-based therapies for COVID-19 and linked those trials to their published outcomes. This "end-to-end" survey of the research field has enabled us to learn new insights not published earlier. First, we found that global registrations of advanced cell-based therapies for COVID-19 were more numerous than previously reported, but that they experienced only one single early surge in trial registrations during a time frame, when global COVID-19 infections went through multiple surges. Our analysis also includes the contribution from registered clinical trials that are not listed on the national registries of the United States and China, with 53 and 43 trials, respectively. Hereby, we have learned that Iran (19 trials) is among the three leading nations running advanced cell therapy trials for COVID-19 and the 2nd in publishing trial outcomes. In turn, Israel, Spain, Iran, Australia, and Sweden are leading in relative contributions to COVID-19 cell therapy trials normalized to population size (N=0.641, 0.232, 0,223, 0.194, and 0.192 trials per million inhabitants).

Although 72% of the COVID-19 cell therapy trials employed tissue-derived MSCs, a significant fraction of clinical trials conducted immunotherapy with blood-derived cells. So far, most of the published trials describe infusions of MSCs, and 75% of those employed MSCs derived from perinatal tissue sources. Throughout these studies there is a strong theme of heterogeneity. The patient groups in the clinical trials are heterogeneous, as are the manufacturing methods used to prepare the MSCs. Most importantly, our statistical analysis shows that infusions of MSCs show a clinical benefit for COVID-19 patients. The risk ratio for all-cause mortality is RR=0.62 [95% CI 0.44 to 0.87] for the 17 MSC studies with control arms, and when we compensated for the missing controls and incorporated all 24 MSC studies the result is RR=0.63 [95% CI 0.46 to 0.85]. Early during the COVID-19 pandemic, it emerged

that the respiratory distress caused by COVID-19 is a substantially different clinical entity compared to classic ARDS [21]. Hence, we cannot assume that the efficacy results obtained here will similarly influence the long-standing quest to improve ARDS mortality. For this answer, we must await the outcomes of large placebo-controlled randomized trials of MSCs for ARDS, such as the REALIST trial in the UK, and the STAT trial in the US, respectively [147, 148]. We close with the concern that there may never be enough data to fully explore the efficacy of cell-based therapy against ARDS from COVID-19. Owing to the evolution of the virus to less lethal variants and the rollout of vaccination, it has recently become difficult to accrue patients for clinical trials that treat severe symptoms of COVID-19 infection. The relative impact of MSC product source, MSC dosing, and the timing and type of MSC delivery, etc., may never be fully explored or known within the current setting of COVID-19.

From a public health perspective, the highest goal is to prevent the development of severe or critical COVID-19 through combined effective pandemic countermeasures (**Figure 1A**) [149]. In this regard, vaccination is the most valuable tool available. Also, the standard-of-care for the treatment of severe and critical COVID-19 is continuously improving. Although we here found that infusions of MSCs confer a reduction in the risk for all-cause-mortality from COVID-19 in the studies published to date, more research is needed to clarify this point. Nonetheless, there will always be high-risk patients who develop severe or critical COVID-19, and for them the existence of adjunct treatment with advanced cell therapy may be beneficial. The target groups for whom this therapy may provide benefit include the elderly, immunocompromised individuals, cancer patients, and transplant patients (both stem cell transplants and solid organ transplants) as well as patients with kidney failure on dialysis [17, 150, 151]. More research on the efficacy of advanced cell therapy for COVID-19 will reveal the degree to which these groups may benefit. Eventually, the cost and access to advanced cell therapy must also be anticipated, typically requiring advanced medical infrastructure. It is our hope that the testing of advanced therapies will be pursued in parallel to the improvement of standard care.

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3.8 SUPPLEMENTARY FILES

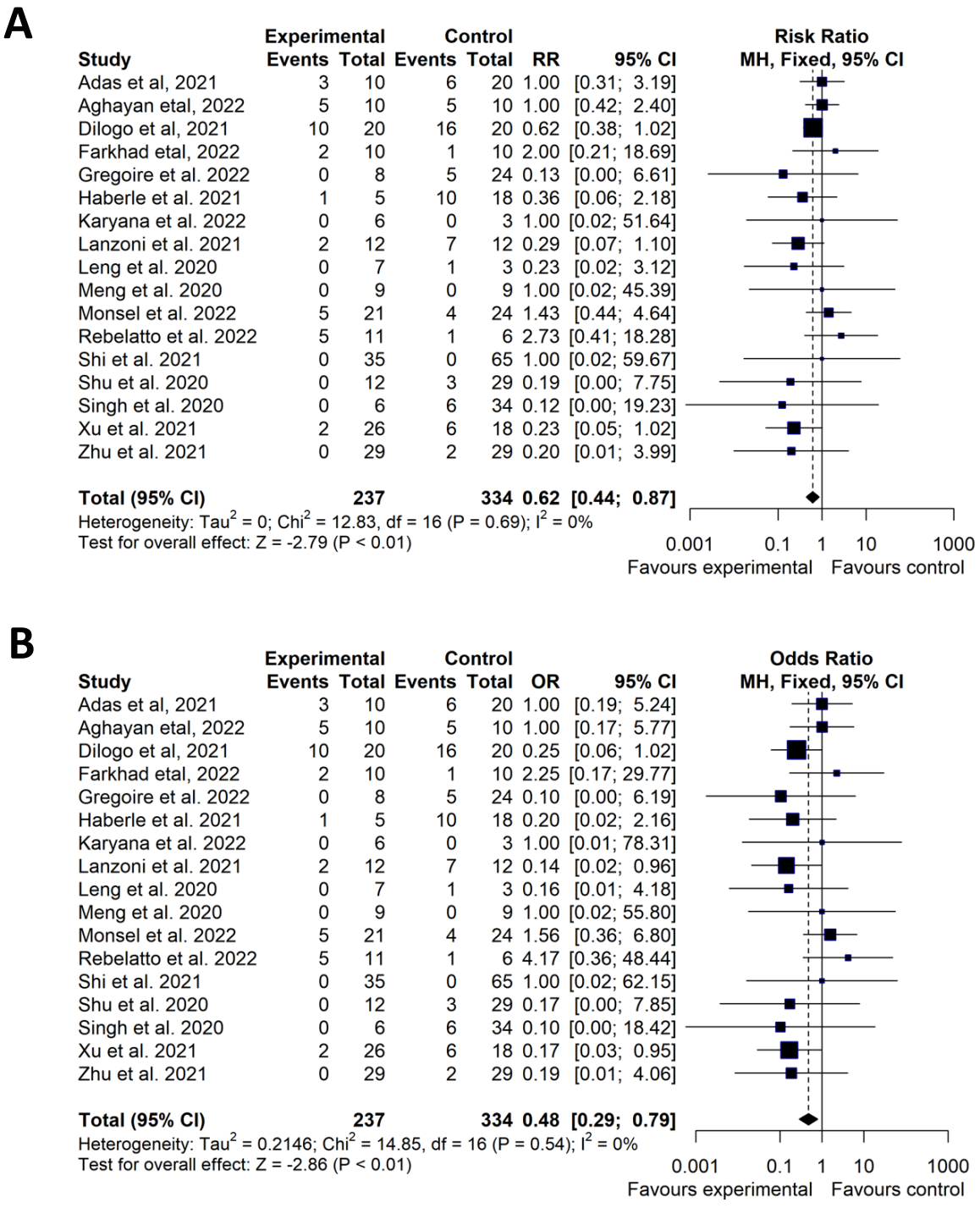

Figure S1: Treatment Efficacy of MSC Therapy for COVID-19 (RR/OR Analysis) controlled studies only (Analysis Supplemental to Figure 4 Part A).

Table 1: National Registries Searched for COVID-19 Advanced Cell Therapy Clinical Trials.

Nation	No. of Trials	Registry Name	Registry URL
Australia & New Zealand	4	Australian New Zealand Clinical Trial Registry (ANZCTR)	**https://www.anzctr.org.au/**
Brazil	2	Registro Brasileiro de Ensaios Clinicos (ReBEC)	**https://ensaiosclinicos.gov.br/**
China	28	Chinese Clinical Trial Registry (ChiCTR)	**http://www.chictr.org.cn/**
Cuba	1	Registro Público Cubano de Ensayos Clínicos	**https://rpcec.sld.cu/**
EU	4	EU Clinical Trials Register (EudraCT)	**https://www.clinicaltrialsregister.eu/**
Germany	0	German Clinical Trials Register (DRKS)	**https://www.drks.de/**
India	3	Clinical Trials Registry-India (CTRI)	**http://ctri.nic.in/**
Iran	18	Iranian Registry of Clinical Trials (IRCT)	**https://www.irct.ir/**
Japan	1	JAPIC Clinical Trials Information	**https://www.clinicaltrials.jp/**
Japan	0	Japan Medical Association Clinical Trial Registry (JMA-CTR)	**http://www.jmacct.med.or.jp/**
Japan	1	Japan Registry of Clinical Trials	**https://jrct.niph.go.jp/**
Japan	0	Japan University hospital Medical Information Network Clinical Trial Registry (UMIN-CTR)	**https://www.umin.ac.jp/ctr/**
Netherlands	0	Netherlands Trial Register (NTR)	**http://www.trialregister.nl/**
Singapore	0	Health Sciences Authority Clinical Trial Registry	**https://www.hsa.gov.sg/clinical-trials/clinical-trials-register**
South Korea	1	Clinical Research Information Service from South Korea (CRiS)	**https://cris.nih.go.kr/**
Thailand	0	Thai Clinical Trials Registry (TCTR)	**https://www.thaiclinicaltrials.org/**
USA	131	ClinicalTrials.gov	**https://clinicaltrials.gov**
WHO	1	World Health Organization International Clinical Trials Registry Platform (ICTRP)	**https://www.who.int/clinical-trials-registry-platform/ also https://www.isrctn.com/**

Legend Table 1: This study covers 195 clinical trials worldwide conducting advanced cell therapy for COVID-19 that were registered during the time period Jan. 2020 to Dec. 2021, with a follow-up until end of July 2022 to detect 26 published outcomes of trials.

Table 2: Previous Compilations of Cell Therapy Trials for COVID-19 Sorted by Search-End-Date.

No. of Trials	Search End	Registries Searched	Authors and Reference
195 (141 MSC)	2021-12	ALL (WHO)	Couto et al. 2023 (this article)
82 MSC	2021-10	ClinicalTrials.gov	**Grumet Sherman Dorf 2022** [66]
51 MSC	2021-10	ClinicalTrials.gov & ChiCTR	**Lu et al. 2022** [67]
185 (134 MSC)	2021-06	ALL (WHO)	**Verter & Couto 2021** [64]
89	2020-12	ClinicalTrials.gov	**Zaki et al. 2021** [68]
22 MSC	2020-11	WHO	**Khoury et al. 2021** [69]
88	2020-08	WHO	**Li et al. 2020** [70]
79	2020-08	ClinicalTrials.gov & ChiCTR	**Kim & Knoepfler 2021** [71]
71	not stated	ClinicalTrials.gov	**Golchin 2021** [72]
111 (85 MSC)	2020-06	WHO	**Verter & Couto 2020** [63]
57	2020-06	ClinicalTrials.gov & ChiCTR	**Choudhery & Harris 2020** [73]
54 MSC	2020-06	ClinicalTrials.gov	**Shetty et al. 2021** [74]
4 NK	2020-05	ClinicalTrials.gov	**Market et al. 2020** [75]
61	2020-04	PubMed & Cochrane	**Rada, Corbalán, Rojas 2020** [76]
54	2020-04	ClinicalTrials.gov & WHO	**Liao et al. 2020** [77]
29 MSC	2020-04	ClinicalTrials.gov & ChiCTR	**Sahu, Siddiqui, Cerny 2021** [78]
28 MSC	2020-04	ClinicalTrials.gov & WHO	**Zumla et al. 2020** [79]
16	2020-04	WHO	**Thorlund et al. 2020** [80]
15	2020-04	ClinicalTrials.gov	**Babaei et al. 2020** [81]
31	2020-03	ClinicalTrials.gov & ChiCTR	**Golchin et al. 2020** [82]
23	2020-03	ClinicalTrials.gov & ChiCTR	**Khoury et al. 2020** [83]
24 MSC	2020-03	WHO	**Ji, Liu, Zhao 2020** [84]
24 MSC	2020-03	WHO	**Lythgoe & Middleton 2020** [85]
5 MSC	2020-02	ClinicalTrials.gov	**Liu et al. 2020** [86]

Legend Table 2: Previous published compilations of clinical trials conducting cell therapy for COVID-19. Publications are sorted according to the cut-off date of trial collection. **Abbreviations:** WHO, world health organization; and ChiCTR, Chinese clinical trials registry.

Table 3: Published Cell Therapy Trials for COVID-19 with Details on Manufacturing.

Trial	Publication Reference	Country	Enrollment Target (Actual)	Principle Cell Type	Cell Dose and Delivery	Manufacturer (Product Name, if any)	Cell Product Manufacturing Method	Quality Control Characterization
NCT04392778	Adas et al. 2021 [37]	Turkey	30 (30)	UC-derived PT-MSCs	3 x 3.0 million cells/kg IV	Liv MedCell, Liv Hospital	Donors: Individuals; Cell isolation: Explants; Expansion: ND; Passage: P7; Medium: ND; and Storage: Frozen	Immunophenotype, Sterility, and Viability
IRCT20200621047859N4	Aghayan et al. 2022 [38]	Iran	20 (20)	Placenta-derived PT-MSCs	1 x 1.0 million cells/kg IV	Motamed Cancer Institute, Tehran Univ of Medicine	Donors: Individuals / C-section; Cell isolation: Enzymatic; Expansion: Monolayer; Passage: P6/P7; Medium: Xeno-Free; Storage: Frozen	Immunophenotype, Trilineage Differentiation, Sterility, Karyotype, and Viability
NCT04457609	Dilogo et al. 2021 [39]	Indonesia	40 (40)	UC-derived PT-MSCs	1 x 1.0 million cells/kg IV	Stem Cells Medical Technology, Cipto Mangunkusumo Hospital	Donors: ND; Cell Isolation: Explants; Expansion: ND; Passage: P6/P7; Medium: ND; and Storage: ND	Immunophenotype
IRCT20160809029275N1	Farkhad et al. 2022 [40]	Iran	20 (20)	UC-derived PT-MSCs	3 x 1.0 million cells/kg IV	Mashhad University	Donors: Individuals; Cell Isolation: Enzymatic; Expansion: Monolayer; Passage: P3; Medium: α-MEM + 20% FBS; and Storage: Fresh	Immunophenotype, Trilineage Differentiation, Sterility, and Viability
NCT04269525	Feng et al. 2020 [54]	China	16 (16)	UC-derived PT-MSCs	4 x 100 million cells IV	Jilin Tuoha Biotech	Donors: ND; Cell Isolation: ND; Expansion: ND; Passage: ND; Medium: ND; Storage/Use: ND	ND
NCT04445454	Gregoire et al. 2022 [41]	Belgium	20 (32)	BM-derived BM-MSCs	3 x 1.5-3.0 million cells/kg IV	University of Liège	Donors: Individuals; Cell Isolation: Adherence; Expansion: Monolayer; Passage: P3; Medium: DMEM + 10% FBS; and Storage: Frozen	Immunophenotype, Morphology, Karyotype, Viability, Immunosuppression
NCT04377334	Häberle et al. 2021 [42]	Germany	40 (23)	BM-derived BM-MSCs	2-3 x 1.0 million	Medac	Donors: 8 pooled; Cell Isolation: Sepax and Adherence; Expansion: Monolayer /	Immunophenotype, Sterility Viability

					cells/kg IV	(MSC-FFM, aka Obnitix)	Quantum Bioreactor; Passage: P3; Medium: DMEM + 10% hPL; and Storage: Frozen	
IRCT20200217046526N2	Hashemian et al. 2021 [55]	Iran	6 (11)	UC-derived vs Placenta-derived PT-MSCs	3 x 200 million cells IV	Royan Institute	Donors: Individuals / Vaginal Delivery; Cell Isolation: UC Enzymatic vs Placenta Explants; Expansion: UC ND vs Placenta Monolayer; Passage: UC-MSCs P4 vs Placenta ND; Medium: DMEM + 10% FBS; Storage: UC-MSCs Frozen vs Placenta MSCs Fresh	Immunophenotype, and Viability
NCT04416139	Iglesias et al. 2021 [56]	Mexico	10 (5)	UC-derived PT-MSCs	1 x 1.0 million cells/kg IV	CBCells Biotech	Donors: ND; Cell Isolation: ND; Expansion: ND; Passage: ND; Medium: ND; Storage: ND	ND
NCT04535856	Karyana et al. 2022 [43]	Indonesia	9 (9)	Embryonic-Cell-derived MSCs	50-100 million cells IV	National Institute of Health, South Korea	Donors: Fetus; Cell Isolation: eSC-line; Expansion: Monolayer; Passage: P12; Medium: Xeno-free; and Storage: Frozen	Immunophenotype, Sterility, Trilineage Differentiation, and Tumorigenesis
NCT04355728	Lanzoni et al. 2021 [44]	USA	24 (24)	UC-derived PT-MSCs Sub-Epithelial	2 x 100 million cells IV	Therapeutic Solutions International (JadiCell)	Donors: Individuals; Cell isolation: Explants; Expansion: Monolayer; Passage: P3; Medium: α-MEM + 10% hPL; and Storage: Frozen	Immunophenotype, Sterility, Viability, and Trilineage Differentiation
ChiCTR2000029990	Leng et al. 2020 [45]	China	120 (10)	UC-derived PT-MSCs ACE2neg	1 x 1.0 million cells/kg IV	Qingdao Co-orient Watson Biotech Group	Donors: ND; Cell Isolation: ND; Expansion: ND; Passage: P3; Medium: DMEM + 2% FBS; and Storage: Frozen	Immunophenotype, Viability, and Trilineage Differentiation
NCT04252118	Meng et al. 2020 [46]	China	20 (9)	UC-derived PT-MSCs	3 x 30 million cells IV	Vcanbio Cell & Gene Engineering	Donors: ND; Cell Isolation: Explants; Expansion: Monolayer; Passage: P5; Medium: Serum-Free; and Storage: ND	Morphology, Immunophenotype, and Trilineage Differentiation
NCT04333368	Monsel et al. 2022 [47]	France	40 (45)	UC-derived PT-MSCs	3 x 1.0 million cells/kg IV	Saint-Louis Hospital Cell Therapy Unit	Donors: Individuals; Cell Isolation: Enzymatic or Explants; Expansion: ND; Passage: P4; Medium: Nutristem® MSC XF + 5% hPL; and Storage: Frozen	Immunophenotype, Sterility, Viability, Karyotype, T-cell-

								Inhibition Proliferation Assay
NCT04578210	Pérez-Martínez et al. 2021 [87]	Spain	58 (9)	Memory T-cells (CD45RA-)	1 x 0.1, 0.5, 1.0 million cells/kg IV	Hospital La Paz	Donors: Convalescent donors 1 HLA match with patient; Cell Isolation: CliniMacsPlus; Expansion: Monolayer; Passage: P12; Medium: SF/XF; and Storage: Frozen	Immunophenotype and Viability
U1111-1254-9819	Rebelatto et al. 2022 [48]	Brazil	15 (17)	UC-derived PT-MSCs	3 x 0.5 million cells/kg IV	Pontifícia Universidade Católica do Paraná	Donors: Individuals / C-section; Cell Isolation: Enzymatic; Expansion: Monolayer; Passage: P3; Medium: IMDM + 20% FBS; and Storage: Frozen	Immunophenotype, Sterility, and Trilineage Differentiation
IRCT2017010531786N1	Sadeghi et al. 2021 [57]	Iran	15 (10)	Placenta Decidua-derived MSCs	1-2 x 1.0 million cells/kg IV	Taleghani Hospital (DSCs)	Donors: Individual / C-section; Isolation: Enzymatic; Expansion: Monolayer; Passage: P4/P5; Medium: ND; and Storage/Use: Frozen	Immunophenotype, Viability, and Karyotyping
IRCT20190717044241N2	Saleh et al. 2021 [58]	Iran	5 (5)	UC-derived PT-MSCs	3 x 150 million cells IV	CellThecPharmed	Donors: Individuals; Cell Isolation: Explants; Expansion: Monolayer; Passage: P5; Medium: ND; and Storage: Fresh	Immunophenotype, and Trilineage Differentiation
2020-001266-11	Sanchez-Guijo et al. 2020 [59]	Spain	100 (13)	AT-derived AT-MSCs (Liposuction)	1-3 x 1.0 million cells/kg IV	Hospitals Salamanca, Navara, Gregorio Marañón	Donors: Individuals Liposuction; Cell Isolation: Enzymatic; Expansion: Monolayer; Passage: ND; Medium: DMEM + 10% FBS; and Storage: Frozen	Morphology, Immunophenotype, Viability, and Trilineage Differentiation
CTRI/2020/08/027043	Sharma et al. 2022 [60]	India	20 (10)	UC- and Placenta-derived PT-MSCs	2 x 100 million cells IV	ReeLabs	Donors: ND; Cell Isolation: ND; Expansion: ND; Passage: ND; Medium: StemPro MSC SFM/XF medium; and Storage: ND	ND
NCT04288102	Shi et al. 2021[49]	China	100 (100)	UC-derived PT-MSCs	3 x 40 million cells IV	Vcanbio Cell & Gene Engineering	Donors: ND; Cell Isolation: Explants; Expansion: Monolayer; Passage: P5; Medium: Serum-Free; and Storage: ND	Morphology, immunophenotype and Trilineage Differentiation
ChiCTR2000031494	Shu et al. 2020 [50]	China	36 (41)	UC-derived PT-MSCs	1 x 2.0 million	Jiangsu Cell Tech Biotech	Donors: ND; Cell Isolation: ND; Expansion: ND; Passage: P3-P5; Medium: ND; and Storage: ND	Immunophenotype

					cells/kg IV			
NCT04338347	Singh et al. 2020 [51]	USA	Unknown (6)	Cardiac-derived Stromal Cells	1-2 x 150 million cells IV	Capricor (CAP-1002)	Donors: Cadaveric Donor Biopsy; Cell Isolation: Explants; Expansion: Monolayer; Passage: ND; Medium: ND; and Storage: Frozen	Immunophenotype
NCT04473170	Ventura-Carmenate et al. 2021 [88]	UAE	146 (139)	Peripheral Blood-derived Non-Hematopoietic Cells	1 x 2.2 million cells via Nebulizer	Abu Dhabi Stem Cells Center	Donors: Autologous; Cell Isolation: Centrifugation; Expansion: ND; Passage: ND; Medium: ND; and Storage: Fresh	Immunophenotype, and Viability
ChiCTR2000029606	Xu et al. 2021 [52]	China	63 (40)	Menstrual Blood-derived MSCs	3 x 30 million cells IV	Innovative Precision Medicine	Donors: Three Individuals; Cell Isolation: Ficoll-Paque Gradient; Expansion: Monolayer; Passage: ND; Medium: ND; and Storage: Frozen	Immunophenotype, Viability, Trilineage Differentiation
NCT04339660	Zhu et al. 2021 [53]	China	30 (58)	UC-derived PT-MSCs ACE2neg	1 x 1.0 million cells/kg IV	Qingdao Co-orient Watson Biotech Group	Donors: ND; Cell Isolation: ND; Expansion: ND; Passage: P3; Medium: DMEM+ 2% FBS; and Storage: Frozen.	Immunophenotype, Viability; Trilineage Differentiation

Legend Table 3: Publications from completed clinical trials of advanced cell therapy for COVID-19 that were registered Jan. 2020 to Dec. 2021 and published by the end of July 2022. The 26 publications are listed alphabetically by the first author. **Abbreviations:** MSCs, mesenchymal stromal/stem cells; ND, not detailed or not determined; AT, adipose tissue; BM, bone marrow; PT, perinatal tissue; and UC, umbilical cord.

Table 4: Summary of Key Manufacturing Parameters from all 26 Published COVID19 Trials.

Manufacturing Parameter	Option 1	Option 2	ND / Other
Manufacturer	18 Commercial Labs	8 Academic Labs	0 ND
Donors	13 Individuals	2 Pooled	11 ND
Cell Isolation	11 Explants	7 Enzymatic	7 ND / 3 Other
Cell Expansion	15 Monolayer	1 Bioreactor	9 ND / 1 None
Cell Passage	1 None	19 report P3 – P12	6 ND
Cell Medium	7 Xenogenic	9 Xeno-free	9 ND / 1 None
Cell Storage	4 Fresh	17 Frozen	6 ND

Legend Table 4: Summary of cell manufacturing parameters in 26 publications from completed cell therapy trials for COVID-19 registered Jan. 2020 to Dec. 2021 and published by the end of July 2022. **Abbreviations:** ND, not detailed or not determined.

Table 5: Survial Outcomes of Published COVID-19 MSC Therapy Trials.

Published Studies MSC IV Therapy for COVID-19	Study Design	Number MSC Patients	Number Control Patients	MSC Survival (%)	Control Survival (%)	Study End Point
Adas et al. 2021 [37]	Randomized controlled	10	20	70%	70%	Survival in ICU
Aghayan et al. 2022 [38]	Randomized placebo controlled	10	10	50%	50%	Survival 28 days
Dilogo et al. 2021 [39]	Randomized controlled	20	20	50%	20%	Survival 40+ days
Farkhad et al. 2022 [40]	Non-randomized placebo-controlled	10	10	80%	90%	Survival 17 days
Gregoire et al. 2022 [41]	Controlled [a]	8	24	100%	79%	Survival 28 days
Häberle et al. 2021 [42]	Placebo-controlled	5	18	80%	44%	Survival in ICU
Karyana et al. 2022 [43]	Randomized Placebo-controlled	6	3	100%	100%	Survival 28 days
Lanzoni et al. 2021 [44]	Randomized controlled	12	12	83%	42%	Survival 28 days
Leng et al. 2020 [45]	Placebo-controlled	7	3	100%	67%	Survival 14 days
Meng et al. 2020 [46]	Controlled	9	9	100%	100%	Discharge from Hospital
Monsel et al. 2022 [47]	Randomized	21	24	76%	83%	Survival 28 days

	Placebo-controlled					
Rebelatto et al. 2022 [48]	Randomized placebo-controlled	11	6	55%	83%	Cytokine markers 4 months
Shi et al. 2021 [49]	Randomized Placebo-controlled [b]	35	65	100%	100%	Decrease in Lung Lesions
Shu et al. 2020 [50]	Randomized controlled	12	29	100%	90%	Survival 28 days
Singh et al. 2020 [51]	Controlled [a]	6	34	100%	82%	Discharge from Hospital
Xu et al. 2021 [52]	Placebo-controlled	26	18	92%	67%	Survival
Zhu et al. 2021 [53]	Randomized Placebo-controlled	29	29	100%	93%	Survival 28 days
Feng et al. 2020 [54]	Single arm	16	0	88%	n/a	Survival 28 days
Hashemian et al. 2021 [55]	Single arm	11	0	55%	n/a	Survival
Iglesias et al. 2021 [56]	Single arm	5	0	60%	n/a	Discharge from Hospital
Sadeghi et al. 2021 [57]	Single-arm [c]	9	0	89%	n/a	Discharge from Hospital
Saleh et al. 2021 [58]	Single arm	5	0	100%	n/a	Survival 28 days
Sanchez-Guijo et al. 2020 [59]	Single arm [d]	12	0	92%	n/a	Survival in ICU
Sharma et al. 2022 [60]	Single arm	10	0	100%	n/a	Discharge from Hospital

Legend Table 5: Summary of study design and survival outcomes in 24 publications from completed MSC therapy trials for COVID-19 registered Jan. 2020 to Dec. 2021 and published by the end of July 2022. Publications are sorted alphabetically according to the first author in two groups: first all studies with controls, then all single arm studies. **Annotations**: a) Control group is retrospective, b) Most patients were convalescent, c) Excludes a patient who left against medical advice, and d) Excludes a patient that died of bleeding caused by a nasal-gastric tube. **Abbreviations**: COVID-19, coronavirus-induced disease 2019; MSC, mesenchymal stromal/stem cells; ICU, intensive care unit; IV, intravenous.

Table 6: Summary of Reported RR/OR in Meta-Analyses of MSC Trials for COVID-19.

Meta-Analysis Study (Author, Year)	No of Studies Included in Meta-Analysis	Risk Ratio [95% CI]	Odds Ratio [95% CI]
Qu et al. 2022 [92]	N=10	0.54 [0.35; 0.85]	---
Kirkham et al. 2022 [93]	N=9	0.50 [0.34; 0.75]	---

Zhang et al. 2022 [94]	N=12 (N=11 MSCs)	---	0.24 [0.13; 0.45]
Taufiq et al. 2023 [95]	N=06	0.65 [0.44; 0.96]	---
Couto et al. 2023* All MSC studies including missing controls	N=24	0.63 [0.46; 0.85]	0.51 [0.33; 0.78]
Couto et al. 2023 MSCs only controlled studies	N=17	0.62 [0.44; 0.87]	0.48 [0.29; 0.79]
Couto et al. 2023 * Perinatal MSCs including missing controls	N=18	0.75 [0.54; 1.02]	0.64 [0.40; 1.03]
Couto et al. 2023 Perinatal MSCs only controlled studies	N=12	0.75 [0.53; 1.07]	0.63 [0.36; 1.11]
Couto et al. 2023 * Non-Perinatal MSCs including missing controls	N=06	0.27 [0.10; 0.69]	0.19 [0.06; 0.57]

Legend Table 6: Meta-Analyses of Risk Ratio (RR) and Odds Ratio (OR) for all-cause mortality when MSCs are administered intravenously to treat COVID-19. In this paper (Couto et al. 2023) the calculation is performed for several sub-groups of the 24 articles published so far. Our results are compared to previous meta-analyses of MSC infusions for COVID-19.

Annotations: Our* represents the second approach in this manuscript, which used reconstructed data where the control group was missing. **Abbreviations**: COVID-19, coronavirus-induced disease 2019; CI, Confidence Interval; MSC, mesenchymal stromal/stem cells.

4 LISTA DE ARTIGOS

4.1 PRIMEIRO AUTOR

1. **Filgueiras IS**, Torrentes de Carvalho A, Cunha DP, Mathias da Fonseca DL, El Khawanky N, Freire PP, Cabral-Miranda G, Schimke LF, Camara NOS, Ochs HD, Peron JPS, Cabral-Marques O, de Vasconcelos ZFM. The clinical spectrum and immunopathological mechanisms underlying ZIKV-induced neurological manifestations. PLoS Negl Trop Dis. 2021 Aug 5;15(8):e0009575. doi: 10.1371/journal.pntd.0009575. PMID: 34351896; PMCID: PMC8341629.

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4.3 MANUSCRITOS EM PREPARO OU EM REVISÃO

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