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MESENCHYMAL STEM CELLS DERIVED EXTRACELLULAR VESICLES FOR COVID-19 TREATMENT

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Porto Alegre

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Mesenchymal stem cells derived extracellular vesicles for COVID-19 treatment

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This doctoral thesis is dedicated to all those affected in some way by the COVID-19 Pandemic.

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"Success is not about how high you climb, but how many people you bring with you."

Wilfred Peterson

SUMMARY

ABBREVIATIONS LIST

2019-nCoV – 2019 novel coronavirus

ACE2 – Angiotensin converting enzyme 2

ARDS – acute respiratory distress syndrome

ASC - apoptosis-associated speck-like protein with a caspase-recruitment domain

COVID-19 – Coronavirus Disease 2019

DAMPs – Damage associated molecular patterns

DNA – Deoxyribonucleic acid

EV – Extracellular vesicles

FDA – Food and Drug Administration

MSCs – Mesenchymal stem cells

PAMPs – Pathogen associated molecular patterns

RNA – Ribonucleic acid

SARS-CoV – severe acute respiratory syndrome coronavirus

SARS-CoV-2 - severe acute respiratory syndrome coronavirus 2

TMPRSS2 – Transmembrane serine protease 2

VOC – Variant of concern

VOI – Variant of interest

VUM – Variant under monitoring

VOC-LUM – Variants of Concern Lineages Under Monitoring

WHO – World Health Organization

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RESUMO

A Coronavirus Disease 2019 (COVID-19), causada pelo SARS-CoV-2, é uma doença sistêmica caracterizada por uma inflamação maciça e um desequilíbrio do sistema imunológico. Apesar dos esforços globais, o acesso a vacinas e tratamentos antivirais continua desigual. Portanto, terapias complementares de múltiplos alvos facilitariam o manejo dos pacientes. As células-tronco mesenquimais (CTMs) são conhecidas pelo seu potencial imunomodulador através da liberação de proteínas e outras moléculas no espaço extracelular, seja como elementos solúveis ou transportados por vesículas extracelulares (VEs). As VEs surgiram como uma opção terapêutica devido às suas propriedades intrínsecas, como o diâmetro nanométrico, e o seu conteúdo heterogêneo, incluindo pequenas sequências não codificantes envolvidas na repressão ou degradação de genes pós-transcricionais, como os microRNAs. O principal objetivo deste doutorado foi avaliar se as VEs das CTMs poderiam desempenhar um papel terapêutico durante o desenvolvimento da COVID-19. Primeiramente, através de análise bioinformática, analisamos quatro conjuntos de dados de miRNA utilizando diferentes fontes de tecido de células-tronco (medula óssea, cordão umbilical e tecido adiposo) e cruzamos seu conteúdo. Cinquenta e oito miRNAs se sobrepuseram nos quatro conjuntos de dados de miRNA analisados. Sequencialmente, esses miRNAs presentes em pelo menos dois conjuntos de dados foram submetidos ao software miRWalk, que previu 258, 267 e 148 miRNAs direcionados ao 3'UTR de citocinas e quimiocinas, genes de morte celular e cascatas de coagulação, respectivamente. Sequencialmente, para validar os achados in silico, obtivemos CTMs de tecido adiposo humano e avaliamos in vitro o potencial anti-inflamatório de suas VEs durante a infecção por SARS-CoV-2. As VEs das CTMs foram isoladas dos sobrenadantes de culturas celulares utilizando centrifugação diferencial seguida de cromatografia de exclusão por tamanho (SEC) e caracterizadas por citometria de fluxo, Análise de Rastreamento de Nanopartículas e imunoblotting. A microscopia confocal confirmou que as VEs das CTMs foram internalizadas pela linha celular alveolar, A549-hACE2, e se localizaram ao longo da via endocítica. Para determinar sua funcionalidade, as células A549-hACE2 foram infectadas com SARS-CoV-2 e tratadas com 3, 10, 30, 100 e 300 VEs das CTMs/célula por 24 horas, e os sobrenadantes das culturas celulares foram coletados. A análise multiplex de 30 VEs das CTMs/célula foi suficiente para alterar 32 de 48 mediadores imunológicos. Entre estes, MCP-1, IL-6 e IL-8 foram confirmados por ELISA, resultando em uma redução dependente da dose. Além

disso, a atividade antiviral das VEs das CTMs foi avaliada. Concentrações mais baixas (3, 10 e 30 VEs das CTMs/célula) não tiveram efeito na redução da replicação viral, embora concentrações mais altas (100 e 300 VEs/célula) mostraram reduções significativas.

Em conclusão, este trabalho mostra que as VEs das CTMs do tecido adiposo têm um potencial terapêutico contra a COVID-19, principalmente no que diz respeito à atenuação da inflamação após a infecção por SARS-CoV-2, possivelmente devido ao conteúdo de miRNA das VEs das CTMs, sendo necessárias mais pesquisas para estabelecer qual mecanismo de ação é responsável por essa ação.

ABSTRACT

The Coronavirus Disease 2019 (COVID-19), caused by SARS-CoV-2, is a systemic disease characterized by massive inflammation and immune system imbalance. Despite global efforts, access to vaccines and antiviral treatments remains unequal. Therefore, complementary multi-target therapies would facilitate patient management. Mesenchymal stem cells (MSCs) are known for their immunomodulatory potential through the release of proteins and other molecules into the extracellular space, either as soluble elements or carried by extracellular vesicles (EVs). EVs have emerged as a therapeutic option due to their intrinsic properties, such as nanosized diameter, and their heterogeneous cargo, including small noncoding sequences involved in post-transcriptional gene repression or degradation, such as microRNAs. The main objective of this PhD was to evaluate whether MSC-EVs could play a therapeutic role during COVID-19 development. Firstly, through bioinformatics analysis, we analyzed four datasets of miRNA using different stem cell tissue sources (bone marrow, umbilical cord, and adipose tissue) and crossed their content. Fifty-eight miRNAs overlapped in the four miRNA analyzed datasets. Sequentially, those miRNAs present in at least two datasets were submitted to miRWalk software, which predicted 258, 267, and 148 miRNAs targeting the 3'UTR of cytokines and chemokines, cell death genes, and coagulation cascades, respectively. Sequentially, to validate *in silico* findings, we obtained MSCs from human adipose tissue and evaluated *in vitro* the anti-inflammatory potential of their EVs during SARS-CoV-2 infection. MSC-EVs were isolated from cell-culture

supernatants using differential centrifugation followed by size exclusion chromatography (SEC) and characterized by flow cytometry, Nano Tracking Analysis, and immunoblotting. Confocal microscopy confirmed that MSC-EVs were internalized by the alveolar cell line, A549-hACE2, and they localized along the endocytic pathway. To determine their functionality, A549-hACE2 cells were infected with SARS-CoV-2 and treated with 3, 10, 30, 100 and 300 MSC-EVs/cell for 24 hours, and cell culture supernatants were collected. Multiplex analysis of 30 MSC-EV/cell, sufficed to alter 32 of 48 immune mediators. Among these, MCP-1, IL-6, and IL-8 were confirmed by ELISA, resulting in a dose-dependent reduction. Also, antiviral activity of MSC-EVs was evaluated. Lower concentrations (3, 10, and 30 MSC-EVs/cell) had no effect on reducing viral replication, although higher concentrations (100 and 300 EVs/cell) showed significant reductions.

In conclusion, this work shows that MSC-EVs from adipose tissue have a therapeutic potential against COVID-19, mainly regarding the attenuation of inflammation after SARS-CoV-2 infection, possibly due to the miRNA content of MSC-EVs and further research is necessary to establish which mechanism of action is responsible for this action.

1. INTRODUCTION

1.1. Epidemiology

At the end of 2019, a new virus from the *Coronaviridae* family was isolated from a series of pneumonia cases in patients in Wuhan, China (Zhu et al., 2020). Due to its genomic similarity to the severe acute respiratory syndrome coronavirus (SARS-CoV), responsible for the 2003 epidemic, this new virus was initially called 2019-novel coronavirus (2019-nCoV). However, its nomenclature was modified by the International Committee on Taxonomy of Viruses to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and the World Health Organization named the disease caused by the viral infection as Coronavirus Disease 2019 (COVID-19). The WHO classified COVID-19 as pandemic on March 11, 2020, and three years later, on May 5, 2023, ended its global emergency status. Although, since its beginning, the disease has infected approximately 775 million people with a mortality rate of 1%, accounting for around 7 million deaths (WHO, 2024).

1.2. SARS-CoV-2

Like other coronaviruses, SARS-CoV-2 is composed of a single strand of positive-sense ribonucleic acid (RNA) (Woo et al., 2009) and it is composed of the structural proteins: Membrane protein (M), Spike protein (S), Nucleocapsid protein (N) and Envelope protein (E) (Figure 1).

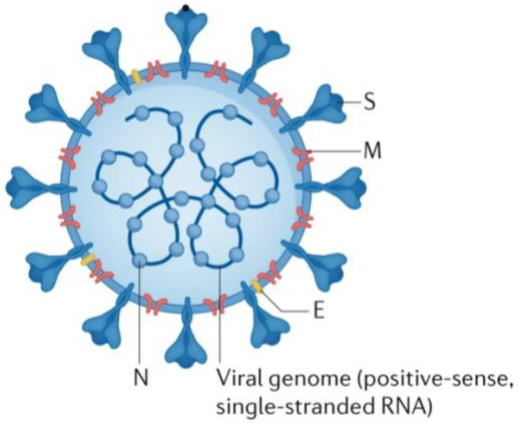

Figure 1. SARS-CoV-2 morphology and structural components. Adapted from (Jackson et al., 2022).

The S protein comprises two subunits, S1 and S2, the S1 has an amino-terminal domain (NTD), the receptor-binding domain (RBD) and two carboxy-terminal (C-terminal) domains (CTD1 and CTD2). The RBD contains the receptor-binding motif (RBM) and, together with the NTD, serves as the primary target for neutralizing antibodies and both are susceptible to immune-evasive mutations. Indeed, ongoing evolution of SARS-CoV-2 has been characterized by the emergence of variants that have mutations mainly in the NTD and RBD, resulting in ACE2 binding changes impacting virus infectivity and transmissibility (Steiner et al., 2024).

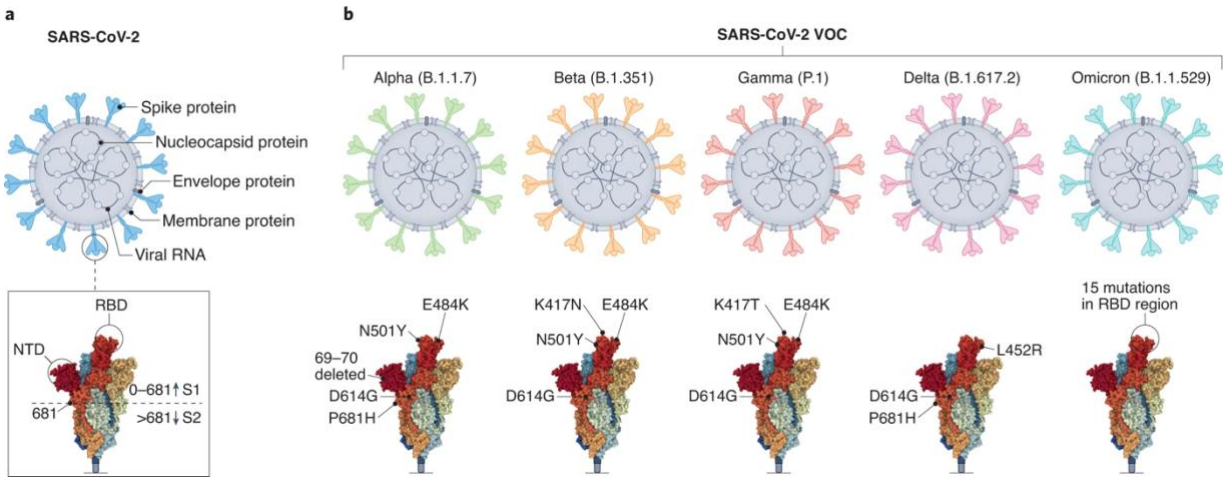

Figure 2. Representation of some SARS-CoV-2 variants of concern with their mutations in the S protein. Adapted from (Huang et al., 2022).

According to the SARS-CoV-2 Interagency Group (SIG), created in the USA, the variants and their lineages are classified in four different groups: Variant of high consequence (VOHC), Variant of concern (VOC), Variant of interest (VOI) and Variants being monitored (VBM). The classification considers the variant impact on vaccines efficacy, treatment response, diagnostics and pathophysiology. This classification has changed dynamically in the last years, and constant surveillance is based on research studies and clinical cases reported to official databases.

Currently, the Centers for Disease Control and Prevention (CDC) classify SARS-CoV-2 variants as listed in Table 1:

Table 1**.** *List of SARS-CoV-2 variants. Adapted from www.cdc.gov/coronavirus.*

WHO Label	**Pango lineage**	**Current status**	**Date of designation**
N/A	Variants containing the F456L spike mutations*	VOI	VOI: September 1, 2023
Omicron	BA.2.86	VBM	VBM: September 1, 2023
Omicron	XBB.1.9.1	VBM	VBM: September 1, 2023
Omicron	XBB.1.9.2	VBM	VBM: September 1, 2023
Omicron	XBB.2.3	VBM	VBM: September 1, 2023
Omicron	XBB.1.16	VBM	VBM: September 1, 2023
Omicron	XBB.1.5	VBM	VBM: September 1, 2023
Omicron	CH.1.1	VBM	VBM: September 1, 2023
Omicron	BA.2.74	VBM	VBM: September 1, 2023
Alpha	B.1.1.7 and Q lineages	VBM	VOC: December 29, 2020 VBM: September 21, 2021
Beta	B.1.351 and descendent lineages	VBM	VOC: December 29, 2020 VBM: September 21, 2021
Gamma	P.1 and descendent lineages	VBM	VOC: December 29, 2020 VBM: September 21, 2021
Delta	B.1.617.2 and descendant lineages	VBM	VOC: June 15, 2021 VBM: April 14, 2022
Epsilon	B.1.427 and B.1.429	VBM	VOC: March 19, 2021 VOI: February 26, 2021 VOI: June 29, 2021 VBM: September 21, 2021
Eta	B.1.525	VBM	VOI: February 26, 2021 VBM: September 21, 2021
Iota	B.1.526	VBM	VOI: February 26, 2021 VBM: September 21, 2021
Kappa	B.1.617.1	VBM	VOI: May 7, 2021 VBM: September 21, 2021
N/A	B.1.617.3	VBM	VOI: May 7, 2021 VBM: September 21, 2021
Omicron (parent lineages)**	B.1.1.529 and descendant lineages	VOC	VOC: November 26, 2021
Zeta	P.2	VBM	VOI: February 26, 2021 VBM: September 21, 2021

Mu	B.1.621, B.1.621.1	VBM	VBM: September 21, 2021

* Many lineages have acquired the F456L mutation and common examples include EG.5, FL.1.5.1, and XBB.1.16.6. ** Omicron parent lineages include BA.1 or similar.

1.3. SARS-CoV-2 replication

To infect the host, the virus enters through the upper airways and, via the Spike protein, attaches to the host cell's ACE2 receptor. The Spike protein is divided into S1 and S2 subunits, with the S1 subunit responsible for docking to the ACE2 receptor. The S2 subunit, after cleavage by the transmembrane serine protease 2 (TMPRSS2) enzyme, enables fusion of the virus's lipid bilayer with the host cell, internalizing its viral RNA (P. Zhou et al., 2020). Another mechanism that can also be used for viral particle internalization is through endocytosis (Lamers & Haagmans, 2022). Other co-receptors, such as neuropilin 1, are also suggested to facilitate entry of SARS-CoV-2 into host cells, but their contribution to pathogenesis needs to be investigated more deeply (Cantuti-Castelvetri et al., n.d.; Daly et al., n.d.; Hoffmann et al., 2021). As depicted in Figure 3, after the internalization of the viral particle by the host cell, SARS-CoV-2 mobilizes the cellular machinery for the assembly of new viral copies that will be released into the extracellular space through exocytosis.

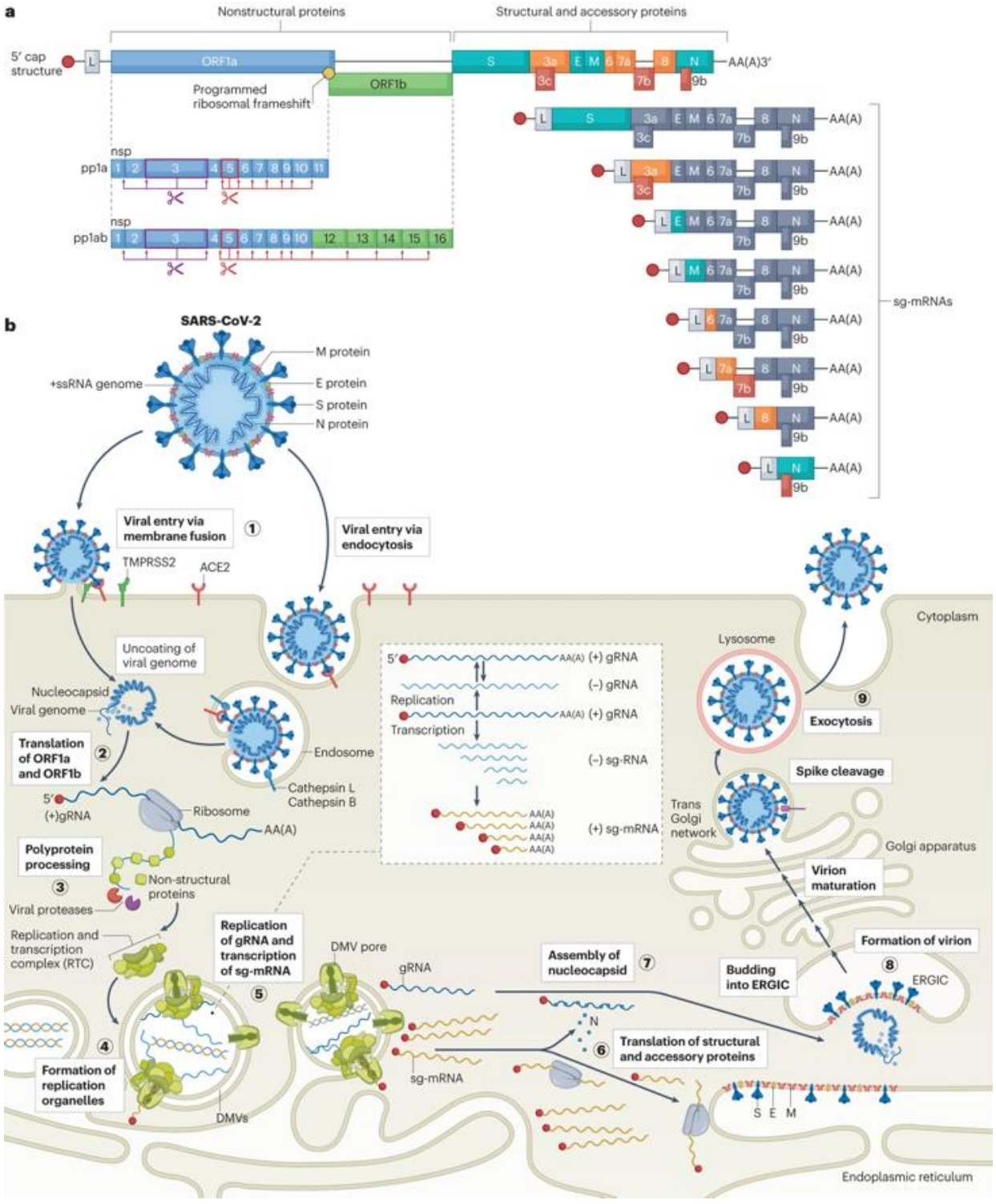

Figure 3. SARS-CoV-2 internalization mechanism and replication process in the host cells. Adapted from (Steiner et al., 2024).

1.4. COVID-19 Pathophysiology

In most cases, after cellular infection occurs, a cascade of signaling is initiated to resolve the infectious process. The interferon response is initiated, activating cells of the innate immune system to combat the external pathogen and perform viral clearance. However, during SARS-CoV-2 infection, some people face alterations in the interferon response, occurring in a reduced or delayed manner, thus allowing prolonged viral replication (L. Yang et al., 2021).

Regardless of the cell type infected by SARS-CoV-2, cell death by highly inflammatory pyroptosis is induced (Figure 4). During pyroptosis, molecules such as interleukin-1β, interleukin-18, and other damage-associated molecular patterns (DAMPs) are released in high amounts into the extracellular environment. Epithelial cells, alveolar cells, and alveolar macrophages detect pathogen-associated molecular patterns (PAMPs), like viral RNA, and DAMPs including ATP, DNA, and ASC oligomers through receptors, initiating the inflammatory process with the secretion of pro-inflammatory cytokines and chemokines such as IL-6, IL-1β, IL-2, IL-7, IL-8, IL-9, IL-10, IL-17, G-CSF, GM-CSF, MCP-1 (CCL2), MIP1α (CCL3), TNF-α, CXCL10 (IP-10) into the bloodstream of affected patients (Can & Coskun, 2020; Cao, 2020a; Rogers et al., 2020; Wiklander et al., 2019a). The synergy between TNF-α and IFN-γ has also been reported as responsible for a PANoptosis (pyroptosis, apoptosis, and necrosis) cell death signature both in vitro and in vivo in COVID-19 (Karki et al., 2021). This secretion of cytokines attracts other immune cells such as monocytes, neutrophils, and T lymphocytes from the bloodstream to the primary site of infection contributing to immunothrombosis formation.

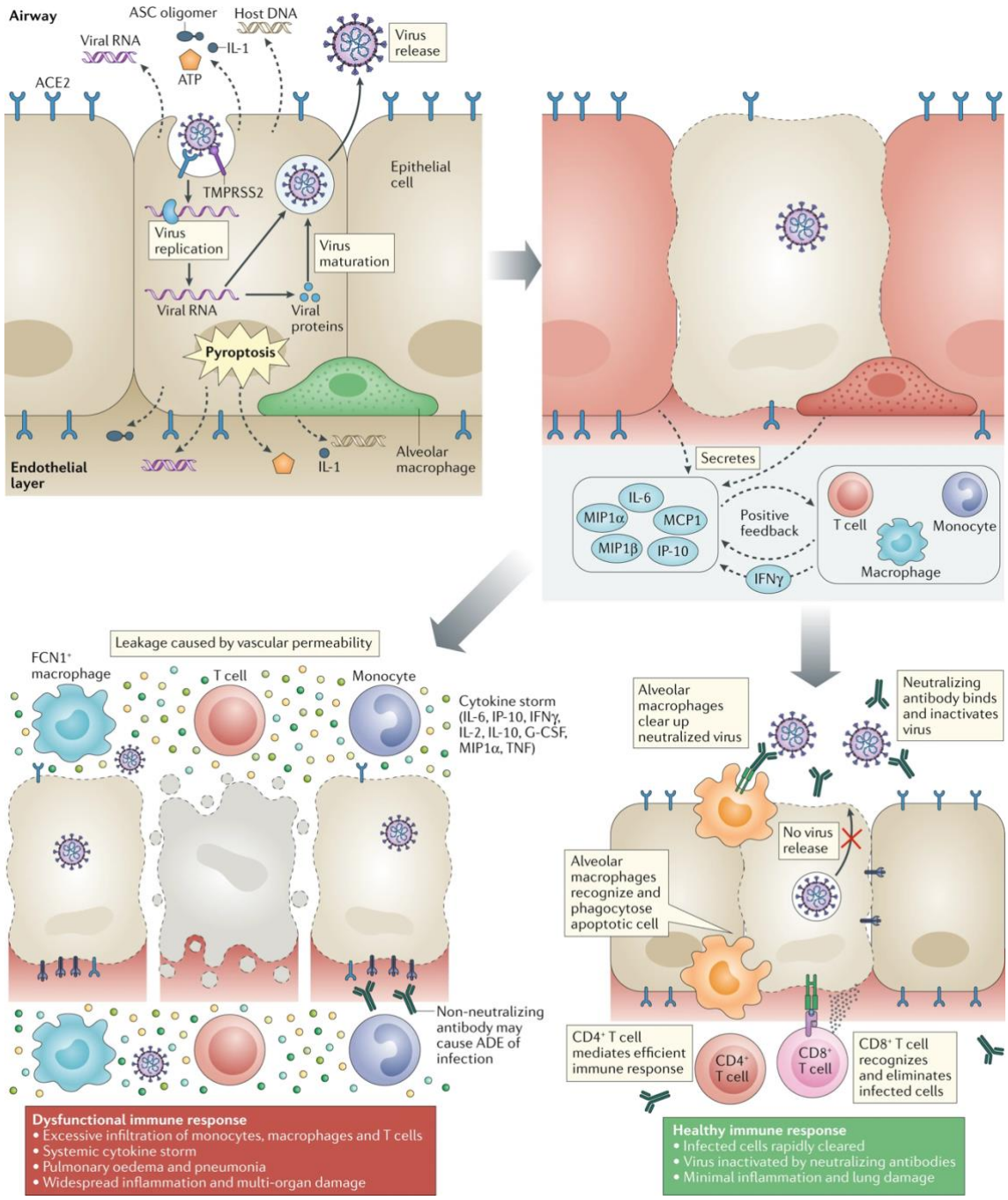

Figure 4. COVID-19 pathophysiology. SARS-CoV-2 infection triggers signaling changes, including interferon alterations, fostering viral persistence. Pyroptosis elicits cytokine release, amplifying inflammation via PAMPs and DAMPs detected by cell receptors. TNF-α and IFN-γ synergize to induce PANoptosis, recruiting immune cells like monocytes and T lymphocytes, promoting immunothrombosis at infection sites. Adapted from (Tay et al., 2020).

1.5. Diagnosis

The main transmission route of SARS-CoV-2 is through physical contact or contact with aerosols released in the air. Different diagnostic methods were suggested, since 2020, to diagnose and monitor the stages of infection. Considered the gold standard method for COVID-19 diagnosis is the reverse transcription followed by real-time polymerase chain reaction (RT-qPCR) (Alcoba-Florez et al., 2020). This is a molecular test which identifies the presence of SARS-CoV-2 proteins in nasopharyngeal samples.

Additionally, to molecular tests, serological tests can be used for retrospective analysis, epidemiological mapping and evaluation of seroconversion rates assessing vaccine efficacy for everyone. It is not recommended as a single method for diagnosis during the acute phase of infection.

Currently, antigen-detection rapid diagnostic tests (Ag-RDTs) are available, and they are affordable, disposable, single-use cassettes that require minimal training and provide the result in 15–20 min. These tests are much more feasible as a screening tool than molecular tests, which are more costly and require technical formation. They were created to help the population to monitor their infective status and manage physical distancing (quarantine) periods and can be used as a complement to molecular assays (Vandenberg et al., 2021; Wells et al., 2022). Therefore, it is recommended for people with low risk of infection, asymptomatic or people with no contact with infected individuals, once it has negative predictive value (NPV) correlated with decreasing disease prevalence (Peeling et al., 2021).

1.6. Risk Factors

Some risk factors such as age, obesity and male sex are correlated to disease severity progression. Also, people with common cardiac, respiratory and metabolic comorbidities are more susceptible to get infected with SARS-CoV-2 and progress to advanced stages of COVID-19 (Guan et al., 2020). Genetic factors, like Down syndrome, could increase viral internalization process once individuals have 60% more TMPRSS2 gene expression (De Toma & Dierssen, 2021). Individuals living with diabetes melitus have higher levels of IL-6 which maintain the platelets in continuous active state contributing to prothrombotic state (Ayres, 2020; Guo et al., 2020). Other risk factors include racial and social aspects with black population and low socio demographics status being more susceptible to get infected with SARS-CoV-2 and progress to severe stages of COVID-19 (Muñoz-Price et al., 2020).

1.7. COVID-19 classification

Due to the vaccination protocols currently underway and episodes of reinfection with different viral variants by the same individual, the symptomatology of COVID-19 is diversified. However, commonly manifested symptoms include fever, cough, headache, anosmia, and in more severe cases, it can progress to pneumonia and escalate to acute respiratory distress syndrome (ARDS).

According to manifested symptoms, the WHO stratified patients as follows (World Health Organization, 2023):

- **Non-severe (mild and moderate):** Defined as the absence of any criteria for severe or critical COVID-19.
- **Severe:** Defined by any of:
  - oxygen saturation < 90% on room air.
  - Signs of pneumonia.
  - Signs of severe respiratory distress (in adults, accessory muscle use, inability to complete full sentences, respiratory rate > 30 breaths per minute; and, in children, very severe chest wall in-drawing, grunting, central cyanosis, or presence of any other general danger signs including inability to breastfeed or drink, lethargy, convulsions or reduced level of consciousness).

- **Critical:** Defined by the criteria for acute respiratory distress syndrome (ARDS), sepsis, septic shock, or other conditions that would normally require the provision of life-sustaining therapies such as mechanical ventilation (invasive or non-invasive) or vasopressor therapy.

The concept of long COVID-19 still remains under discussion and investigation and its pathophysiology remains elusive. However, multiple studies showed significant consequences in patients after disease resolution. About 10% of patients report the persistence of at least 1 symptom manifested in the acute phase of COVID-19 (Robineau et al., 2022). The incidence is estimated at 10–30% of non-hospitalized cases, 50–70% of hospitalized cases and 10–12% among vaccinated cases. It is associated with all ages, with the highest number of diagnoses between 36 and 50 years old, and most cases are in non-hospitalized patients with a mild acute illness (Altmann et al., 2023; Davis et al., 2023).

1.8. Treatment and vaccines against SARS-CoV-2

There are ten COVID-19 vaccines approved by the World Health Organization, for which usage recommendations have been issued and which are produced by the following manufacturers: Pfizer/BioNTech, AstraZeneca/Oxford, Janssen, Moderna, Sinopharm, Sinovac, Bharat, Novavax, Casino, and Valneva. Although the vaccines are available, their adherence rates and distribution among countries remains unequal. According to WHO, 67% of the world's population have the 1st dose of the vaccine and 30% have a boost dose (WHO, 2024). In Brazil, 85% have the 1st and 2nd dose of the vaccine against COVID-19 and 50% have a boost dose (CVI, 2024).

According to current WHO guidelines (November 2023), approved treatments for non-severe, severe and critical COVID-19 patients and its specific indications are represented in Figure 5 (World Health Organization, 2023).

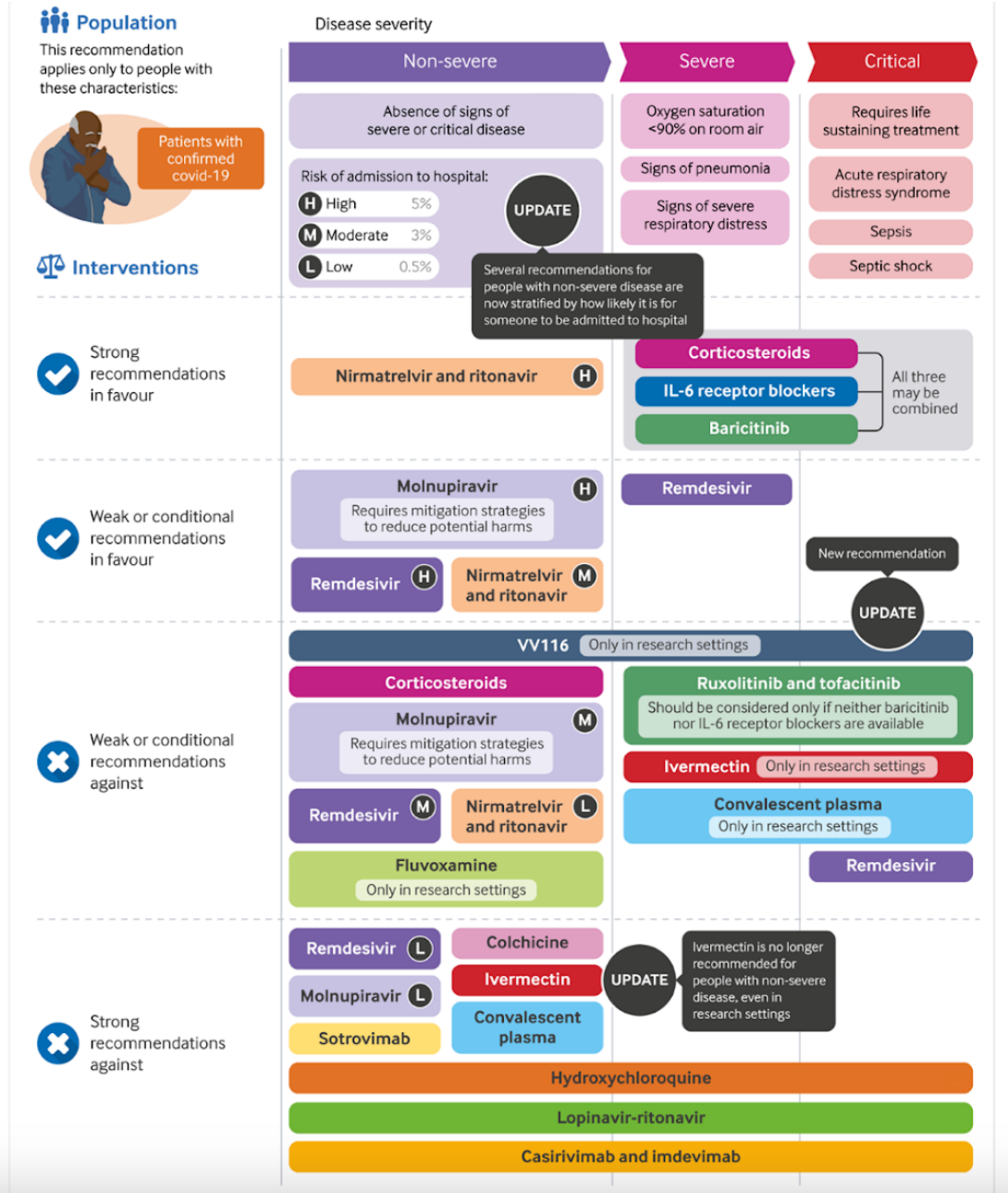

Figure 5. World Health Organization treatment guidelines on treatments for COVID-19 patients. Adapted from (World Health Organization, 2023).

In Brazil, the ANVISA approved 6 approved medications to treat COVID-19:

- Remdesivir (antiviral)
- Sotrovimab (monoclonal ab anti-S protein)
- Baracitinib (selective inhibitor JAK 1 e 2)
- Paxlovid (antiviral)
- Molnupiravir (antiviral)

- Tocilizumab (monoclonal ab anti-IL-6R)

1.9. Mesenchymal Stem cells

Considering that at the onset of COVID-19 there were no specific medications for its treatment and that during the development of COVID-19 disease, there is an excessive inflammatory process culminating in tissue damage, the therapeutic use of mesenchymal stem cells (MSCs) was suggested.

MSCs are known for their multipotent potential with differentiation into multiple lineages (adipocytes, chondrocytes, and osteocytes) (Ghannam et al., 2010). They have been extensively studied in recent decades due to their immunomodulatory activity in the innate and adaptive immune system, including the phenotype shift from pro-inflammatory M1 macrophages to anti-inflammatory M2 macrophages, which are important in resolving exacerbated inflammatory processes. To exert this immunomodulatory activity, MSCs release molecules into the extracellular environment either freely or encapsulated in extracellular vesicles. Some of the anti-inflammatory molecules released by MSCs are IL-4, IL-10, TGF-α, CCL18, prostaglandin E2, IDO, nitric oxide, and lipoxin A4 (Mao et al., 2015; Zheng et al., 2018a).

Due to these intrinsic characteristics, the safety and effectiveness of MSCs have been investigated in various clinical trials for the treatment of multiple diseases, including graft-versus-host disease, inflammatory bowel disease, osteoarthritis, rheumatoid arthritis, and multiple sclerosis (Xu et al., 2022). In COVID-19, multiple clinical trials were registered on clinicaltrials.gov to evaluate their safety and efficacy, and there are some case reports of clinical use of MSCs from different tissue sources in severe COVID-19 patients in the first year of the pandemic (Leng et al., 2020a; Liang et al., 2020; L. Shu et al., 2020; Soetjahjo et al., 2023). After the administration of cell therapy to these severe patients, researchers showed that inflammatory markers that were previously elevated in the patients, such as IL-6 and C-reactive protein, decreased after treatment. Furthermore, a follow-up of patients in a randomized clinical trial after two years of MSC treatment showed a sustained reduction in these inflammatory markers (T. T. Li et al., 2023a).

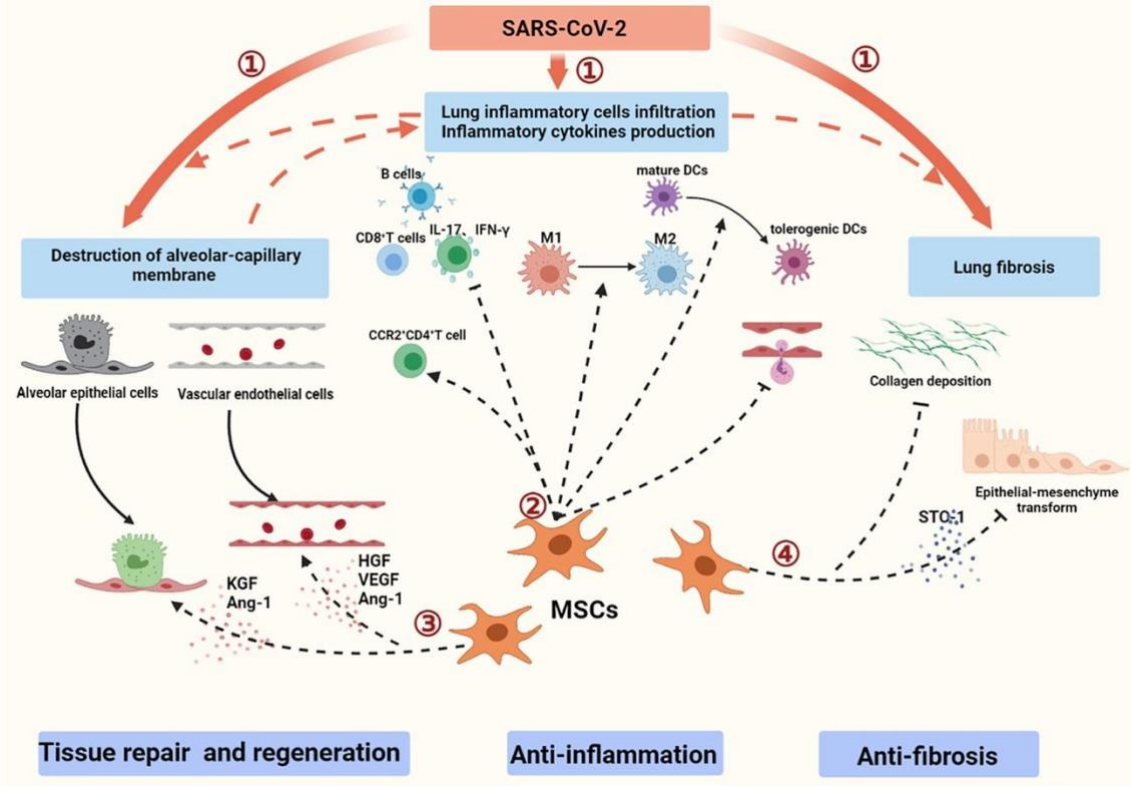

Figure 6. Schematic representation of Mesenchymal stem cells performing their immunomodulatory and tissue repair actions in COVID-19. Adapted from (Xu et al., 2022).

1.10. Extracellular vesicles

Extracellular vesicles (EVs) are spherical structures of nanometric and micrometric scale that are released by all cell types (Wiklander et al., 2019a). They are classified according to their biogenesis pathway as shown in Figure 7:

Vesicle types	Characteristics			
	Origin	Size	Markers	Contents
Exosomes	Endolysosomal pathway; intra-luminal budding of multivesicular bodies and fusion of multivesicular body with cell membrane	40–120 nm	Tetraspanins (such as TSPAN29 and TSPAN30), ESCRT components, PDCD6IP, TSG101, flotillin, MFGE8	mRNA, microRNA (miRNA) and other non-coding RNAs; cytoplasmic and membrane proteins including receptors and major histocompatibility complex (MHC) molecules
Microvesicles	Cell surface; outward budding of cell membrane	50–1,000 nm	Integrins, selectins, CD40 ligand	mRNA, miRNA, non-coding RNAs, cytoplasmic proteins and membrane proteins, including receptors
Apoptotic bodies	Cell surface; outward blebbing of apoptotic cell membrane	500–2,000 nm	Extensive amounts of phosphatidyl-serine	Nuclear fractions, cell organelles

Figure 7. Extracellular vesicles classification in exosomes, microvesicles and apoptotic bodies according to their biogenesis route, size, protein markers and contents. Adapted from Ozturk 2020.

Due to their intrinsic characteristics, such as their reduced size, EVs have been explored as diagnostic and prognostic biomarkers, as well as for molecule transport and drug delivery (Kumar et al., 2024; Morrison et al., 2017). Because they contain a similar cargo to their parent cells that release them, EVs contain proteins, miRNAs, mRNAs, long non-coding RNAs, DNA, lipids, and organelles such as mitochondria, which shape the behavior of target cells. In the case of MSC-derived EVs, this contributes to immunomodulatory and regenerative activities (Bulut & Gürsel, 2020).

Compared to their parent cells, EVs have several advantages for therapeutic use. Their reduced size allows them to reach distant locations without getting trapped in the bloodstream's microcirculation. EVs can overcome natural defense barriers like the blood-brain barrier, reaching distant sites that cells cannot reach. Additionally, EVs are considered acellular products, meaning they cannot differentiate into other cell types and do not carry the risk of malignant transformation (Majolo et al., 2020; Monsel et al., 2016; Öztürk et al., 2020a).

Table1 Advantages and disadvantages of stem cell and extracellular vesicle therapies

	Advantages	Disadvantages
Stem cells	-Living cells -Potency to differentiate and/or regenerate into the tissue of interest. -Ability to be reprogrammed into pluripotent cells -Well-defined isolation, characterization and expansion procedures -Potential for bioengineering and/or conditioning -Fabrication as "off-the-shelf" products in large quantities -Availability of clinical trials -Approved by federal agencies for the treatment of certain diseases	-Risk of malign transformation -Trap in lungs and elimination from the vasculature -Risk for vascular obstruction -Minimal homing, migration and differentiation capacity -Risk for immune rejection -Insufficient therapeutic effects in clinical trials -Lack of optimal dosage, route of administration, and timing -Necessity for fabrication under GMP conditions -Risk for altered viability during cryopreservation
Extracellular vesicles	-Cell-free agents -Minimal risk of malign transformation -Minimal risk of trap in lungs -Ability to pass blood-brain barrier -Minimal risk of vascular obstruction -Non-immunogenic profile -Secreted by all cell types -Detected in all body fluids -Availability as biomarkers -Ideal candidates for drug delivery -Potential for bioengineering and/or conditioning -Fabrication as "off-the-shelf" products in large quantities -Presence of databases to provide information about their composition and functions -Demonstrated efficacy in case studies of certain diseases	-Inability to differentiate into any cell -Lack of understanding of mechanism of action -Systemic and diverse effects of miRNAs -Lack of standardization regarding nomenclature -Risk of tumor growth, autoimmunity, neurodegenerative diseases, prion diseases or viral infections -Lack of standardization for fabrication procedures -Very short half-life in the blood after application -Lack of well-designed clinical trials -Lack of optimal dosage, route of administration, and timing -Necessity for fabrication under GMP conditions

Figure 8. Table of advantages and disadvantages of using extracellular vesicles comparatively to their parental cells, the stem cells, as therapies. Adapted from (Öztürk et al., 2020b).

1.11. Micrornas

One of the cargoes encapsulated by extracellular vesicles is microRNAs (miRNAs). miRNAs are small structures of genetic material synthesized in the cell nucleus and composed of 18-25 nucleotides. As shown in Figure 9, after synthesis, they are transported to the cell cytoplasm where they are responsible for post-transcriptional modifications by binding to messenger RNAs (mRNAs), preventing the translation of functional proteins (Bartel, 2004a; He & Hannon, 2004; J. O'Brien et al., 2018; Shang et al., 2023).

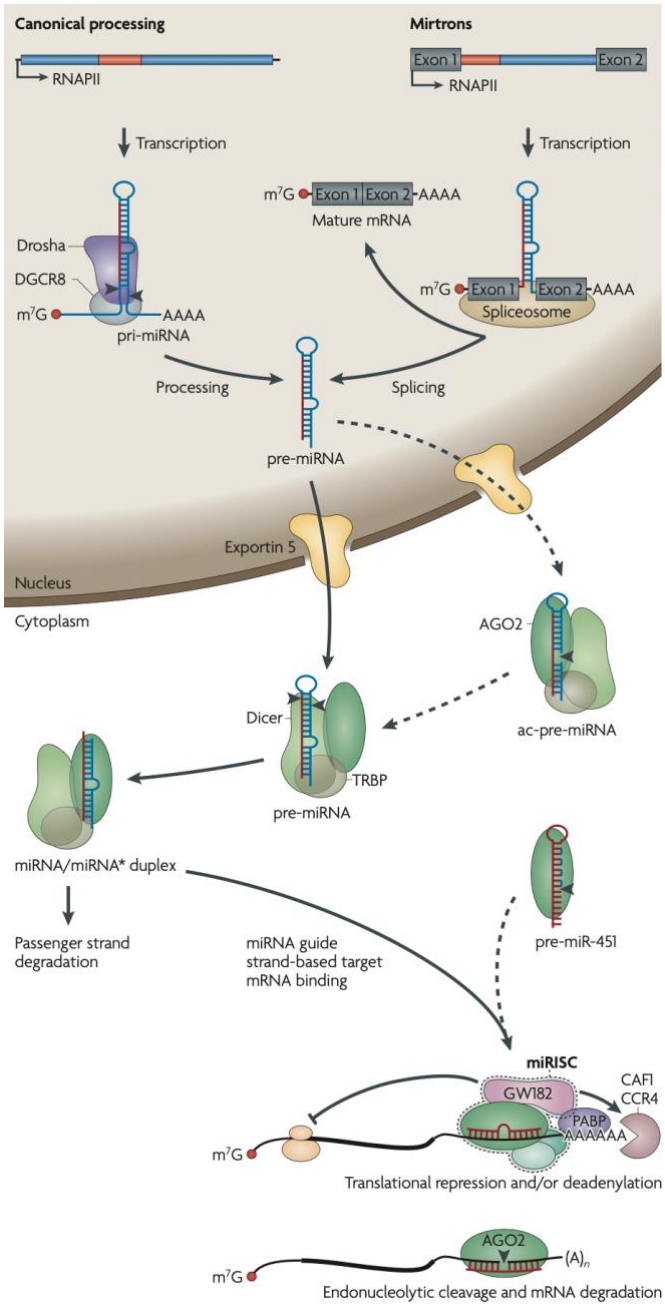

Figure 9. MicroRNA biogenesis pathway. It begins with the transcription of miRNA genes by RNA polymerase II into primary miRNA (pri-miRNA) transcripts. These pri-miRNAs are typically several kilobases long and undergo cleavage by the microprocessor complex, composed of Drosha and DGCR8, in the cell nucleus. This cleavage generates precursor miRNAs (pre-miRNAs), which are approximately 70 nucleotides in length and feature a stem-loop structure. Following export to the cytoplasm by Exportin-5, pre-miRNAs are further processed by Dicer, an RNase III enzyme, into mature miRNA duplexes. One strand of the duplex, known as the guide strand, is preferentially selected to form the RNA-induced silencing complex (RISC) with Argonaute proteins. The guide strand directs RISC to target mRNAs based on sequence complementarity, leading to mRNA degradation or translational repression, thereby regulating gene expression. Adapted from (Krol et al., 2010).

Over the last decade, miRNAs have been studied for their use as diagnostic and prognostic tools in oncology (B. Zhou et al., 2020). The number of miRNAs circulating in the bloodstream can indicate the presence and extent of tumoral development (Bertoli et al., 2015). In inflammatory and respiratory diseases like COVID-19, miRNAs could have therapeutic effects by binding to inflammatory cytokines and chemokines mRNAs, reducing the translation of their functional proteins (Bayraktar et al., 2019; Chauhan et al., 2020; Girardi et al., 2018; Tahamtan et al., 2018; Weidner et al., 2020; Ying et al., 2021). For that, in animals, most miRNAs bind to the target 3′ UTR (untranslated region) with perfect or imperfect complementarity (Bartel, 2004b). Although less common, miRNAs also bind to other parts of the mRNAs such as the CDS (coding sequence) and the 5'UTR of the mRNAs. This multi-targeting binding potential allows the same miRNA to interact with different regions of the same mRNA but also interact with more than one mRNA, interacting with multiple pathways at the same time (H. Zhou et al., 2023).

Therefore, considering COVID-19 pathophysiology and the intrinsic characteristics of MSCs and their derived EVs, the aim of this doctoral project is to evaluate the anti-inflammatory potential of MSC-EVs during SARS-CoV-2 infection. For that, an *in silico* analysis will be performed to evaluate the miRNA content of MSC-EVs and the treatment of lung cells infected with SARS-CoV-2 with MSC-EVs to assess therapeutic potential.

2. OBJECTIVES

Main objective

Evaluate therapeutic potential of Mesenchymal stem cells derived extracellular vesicles against COVID-19.

Specific Objectives

I. Perform an *in silico* analysis to evaluate targeting potential of MSC-EVs miRNA to relevant genes involved in COVID-19 development.
II. Isolate and culture mesenchymal stem cells from adipose tissue.
III. Isolate and characterize the extracellular vesicles from adipose MSCs.
IV. Evaluate the anti-inflammatory potential of the MSC-EVs during SARS-CoV-2 infection.

3. CHAPTER 1: *Mesenchymal Stem Cell-derived extracellular vesicles carrying miRNAs as a potential multi-target therapy to COVID-19: an in silico analysis*

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Mesenchymal Stem Cell-Derived Extracellular Vesicles Carrying miRNA as a Potential Multi Target Therapy to COVID-19: an In Silico Analysis

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Abstract
In the end of 2019 COVID-19 emerged as a new threat worldwide and this disease present impaired immune system, exacerbated production of inflammatory cytokines, and coagulation disturbs. Mesenchymal stem cell (MSC) derived extracellular vesicles (EVs) have emerged as a therapeutic option due to its intrinsic properties to alleviate inflammatory responses, capable to promote the restoring of injured tissue. EVs contain heterogeneous cargo, including active microRNAs, small noncoding sequences involved in post-transcriptional gene repression or degradation and can attach in multiple targets. This study investigated whether the MSC-EVs miRNA cargo has the capacity to modulate the exacerbated cytokines, cell death and coagulation disturbs present in severe COVID-19. Through bioinformatics analysis, four datasets of miRNA, using different stem cell tissue sources (bone marrow, umbilical cord and adipose tissue), and one dataset of mRNA (bone marrow) were analyzed. 58 miRNAs overlap in the four miRNA datasets analyzed. Sequentially, those miRNAs present in at least two datasets, were analyzed using miRWalk for the 3'UTR binding target mRNA. The result predicted 258 miRNAs for exacerbated cytokines and chemokines, 266 miRNAs for cell death genes and 148 miRNAs for coagulation cascades. Some miRNAs may simultaneously attenuate inflammatory agents, inhibit cell death genes and key factors of coagulation cascade, consequently preventing tissue damage and coagulation disturbs. Therefore, the MSC-derived EVs due to their heterogeneous cargo are a potential multitarget approach able to improve the survival rates of severe COVID-19 patients.

Keywords Sars-Cov-2 · ARDS · miRNA · Mesenchymal stem cell · MSC · Microvesicles · Exosomes · COVID-19 · Bioinformatics

Introduction

In December 2019 a new β-virus from the family *Coronaviridae* was first isolated from pneumonia cases in Wuhan city, China, and started a pandemic affecting the population worldwide [1]. Due to the genomic similarity of 79% to Sars-CoV [2], also a β-virus, this pathogen was first called of 2019 novel coronavirus (2019-nCoV) and then officially called as Sars-CoV-2, while the WHO named the disease caused by the virus, Coronavirus disease-19 (COVID-19) [3]. To date, January 2021, over 88 million cases have been reported across the globe, resulting more than 1.9 million deaths (covid19.who.int). The genome of Sars-CoV-2, similarly to other coronaviruses, is a single-stranded ribonucleic acid (RNA) with positive polarity [4] and the viral membrane contains 4 structures: membrane protein (M), Spike protein (S), Nucleocapsid protein (N) and Envelope protein (E). Similar to Sars-CoV infection, the spike of Sars-CoV-2 dictates the host tropism and pathogenicity [5]. The main mechanism of transmission is human to human contact or contact with virus aerosolization. Once inside the human body, the receptor binding domain (RBD) of Sars-CoV-2 spike glycoprotein attaches to the Angiotensin Converting Enzyme 2 (ACE2) receptor and is cleavage by Transmembrane Serin Protease 2 (TMPRSS2) helping the

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viral internalization mainly of type II alveolar cells. The respiratory tract have a widely expression of the ACE2 receptors and the airways are the main entrance of the virus [6]. Other tissues such as cardiac, kidney, bowel, brain, endothelial cells and others also express the ACE2 receptor [7]. Other proteases expressed in various tissues may also cleave the Sars-CoV-2 spike protein [8] and one of them, as showed in previous studies with Sars-CoV, is the coagulation Factor Xa, colocalized with TMPRSS2 in the cell membrane [9, 10]. Patients with preexisting conditions are more vulnerable of getting infected and develop a poor prognosis of COVID-19 [11]. Preexisting conditions such as pulmonary and cardiac diseases emerge as the highest risk groups for severe COVID-19 [12] and these patients may have a higher baseline expression of the receptor ACE2 and Factor X in these cell populations, what could put them at increased risk of host cell infection by Sars-CoV-2, with Factor X serving as one of the cleavage proteases for spike protein [9].

A major part of COVID-19 cases are asymptomatic, although around 20% of patients are severely or critically unwell [13, 14]. In those individuals, who present symptoms, common clinical manifestations of COVID-19 include fever, cough, fatigue, sputum production, shortness of breath, sore throat, headache, loss of taste and smell, pneumonia and more advanced cases progress to Acute Respiratory Distress Syndrome (ARDS), coagulation disturbs [15], multiple organ failure, septic shock and death [16]. The patients who progressed to ARDS are evaluate following the Berlin definition [17]. Overall, around 36% of the patients died within 28 days of intensive care unit (ICU) admission [18].

ICU patients admitted with pneumonia who progress to ARDS have systemic inflammation, and present altered plasma levels of lymphocytopenia, D-dimer, C-reactive protein, LDH and inflammatory cytokines such as IL-6, IL-1β, IL-2, IL-7, IL-8, IL-9, IL-10, IL-17, G-CSF, MCP-1 (CCL2), MIP-1α (CCL3), MCP-3 (CCL7) and CXCL10 (IP-10), IL-18, IL-33, IL1- α, IL-15. D-dimer, a product of fibrin degradation, is consider a biomarker of poor prognosis in COVID-19 when the measurements are >1 μg/mL [19, 20]. Altered prothrombin time was also positively correlated with patient mortality [21]. Tissue factor, usually, is not express by endothelial cells and leukocytes, but the expression can be induced by inflammatory stimuli such as cytokines TNF-α and IL-1β. High levels of pro-inflammatory agents, specially IL-6, IL-1β and TNF-α were associated to the harshness and progression of the disease [13, 19, 22–24]. Although, despite the elevated baseline levels of the proinflammatory agents in COVID-19, they are significantly lower than non-COVID-19 related ARDS patients [25]. Recently, in vitro and in vivo evidence-based, Karki and colleagues, 2020 showed that TNF-α and INF-γ together can induce cell death through PANoptosis (pyroptosis, apoptosis and necrosis) [26]. In addition, they evidenced that the other cytokines, despite exacerbated, are not related to cell death in the acute inflammation of COVID-19.

In the end of 2020, vaccines for COVID-19 have been approved in a few countries. So far, two vaccines were approved in the USA and three in the UK by their respective regulatory agencies, but no specific treatment has yet been officially approved to treat COVID-19. However, prophylactic doses of low molecular weight heparin are recommended by the International Society on Thrombosis and Hemostasis for all hospitalized COVID-19 patients, except for those with active bleeding or low platelet counts, aiming the reduction of coagulation disturbs as the venous thromboembolic event (VTE) [27]. Heparin is an inhibitor mainly of the Factor Xa and IIa, components of the coagulation cascade [28]. Treatment of hospitalized COVID-19 patients with heparin improved the outcomes and survival rates compared to those patients who did not receive the anticoagulant treatment [21]. However, a few patients shows heparin resistance and have extremely high levels of Factor VIII, fibrinogen and D-dimer [29]. Yet, doses and time of administration of anticoagulant treatment or prophylaxis still diverge between hospitals and medical institutions [18, 30].

A considerable number of studies are being carried out to test different therapeutic approaches [31]. One of these approaches is mesenchymal stem cells (MSCs), a therapy reported as a potent agent to attenuate inflammation, due to intrinsic characteristics of immunomodulation, and leading to lung tissue regeneration, useful features in severe COVID-19 cases clinical management. Still, after decades of the discovery of these cells, the definition of the nomenclature used to identify MSCs remains controversial and different terms have been used to name these cells. While a definition of the use of "stem" or "stromal" is not decided by International Society for Cellular Therapy, in the present study was used the term "Mesenchymal stem cells" to simplify the bibliographic search [32].

Until the submission of this paper a total of 46 clinical trials using mesenchymal stem cells to treat ARDS derived of COVID-19, 39 of these clinical trials are currently ongoing and six are completed (clinicaltrials.gov). A few clinical interventions using intravenous MSCs treatment in COVID-19 showed improvement in clinical outcomes of treated patients with no adverse reactions [33–35]. Another therapeutic intervention is using the MSC-derived exosomes in cases of COVID-19. On clinicaltrals.gov there are three studies using this approach registered, NCT04276987 (a pilot study completed) using aerosol inhalation of the exosomes derived from allogenic adipose mesenchymal stem cells; NCT04491240 (completed and has positive results about safety and efficiency of clinical use) based on the NCT04276987 and the literature; and NCT04602442 (enrolling by invitation) also based on the NCT04276987, using aerosol inhalation of the exosomes derived from

allogenic MSCs. Moreover, there is one report of clinical use of commercial MSC-derived exosomes intravenously (ExoFlo™), obtained from allogenic bone marrow mesenchymal stem cells in COVID-19 patients [36].

In 2018, the International Society for Extracellular Vesicles (ISEV) updated their guidelines with basic information to help researchers in the communication process of their results with the extracellular vesicles (EVs). ISEV also encouraged the authors to submit their results in other database as EV-TRACK [37, 38].

All types of cells shed EVs to cell-to-cell communication [39, 40]. EVs can be subdivided in exosomes, microvesicles and apoptotic bodies. Exosomes, 40-120 nm, resulted from intraluminal budding of multivesicular bodies and fusion of these multivesicular bodies with cell membrane via the endosomal pathway. Microvesicles, 50–1.000 nm, are structures released from the outward budding and fission of the plasma membrane. Apoptotic bodies, 50-2000 nm, are vesicles released from the cell surface through outward budding of apoptotic cell membrane [41]. The MSCs-derived EVs can carry inside their lipidic bilayer, proteins, messenger RNAs (mRNAs), small and long non-coding RNAs (ncRNAs), DNA, lipids, and carbohydrates from parental cells which could shape the behavior of target cells contributing to the angiogenic, immunomodulatory and regenerative effect [42–44]. Thereby, the MSC-derived EVs present themselves as potentially cell-free agents on account of their similar characteristics with the parental cells. The benefits of EVs are the minimal risk of getting trapped in the lungs, production as "off-the-shelf" products in large quantities, minimal risk of tumor formation, non-immunogenic profile, ability to cross natural barriers and deliver bioactive compounds and others [44]. Studies using EVs in ARDS are incipient, but there are studies showing MSC-derived EVs present similar effects when compared to administration of MSCs [45, 46]. Gardin and collaborators, in a recent review, presented the treatment scenario and potential of exosomes in patients with heart and lung injuries [47]. MicroRNAs (miRNAs) are endogenous small ncRNAs that can be found within MSC-derived EVs and are associated with post-transcriptional gene repression or degradation [48]. Growing evidence shows that miRNAs could alleviate antiviral responses [49] since a single miRNA could target multiple genes. Functionally, miRNAs guides proteins of the Argonaute family to form a silencing complex through base pairing between the 5′ portion of miRNA "seed sequence" (miRNA nucleotides 2–7/8) and complementary sites within the coding sequence (CDs), 3′ or 5′ untranslated regions (UTRs) of target RNAs [50].

The main objective of this study was to analyze the rationale behind the use of this biologic, cell-free structure, the MSC-derived extracellular vesicles, carrying heterogeneous inner cargo. In addition, using a bioinformatics approach, we analyzed evidence of vesicles carrying miRNAs content as modulators of acute inflammation, the PANoptosis cell death signature and the coagulation disturbs found in severe COVID-19 patients which resulted in an in silico prediction.

Material and Methods

A search of mRNA and miRNAs expression profiles in the MSC-derived EVs (microvesicles and exosomes) was performed using Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) restricting the search to *Homo sapiens*. The target prediction miRWalk 3.0 server (http://mirwalk.umm.uni-heidelberg.de/) was used to identify potential miRNAs binding sites within the mRNA sequences and to verify which miRNAs are experimentally validated based on its corresponding miRTarBase ID. The score ≥ 0.95 and more than 10 pairs were considered as the critical criteria for the predictive analysis. Considering the different platforms and methods between used datasets, the expression of miRNAs data was filtered based as follows: I) GSE69090 and GSE81151: miRNAs with zero counts were removed; II) GSE78865: miRNAs with same negative control values were removed; III) GSE71241: miRNAs that did not appear in at least one replicate sample were removed. A qualitative global score was generated to compare the levels of miRNA expression on each dataset (Sup.Table S1). The 5′ and 3′ mature miRNAs (miR-5p and miR-3p) were considered separately, except in the GSE78865 dataset that only miRNA precursor was available for analysis.

Results

To investigate the therapeutic potential of the MSC-derived EVs against COVID-19 lung and systemic inflammation, we conducted an in silico analysis of microRNA and mRNA profiles of human MSC-derived EVs in GEO database using GSE12243, GSE71241, GSE78865, GSE81151 and GSE69909 datasets. The main features of the used datasets are shown in Sup.Table 2.

The MSC-derived EVs cargo can vary depending on the biological source where they are extracted. Sup.Table 2 shows an overview of the dataset's characteristics used in this study. One dataset isolated MSCs from human umbilical cord [51, 52], three datasets used MSCs from human bone marrow [53–57] and one dataset used MSCs from human adipose tissue [58]. Also, there were differences regarding the technique of EVs isolation: two datasets used a commercial isolation kit (ExoQuick-TC kit) and the other three used serial and ultracentrifugation processes. As final product, one dataset tested only microvesicles, one dataset tested microvesicles and exosomes and three datasets used exclusively exosomes in their analysis.

The integrative analysis revealed that 58 miRNAs were commonly expressed in all four collected miRNA datasets, however with different expression levels (Table 1). Among these 58 miRNAs, using the miRWalk server, we searched for the experimentally validated ones, which resulted in 27 miRNAs. They are highlighted in the Sup. Table 4, Figs. 1 and 2 with their respective miRTarBase IDs. Also, we considered as potential targets 361 miRNAs that were commonly shared in at least two datasets (Sup.Table 3). The miRNAs that were expressed only in one dataset or were absent in all datasets were not considered as potential targets. Next, we used miRWalk server to predict miRNAs binding sites, from those present in at least two datasets, in the 3'UTR region of CCL2, CCL3, CCL7, CSF3, CXCL10, CXCL8 (IL8), IL1B, IL1A, IL2, IL6, IL6R, IL7, IL9, IL10, IL15, IL17A, IL33, IL18 genes. As result, a total of 258 miRNAs were predicted to target the 3'UTR region of the mRNAs and are listed in (Sup.Table 4). The miRNAs that may interact concomitant with more than one mRNA target also shown in (Sup.Table 4).

In a separate analysis, aiming to investigate the interaction of the MSC-derived EVs miRNA cargo and the PANoptosis cell death pathway, suggested by [26], we executed the same previous protocol, only changing the target genes to TNF, IFNG, STAT1, IRF1, NOS2, RIPK1, RIPK3, FADD, GSDME, CASP3, CASP7, CASP8, MLKL, JAK1, JAK2. A total of 266 miRNAs were predicted to target the 3'UTR region of the mRNAs and are listed in Fig. 1**.** The miRNAs that may interact concomitant with more than one mRNA target also shown in Fig. 1.

Since coagulation disturbs has a direct relation with patients' mortality, a third analysis was performed to evaluate the interaction of the MSC-derived EVs miRNA cargo and genes involved in the coagulation cascade. Therefore, applying the same previous analysis, for the genes F2, F3, F5, F7, F8, F9, F10, F11, F12 and F13 a total of 148 miRNAs were predicted to target the 3'UTR region of the mRNAs which are listed in Fig. 2. The miRNAs that may interact concomitant with more than one mRNA target also shown in Fig. 2.

We also investigated the profile of cytokines and chemokines, cell death and coagulation genes through the mRNA content from MSC-derived microvesicles in the GSE12243 dataset. In this dataset, three different quantities (0.25 μg, 0.5 μg and 1 μg) of total RNA from vesicles were submitted to microarray analysis [57]. As shown in Supplementary Table 5, the microvesicles did not contain significant ($p \leq 0.05$) or positive Pearson's correlation coefficient ≥ 0.8 for our targets, suggesting that no functional protein will be produced.

Discussion

The pathogenesis of COVID-19, as shown in Fig. 3, is based on a dysregulation of innate and adaptative immune systems response after the infection mainly of the alveolar cells and endothelial cells by Sars-CoV-2, through specific receptor ACE2. Thus, the direct viral infection and the immune system dysregulation causes an acute inflammation and will result in alveolar and endothelial tissue damage. Furthermore, subsequently to acute inflammation and infected cell death the coagulation cascade will be triggered causing thrombotic events. Taken together, the blood clot formations and the upregulation of proinflammatory agents, the severe COVID-19 patients will develop the acute respiratory distress syndrome (ARDS), leading to organ failure and a fraction of them eventually will die [26, 59–62].

The magnitude of the "cytokine storm" has a direct relation with the harshness of COVID-19. Since the beginning of the pandemic, several studies have been published about COVID-19 due to its importance and consequences globally. Like every new disease, in the beginning the biologic mechanisms, concepts and exact consequences are not well elucidated. Multiple papers refers to the exacerbated cytokines and chemokines levels as cytokine storm [42, 60, 63–69]. However, some reports affirm that this term cytokine storm might has been misused [25, 70, 71]. Despite the elevated levels of the cytokines and chemokines in COVID-19 derived ARDS, these levels are lower than those present in non-COVID related ARDS or sepsis [25]. Yet, the authors found that despite the lower levels of cytokines compared to other diseases, D-dimer amounts in COVID-19 related ARDS are higher than non-COVID-19 related ARDS [25].

The coagulation and the immune system are directly linked [72]. COVID-19 may predispose to venous thromboembolic events due to inflammation, hypoxia and tissue damage. The most typical findings of severe COVID-19 patients are an increased concentration of D-dimer, a slight decrease in platelet counts and a prolongation of prothrombin time. Direct viral infection as well as inflammation-induced endothelial cell injury could result in a massive release of plasminogen activators, which could explain the higher concentration of D-dimer and fibrin degradation products found in the patients peripheral blood circulation [73, 74]. A cohort study shows that venous thromboembolism (VTE) in COVID-19 ICU patients are high and the rates of affected patients ranges between 35 and 45% [75]. Also, postmortem lung analysis, from patients who had died from COVID-19, revealed the presence of microthrombus in lung vascularity [76].

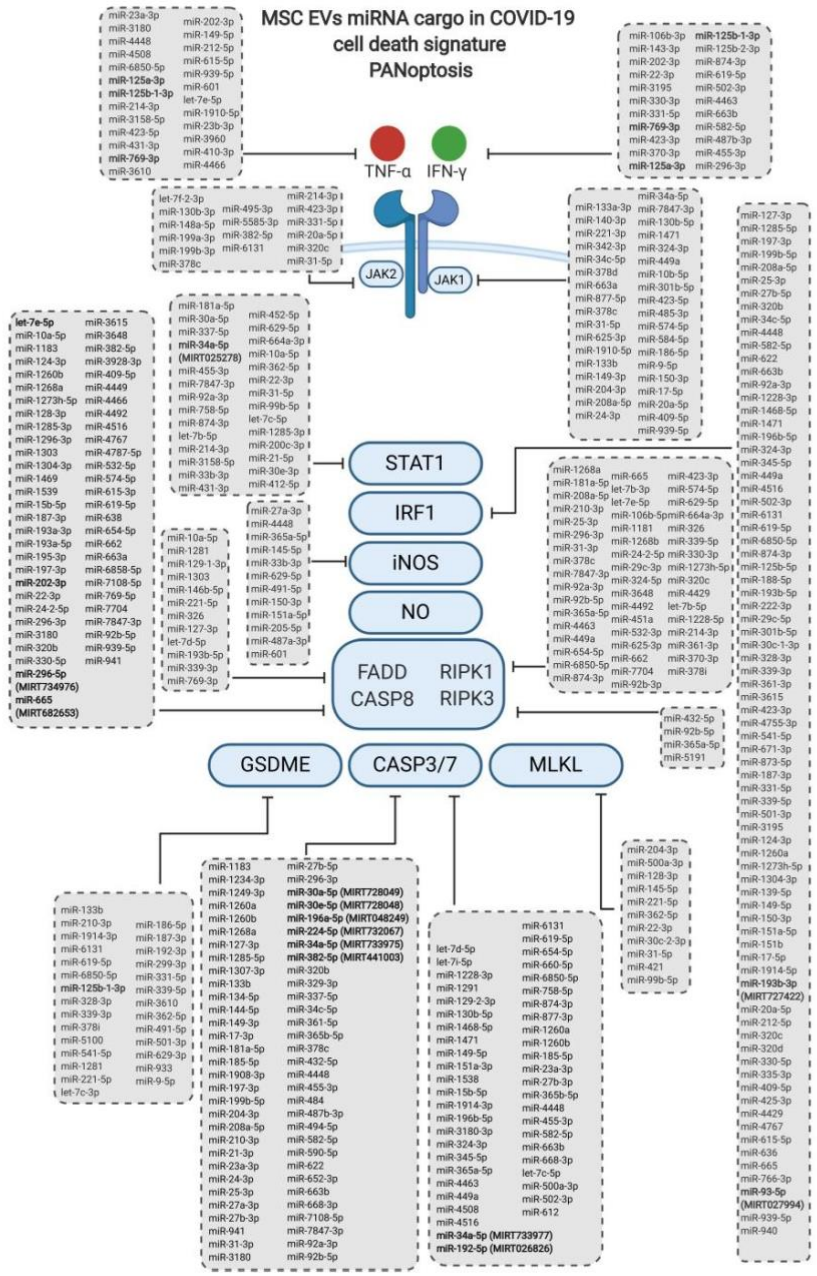
Interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor (TNF) have all been shown to trigger acute endothelial cell activation. First, it was thought that TNF had a central role in coagulation activation in cases of sepsis. However, after studies using TNF blockers no significant decrease in coagulation was observed [77]. Then, studies proved that blocking IL-6 attenuates the coagulation activation [78]. Therefore, modulation of some cytokines could ameliorate the outcome of patients with coagulation disturbs [79, 80].

Table 1 58 miRNAs of MSC-derived extracellular vesicles were commonly shared in all four analyzed datasets GSE71241, GSE78865, GSE81151 and GSE69909 regardless of the expression amount

Mature miRNA	Precursor	GSE81151	GSE69909	GSE71241	GSE78865[#]	
hsa-let-7a-5p	hsa-let-7a-1/7a-2/7a-3	***	*****	***	hsa-let-7a	****
hsa-let-7e-5p	hsa-let-7e	**	****	***	hsa-let-7e	**
hsa-let-7f-5p	hsa-let-7f-1/7f-2	***	*****	***	hsa-let-7f	****
hsa-let-7 g-5p	hsa-let-7 g	**	****	***	hsa-let-7 g	***
hsa-miR-10a-5p	hsa-mir-10a	**	****	*	hsa-miR-10a	****
hsa-miR-16-5p	hsa-mir-16-1/16–2	***	*****	***	hsa-miR-16	***
hsa-miR-17-3p	hsa-mir-17	**	***	***	hsa-miR-17	**
hsa-miR-23a-3p	hsa-mir-23a	**	*****	***	hsa-miR-23a	****
hsa-miR-19b-3p	hsa-mir-19b-1/19b-2	**	****	*	hsa-miR-19b	**
hsa-miR-25-3p	hsa-mir-25	**	****	***	hsa-miR-25	****
hsa-miR-26a-5p	hsa-mir-26a-1/26a-2	****	*****	***	hsa-miR-26a	****
hsa-miR-27b-3p	hsa-mir-27b	***	****	***	hsa-miR-27b	****
hsa-miR-28-3p	hsa-mir-28	***	***	***	hsa-miR-28-3p	****
hsa-miR-29a-3p	hsa-mir-29a	**	*****	***	hsa-miR-29a	****
hsa-miR-30c-5p	hsa-mir-30c-1/30c-2	**	****	*	hsa-miR-30c	**
hsa-miR-29c-3p	hsa-mir-29c	**	***	**	hsa-miR-29c	**
hsa-miR-31-5p	hsa-mir-31	**	*****	*	hsa-miR-31	**
hsa-miR-99a-5p	hsa-mir-99a	**	****	**	hsa-miR-99a	**
hsa-miR-99b-5p	hsa-mir-99b	***	***	*	hsa-miR-99b	****
hsa-miR-100-5p	hsa-mir-100	***	*****	***	hsa-miR-100	***
hsa-miR-103a-3p	hsa-mir-103a-1/103a-2	***	****	**	hsa-miR-103	****
hsa-miR-124-3p	hsa-mir-124-1	***	**	****	hsa-miR-124	****
hsa-miR-125a-5p	hsa-mir-125a	***	****	*	hsa-miR-125a-5p	****
hsa-miR-125b-1-3p	hsa-mir-125b-1	**	****	*	hsa-miR-125b	**
hsa-miR-126-3p	hsa-mir-126	**	****	***	hsa-miR-126	****
hsa-miR-127-3p	hsa-mir-127	****	****	**	hsa-miR-127-3p	****
hsa-miR-130a-3p	hsa-mir-130a	**	****	**	hsa-miR-130a	****
hsa-miR-130b-3p	hsa-mir-130b	**	***	*	hsa-miR-130b	****
hsa-miR-134-5p	hsa-mir-134	**	****	***	hsa-miR-134	****
hsa-miR-139-5p	hsa-mir-139	**	***	*	hsa-miR-139-5p	***
hsa-miR-138-5p	hsa-mir-138-1/2	***	**	*	hsa-miR-138	***
hsa-miR-140-3p	hsa-mir-140	**	****	**	hsa-miR-140-3p	****
hsa-miR-140-5p	hsa-mir-140	**	***	*	hsa-miR-140-5p	****
hsa-miR-142-5p	hsa-mir-142	**	***	**	hsa-miR-142-5p	****
hsa-miR-143-3p	hsa-mir-143	***	****	*	hsa-miR-143	****
hsa-miR-148b-3p	hsa-mir-148b	**	***	*	hsa-miR-148b	****
hsa-miR-193a-3p	hsa-mir-193a	**	**	*	hsa-miR-193a-3p	****
hsa-miR-193a-5p	hsa-mir-193a	**	****	*	hsa-miR-193a-5p	****
hsa-miR-193b-3p	hsa-mir-193b	**	***	**	hsa-miR-193b	**
hsa-miR-199a-3p	hsa-mir-199a-1/199a-2/199b	**	*****	***	hsa-miR-199a-3p	****
hsa-miR-214-3p	hsa-mir-214	**	*****	**	hsa-miR-214	****
hsa-miR-222-3p	hsa-mir-222	**	*****	*	hsa-miR-222	**
hsa-miR-223-3p	hsa-mir-223	**	****	***	hsa-miR-223	**
hsa-miR-335-5p	hsa-mir-335	**	*****	*	hsa-miR-335	****
hsa-miR-320a	hsa-mir-320a	*****	****	***	hsa-miR-320	***
hsa-miR-328-3p	hsa-mir-328	**	**	****	hsa-miR-328	****
hsa-miR-345-5p	hsa-mir-345	**	***	***	hsa-miR-345	****
hsa-miR-361-5p	hsa-mir-361	**	***	*	hsa-miR-361-5p	****
hsa-miR-370-3p	hsa-mir-370	***	***	**	hsa-miR-370	****
hsa-miR-376c-3p	hsa-mir-376c	**	****	**	hsa-miR-376c	****
hsa-miR-381-3p	hsa-mir-381	**	***	*	hsa-miR-381	****
hsa-miR-382-5p	hsa-mir-382	**	****	*	hsa-miR-382	****
hsa-miR-410-3p	hsa-mir-410	**	***	*	hsa-miR-410	****
hsa-miR-423-5p	hsa-mir-423	****	****	**	hsa-miR-423-5p	****
hsa-miR-484	hsa-mir-484	**	****	*	hsa-miR-484	***
hsa-miR-486-5p	hsa-mir-486-1	****	****	***	hsa-miR-486-5p	****
hsa-miR-487b-3p	hsa-mir-487b	**	****	*	hsa-miR-487b	****
hsa-miR-495-3p	hsa-mir-495	**	****	*	hsa-miR-495	****

only miRNA precursor was available for analysis

* global score of miRNA expression from weak (*) to strong (****)


MSC EVs miRNA cargo in COVID-19
cell death signature
PANoptosis
TNF-α
IFN-γ
JAK2
JAK1
STAT1
IRF1
iNOS
NO
FADD
CASP8
RIPK1
RIPK3
GSDME
CASP3/7
MLKL

◀ **Fig. 1** Prediction of miRWalk for 3'UTR binding site of the 266 miRNAs from the MSC-derived EVs, shared in at least two datasets, in one or multiple targets of the PANoptosis cell death (pyroptosis, apoptosis and necrosis) key genes suggested by Karki et al.2020 [26]

The blockade of the cytokines and their receptors associated with hyperinflammation during COVID-19 can be a more rational targeted therapy [5]. For example, Tocilizumab is a monoclonal antibody anti-IL-6R, which blocks the IL-6/IL-6R complex formation [81, 82]. However, a randomized trial showed that the single target approach with the anti-IL-6R Tocilizumab did not show a significant improvement on COVID-19 patients outcome compared to standard care [83].

The approach using mesenchymal stem cells as therapy can be helpful once they have migration and homing ability, anti-inflammatory, immunomodulatory, regenerative, pro-angiogenic and anti-fibrotic properties [33, 84]. Another relevant consideration is the increasing number of caesarian deliveries and aesthetic procedures, as liposuction, performed at the hospitals which increases the availability of discard materials such as umbilical cord and adipose tissue. These biological materials can be use as tissue source of MSC and before would be discarded and now can be reused. The homing ability of MSCs, in the case of ARDS due to COVID-19, may happen because the cells have membrane receptors to cytokines present in high levels, such as MCP-1(CCL2) and IL-8, chemoattracting the MSCs to the injury site [85, 86]. Preclinical studies showed high efficacy of MSCs cell-therapies allowing their clinical use [87]. Completed and ongoing clinical trials demonstrated the feasibility, safety, and tolerability of MSCs use in respiratory disorders, including ARDS [88]. Also, Emukah et al., reviewed the effects of MSC conditioned medium (MSC-CM) on many lung diseases and the results demonstrated that MSC-CM minimized the inflammation and was as similar as MSCs [89]. Further, it was identified that MSC-CM effects on cell proliferation, regeneration angiogenesis and others were partially due to the extracellular vesicles, drawing the scientific community attention to explore the EVs in translational medicine [39].

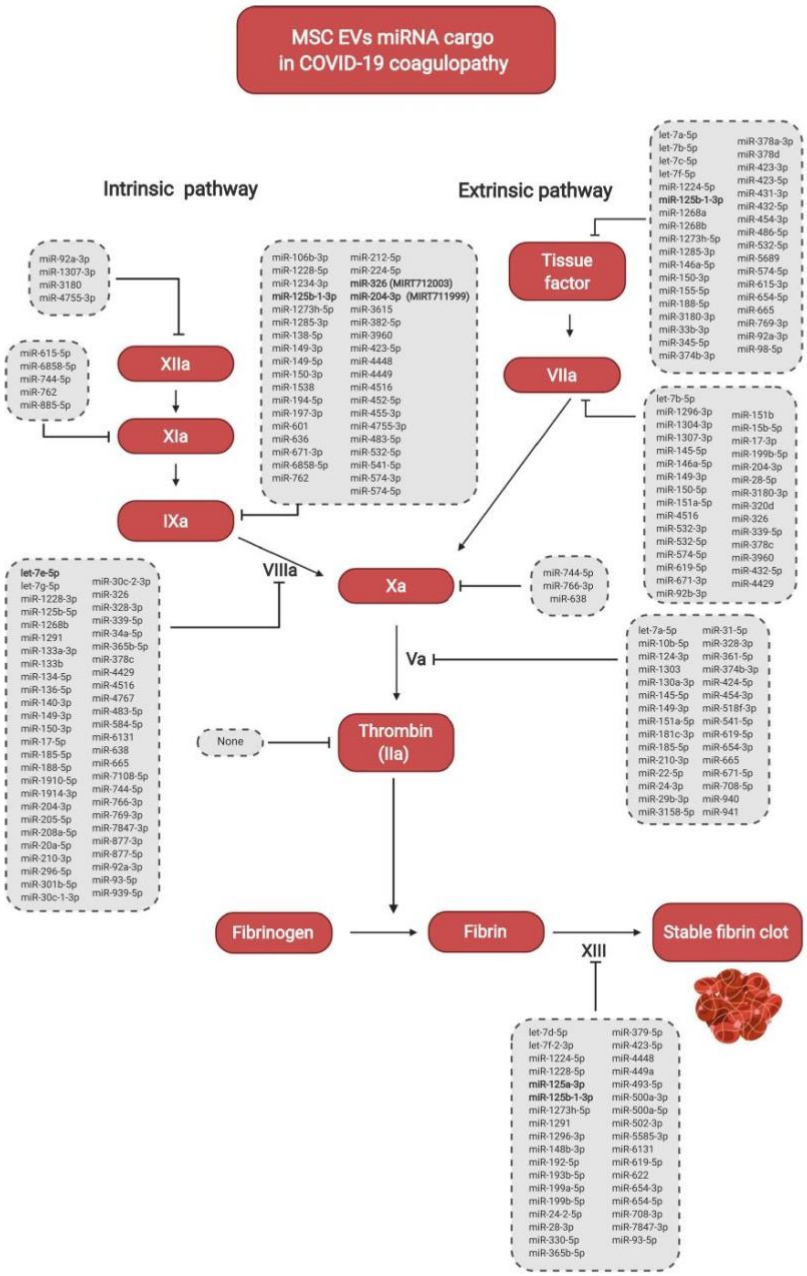
Despite of the establishment of the ISEV guidelines, in 2014 and updated in 2018, the studies with extracellular vesicles still present differences. As recently reviewed by Tieu et al., 2020, there are discrepancies according to the source, method of isolation, characterization of the EVs, nomenclature, treatment doses, administration route and experimental design. The authors also evidenced the scientific community interest showing the crescent curve of published papers since the first report of extracellular vesicle used as treatment for kidney injury model in 2009 [56, 90].

MSC-derived EVs are heterogeneous particles and its inner cargo can vary according to the source and physiological/pathologic conditions at the time of EVs isolation [43]. In pathologic conditions, as viral infections, the EVs can carry in their cargo viral particles which could help to spread the pathogen throughout the body [91]. Therefore, when considering treatment, it is important use only cells derived from tissues of healthy donors, without any previous infectious event, preventing the viral transmission to treated patients. Also, differences in proteomics between MSC from adipose tissue, umbilical cord and bone marrow were already reported [92]. Moreover, differences about donors age shapes the EVs inner cargo and activity, except in MSCs from umbilical cord, presenting strongly immunomodulatory effects in healthy younger people [93]. Thus, we excluded the data of MSC-derived EVs from elderly donors, using just healthy and young donors available data. Therefore, this higher immunomodulatory effect of MSC-derived EVs from healthy young donors with low immunogenicity, scalable production, storage for further use, could make it possible the creation of a "cell-free therapy bank". By using these healthy tissues discharged in medical procedures, it would turn this "multi-target treatment ready to use" available to patients, at the moment they arrive in the health system units, with high inflammatory and coagulant diseases.

The first bioinformatics analysis to predict microRNA targets was based on sequences complementarity between plant miRNAs and their targets. It has guided functional studies of several miRNAs [94]. Chauhan et al., through computational analysis, identified miRNAs that target Sars-CoV-2 viral genes (e.g. reducing the spike protein) avoiding the viral connection with the receptors in the host cells. Also, the authors identified miRNAs acting inside the host cells reducing the expression of receptors, preventing the entrance and viral replication, minimizing the spread of the infection [95]. MicroRNAs are small and unstable structures and possess a short half-life when are free in blood circulation and, for this reason, methods that could improve the half-life and delivery in specific sites improving their activity are necessary [96]. This highlights the benefits of use the EVs as a transportation carrier for this type of cargo.

As shown in Fig. 4, after the internalization of the EVs by the target cell (Fig. 4a), the miRNAs are released in the target cell cytoplasm (Fig. 4b) and, with assistance of enzyme complexes, the miRNA will attach in the target mRNA through a perfect or imperfect base-pairing in the 5'UTR, CD or 3'UTR gene regions (Fig. 4c). The imperfect attachment will result in an inhibition of the translation, while the perfect attachment will result in degradation of the mRNA [97].

We focused on this paper on the miRNAs related to the exacerbated cytokines and chemokines genes of severe COVID-19 patients reported elsewhere. Furthermore, we focused on analyze the interaction of the MSC-derived EVs miRNA cargo with the genes reported by Karki et al., 2020 [26] as responsible for cell death signature through PANopotis and the genes from the coagulation cascades. Our analysis


MSC EVs miRNA cargo in COVID-19 coagulopathy
Intrinsic pathway
Extrinsic pathway
Tissue factor
VIIa
XIIa
XIa
IXa
VIIIa
Xa
Va
Thrombin (IIa)
None
Fibrinogen
Fibrin
XIII
Stable fibrin clot
miR-92a-3p
miR-1307-3p
miR-3180
miR-4755-3p
miR-615-5p
miR-6858-5p
miR-744-5p
miR-762
miR-885-5p
miR-106b-3p
miR-1228-5p
miR-1234-3p
miR-125b-1-3p
miR-1273h-5p
miR-1285-3p
miR-138-5p
miR-149-3p
miR-149-5p
miR-150-3p
miR-1538
miR-194-5p
miR-197-3p
miR-601
miR-636
miR-671-3p
miR-6858-5p
miR-762
miR-212-5p
miR-224-5p
miR-326 (MIRT712003)
miR-204-3p (MIRT711999)
miR-3615
miR-382-5p
miR-3960
miR-423-5p
miR-4448
miR-4449
miR-4516
miR-452-5p
miR-455-3p
miR-4755-3p
miR-483-5p
miR-532-5p
miR-541-5p
miR-574-3p
miR-574-5p
let-7a-5p
let-7b-5p
let-7c-5p
let-7f-5p
miR-1224-5p
miR-125b-1-3p
miR-1268a
miR-1268b
miR-1273h-5p
miR-1285-3p
miR-146a-5p
miR-150-3p
miR-155-5p
miR-188-5p
miR-3180-3p
miR-33b-3p
miR-345-5p
miR-374b-3p
miR-378a-3p
miR-378d
miR-423-3p
miR-423-5p
miR-431-3p
miR-432-5p
miR-454-3p
miR-486-5p
miR-532-5p
miR-5689
miR-574-5p
miR-615-3p
miR-654-5p
miR-665
miR-769-3p
miR-92a-3p
miR-98-5p
let-7b-5p
miR-1296-3p
miR-1304-3p
miR-1307-3p
miR-145-5p
miR-146a-5p
miR-149-3p
miR-150-5p
miR-151a-5p
miR-4516
miR-532-3p
miR-532-5p
miR-574-5p
miR-619-5p
miR-671-3p
miR-92b-3p
miR-151b
miR-15b-5p
miR-17-3p
miR-199b-5p
miR-204-3p
miR-28-5p
miR-3180-3p
miR-320d
miR-326
miR-339-5p
miR-378c
miR-3960
miR-432-5p
miR-4429
miR-744-5p
miR-766-3p
miR-638
let-7e-5p
let-7g-5p
miR-1228-3p
miR-125b-5p
miR-1268b
miR-1291
miR-133a-3p
miR-133b
miR-134-5p
miR-136-5p
miR-140-3p
miR-149-3p
miR-150-3p
miR-17-5p
miR-185-5p
miR-188-5p
miR-1910-5p
miR-1914-3p
miR-204-3p
miR-205-5p
miR-208a-5p
miR-20a-5p
miR-210-3p
miR-296-5p
miR-301b-5p
miR-30c-1-3p
miR-30c-2-3p
miR-326
miR-328-3p
miR-339-5p
miR-34a-5p
miR-365b-5p
miR-378c
miR-4429
miR-4516
miR-4767
miR-483-5p
miR-584-5p
miR-6131
miR-638
miR-665
miR-7108-5p
miR-744-5p
miR-766-3p
miR-769-3p
miR-7847-3p
miR-877-3p
miR-877-5p
miR-92a-3p
miR-93-5p
miR-939-5p
let-7a-5p
miR-10b-5p
miR-124-3p
miR-1303
miR-130a-3p
miR-145-5p
miR-149-3p
miR-151a-5p
miR-181c-3p
miR-185-5p
miR-210-3p
miR-22-5p
miR-24-3p
miR-29b-3p
miR-3158-5p
miR-31-5p
miR-328-3p
miR-361-5p
miR-374b-3p
miR-424-5p
miR-454-3p
miR-518f-3p
miR-541-5p
miR-619-5p
miR-654-3p
miR-665
miR-671-5p
miR-708-5p
miR-940
miR-941
let-7d-5p
let-7f-2-3p
miR-1224-5p
miR-1228-5p
miR-125a-3p
miR-125b-1-3p
miR-1273h-5p
miR-1291
miR-1296-3p
miR-148b-3p
miR-192-5p
miR-193b-5p
miR-199a-5p
miR-199b-5p
miR-24-2-5p
miR-28-3p
miR-330-5p
miR-365b-5p
miR-379-5p
miR-423-5p
miR-4448
miR-449a
miR-493-5p
miR-500a-3p
miR-500a-5p
miR-502-3p
miR-5585-3p
miR-6131
miR-619-5p
miR-622
miR-654-3p
miR-654-5p
miR-708-3p
miR-7847-3p
miR-93-5p

◀ **Fig. 2** Prediction of miRWalk for 3'UTR binding site of the 148 miRNAs from the MSC-derived EVs, shared in at least two datasets, in one or multiple targets of intrinsic and extrinsic coagulation cascades

results allow us to suggest that the miRNA from the MSC-derived EVs has a therapeutic potential for investigation. As mentioned before, regardless the MSC-EVs have been derived from different tissue sources (adipose tissue, umbilical cord and bone marrow), there was an overlap of 58 miRNAs, among the four datasets of miRNA analyzed. The analysis of 3'UTR binding site target, from those miRNAs shared in at least two datasets, showed that 258 miRNAs could attach in this target region for all analyzed cytokines and chemokines. The extracellular vesicles carry not only different miRNAs, but also other molecules, characterizing the cargo as a heterogeneous material capable to interact with multitargets.

As mentioned, in pathological conditions, the EVs released can help to disseminate the disease throughout the organism, they can carry the pathogen responsible, or its structures inside the EVs. Although, based on the GSE12243 dataset analysis, the expression of the two receptors used by the Sars-CoV-2 to enter the host cell, toll like receptor 4 (TLR4) and Angiotensin-Converting enzyme 2 (ACE2), the TLR4 mRNA inside the EVs was not significant (Pearson's $r = 0.763$, p value = 0.0776), as well as the ACE2 mRNA (Pearson's $r = -0.2717$ and p value = 0.6025). Based on these results, there is not enough mRNA to produce these proteins in the EVs. Therefore, MSC-derived EVs, unlike their MSCs parental cells, would not be involved in the spread of the Sars-CoV-2 and wouldn't increase the COVID-19 development.

As highlighted in Sup.Table 4, Figs. 1 and 2, one miRNA binds to multiple mRNAs. For example, **miR-125a-3p** binds to the portion 3'UTR of IL2, CXCL10, IL7, IL10 and IL15.

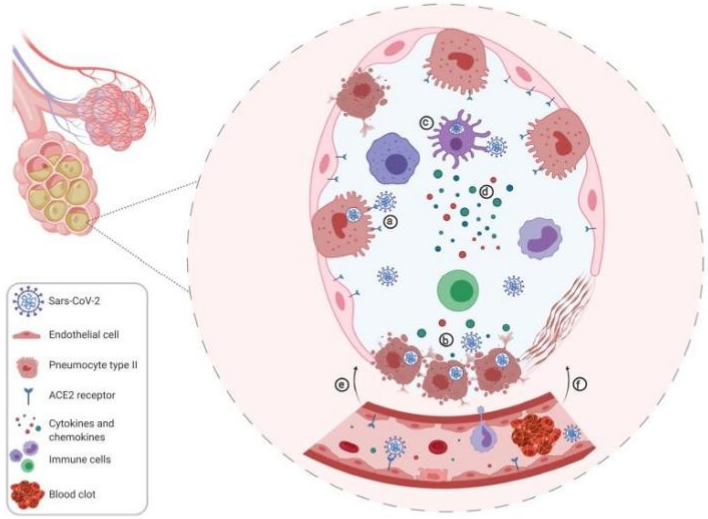


Fig. 3 A representative alveoli cross-section with adjacent blood capillary presenting the events of COVID-19 development. **(a)** The Receptor-Binding Domain (RBD) of Sars-CoV-2 binds to the ACE2 receptor in the membrane of pneumocytes type II, endothelial cells and others. The viral spike protein is cleveaged by Transmembrane Protease Serine 2 (TMPRSS2), a protease present in the surface of the host cells. **(b)** Viral replication is performed originating new copies to be released in the extracellular environment. Also, the virus induce the cell death by PANoptosis (pyroptosis, apoptosis and necrosis) causing the release of damage associated molecular patterns (DAMPs), which will be recognized by alveolar epithelial cells and alveolar macrophages with the pattern recognition receptors (PRRs). **(c)** The antigen recognition will trigger an immune response where dendritic cells, monocytes, macrophages, neutrophils and T cells will be atracted to the infection site. These cells have the stimuli to express tissue factor in their membrane starting coagulation activation **(d)** ICU patients with severe COVID-19 present higher baseline levels of IL-6, IL-1β, TNF-α, IL-2, IL-7, IL-8, IL-9, IL-10, IL-17, G-CSF, MCP-1 (CCL2), MIP-1α (CCL3), MCP-3 (CCL7) and CXCL10 (IP-10), IL-18, IL-33, IL1- α, IL-15. **(e)** Direct cell death, caused by Sars-CoV-2, and the inflammatory cell death increase vascular permeability and cause fluid efflux from blood vessels and capilaries into the lungs interfering in the gas exchange and consequently damaging lung tissue. Also, blood clot formation interfere in the organs homeaostasis. Thus, the patient clinically progress to an acute respiratory distress syndrome requiring mechanical ventilation. **(f)** Areas of consolidation by fibroblastic proliferation and deposition of extracellular matrix and fibrin in the alveolar spaces can be detected by CT scans

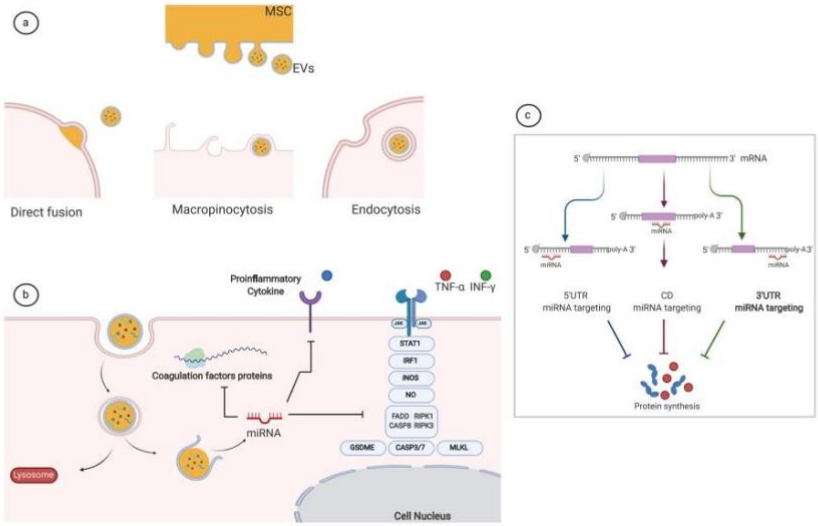


Fig. 4 Mechanisms of extracellular vesicles release, internalization and action of miRNA cargo in the target cell genes. **(a)** The internalization of the EVs in the target cell occurs by multiple mechanisms, such as membrane direct fusion, macropinocytosis and endocytosis. **(b)** After EVs internalization, through endolysosomal pathway, the lipidic bilayer of the EVs are degraded and their cargo is released in the cell cytoplasm allowing the performance of each cargo specific action. **(c)** In mammalians, the miRNA binds through perfect and imperfect base-pairing on different regions of target mRNA 3'UTR, repressing translation of the protein

Moreover, **miR-125a-3p** binds to the 3'UTR region of TNF, IFN and binds also to the 3'UTR of Factor XIII gene. This multitargeted approach could minimize cell death, alleviate the systemic inflammation and coagulation disturbs in severe COVID-19 patients improving their clinical outcome. Another isoform, expressed in the four analyzed miRNA datasets, is the **miR-125b-1-3p** which targets the 3'UTR region of the CXCL10, IL17A, IL10, CCL3, IL18 and IL33 and targets the 3'UTR region of TNF, IFN, and GSDME genes. In addition, **miR-125b-1-3p** also binds to the 3'UTR region of Factor III, IX and XIII. One study performed by Fujii and collaborators showed the Graft-Versus-Host Disease (GVHD) amelioration in vitro and in vivo through, at least partially, by the multitarget potential of BM-MSC-derived EVs miRNA content, including miR-125a-3p [98]. Two other miRNAs, **miR-769-3p** and **miR-202-3p,** may attenuate the cell death avoiding tissue damage by targeting synergistically the 3'UTR region of the TNF e IFN genes inhibiting their protein translation.

The target of IL-1 and IL-6 pathways could attenuate the coagulation activation manifested by admitted severe COVID-19 ICU patients [72, 75, 99]. Thus, the miRNA, highly expressed in the four analyzed datasets, **let-7e-5p** could promote this action by binding in the 3'UTR region of IL1A, IL1B, IL6R, IL15, IL10 and CSF3 genes. In addition, the **let-7e-5p** binds to the 3'UTR region of TNF, RIPK1 and CASP8 genes that are participants of the cell death signature pathway. In addition, it directly targets the 3'UTR portion of Factor VIII gene from the coagulation cascade.

Let-7 was firstly found in *C. elegans* and it was the second miRNA discovered [100]. The functions of this microRNA family are the most studied. There are reports in the literature about their relation with inflammation [101, 102], cancer [103] and other conditions. Among our 27 experimentally validated miRNAs, 7 target the IL-6R of which 3 are members of the let-7 family. This relation is corroborated by the literature, as Sung et al. (2018) showed that in a coculture of MSCs derived from bone marrow and prostate cancer cells, the loss of let-7 leads to an upregulation of IL-6 expression [104]. Moreover, Di et al.(2020) evaluated the let-7 in airway remodeling in chronic obstructive pulmonary disease (COPD) via the regulation of IL-6 mRNA by targeting and silencing its 3'UTR region. Also, let-7 works as a regulator of myofibroblast differentiation, through the regulation of this cytokine expression [105].

Some studies suggest that using an approach with a combination of treatments, or a multitarget therapy, versus monotherapy is preferable [106, 107]. Several reviews and reports already discussed the potential of investigation of MSC-derived EVs and its cargo, particularly miRNA, in COVID-

19 as well as other lung and heart injuries [41, 108–114]. Based on our analysis, it is possible to suggest that the multi-target characteristic of the MSC-derived EVs miRNA cargo could be an advantage, in comparison to therapies with a single target, such as Tocilizumab or Anakira (anti-IL1R). These therapies are used to decrease inflammatory biomarkers, due to interactions with multiple pathways. They improve the patient outcome by minimizing tissue damage and mitigating the coagulation activation and thrombi formation. Although, more studies of fully characterization, evaluation and functionality of the MSC-EVs content are required to confirm their benefits.

The therapeutically potential of the MSC-derived EVs against COVID-19 and other high inflammatory lung injuries is corroborated by other reports showing important results such as: the polarization of macrophages M1 (pro-inflammatory) to a M2 (anti-inflammatory) phenotype, activation and regulation of T cells [115]; MSC-derived exosomes prevent the recruitment of monocytes and reduces the secretion of pro-fibrotic IL10 and TGF-β by these cells in the lung of silica-exposed mice [55]. Also, the use of MSC-derived EVs ACE2+ to compete with Sars-CoV-2 to the host cells receptor binding [116]. Therefore, the miRNA cargo of the MSC-derived EVs as therapy could be useful, not only in COVID-19, but also in other viral infections that present high levels of inflammatory cytokines, as influenza.

Conclusion

The analysis of bioinformatics prediction showed that miRNA inner cargo of MSC-derived EVs may attenuate the production of excessive inflammatory cytokines and chemokines, coagulation cascade and cell death by multitargeting the 3'UTR region of several mRNA. Regardless the differences among tissue sources, there was miRNA overlap. Thus, even without an establishment of which tissue is the best source to extract the mesenchymal stem cells, or methods of the EVs isolation and characterization, the positive effect against the exacerbated inflammatory agents and coagulation disturbs present in severe COVID-19 may be achieved. Multiple clinical trials using MSC against COVID-19 are currently ongoing, some of them already are completed and showed the feasibility, safety and low adverse effects in COVID-19 treated patients. As the extracellular vesicles share some intrinsic characteristics with their parental cells and have the advantage to be small sized, non-proliferation activity and low immunogenicity more published outcomes of currently ongoing randomized and future clinical trials, with a larger number of enrolled patients, testing exosomes in COVID-19 are well awaited.

The research for a specific antiviral to Sars-CoV-2 and the development of a functional vaccine are the goals in this pandemic period. However, a long period can be taken until the achievement of satisfactory results and an equal distribution worldwide. Therefore, the development of anti-inflammatory and anticoagulant therapies, such as the MSC-derived EVs and its inner natural cargo from healthy donors, deserves attention and investigation, aiming the reduction of death rates due to the COVID-19 around the world. Besides that, there is a possibility to create in health units, a cell-free therapy bank that could provide treatment for immediate application that could also benefit other patients, with high inflammatory and coagulant diseases. Furthermore, more studies of full characterization and evaluation of the mesenchymal stem cells derived extracellular vesicles and its cargo functions in COVID-19, as well as in other infectious events, are still necessary.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12015-021-10122-0.

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Authors' Contribution ICS – data analysis and manuscript writing.
APS – data analysis and manuscript writing.
MW - reviewed the manuscript.

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Data Availability Not applicable.

Code Availability Not applicable

Compliance with Ethical Standards

Conflict of Interest The authors declared no conflict of interest.

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

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In view of the results obtained in Chapter 1, with the *in silico* analysis, and considering the emergence of notifications of consequences in the central nervous system, we decided to suggest that extracellular vesicles could be applied for the clinical management of the patient attenuating the damage cause in the CNS by the exacerbated inflammation and preventing neurologic sequelae.

4. CHAPTER 2: *MSC-Exosomes Carrying miRNA - Could they Enhance Tocilizumab Activity in Neuropathology of COVID-19?*

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MSC-Exosomes Carrying miRNA – Could they Enhance Tocilizumab Activity in Neuropathology of COVID-19?

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Introduction

It is known that the central nervous system (CNS) is affected by viral infections. The Coronavirus disease 2019 (COVID-19) is a systemic disease that compromises the primary site of infection, the lungs, and then spreads throughout the body, including the CNS [1, 2]. Like other organs, the CNS is affected by the overproduction of pro-inflammatory cytokines and chemokines [3] and not only severe patients suffer from neurologic disturbances. Milder cases have shown that these patients also manifest headache, anosmia, nausea, and vomiting, gray matter loss and abnormalities in the brain after COVID-19 recover [4, 5]. Recent findings suggest that the blood-brain and blood-cerebrospinal fluid (CSF) barriers are disrupted in severe COVID-19 patients and this allows the infiltration of infected leukocytes and IL-6 inside the brain interstitial system [6, 7]. Also, CSF lumbar punction samples indicated an increase in the CSF/serum ratio for albumin (QAlb), suggesting a dysfunction of the blood-cerebrospinal fluid barrier (BCB) in around of 50% collected samples [8].

Interleukin-6 has been suggested as a predictive biomarker of severity and progression of this disease [9–12]. Both detrimental and beneficial functions of this cytokine have been reported. When compared to other cytokines, IL-6 remains for a longer period in the blood circulation, causing organ damage but also becoming a potential target for new therapies [13]. Opinions diverge about the occurrence of the "cytokine storm" in COVID-19 and the presence of the high levels of IL-6 in comparison to other respiratory diseases [14]. Italian researchers reported that COVID-19 patients treated with one infusion of anti-IL-6 receptor Tocilizumab survived more than those patients who didn't and this was correlated with IL-6 serum levels [15]. Then, many clinical trials testing blockers against the IL-6R, such as Tocilizumab and Sarilumab, in COVID-19 patients, are currently completed or ongoing. Among them, the large and randomized clinical trials, REMAP-CAP, RECOVERY, and PROSPERO showed an improvement in survival rate and patients' clinical outcomes when they were treated with IL-6R blockers [16–18].

The mesenchymal stem cells (MSCs) can be isolated from different adult tissues and they have the capability of modulating other cells, like immune cells, to promote anti-inflammatory activity and tissue regeneration for clinical applications [19]. Several clinical trials evaluating the efficacy and safety of the MSCs, such as the START study in COVID-19, and other respiratory distress, were registered and interventional uses were reported [20–23]. In example, a double-blind, phase 1/2a, randomized, controlled trial was performed by Lanzoni et al., where patients suffering with acute respiratory distress syndrome (ARDS) induced by COVID-19 (n = 12) were treated with two infusions of umbilical cord MSCs (UC-MSCs). The endpoints of the study showed that the cell therapy infusions were safe and no adverse effects was registered. The inflammatory biomarkers, including the IL-6, decreased its levels after 6 days of the first cell infusion, leading to a positive prognosis for the patients [24]. In this line, a case report made by Senegaglia et al., showed an innovative combination, in alternate days, of these two potential therapies, Tocilizumab and UC-MSCs. They treated a severe COVID-19 patient admitted to the intensive care unit, showing an improvement of the patient's clinical outcome resulting in survival, faster recovery, and clinical discharge [25].

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Although it was only one patient that received the combination of treatments, the authors opened a new and promising investigation path.

Despite the mesenchymal stem cells being an alternative treatment for cell therapy, they have some disadvantages for the clinical use. Some of these are their size, their differentiation into other cell lines, tumorgenicity capacity, be trapped in the lungs vascularization, the incapacity to cross the blood brain barrier (BBB) and others [26]. Therefore, elements comprised in the secretome released by the MSCs such as the extracellular vesicles (EVs) could be an alternative. The EVs group are comprised by the exosomes, microvesicles and apoptotic bodies and they are released by the parental cells carrying specific cargos and they differ regarding their biogenesis. Here, we focus on the exosomes, the smallest type of EV, with 30-150 nm, formed in the endolysosomal pathway, and with similar activities as their parental cells. They encapsulate proteins, lipids, miRNAs, mRNA, and other important molecules used for communication between cells [27, 28]. In spite of most studies investigate the benefit of the MSCs and their exosomes in the lungs, due to their nanosized and lipophilic structure, the exosomes could also be used to reach and treat the symptoms in the CNS of COVID-19 patients, thanks to their ability to cross the BBB.

Furthermore, Tocilizumab, a large neutralizing monoclonal antibody (mAb), has a minimal crossing capacity through the natural barriers in hemostatic conditions [29]. In pathological conditions, the patients have a disrupted barrier that could allow the anti-IL-6R to cross it and reach the CNS microenvironment. However, as aforementioned, it is not all barriers that are disrupted in COVID-19, therefore, not every patient would beneficiate of this therapy in the brain [8]. Likewise, it is known that after the first infusion of Tocilizumab, there is an elevation of circulating IL-6 levels due to saturation of the IL-6 receptors by the mAb [30]. This transient elevation of IL-6 has been associated in Chimeric Antigen Receptor (CAR) neuro-toxicity, which can occurs independently from Cytokine Release Syndrome (CRS) [31]. Thus, a second infusion of Tocilizumab might be hazardous for the CNS, although in ARDS patients this therapeutic scheme proved to be safe [24]. Consequently, due to their nanosized diameter and their lipophilic bilayer structure, the exosomes can easily cross the natural barriers and attenuate neurologic symptoms, by acting in resident immune cells, such as astrocytes and microglial cells, which are activated by the IL-6. Meaning another advantage of using the MSC-exosomes in COVID-19 patients with neurologic symptoms. Yet, it was suggested that the IL-6 could be used as a biomarker of long-term neuropsychiatric symptoms, such as fatigue, depression, and anxiety in COVID-19 survivors, usually classified with long COVID-19 or post-COVID-19 syndrome [32, 33]. Therefore, the use of IL-6/IL-6R blockers in association with MSCs-exosomes, on these patients could also attenuate these late symptoms and sequelae [34].

MicroRNAs (miRNAs) are non-coding RNAs and are associated with post-transcriptional gene repression or degradation [35] and it is well known that IL-6 is modulated by miRNAs at multiple levels. Previously, we performed the prediction of the miRNAs targeting the cytokines involved in the PANoptosis pathway [36] and genes of coagulation cascades, present in severe COVID-19 patients, with ARDS only for a 3′ untranslated region (3'UTR) binding site [37]. For that, we analyzed 4 available datasets of MSCs-derived extracellular vesicles data from different tissues and found an overlay of 58 miRNAs, which indicate that MSC-derived EVs from different tissues share a common cargo. Considering that the IL-6 has been used as a biomarker of severity and mortality in COVID-19, we can infer that targeting the IL-6 complex (*IL6, IL6R,* and *IL6ST*), not only in the 3'UTR, but also in the coding sequence (CDS) and 5′ untranslated region (5'UTR), would increase its effectivity. Targeting these three regions could increase the efficacy of degradation and stop the translating actions of proteins through a perfect or imperfect base-pairing in the regions 5'UTR, CDS, or 3'UTR of the mRNA [37]. In this line, our intention is to reinforce the potential use of the MSC-exosomes containing miRNAs for COVID-19 as monotherapy, or in combination with other treatments, like Tocilizumab, enhancing their effects against COVID-19 as proposed in Fig. 1.

There are a few successful case reports of patients treated with Tocilizumab [38], MSCs [39], or as performed by Senegaglia et al., a combination of both [25]. Despite of we have the evaluation of these approaches as monotherapy, there is no study evaluating the combination of these therapies in the CNS deeply. Notwithstanding the intravenous administration (IV) of the MSC-derived exosomes allow their distribution systemically, the intranasal administration (IN) of the MSC-exosomes may be an alternative route to be considered. This is a shorter route to the CNS, and the exosomes would arrive faster to the primary site of infection, the respiratory system [40]. Accordingly, clinical trials are evaluating this administration method and are registered on clinicaltrials.gov and positive results are available.

As mentioned above, the MSC-exosomes carry several molecules in addition to miRNAs, which characterize their cargo as a heterogeneous material. Thus, the miRNAs and other elements present in their inner core can modulate multiple targets besides the *IL6* complex. The diversity of modulators presented inside this biological product can be an advantage in comparison to therapies focusing on a single target anti-IL-6R. Moreover, besides the benefits of the MSC-exosomes promoting immunomodulatory effects to the lungs and the heart [41], leading to attenuation of fibrosis and tissue damage, this therapy could also be useful

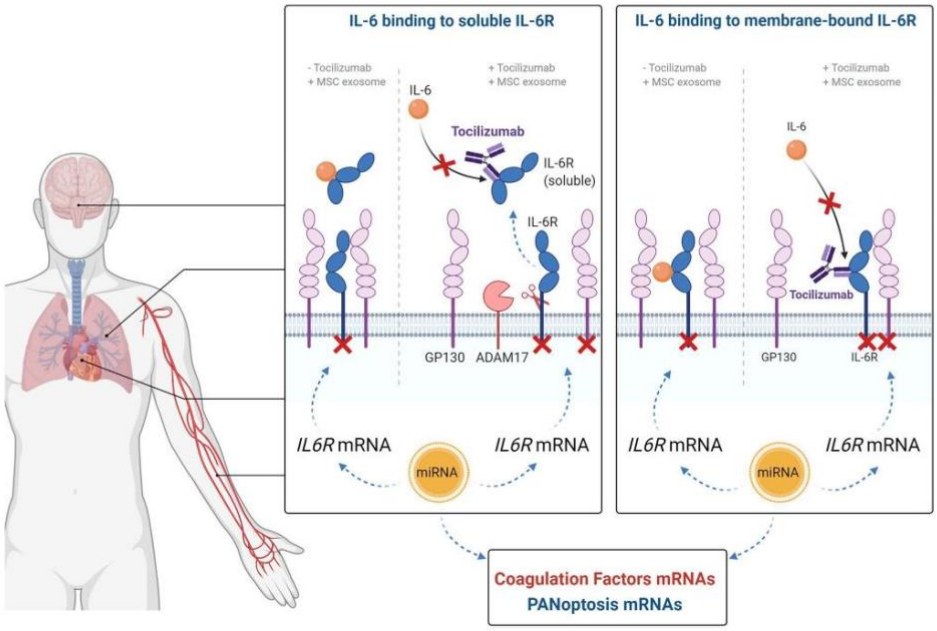

Fig. 1 Mechanism of action of the monoclonal antibody anti-IL-6R (Tocilizumab) and the mesenchymal stem cells derived exosomes carrying microRNAs (miRNAs). The neutralizing antibody Tocilizumab attach and inhibit the activation of the signaling pathway started by the IL-6 reducing the patient inflammatory state. Due to their nano-sized diameter, the exosomes can cross the central nervous system natural barriers (BBB and CSF) and release the small and non-coding miRNAs inside the target cell cytoplasm. The miRNAs perform a post-transcriptional activity by binding to the IL-6 complex mRNAs (*IL6, IL6R and IL6ST*) through perfect and imperfect base pairing stopping protein translation and attenuate the inflammation and consequently blood coagulation inside microvasculation of the CNS

to neuro COVID-19 management. When associated with Tocilizumab, the exosomes could improve the action of this monoclonal antibody by targeting the *IL6*, *IL6R,* and *IL6ST*. The combination of these therapies can act systemically and target other genes from PANoptosis and coagulation pathways throughout the body. Before going further into clinical trials, a pre-clinical analysis may prove this theory's potential. The use of transgenic mice expressing human angiotensin-converting enzyme 2 receptor has been well accepted to study COVID-19 development and treatments [42, 43]. Therefore, infecting animals with SARS-CoV-2 and then treating them with the combination of Tocilizumab and the MSCs-derived exosomes could be a smart choice for an initial step to evaluate the impact of treatment on the inflammation and blood coagulation.

Even so the debate around IL-6 blockage continues, the rationale of investigating the use of the MSC-derived exosomes against COVID-19 and especially in the CNS has great potential and deserves deeper investigations. However, some questions remain open: could miRNAs of the MSCs-exosomes prevent the IL-6 complex formation and prevent the transient elevation after Tocilizumab infusion in COVID-19 patients? Can we count with a disrupted BBB during COVID-19 for Tocilizumab to perform its crossing activity and decrease the IL-6 levels and consequently damage inside the CNS? Or the use of MSC-exosomes in combination with the IL-6R blocker would be a more guaranteed way of effectiveness?

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Declarations

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To corroborate the *in silico* findings, we evaluated the anti-inflammatory potential of MSC-EVs in vitro by infecting an alveolar cell line with SARS-CoV-2, treating it with MSC-EVs, and sequentially quantifying the panel of inflammatory markers.

5. CHAPTER 3: *Targeting cytokines: Evaluating the potential of Mesenchymal Stem Cells derived extracellular vesicles in the Management of COVID-19*

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Targeting cytokines: Evaluating the potential of Mesenchymal Stem Cells derived extracellular vesicles in the Management of COVID-19

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6. CONCLUSION

After 4 years since its emergence in 2019, COVID-19 has impacted the lives of the global population by infecting approximately 775 million people with SARS-CoV-2, resulting in the death of approximately 1% of these individuals. Vaccines and treatments were developed in record time; however, their adherence and distribution are not equal worldwide. In this doctoral work, the therapeutic potential of extracellular vesicles derived from mesenchymal stem cells against COVID-19 was evaluated.

Using bioinformatics analyses, predictions of interactions of microRNAs contained in EVs with inflammation modulators, PANoptosis cell death signature, and coagulation cascades, important for COVID-19 development, were performed. Fifty-eight miRNAs shared among 4 different tissue source databases were identified. Additionally, miRNAs capable of binding to the 3'UTR portion of cytokines and chemokines, PANoptosis cell death pathway, and coagulation cascades were identified. This highlights the potential use to reduce the systemic inflammation process and coagulation disorders presented by severe COVID-19 patients. Subsequently, considering studies associating inflammatory markers, such as IL-6, with neurological and neuropsychiatric consequences, we suggest that treatment with EVs containing their miRNAs in combination with Tocilizumab could be advantageous for patients, as our *in silico* analyses showed the potential interaction of miRNAs with IL-6, IL-6R, and IL-6ST.

To evaluate the functionality of EVs, lung epithelial cells that overexpress the ACE2 receptor, A549-hACE2, were infected with active SARS-CoV-2 and subsequently treated with a concentration curve of MSC-EVs derived from adipose tissue. After treatment, a screening of the supernatant from the 30EVs/cell point on the curve was performed using multiplex analysis, which detected modulation of 32 out of 48 elements in the cytokines and chemokines panel. Then, severity and mortality biomarkers IL-6, IL-8, and MCP-1 were tested with the concentration curve of 3, 10, and 30 EVs/cell, showing a dose-dependent reduction. MSC-EVs also showed anti-SARS-CoV-2 activity. However, antiviral activity was not detected at lower concentrations where anti-inflammatory activities were observed (3, 10, and 30EVs/cell), but rather at higher concentrations on the concentration curve (100 and 300EVs/cell).

The mechanism of action in anti-inflammatory and antiviral activities has not been thoroughly elucidated. However, confocal microscopy images show that MSC-EVs were internalized by lung epithelial cells and were located along the endosomal route, visually

more abundant in the early endosome compartment. This is where many studies report the fusion of endosomal membranes with EVs and the release of EV cargo into the cytoplasm, enabling their action. Therefore, it can be affirmed that extracellular vesicles derived from adipose tissue mesenchymal stem cells are internalized by lung epithelial cells, located along the endosomal route, and possibly release their miRNA cargo into the cytoplasm of cells, leading to the reduction of experimentally quantified inflammatory markers and viruses. However, further research is needed to validate the mechanism and hypothesis of miRNA targeting inflammatory and viral markers of COVID-19.

7. Complementary work: Purinergic signaling elements are correlated with coagulation players in peripheral blood and leukocyte samples from COVID-19 patients

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ORIGINAL ARTICLE

Purinergic signaling elements are correlated with coagulation players in peripheral blood and leukocyte samples from COVID-19 patients

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Abstract

For over a year, the coronavirus disease 2019 has been affecting the world population by causing severe tissue injuries and death in infected people. Adenosine triphosphate (ATP) and the nicotinamide adenine dinucleotide (NAD+) are two molecules that are released into the extracellular microenvironment after direct virus infection or cell death caused by hyper inflammation and coagulopathy. Also, these molecules are well known to participate in multiple pathways and have a pivotal role in the purinergic signaling pathway. Thus, using public datasets available on the Gene Expression Omnibus (GEO), we analyzed raw proteomics data acquired using mass spectrometry (the gold standard method) and raw genomics data from COVID-19 patient samples obtained by microarray. The data was analyzed using bioinformatics and statistical methods according to our objectives. Here, we compared the purinergic profile of the total leukocyte population and evaluated the levels of these soluble biomolecules in the blood, and their correlation with coagulation components in COVID-19 patients, in comparison to healthy people or non-COVID-19 patients. The blood metabolite analysis showed a stage-dependent inosine increase in COVID-19 patients, while the nucleotides ATP and ADP had positive correlations with fibrinogen and other coagulation proteins. Also, ATP, ADP, inosine, and hypoxanthine had positive and negative correlations with clinical features. Regarding leukocyte gene expression, COVID-19 patients showed an upregulation of the *P2RX1*, *P2RX4*, *P2RX5*, *P2RX7*, *P2RY1*, *P2RY12*, *PANX1*, *ADORA2B*, *NLPR3*, and *F3* genes. Yet, the ectoenzymes of the canonical and non-canonical adenosinergic pathway (*ENTPD1* and *CD38*) are upregulated, suggesting that adenosine is produced by both active adenosinergic pathways. Hence, approaches targeting these biomolecules or their specific purinoreceptors and ectoenzymes may attenuate the high inflammatory state and the coagulopathy seen in COVID-19 patients.

Key messages

- Adenosinergic pathways are modulated on leukocytes from COVID-19 patients.
- Plasmatic inosine levels are increased in COVID-19 patients.
- ATP, ADP, AMP, hypoxanthine, and inosine are correlated with coagulation players.
- The nucleotides and nucleosides are correlated with patients' clinical features.
- The P2 receptors and ectoenzymes are correlated with Tissue factor in COVID-19.

Keywords COVID-19 · SARS-CoV-2 · Leukocyte · Purinergic signaling · Inosine

Introduction

Since the end of 2019, the world has been affected by the global pandemic of coronavirus disease 2019 (COVID-19), caused by a new β-virus called severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [1]. So far, more than 228 million people have been infected and 4.6 million have died worldwide (covid19.who.int). This single-stranded RNA virus has a similarity of 79% with the previous SARS-CoV [2], and variants of the new coronavirus

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have already appeared worldwide [3–5]. This virus uses the angiotensin-converting enzyme 2 (ACE2) receptor to enter a wide range of cell types, including alveolar, endothelial, kidney, heart, and brain cells [6–10], with the assistance of the transmembrane protease serine 2 (TMPRSS2) and possibly other proteases [11].

Around 20% of infected people are symptomatic, and the most common manifestations are fever, cough, loss of taste and smell, and fatigue. According to the symptoms, the patients are stratified as having asymptomatic, mild, moderate, or severe COVID-19 [12, 13]. Within days after the viral infection, the severely ill patients develop pneumonia that usually progresses to acute respiratory distress syndrome (ARDS), requiring admission to the intensive unit care (ICU) and supplementation with exogenous oxygen [14]. The ICU patients have alterations in blood biomarkers associated with the severity and progression of the disease, such as elevated levels of circulating D-dimer (> 1 μg/mL) [15–17], prolonged prothrombin time [18], elevated levels of pro-inflammatory cytokines, and chemokines [15, 19], especially IL-6, IL-1β and TNF-α [15, 20–23], C-reactive protein (CRP), lactate dehydrogenase (LDH), and the activation of the *NLRP3* inflammasome [24].

Immunothrombosis is a defensive effector of the innate immune system. However, if uncontrolled, pathological thrombosis can emerge, mainly inside the microvessels [25]. Thrombotic complications are a major cause of morbimortality in COVID-19 patients, although the mechanism remains under investigation [26]. Around 45% of ICU patients present with venous thromboembolic events (VTE) [27]. Post-mortem analysis of COVID-19 patients has revealed the presence of microthrombus in the lung vasculature [6], including in children [28]. Thus, the International Society on Thrombosis and Hemostasis (ISTH) recommended the administration of low molecular weight heparin to COVID-19 patients to attenuate the lethality caused by these coagulopathies [29, 30].

Several research groups, including ours, have been repurposing approved medications [31] or indicating novel molecules and approaches to treat this new disease [10, 32–37]. Several vaccination protocols against COVID-19 are ongoing worldwide, but a satisfactory vaccination rate has not been achieved yet.

Due to viral infection, systemic inflammation, and coagulation disturbance, tissues are constantly damaged, releasing pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), including the extracellular molecule adenosine triphosphate (ATP) [19, 38, 39]. Extracellular ATP acts through paracrine and autocrine signaling pathways, acting as a strong "find me" signal for immune cell recruitment to damaged sites [40]. In physiological conditions, the release of ATP occurs by membrane pore formation or through pannexin channels and connexin hemichannels [41–43]. In the extracellular space, ATP acts as a signaling molecule through the P2 receptor (P2R) family, the subfamily of G protein-coupled metabotropic P2Y ($P2Y_{1,2,4,6,11-14}$), and the ligand-gated ionotropic P2X ($P2X_{1-7}$) [44]. In addition to receptor activation, ATP is hydrolyzed to adenosine diphosphate and monophosphate (ADP and AMP) by the ectonucleoside triphosphate diphosphohydrolase 1 (CD39/*ENTPD1*), and the ecto-5′-nucleotidase (CD73/*NT5E*) converts the AMP to adenosine. Inosine is then formed through adenosine deaminase (ADA) (Fig. 1), completing the canonical adenosinergic pathway. However, there is also a non-canonical adenosinergic pathway that uses the extracellular nicotinamide adenine dinucleotide (NAD^+) molecule. This alternative pathway follows the axis of the CD38/CD203a(*ENPP1*)/CD73/ADA ectoenzymes, which catabolize the NAD^+ into ADP-ribose (ADPR), AMP, adenosine, and inosine, sequentially (Fig. 1) [45].

Adenosine is known as an anti-inflammatory and healing molecule of the purinergic system [46]. It activates the adenosine receptors (P1 receptors) A_1, A_{2A}, A_{2B}, and A_3, encoded by the *ADORA1*, *ADORA2*, *ADORA2A*, *ADORA2B*, and *ADORA3* genes, respectively. In COVID-19, researchers suggest that targeting the adenosine receptors, specifically A_{2A}, may be an alternative to attenuate lung inflammation and thrombotic consequences of the disease [47]. Also, adenosine is metabolized by ADA, resulting in inosine, hypoxanthine, xanthine, and uric acid. Regarding these sequential metabolites, bronchoalveolar lavage fluid (BALF) and blood samples of COVID-19 patients showed alterations in the metabolization of adenosine to inosine [48] and inosine to hypoxanthine [49], indicating the relevant role of purines in the pathophysiology of COVID-19.

Therefore, the main objective of this study was to analyze whether alterations in the purinergic system profile of leukocytes and soluble blood metabolites exist, as well as their relationship with key points of coagulopathy and clinical features manifested by COVID-19 patients. In order to do this, available public datasets were employed to analyze genomic and metabolomic data from peripheral blood samples from patients with and without COVID-19, to assess positive and negative correlations between the purinergic elements, coagulation, and inflammatory players, in order to establish a purinergic profile of COVID-19 patients.

Materials and methods

To analyze the adenine nucleotides (ATP, ADP, AMP), adenosine, and its sequential metabolites (inosine, hypoxanthine, xanthine), two datasets were downloaded and used in the present study. The first dataset, entitled "Large-scale Multi-omic Analysis of COVID-19 Severity," was

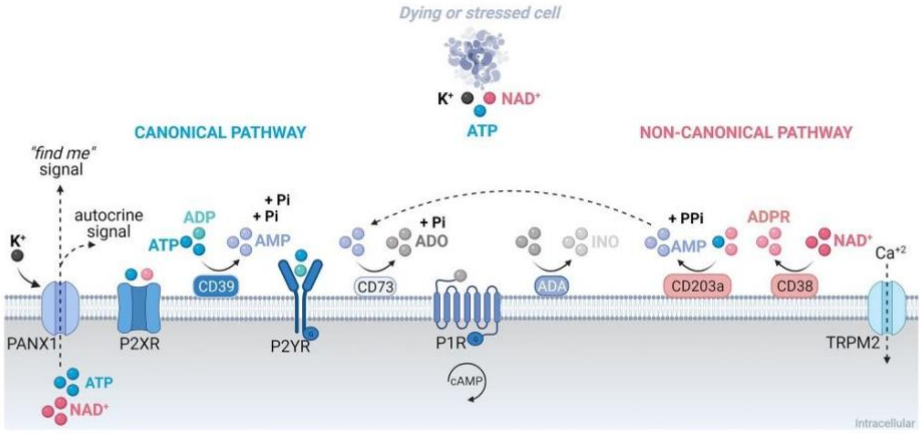

Fig. 1 Purinergic signaling comprises the canonical and non-canonical adenosinergic pathways. The canonical pathway is responsible for the production of the anti-inflammatory biomolecule adenosine (ADO) starting from the adenosine triphosphate (ATP) which is hydrolyzed to adenosine diphosphate (ADP) and adenosine monophosphate (AMP) by the ectoenzymes following the axis CD39/CD73. The non-canonical adenosinergic pathway has the same function of adenosine formation, although this alternative way uses the NAD^+ which is metabolized by the axis CD38/CD203a/CD73. After the adenosine is formed, it can activate the P1 receptors or be metabolized by the adenosine deaminase (ADA) and originate the inosine

generated by Katherine Overmyer et al. in 2020 and is composed of blood samples from 102 COVID-19 and 26 non-COVID-19 patients from Albany Medical Center in Albany, NY, USA [50]. The raw data on nucleotide and nucleoside levels are available and were obtained via GitHub (https://github.com/ijmiller2/COVID-19_Multi-Omics/), and the quantitative measurements of plasma metabolites were analyzed by liquid chromatography coupled to mass spectrometry (LC–MS). From this study, data on ATP, ADP, AMP, and hypoxanthine levels were used, and clinical features were also used to correlate with these biomolecules. The second dataset was provided by Shen et al. [51], and the data on AMP, adenosine, hypoxanthine, inosine, and xanthine levels from 25 healthy, 25 non-COVID-19, 21 severe COVID-19, and 25 non-severe COVID-19 patients are available in Supplemental Information Table S2 of the study entitled "Proteomic and Metabolomic Characterization of COVID-19 Patient Sera" [51].

For Overmyer's study, we also downloaded the GSE157103 dataset from Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE157103), which provided the transcriptomic data for total blood leukocytes from 101 COVID-19 and 149 non-COVID-19 patients. Another two datasets available on the GEO, GSE154998 [52] and GSE160351 [53], were also downloaded to perform the analysis of leukocyte genomic profiles from patients with COVID-19.

To perform the statistical analysis, the Shapiro–Wilk test was applied to determine sample normality; multiple groups were compared using one-way ANOVA and the Tukey post hoc test. Mann–Whitney *U* test, Student's *t*-test, and paired Student's *t*-test were used, as appropriate, implemented using SPSS software (Version 21). Correlations between the levels of biomolecules and clinical features or protein levels were assessed using Pearson's correlation. Graphs were created with GraphPad Prism (Version 7).

Results

Identification of plasma nucleotide and nucleoside levels

To analyze the profile of plasma nucleotides and nucleosides of COVID-19 patients, we used two studies measuring their levels using high-resolution mass spectrometry. This methodology, due to its accurate measurements and high sensitivity, has emerged as an efficient alternative to identify and quantify plasma metabolites even at low concentrations.

Figure 2A shows the levels of ATP, ADP, AMP, and hypoxanthine in both groups, non-COVID-19 and COVID-19, stratified by Overmyer et al. In Fig. 2B, the analysis of these biomolecules shows no statistical significance between the two groups. However, among the biomolecules, the analyses showed a higher correlation between ATP and ADP

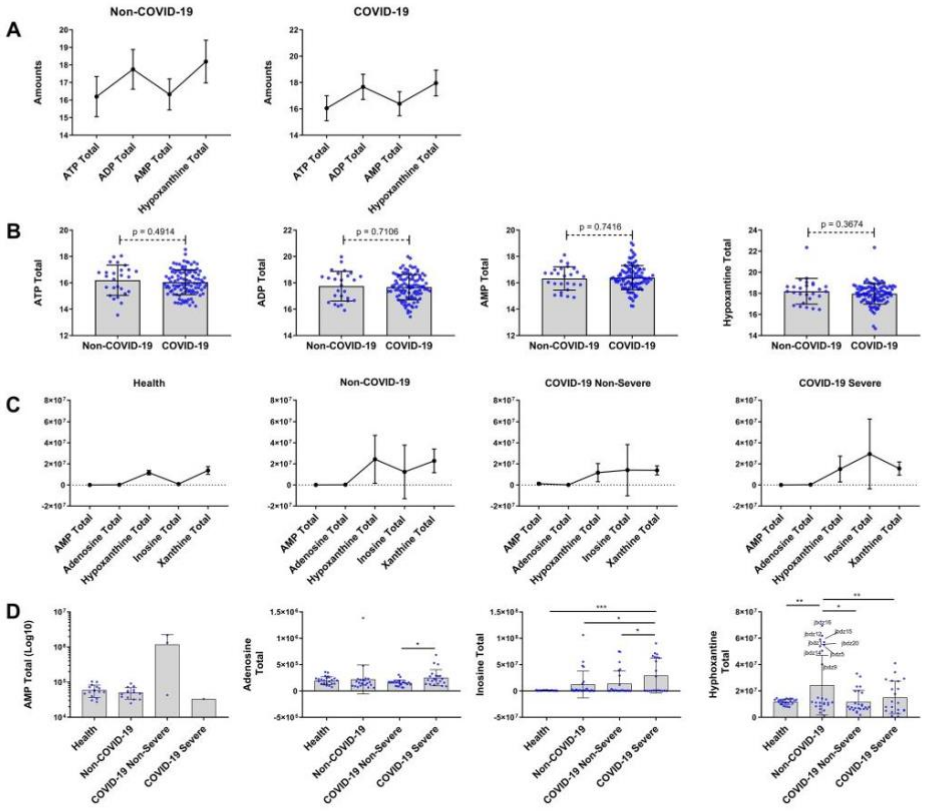

Fig. 2 Nucleotides and nucleosides levels and their comparison among the analyzed groups in each study performed by Overmyer et al. [50] (**A** and **B**) and Shen et al. [51] (**C** and **D**)

($r = 0.931$; $n = 103$; $p < 0.0001$), and with AMP ($r = 0.735$; $n = 103$; $p < 0.0001$), but not with hypoxanthine ($r = -0.003$; $n = 103$; $p = 0.972$).

Using the data available from the study performed by Shen et al., Fig. 2C shows the measurements of AMP, adenosine, hypoxanthine, inosine, and xanthine from four groups, healthy, non-COVID-19, non-severe COVID-19, and severe COVID-19, stratified by the authors of the study. There is no significant difference between the biomolecule levels in each group. On the other hand, when compared between the four groups, as shown in Fig. 2D, the inosine levels were significantly increased in the severe COVID-19 group, when compared to the healthy, non-COVID-19, and non-severe COVID-19 groups (respectively, $p < 0.0001$, $p = 0.0169$; and $p = 0.0327$). For hypoxanthine, it was possible to note that the non-COVID-19 group comprises two distinct clusters. One is composed of eight samples (8/25; 32%), which were responsible for the significant increase in the amounts of hypoxanthine when compared with the healthy group ($p = 0.0016$), non-severe COVID-19 ($p = 0.0015$), and severe COVID-19 ($p = 0.0253$) groups. The other cluster was composed of 17 samples (17/25; 68%) and shows no significant difference from the other groups.

Correlations of clinical features with purine nucleotide and nucleoside levels

Considering that purine biomolecules are associated with blood coagulation, platelet activation, and leukocytes

chemotaxis, we analyzed whether they could be associated with the different stages or clinical features of COVID-19 patients.

In Table 1, we show the correlations between purine biomolecules and clinical features presented by the patients of each group. Using the data from Shen et al., inosine showed negative correlations with white blood cells (WBC), monocyte count, and platelet count in the non-severe COVID-19 group. In the severe COVID-19 group, the levels of inosine showed a negative correlation with monocyte count. Adenosine showed a positive correlation with platelet count in the non-COVID-19 group, while hypoxanthine showed a negative correlation in the non-severe COVID-19 group.

Aiming to analyze the relationship between the purines and coagulation system components, and two severity and mortality scores, we used the metabolomics data from a study by Overmyer et al. This data regarding the amounts of ATP, ADP, and AMP was compared with the information on fibrinogen, Acute Physiology and Chronic Health Evaluation II (APACHE II) and Sequential Organ Failure Assessment (SOFA), C-reactive protein, and D-dimer from both groups (Table 1). Positive correlations between ATP × fibrinogen and ADP × fibrinogen are presented by both groups. The correlation of AMP × fibrinogen is presented only by the non-COVID-19 group. ATP and ADP presented a negative correlation with SOFA in the non-COVID-19 group. Also, in the non-COVID-19 group, ATP and ADP presented a negative correlation with APACHE II. Hypoxanthine showed a positive correlation with APACHE II in both groups. The samples of the COVID-19 group showed positive correlations between ATP and AMP and C-reactive protein, and the hypoxanthine levels showed a positive correlation with D-dimer levels. It is important to note that Overmyer et al., unlike Shen et al., stratified the patients in their study into only two groups and both are comprised of patients admitted to the hospital with COVID-19 symptoms. The difference between them is that the COVID-19 groups are those patients with molecular diagnosis real-time quantitative polymerase chain reaction (RT-qPCR) positive for SARS-CoV-2 infection.

There were positive correlations between inosine and coagulation metabolites such as fibrinopeptide A516 ($n=19$; $p=0.0291$; $r=0.5005$) and two other peptides derived from fibrinopeptide A, ADSGEGDFXAEGGGVR ($n=14$; $p=0.0272$; $r=0.5873$) and DSGEGDFXAEGGGVR ($n=22$; $p=0.0158$; $r=0.5081$), in the non-severe COVID-19 group.

Table 1 Correlation of purine biomolecules with clinical features presented by the patients of each group in their respective study

Overmyer data		**ATP**	**ADP**	**AMP**	**Hypoxanthine**
Fibrinogen	Non-COVID-19	0.618* ($n=14$)	0.632*** ($n=14$)		
	COVID-19	0.358** ($n=81$)	0.410*** ($n=81$)	0.328* ($n=81$)	
SOFA	Non-COVID-19	-0.593* ($n=17$)	-0.495* ($n=17$)		
	COVID-19				
APACHE II	Non-COVID-19	-0.643* ($n=17$)	-0.601* ($n=17$)		0.598* ($n=17$)
	COVID-19				0.277* ($n=58$)
CRP	Non-COVID-19				
	COVID-19	0.206* ($n=94$)		0.251* ($n=94$)	
D-dimer	Non-COVID-19				
	COVID-19				0.317* ($n=87$)
Shen data		**Adenosine**	**Inosine**	**Hypoxanthine**	**Xanthine**
Platelet count ($\times 10^9$/L)	Non-COVID-19	0.443* ($n=22$)			
	COVID-19_Non-Severe		-0.5121* ($n=24$)		
	COVID-19_Severe				
WBC count ($\times 10^9$/L)	Non-COVID-19				
	COVID-19_Non-Severe		-0.409* ($n=25$)		
	COVID-19_Severe				
Monocyte count ($\times 10^9$/L)	Non-COVID-19				
	COVID-19_Non-Severe		-0.459* ($n=25$)	-0.466* ($n=25$)	
	COVID-19_Severe				
Lymphocyte count ($\times 10^9$/L)	Non-COVID-19				
	COVID-19_Non-Severe				
	COVID-19_Severe				

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

In addition, Overmyer et al. [50] present in their study a representative table with Kendall Tau correlation coefficients between total ATP and total ADP with peripheral blood metabolites. These correlations show that the nucleotides may be involved in blood coagulation and, therefore, we adapted and represented their results in Table S1 of the current study. Interestingly, we highlight that, among the molecules with a significant correlation with the nucleotides, there are molecules directly linked to the function of platelets, the major players in blood coagulation, such as ITGA2B, TUBB1, ITGB3, and PLEK.

Correlations of circulating immune cells with purine nucleotide and nucleoside levels

Another important question to investigate, regarding the modulation of plasma nucleotide and nucleoside levels, is the profile of the purine ectoenzymes and purinoreceptors on the peripheral blood immune cells.

Using transcriptomics data from the total leukocyte population, published by Overmyer et al. and available on GEO database (code GSE157103), we analyzed the expression of the purinergic system elements in the immune cells, as shown in Table 2, and their correlations with plasma levels of ATP, ADP, AMP, and hypoxanthine. In the study by Overmyer, there is no information about adenosine levels. As shown in Table S2, no correlation was observed between extracellular ATP, ADP, AMP, and hypoxanthine levels and the expression of ENTPD or ENPP ectoenzymes in leukocyte samples from COVID-19 patients. These results suggest that the catalysis of these biomolecules could be performed by other cells or by other ectoenzymes. Another point to consider is that it is possible that there is involvement and modulation of adenosine kinase (*ADK*) activity and the equilibrative (*SLC28A1*, *SLC28A2*, and *SLC28A3*) and even the concentrative (*SLC29A1*, *SLC29A2*, *SLC29A3*, and *SLC29A4*) adenosine transporters [54, 55]. Indeed, adenosine could be produced by catalysis of the reversible hydrolysis of S-adenosylhomocysteine (SAH) by S-adenosylhomocysteine hydrolase (SAHH), which is encoded by the *AHCY* gene [56].

Considering the last hypothesis, in addition to the investigation of the CD39 (*ENTPD1*)/CD73 (*NT5E*)/ADA axis, we also looked at the expression of the ectonucleotidases that comprise the non-canonical adenosinergic pathway. This non-canonical pathway comprises the *CD38*/CD203a (*ENPP1*)/CD73/*ADA* ectoenzymes, which catabolize the extracellular NAD^+ into ADPR by CD38, followed by ADPR hydrolysis to AMP by CD203a, with sequential production of adenosine by CD73.

The leukocyte blood samples from COVID-19 patients showed a significant upregulation of *ENTPD1* (55.64 ± 2.69; $n = 100$ vs 37.79 ± 4.592; $n = 26$; $p = 0.0017$), *CD38* (7.021 ± 0.6108; $n = 100$ vs 1.458 ± 0.1702; $n = 26$; $p < 0.0001$), and *ADA* (15.3 ± 1.135; $n = 100$ vs 10.54 ± 1.422; $n = 26$; $p = 0.0113$) ectoenzymes, when compared with non-COVID-19 samples, therefore suggesting that both canonical and non-canonical pathways may be activated in these leukocytes. *ADA* showed a relevant correlation with *CD38* ($r = 0.656$; $p < 0.0001$), but not with *ENTPD1* ($r = -0.289$; $p = 0.004$). A strong correlation with *ADA* was observed for *ENTPD6* ($r = 0.897$; $p < 0.0001$) and with the concentrative nucleoside transporter *SLC29A3* ($r = 0.853$; $p < 0.0001$). Also, intermediate significant correlations ($r > 0.600$) were revealed between *ADA* and the *DPP4*, *AHCY*, *GSDMB*, *ENTPD5*, *P2RY6*, *P2RY8* (the function of which is unknown in humans), *P2RY10*, *P2RY11*, *ENPP5*, *P2RX4*, *NT5C*, *NT5C3B*, *SLC29A1*, and *SLC29A2* genes. A further important correlation was observed between *CD38* and the pannexin channel *PANX1* ($r = 0.730$; $p < 0.0001$), while *ENTPD1* showed a weak correlation with *PANX1* ($r = 0.302$; $p = 0.002$).

Interestingly, no modulation of CD73 (*NT5E*) expression was observed. Also, the expression of CD203a (*ENPP1*) showed no modulation, while significant upregulations of *ENPP4* (4.953 ± 0.3185; $n = 100$ vs 2.369 ± 0.27; $n = 26$; $p < 0.0001$) and *ENPP5* (0.7706 ± 0.08098; $n = 99$ vs 0.3904 ± 0.0728; $n = 26$; $p = 0.021$) were observed. However, it is important to highlight that, in these analyzed leukocyte samples, the expression of *ENPP1* was about 10, 4, 70, and 11 times lower than expression of *ENPP2*, *ENPP3*, *ENPP4*, and *ENPP5*, respectively.

Indeed, no difference was observed in *ADK* or *AHCY* expression, but there were significant increases in the levels of *AHCY* isoforms *AHCYL1* (22.95 ± 0.7359; $n = 100$ vs 18.28 ± 0.9089; $n = 26$; $p = 0.0026$) and *AHCYL2* (3.154 ± 0.1975; $n = 100$ vs 1.953 ± 0.2109; $n = 26$; $p = 0.0035$) in samples from COVID-19 patients.

Considering the high levels of inosine, we also investigated the expression of the purine nucleoside phosphorylase (*PNP*) enzyme. As expected, the expression of this enzyme was higher in COVID-19 patients than in non-COVID-19 patients (respectively, 70.01 ± 2.64; $n = 100$ vs 50.18 ± 4.36; $n = 27$; $p = 0.001$).

The tissue factor expression (encoded by the *F3* gene) was also analyzed in leukocytes from the GSE157103 in the COVID-19 and non-COVID-19 groups, due to its essential role in blood coagulation. As shown in Fig. 3, higher expression ($p = 0.0004$) of this gene was observed in COVID-19 patients.

Analyzing the data available in the GSE154998 dataset, which is composed of COVID-19 ($n = 7$) and non-COVID-19 ICU patients ($n = 7$) [52], no significant difference was found for the purinergic enzymes or receptors (Table 2).

Table 2 Relative expression of purinergic system components in total leukocytes with statistical differences between non-COVID-19 and COVID-19 patients

Gene	GSE157103 PMID: 33096026 Sample: leukocytes from whole blood Non_COVID ($n = 26$) vs COVID ($n = 100$)	GSE154998 PMID: 33306162 Sample: buffy coat cells Non_COVID ($n = 7$) vs COVID ($n = 7$)	GSE160351 PMID: 33208929 Sample: peripheral monocytes + CD14 Health ($n = 3$) vs COVID ($n = 6$)
ADORA1	Ø	ND	Ø
ADORA2A	Ø	Ø	Ø
ADORA2B	↑	Ø	↑
ADORA3	Ø	Ø	Ø
ENPP1	Ø	ND	Ø
ENPP2	Ø	Ø	Ø
ENPP3	Ø	Ø	Ø
ENPP4	↑	Ø	Ø
ENPP5	↑	Ø	Ø
ENPP6	Ø	ND	Ø
ENPP7	Ø	ND	ND
ENTPD1	↑	Ø	↓
ENTPD2	Ø	ND	Ø
ENTPD3	ND	ND	Ø
ENTPD4	↑	Ø	Ø
ENTPD5	↑	Ø	Ø
ENTPD6	Ø	Ø	Ø
ENTPD7	Ø	Ø	Ø
ENTPD8	Ø	ND	Ø
NT5E	Ø	Ø	Ø
P2RX1	↑	Ø	Ø
P2RX2	ND	ND	Ø
P2RX3	ND	ND	Ø
P2RX4	Ø	Ø	↑
P2RX5	↑	Ø	↓
P2RX6	ND	ND	Ø
P2RX7	↑	Ø	Ø
P2RY1	↑	Ø	↑
P2RY2	Ø	ND	Ø
P2RY4	ND	Ø	Ø
P2RY5	ND	ND	Ø
P2RY6	Ø	Ø	Ø
P2RY8	Ø	Ø	Ø
P2RY10	Ø	Ø	Ø
P2RY11	ND	ND	Ø
P2RY12	↑	Ø	Ø
P2RY13	Ø	Ø	↓
P2RY14	Ø	Ø	Ø
PANX1	↑	Ø	Ø
ADA	↑	Ø	Ø
DPP4	↑	Ø	Ø
CD38	↑	Ø	Ø
SLC28A1	NA	NA	Ø
SLC28A2	↑	NA	Ø
SLC28A3	↑	Ø	↓

Table 2 (continued)

Gene	GSE157103 PMID: 33096026 Sample: leukocytes from whole blood Non_COVID ($n=26$) vs COVID ($n=100$)	GSE154998 PMID: 33306162 Sample: buffy coat cells Non_COVID ($n=7$) vs COVID ($n=7$)	GSE160351 PMID: 33208929 Sample: peripheral monocytes + CD14 Health ($n=3$) vs COVID ($n=6$)
SLC29A1	Ø	Ø	Ø
SLC29A2	Ø	NA	Ø
SLC29A3	Ø	Ø	Ø
SLC29A4	↑	NA	Ø
ADK	Ø	Ø	Ø
AHCY	Ø	Ø	↑
AHCYL1	↑	Ø	Ø
AHCYL2	↑	Ø	↓
NLRP3	↑	Ø	Ø
GSDMA	Ø	NA	Ø
GSDMB	↑	Ø	Ø
GSDMC	↓	Ø	NA
GSDMD	Ø	Ø	NA
GSDME	Ø	NA	NA
TRPM1	NA	NA	Ø
TRPM2	↓	Ø	Ø
TRPM3	NA	NA	Ø
TRPM4	↓	Ø	Ø
TRPM5	NA	NA	Ø
TRPM6	↑	Ø	Ø
TRPM7	↑	Ø	Ø
TRPM8	NA	NA	Ø

↑ gene upregulated, ↓ gene downregulated, *NA* not available, *ND* not detected, *Ø* no gene expression variation

In the GSE160351 dataset, data obtained from monocyte samples of COVID-19 patients ($n=6$) and healthy controls ($n=3$) was analyzed regarding the purinergic profile, as shown in Table 2. In this dataset, there was a higher expression of *P2RX4* ($p<0.001$), *P2RY1* ($p=0.0175$), *ADORA2B* ($p=0.043$), and *AHCY* ($p<0.001$) in the COVID-19 group. On the other hand, there was reduction of expression of the *P2RY13* ($p=0.0157$), *P2RX5* ($p=0.0389$), *ENTPD1* ($p<0.001$), *ENTPD4* ($p=0.0146$), *SLC29A3* ($p=0.0204$), and *AHCYL2* ($p=0.0021$) genes.

Discussion

In recent years, the use of bioinformatic approaches has increased and is now recognized as important tool in scientific research. According to the current literature, bioinformatic analysis of multi-omics data has the advantage of building networks in a faster and more integrative way, making protein–protein or protein-gene links, and providing more comprehensive insights about possible mechanistic pathways. Thus, combining data from multiple datasets is an alternative and reliable way to expand these connections, obtaining data from different layers and finding answers in a time- and money-saving way, which is necessary in the setting of the COVID-19 pandemic. It is important to highlight that the present paper is not based on prediction analysis. We performed our analysis entirely based on the raw proteomics data collected by mass spectrometry (the gold standard method) and raw genomics data obtained by microarray from COVID-19 patients' samples.

Multiple pathways show alterations in COVID-19 patients [57], including a prominent difference in purine metabolism, identified through targeted and untargeted metabolomics studies [49, 58]. After infection with SARS-CoV-2, cells expressing the ACE2 receptor are infected and COVID-19 develops, damaging the tissue and releasing a range of molecules throughout the body. These

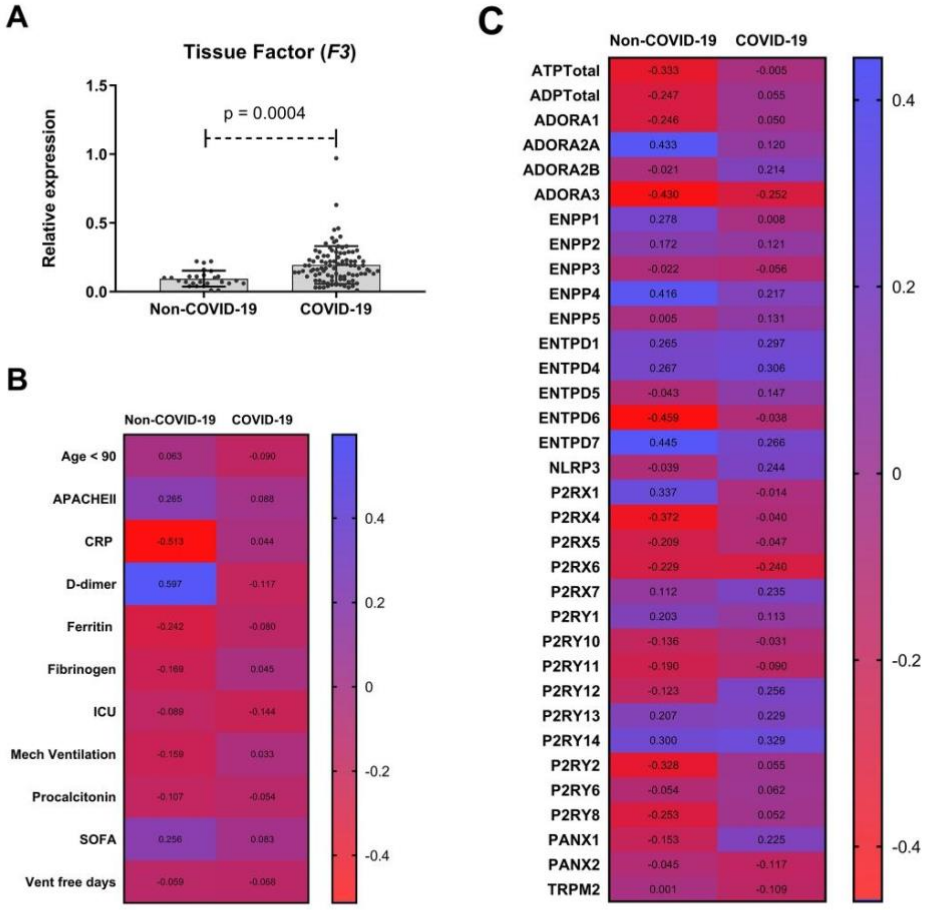

Fig. 3 Relative expression of tissue factor (*F3* gene) in the population of total leukocytes in the two groups of non-COVID-19 and COVID-19 patients (**A**) and its correlation with available clinical, metabolomics (**B**), and transcriptomics features (**C**). The intensity of the colors is proportional to the positive (blue) and negative (red) correlation coefficient

damage-associated molecular patterns (DAMPs) include ATP, NAD^+, and K^+, which have a high inflammatory potential and are important players in purinergic signaling (Fig. 4).

Correlations between nucleotide and nucleoside levels and clinical features

The high inflammatory state of COVID-19 promotes platelet activation and thromboinflammation [26, 59–63]. In this study, we focused on the analysis of the nucleotides/nucleosides ATP, ADP, AMP, adenosine, inosine, hypoxanthine, and xanthine present in the peripheral blood. We also focused on their correlations with other elements of blood coagulation, such as fibrinogen, fibrinopeptides, and coagulation cascade components. These correlations may lead to helpful biomarkers for predicting disease severity, improving the clinical management of infected patients and providing more information about the pathogenesis of hypercoagulability present in COVID-19 patients.

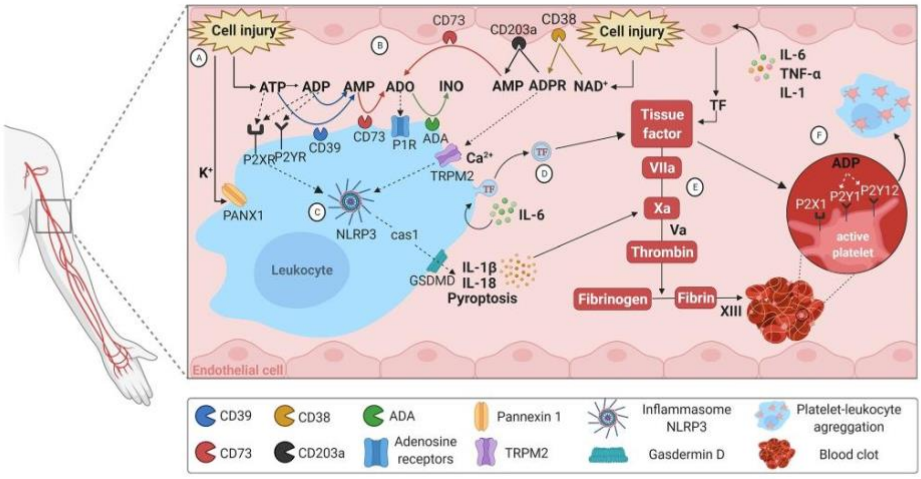

Fig. 4 **a** Direct viral infection of the host cell by Sars-CoV-2 or cell injury caused by thrombosis and inflammation release damage molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) to the extracellular microenvironment. **b** Among the DAMPs released, the adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NAD^+) play a pivotal role as signaling molecules in the purinergic system. **c** The activation of the P2X7 receptor by ATP is the main way of activation of the NLRP3 inflammasome. Other pathways may also perform this activity, such as ADP-ribose and the increase of intracellular Ca^{2+} levels due to the TRPM2 channel, activated by ADPR. The NLRP3 inflammasome activates caspase 1 that cleaves pro-IL1β and IL-18, releasing these cytokines resulting in pyroptosis cell death. **d** The tissue factor (F3) gene upregulation by the immune cells is directly linked to circulating levels of extracellular vesicles containing TF (EV-TF). **e** This high circulating TF stimulates the platelets, leading to the formation of blood clots. **f** Also, high levels and upregulation of TF promote platelet-leukocytes aggregation causing the immunothrombosis

We showed negative correlations between inosine and platelet, WBC, and monocyte counts in the non-severe COVID-19 group. The patients from this group usually recover from the disease, and, for that, these negative correlations could be explained by the immunothrombosis [25]. Also, the increased levels of inosine could be caused by the body's attempt to resolve the immunothrombosis, acting on immune cells and fighting against the excessive inflammation. These actions of inosine have been previously reported, but the mechanism of action remains unclear [64–66]. Recently, Xiao et al. showed, through an integrated study of metabolomics (targeted and untargeted) and cytokine/chemokine profiling, the relationship between the cytokines and metabolites present in samples from COVID-19 patients. Their analysis demonstrated a reprogramming of immunometabolism where, after targeting elements of pathways including purine metabolism, pro-inflammatory cytokine release is altered in PBMCs. They also showed that purine levels increase gradually over time and then decrease in the late phases of the disease in follow-up of hospitalized patients with mild disease (4–36 days after symptom onset). Moreover, when the different groups were analyzed, there was an increase in adenosine, hypoxanthine, and xanthine in the severe group of patients compared to healthy volunteers [58].

A review published by Sliva et al. discussed the antiviral and immunomodulatory actions of isoprinosine (inosine pranobex; IPNX) against multiple diseases and infections. Various effects of IPNX have been suggested, such as the blockage of viral RNA transcription, production of cytokines, differentiation and expansion of T cells, and modulation of NK cells, enhancing their activity [64]. Therefore, inosine has been evaluated in clinical trials as an alternative to prevent or treat COVID-19. At the time of submission of this paper, there are three registered phase III clinical trials, two using isoprinosine (NCT04383717 and NCT04360122) as prophylaxis and one using Molixan (NCT04780672) as a treatment measure. Yet, the preliminary clinical use of inosine pranobex significantly decreases the mortality and infection rates caused by SARS-CoV-2 in elderly patients from three different locations in the Czech Republic [67].

In the COVID-19 group, our results showed positive correlations of ATP and AMP with CRP, a clinical marker for inflammation in peripheral blood with pleiotropic function. Considering that the production of CRP occurs mainly in the liver in response to elevated circulating levels of IL-1β

and IL-6 [68], we suggest that ATP and AMP may trigger the release of IL-1β, resulting in P2 receptor and inflammasome *NLRP3* activation, in a similar mechanism to that demonstrated by Bian et al. [69].

Regarding ATP, ADP, AMP and hypoxanthine, our results suggest that these molecules can activate blood coagulation, considering their positive correlations with the protein necessary for clot formation, fibrinogen, and the protein originating from blood clot lysis, the D-dimer. Yet, severe COVID-19 patients show critical hypoxemia, possibly due to vessel occlusion by these clots. The levels of hypoxemia could be measured using the circulating amounts of plasma hypoxanthine as a point-of-care measurement, as mentioned in the literature [70]. Levels of hypoxanthine and its oxidized metabolite xanthine are indeed present in higher concentrations in COVID-19 patients' peripheral blood circulation, as recently shown by other metabolomics studies. Danlos et al. and Páez-Franco et al. reported statistical analysis that confirms alterations in purine metabolism [71, 72]. Another purine metabolite that could be used as a biomarker to investigate ischemic events, due to its long blood half-life, is the precursor of hypoxanthine, inosine (see below).

Purinergic profile of circulating immune cells

In physiological conditions, the crosstalk between circulating metabolites and cells is essential for the maintenance of metabolism. In COVID-19, due to multiple factors, the cells are damaged and release DAMPs and PAMPs into the extracellular microenvironment. These components are recognized by immune cells, which initiate signaling pathways that aim to clear the infection. In our three analyzed datasets from the GEO database, we have different immune cell populations: total leukocytes, buffy coat population (WBC + platelets), and CD14 + monocytes. For the latter, the literature reports that BALF and peripheral blood single-cell RNA sequencing analysis from severe COVID-19 patients presented an expansion of the CD14 + monocyte population when compared to mild cases or healthy controls [73–75]. Moreover, Wilk et al. created the *cellxgene* application, confirming that there is an increase in CD14 + monocytes, with high inflammatory gene expression [76]. Therefore, we can conclude that our three analyzed leukocyte populations are mostly comprised of CD14 + monocytes.

In Fig. 4, we schematically show platelet activation and leukocyte-platelet aggregation by autocrine and paracrine biomolecules, such as ADP, through the specific receptors P2Y1 and P2Y12 [25, 77]. In addition, it highlights the upregulation of monocyte tissue factor as an important event in starting the coagulation cascade [78]. In accordance, our analysis showed the upregulation of TF in the COVID-19 group. Consequently, this upregulation promotes an elevation of extracellular vesicles with circulating TF (EV-TF), the levels of which positively correlate with clinical features, including leukocyte levels and COVID-19 stages [79]. High levels of these EV-TFs also show positive correlations with other coagulation proteins such as fibrinogen, D-dimer, and Von Willebrand factor [80]. Moreover, our analyzed datasets showed an upregulation of *P2RY1* and *P2RY12* on leukocytes, and the heatmap showed their correlation with TF levels (Fig. 3C). Therefore, the inhibition of the interaction between ADP and the P2Y receptors, as suggested by recent studies [26, 63], could indeed decrease TF expression, EV-TF circulation, and levels of platelet-leukocyte and platelet-platelet conjugates, consequently reducing the risk of immunothrombosis development in COVID-19.

Extracellular ATP and NAD^+ levels can be enhanced by the pannexin 1 channel, encoded by the *PANX1* gene [81]. This channel is located on the plasma membrane surface, allowing the leakage of small biomolecules such as ATP, NAD^+, PGE_2 and glutamate upon physiological stimuli, as increase in intracellular calcium (Ca^{2+}_i) [82] extracellular K^+ [43], activation by TNF-α [83]; or mechanical stimuli, as plasma membrane stretch. The ATP released into the extracellular microenvironment acts as a "danger signal" and promotes migration of leukocytes, especially phagocytes, to control the inflammation and clear cell and pathogen debris [83, 84]. For example, macrophages release ATP by exocytosis and through pannexin channels in response to viral infections [85]. Thus, the prospect of targeting this channel as a therapy for fighting COVID-19 was recently raised and discussed by Swayne et al. [86]. In accordance, our results showed higher expression of the pannexin 1 channel in COVID-19 patients. Therefore, this approach could be a good avenue to be investigated, in an attempt to avoid excessive leakage of ATP and subsequent immune cell infiltration into the lungs, the primary site of infection.

Among the P2X subfamily, the P2X7 receptor (encoded by the *P2RX7* gene) is one of the most studied receptors in inflammation and immunity [87]. It is specialized in detecting high levels of eATP [88, 89]. The P2X7 receptor is significantly expressed in alveolar cell type I [90]; therefore, these cells are able to receive the ATP released by the alveolar cell type II, the main cell type infected by SARS-CoV-2, and become activated. The activation of the P2X7 receptor may have ambiguous functions; it can decrease viral replication and infection, but, if uncontrolled, it also can boost inflammation and may potentially contribute to an exacerbated immune response, depending on the virulence of the pathogen and severity of the infection [91]. To date, the P2X7 receptor has been shown to be a strong activator of the NLRP3 inflammasome and therefore of caspase-1 cleavage and release of mature IL-1β and IL-18 [87]. In addition, the notable involvement of the inflammasome in COVID-19 has been recently discussed in multiple reports [24, 87, 92–96].

Yet, the P2X7 receptor has been suggested to be associated with inflammation and coagulation, since its stimulation of macrophage and dendritic cells upregulates the expression and release of microvesicles containing tissue factor, thus producing a pro-thrombotic response, as discussed above [97, 98]. Genetic depletion or pharmacologic blockade of the P2X7 receptor improved the outcome of animals with acute respiratory distress syndrome. One explanation for this could be a reduction in inflammatory markers such as IL-6 and IL-1β, as well as the reduction of neutrophil infiltration into the lungs.

Recently, Klaver and Thurnher reviewed the influence of P2Y receptors on inflammatory processes of monocytes and macrophages in physiological settings and infectious diseases including COVID-19 [99]. Although we did not see any significant modulation of *P2YR14*, another study has suggested that by targeting this receptor, neutrophilia and NETosis formation, an important event in immunothrombosis, could be attenuated, minimizing thrombotic complications of COVID-19 [100].

As our results showed higher expression of *CD38* and its positive correlation with *P2RX7* in COVID-19 samples, we suggest that ADPR may participate in the activation of this purine receptor. ADPR is a biomolecule that comes from the metabolization of NAD^+ by *CD38* and can activate the P2X7 receptor, although in a weaker manner than ATP [43, 101]. The TRPM2 is a Ca^{2+} permeable, non-selective cation channel, which is activated by ADPR, temperature, oxidative stress, and Ca^{2+} levels [102], and works as a controller of chemotaxis of neutrophils, macrophages, and DCs to infection sites [103, 104]. In a recent review, Wang et al. discussed the role of TRPM2 in NLRP3 inflammasome activation and demonstrated an interplay of Ca^{2+} influx, reactive oxygen species production, and, consequently, the activation of the NLRP3 inflammasome [105]. Thus, as the *TRPM2* is downregulated, the activation of the inflammasome NLRP3 may not be through this axis and reinforce that the major activation of NLRP3 is via P2X7 activation, although further studies are still necessary.

As discussed above, COVID-19 patients have high levels of inosine in the peripheral blood circulation in a disease severity-dependent manner. One reason for this could be the longer half-life of this biomolecule compared to other nucleotides and nucleosides. The blood half-life of inosine is around 15 h, compared to the blood half-life of adenosine of around 10 s [106]. Considering the upregulation of two ectoenzymes involved in the adenosinergic pathway (*ENTPD1* and *CD38*) and the low half-life time of adenosine, we may surmise that the levels of adenosine are higher than those quantified by the studies we analyzed. Furthermore, in our analysis, we show the following: (1) a strong correlation between *CD38* and *PANX1* levels ($r = 0.730$; $p < 0.0001$); (2) a weak correlation between *ENTPD1* and *PANX1* levels ($r = 0.302$; $p = 0.002$); and (3) upregulation of *ADA* and its correlation with *CD38*, but not with *ENTPD1*. Thus, taken altogether, it is possible to suggest that NAD^+ is released by the pannexin 1 channel at higher levels than ATP. These higher amounts of NAD^+ are metabolized by *CD38*, following the non-canonical adenosinergic axis, until originate adenosine and sequentially inosine by upregulated *ADA* (Fig. 1).

Additionally, as mentioned before, inosine is present at high circulating levels, associated with its long half-life and the upregulation of the PNP enzyme that is responsible for the degradation of inosine into hypoxanthine. Inosine also participates in the nucleotide salvage pathway, leading us to hypothesize that the excessive levels of inosine act to boost the generation of ATP and ADP in the immune cells, releasing these nucleotides into the extracellular space and causing a signaling loop and chemotaxis of more immune cells to the inflammation site, and initiating platelet aggregation through the stimulation of specific receptors.

Reports are suggesting that targeting the adenosine pathway could help to treat COVID-19 [93, 107]. Abouelkhair raised the hypothesis that the modulation of CD39, CD73, and A2AR could be a good therapeutic option for treating COVID-19. The author suggested the use of anti-CD39 and anti-CD73 monoclonal antibodies to avoid eATP hydrolysis by the ectoenzymes, maintaining the nucleotide at high levels in the microenvironment, and consequently maintaining IFN-I production, resulting in an "antiviral state." Another suggestion was the inhibition of A2AR (*ADORA2A*) using a receptor antagonist to avoid the immunosuppressive effect of adenosine in the immune cells [108]. However, as we show, the non-canonical adenosinergic pathway is also activated. Therefore, the approach proposed could help, but it is not the only or best alternative, as the analysis did not show an upregulation of A2AR and CD73, but only of CD39 (*ENTPD1*) expression.

Lastly, Arunachalam et al. analyzed blood samples from COVID-19 patients from two different cohorts and compared them to healthy controls. The authors split the total leukocyte population into clusters according to specific features and analyzed the differentially expressed genes in each cluster, compared with all other cells. Multiple cell clusters from COVID-19 patients, when compared to the same cell cluster from healthy individuals, showed an upregulation of *CD38*, *ENTPD1*, *GSDMD*, *P2RX5*, *NT5C*, *NT5C2*, and *NT5C3A*. However, *P2RY13*, *NLRP3*, and *NT5C* were found to be downregulated in three different clusters of monocytes and one cluster of T cells [109]. These findings corroborate some of our own, for example, the upregulation of *CD38* and *ENTPD1* in the total leukocyte population.

Although these findings open new avenues for understanding the mechanisms underlying the role of purinergic

signaling in the main physiological disturbances associated with the severity of COVID-19, many questions are still open. Therefore, the main limitations of our study are: (1) The datasets used have complementary information, so each dataset does not have all the data needed to assess all correlations; (2) as the blood samples were collected by the authors from other studies and we used the publicly-available raw data, we could not control which medications the patients were taking at the time of sample collection, which may act as a bias in our correlation analysis; (3) none of the analyzed public datasets have the quantification of NAD^+ and ADPR, which makes it impossible to correlate these parameters with the clinical data or the other nucleotides and nucleosides. In addition, we must consider the low stability of ATP, AMP, ADP, and adenosine, which means that the pre-analytical handling of the samples could interfere in the metabolite quantification, for example due to hemolysis causing the release of intracellular nucleotides; and (4) despite our analysis being fully based on patient data, it was based on a general population of leukocytes, with only one dataset showing data from a specific cell type (CD14 + monocytes). Thus, further studies are still required to fully understand the mechanism of action of each biomolecule, their interplay, and the signaling pathways involved with each specific cell type.

In conclusion, in this study, we explored the nucleotides and nucleosides, purinoreceptors, and ectoenzymes of the canonical and noncanonical adenosinergic pathway in the blood and immune cells of COVID-19 patients. The main finding of our study is that inosine levels are increased in COVID-19, in a severity-dependent manner. Inosine levels are associated with the levels of the pannexin 1 channel, ATP, NAD^+, and the ectoenzymes CD38, CD39, and ADA. As discussed, ATP, ADP AMP, inosine, hypoxanthine, purinoreceptors, and ectoenzymes play roles in the disturbances of inflammation and coagulation present in COVID-19. Therefore, approaches targeting these biomolecules or their specific purinoreceptors and ectoenzymes may attenuate the high inflammatory state and coagulopathy seen in COVID-19 patients.

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Availability of data and material All data are available in the main text or the supplementary materials.

Code availability Not applicable.

Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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8. Complementary work: A Systematic Review of the Role of Purinergic Signalling Pathway in the Treatment of COVID-19

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Review

A Systematic Review of the Role of Purinergic Signalling Pathway in the Treatment of COVID-19

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Abstract: The coronavirus disease 2019 (COVID-19) pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has become a global health concern. Three years since its origin, despite the approval of vaccines and specific treatments against this new coronavirus, there are still high rates of infection, hospitalization, and mortality in some countries. COVID-19 is characterised by a high inflammatory state and coagulation disturbances that may be linked to purinergic signalling molecules such as adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine (ADO), and purinergic receptors (P1 and P2). These nucleotides/nucleosides play important roles in cellular processes, such as immunomodulation, blood clot formation, and vasodilation, which are affected during SARS-CoV-2 infection. Therefore, drugs targeting this purinergic pathway, currently used for other pathologies, are being evaluated in preclinical and clinical trials for COVID-19. In this review, we focus on the potential of these drugs to control the release, degradation, and reuptake of these extracellular nucleotides and nucleosides to treat COVID-19. Drugs targeting the P1 receptors could have therapeutic efficacy due to their capacity to modulate the cytokine storm and the immune response. Those acting in P2X7, which is linked to NLRP3 inflammasome activation, are also valuable candidates as they can reduce the release of pro-inflammatory cytokines. However, according to the available preclinical and clinical data, the most promising medications to be used for COVID-19 treatment are those that modulate platelets behaviour and blood coagulation factors, mainly through the P2Y12 receptor.

Keywords: COVID-19; SARS-CoV-2; purinergic signalling; purinergic receptors

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1. Introduction

In December 2019, a new single-stranded RNA virus from the Coronaviridae family emerged. It was named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and is the aetiological pathogen of coronavirus disease 2019 (COVID-19). Due to non-existent immunity at the time, the new virus quickly spread worldwide, progressing to a pandemic, as declared by the World Health Organization on 11 March 2020 [1]. Since 2021, vaccines and medications against the original form of SARS-CoV-2 have been approved, although their distribution has not been equal among countries. On the one hand, the United States has approved multiple vaccines and medications such as tocilizumab, remdesivir, and baricitinib. On the other hand, in low-income countries, vaccination protocols have been affected by the lack of the immunisation products, resulting in higher mortality rates.

SARS-CoV-2 enters a person through the upper respiratory tract. Infection of the host cell mainly occurs by the viral spike protein binding to the receptor-binding domain (RBD) of membrane-bound angiotensin-converting enzyme 2 (ace2). People infected with SARS-CoV-2 can develop asymptomatic, mild, moderate or severe forms of COVID-19.

These last two stages manifest as an intense systemic inflammation characterised by the excessive release of pro-inflammatory molecules. These high levels cause tissue damage and dysregulate the coagulation cascade in multiple organs, leading to acute respiratory distress syndrome (ARDS), organ failure, and possibly the death of the infected patient [2,3].

One of the main players of the systemic inflammation manifested in COVID-19 is adenosine triphosphate (ATP). This pro-inflammatory component of the purinergic system is released during tissue damage and modulates other pathways, including cytokine release through the P2X7 receptor and the NLRP3 inflammasome activation [4]. Adenosine diphosphate (ADP), a product of ATP metabolism, can also exacerbate the disease by activating purine receptors on platelets, leading to thrombus formation [5].

These nucleotides and nucleosides are recognised by cells via the P2 and P1 purinergic receptors, which contain different subunits and have distinct affinities for these molecules, leading to different cellular effects [6]. The P2 receptors are divided into P2X1–P2X7, which respond exclusively to ATP, P2Y1–P2Y13 (which have affinity for both ATP and ADP), and pyrimidines (UTP and UDP). The P1 receptors—A1, A2A, A2B and A3—are responsive mainly to ADO, although they may also respond to INO [7]. On the other hand, concentrative nucleoside transporters (CNT1–CNT3) and equilibrative nucleotide transporters (ENT1–ENT4) help to regulate the transport of ADO between intracellular and extracellular spaces [8]. In the intracellular space, ADO can be converted into INO by cytoplasmic ADA or transformed into AMP by adenosine kinase (ADK), which results in the formation of ATP by adenylate kinase. This intracellular ATP can be released to extracellular space by transporter channels, catabolised to cyclic AMP (cAMP) by adenylate-cyclase and phosphodiesterase (PDE), or degraded to ADO by ATP/ADPase and CD73 [8] (Figure 1).

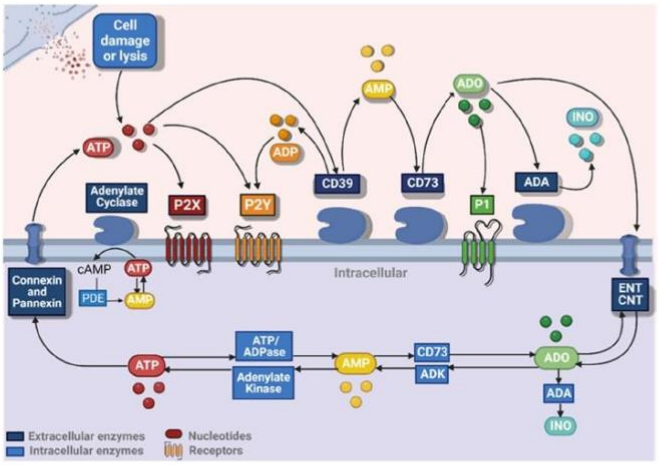

Figure 1. The purinergic signalling cascade. The release of ATP in the extracellular space leads to the activation of P2X and P2Y receptors and it is hydrolyzed by CD39 to ADP, which also activates P2Y receptors. The CD39 also hydrolyzes ADP to AMP, which is sequentially hydrolyzed to adenosine (ADO) by the CD73. ADO activates the P1 receptors and can return to the intracellular space by the ENT/CNT or be hydrolyzed by adenosine desaminase (ADA) into inosine (INO). Once the ADO is inside de cellular space, it can be converted to inosine (INO) by intracellular ADA or can be transformed into AMP by the Adenylate Kinase forming ATP by Adenylate cyclase (AC), which can be released into extracellular space by connexins and pannexins. The intracellular ATP can also be hydrolyzed to AMP by the intracellular ATP/ADPase and then again into ADO by CD73, being released again in the extracellular space by the CNT and ENT. Figure constructed using Biorender.

Many research groups, including ours, have hypothesised that purinergic signalling affects the course of COVID-19 infection by affecting blood flow, coagulation, and immunomodulation [9]. Some studies have indicated that the P1 and P2 receptors [10], pannexins [11], and CD39 and CD73 activity [12] influence the pathogenesis of COVID-19. As is the case with other infectious events, this disease causes damage to the host's tissue due to the viral infection per se or because of exacerbated inflammation. With this injury, the levels of ATP in the extracellular space increase, consequently attracting more immune cells to the infection site and perpetuating the purinergic signalling cascade through the activation of its associated receptors and enzymes. Thus, the use of agonists and antagonists to these receptors and enzymes has been intensively evaluated for COVID-19 treatment.

Based on this background, in this review, we address the main preclinical and clinical scientific findings to date on pharmacological approaches targeting the purinergic signalling pathway as a rational alternative for COVID-19 treatment and its complications, such as blood coagulation, inflammation, vasodilation, and immunological processes, which are strongly affected during infection.

2. Methods

As shown in Figure 2, we searched the PubMed and Scopus databases in addition to clinicaltrials.gov with the keywords 'COVID-19' AND 'purinergic'. We used 'purinergic' as a keyword to collect all published papers about the specific subject. Variations such as 'purinergic signalling', 'purinergic system', or 'purinergic pathway' are used by researchers throughout the literature. The inclusion criteria were: (1) COVID-19 is the main subject of study, and (2) analysis of the purinergic signalling components as a target for therapy instead of prognosis. The exclusion criteria were: (1) reviews, comments or hypotheses; (2) duplicate papers; and (3) no experiments to test the hypotheses.

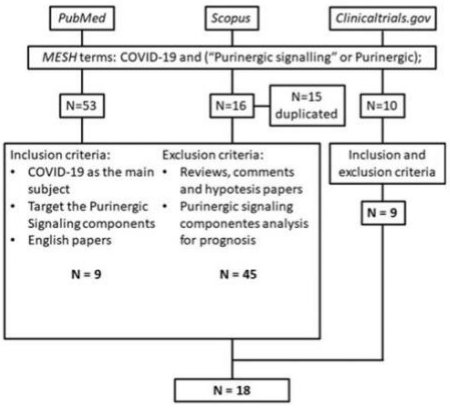

Figure 2. Visual abstract of methodology showing the database, keyword, inclusion and exclusion criteria, and final results from the systematic review.

3. Results and Discussion

Our search yielded 53 papers from PubMed, 16 papers from Scopus (although 15 were duplicates of papers in PubMed, result in 1 paper), and 10 clinical trials from clinicaltrials.gov (Figure 2). Of these 54 papers, only eight experimentally targeted purinergic signalling components for COVID-19 treatment (Table 1). The remaining 45 papers are reviews, commentaries, perspectives, and suggestions, and did not involve experiments to prove the hypotheses; hence, we excluded them. Note that we searched clinicaltrials.gov by using the same keywords as we used to search the PubMed and Scopus databases. We

found 10 clinical trials, but only 9 of these met the inclusion criteria. Table 2 provides the details of these trials.

Table 1. Articles results from the systematic review from the Scopus and PubMed database as described in the Methods section.

PMID	Title	Year of Publication	Purinergic Signalling Target	Drug	Purpose	Results
36268115	Effects of Purinergic Receptor Deletion or Pharmacologic Modulation on Pulmonary Inflammation in Mice [13]	2022	P2Y14R, P2X7R, 2Y14R and A3AR	P2Y14R, P2X7R genetic deletion and modulation with 2Y14R antagonists, A3AR agonists	Treatment	The extent of these responses was diminished by genetic deletion (P2Y14R, P2X7R) or pharmacologic modulation (P2Y14R antagonists, A3AR agonists) of purinergic receptors
35790489	Istradefylline, an adenosine A2a receptor antagonist, inhibits the CWHID4+ T-cell hypersecretion of IL-17A and IL-8 in humans [14]	2022	A2A	A2A antagonist (istradefylline)	Treatment	Attenuation of IL-8 and IL-17A release
35754396	Alterations in CD39/CD73 axis of T cells associated with COVID-19 severity [15]	2022	CD39 and CD73	Adenosine	Prognosis and Treatment	PBMC from severe COVID-19 patients treated with adenosine reduced the NF-κB activation in both CD3+ T cells and CD14+ monocytes. Lower levels of IL-1β and IL-17a were found in the culture supernatant of PBMC treated with adenosine, despite no changes in IL-10 and TNF-α production
35623041	Signalling via dopamine and adenosine receptors modulate viral peptide-specific and T-cell IL-8 response in COVID-19 [14]	2022	A2A	A2A antagonist (istradefylline)	Treatment	Attenuation of Il-8 release
35315874	Effect of Antiplatelet Therapy on Survival and Organ Support-Free Days in Critically Ill Patients with COVID-19: A Randomized Clinical Trial [13]	2022	P2Y12	Clopidogrel, prasugrel and ticagrelor;	Treatment	Among critically ill patients with COVID-19, treatment with an antiplatelet agent, compared with no antiplatelet agent, had a low likelihood of providing improvement in the number of organ support-free days within 21 days
35040887	Effect of P2Y12 Inhibitors on Survival Free of Organ Support Among Non-Critically Ill Hospitalized Patients with COVID-19: A Randomized Clinical Trial [16]	2022	P2Y12	Ticagrelor	Treatment	Among non-critically ill patients hospitalized for COVID-19, the use of a P2Y12 inhibitor in addition to a therapeutic dose of heparin, compared with a therapeutic dose of heparin only, did not result in increased odds of improvement in organ support-free days within 21 days during hospitalization.

Table 1. *Cont.*

PMID	Title	Year of Publication	Purinergic Signalling Target	Drug	Purpose	Results
34867791	Follow Your Nose: A Key Clue to Understanding and Treating COVID-19 [17]	2021	ATP	Dexamethasone and spironolactone	Treatment and Patho-physiology	Mineralocorticoid Receptor blockade can inhibit the release of ATP
33249452	New Horizons: Does Mineralocorticoid Receptor Activation by Cortisol Cause ATP Release and COVID-19 Complications? [18]	2021	Mineralocorticoid receptor, ATP and P2X3	Dexamethasone	Treatment	COVID-19 cough symptom is caused by the activation of purinergic receptors in the lungs following ATP release from virus-infected type II alveolar cells. This raises the question as to when treatment with dexamethasone and spironolactone should be started

Table 2. Results from the systematic review from the Clinical Trials.gov database as described in the Methods section.

Purinergic Element	Pharmacological Mechanism	Medication	Original Therapeutic Use	Clinical Application on COVID-19	Clinical Trial
P2Y12	Antagonist	Clopidogrel	Antiplatelet	Antiplatelet	NCT04333407 (N = 320) NCT02735707 (N = 10.000)
				Antithrombotic and antiplatelet	NCT04368377 (N = 5) NCT04505774 (N = 3.000) NCT04409834 (N = 390)
					NCT02735707 (N = 10.000)
	Antagonist	Prasugrel		Antiplatelet	NCT04445623 (N = 128)
				Antithrombotic and antiplatelet	NCT04505774 (N = 3.000)
	Inhibitor	Ticagrelor		Antiplatelet	NCT02735707 (N = 10.000)
A2A	Agonist	Regadenoson	Vasodilator used in radionuclide myocardial perfusion imaging	Anti-inflammatory	NCT04606069 (N = 40)
A1, A2A, A2B PDE3A *, PDE4A *, PDE5A *	Antagonist	Theophylline	Treat airflow obstruction associated with chronic lung diseases		NCT04789499 (N = 62)
A1, A2A, A2B, A3	Agonist	Adenosine	Myocardial perfusion scintigraphy and converts sinus rhythm of paroxysmal supraventricular tachycardia		NCT04588441 (N = 30)
A1, A2A, A2B, A3 PDE enzymes *	Antagonist	Caffeine	Stimulant, and prevents and treats pulmonary complications of premature birth		NCT05594615 (N = 24)
A2A	Potentiator	Midazolam	Benzodiazepine with rapid onset that is used in seizures, anesthesia, and anxiety disorders		

* Biological targets that also are trigger by the respective drugs.

We focus on highlighting the influence of the purinergic signaling cascade on the immune system and blood coagulation cascades, as both areas are a major concern due to their deregulation during SARS-CoV-2 infection. This imbalance in the immune system

is, for the most part, linked to a cytokine storm, which is an exacerbated release of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α), interleukin 6 (IL-6), IL-1β, and others. This inflammatory explosion shapes immune cell behaviour and activates platelets and other proteins responsible for blood coagulation, resulting in dysregulation of events and immune thrombosis formation. Blood coagulation is directly linked to the activation and aggregation of platelets, resulting in the formation of blood clots, a low platelet count, higher D-dimer levels, elevated prothrombin activity time (TAP), and activated partial thromboplastin time (PTT) [19]. These clinical alterations can persist for 44–155 days after the onset of COVID-19 symptoms [20]. Hence, in 2021, the International Society on Thrombosis and Haemostasis (ISTH) recommended using low molecular–weight heparin to treat patients with moderate and severe COVID-19 [21]. Indeed, clinical trials have shown the benefits of treating patients with COVID-19 with anticoagulants. Higher doses of anticoagulants were associated with lower mortality in hospitalised patients with COVID-19 [22], and there was a dose-dependent delay in death due to COVID-19 [23]. These drugs are used to treat pathologies such as myocardial infarction, stroke, and arterial occlusive diseases [24,25].

Given the dysregulation of these events in patients with COVID-19, it is rational to target these purinergic receptors and enzymes. There has been preclinical and clinical research targeting the P2X3, P2X7, P2Y12, P2Y14, A2A, and A3A receptors, and the enzymes CD39 and CD73 (Table 1).

3.1. P2 Receptors in COVID-19

3.1.1. P2Y Receptors

P2Y12

Based on our search, the P2Y12 receptor stands out as the most promising target for pharmacological modulation of COVID-19 to minimise one of the major culprits of this disease, namely, dysregulation of blood coagulation. Considering the positive results of anticoagulants, researchers began to evaluate the use of P2Y12 antagonists to manage patients with COVID-19. These antithrombotic agents, including clopidogrel, prasugrel, cangrelor, and ticagrelor, have antiplatelet and vasodilatory actions.

The mechanism of action of those drugs involves P2Y12 receptor blockade on platelets, the main players in blood coagulation. Blood coagulation is initiated by cellular signalling of GpIb-IX-V, resulting in the secretion of agonists such as ADP. This nucleotide binds to P2Y1 and P2Y12 receptors, activating thromboxane A2 (TxA2) formation and cyclooxygenases (COXs), and thus triggering inflammatory processes and platelet activation and aggregation [26]. Therefore, P2Y12 receptor blockade attenuates the action of ADP on the formation of platelet aggregates, minimising the interaction with other platelet molecules, such as collagen and thrombin, among others [24].

Clopidogrel is a prodrug that is activated by metabolism to 2-oxo-clopidogrel and later the active thiol metabolite. After activation, clopidogrel irreversibly binds to the P2Y12 receptor on platelets and exerts a therapeutic effect lasting for 5–10 days, based on how long the platelet survives. Thus, this drug has the advantage of being long acting [25,27]. Prasugrel is also a prodrug; it is activated by specific cytochrome P450s (CYPs) [25]. Prasugrel begins working after 30 min, much faster than clopidogrel. This quicker bioavailability and platelet reactivation reduce the risk of extensive bleeding in patients [28]. Cangrelor and ticagrelor are ATP analogues with structural modifications to allow specific binding to the P2Y12 receptor. Cangrelor undergoes non-hepatic metabolism, while ticagrelor is metabolised in the liver by CYP3A4 [25,29]. These drugs have different administration routes: via a nasogastric tube for oral tablets for ticagrelor and intravenously for cangrelor. Cangrelor has a faster action and functional recovery of platelets, around 60–90 min, thus reducing the risk of major bleeding [25,30].

Table 2 lists clinical trials with P2Y12 antagonists that have been conducted in patients infected with SARS-CoV-2. Among them, clopidogrel stands out with five clinical trials investigating antithrombotic and antiplatelet actions (NCT04518735, NCT04368377,

NCT04409834, NCT04505774 and NCT04333407) and evaluation of antiplatelet therapy in COVID-19 pneumonia (NCT02735707).

NCT04409834 (COVID-PACT) evaluated the efficacy and safety of a prophylactic dose of anticoagulation and antiplatelet therapies. The study concluded that compared with placebo treatment, clopidogrel and four heparin variations may reduce all-cause mortality. However, these treatments have an uncertain influence on the necessity for additional respiratory support, COVID-19-related mortality, and quality of life [31]. Moreover, the trial showed that a full dose of an anticoagulant, except clopidogrel, reduced thrombotic complications in critically ill patients. There was an increase in bleeding in haemodynamically stable patients, but with no fatal outcomes [32].

NCT04368377 (PIC-19) analysed the prophylactic use of tirofiban, acetylsalicylic acid, clopidogrel, and fondaparinux. There was an improvement in peripheral oxygenation and a decrease in the need for mechanical respiratory support, with no adverse events reported [33]. In contrast, NCT04505774 (ACTIV-4A) showed that compared with heparin alone, clopidogrel together with heparin was not correlated with improvements in organ support–free days, up to 21 days, during hospitalisation [16].

NCT04333407 established the first endpoint of preventing cardiac complications of COVID-19, but it was terminated due to difficulty in recruiting eligible participants.

NCT04445623 is a phase III double-blind trial comparing the use of placebo with prasugrel in patients with COVID-19. The primary endpoint is to compare the efficiency index of pulmonary gas exchange. The partial pressure of oxygen arterial oxygen (PaO2) divides by the fraction of inspired oxygen (FiO2) known as PaO2/FiO2 ratio (PaO2/FiO2) or ROX index [34], and it was detected 7 days after treatment. The current status of this trial is unknown, and no results have been posted on clinicaltrials.gov. As mentioned for clopidogrel, NCT04505774 evaluated prasugrel together with heparin, but there was no improvement in organ support-free days [35].

A case report evaluating cangrelor and ticagrelor showed better results when compared with clopidogrel, mainly due to the reversibility of the antiplatelet effect [36]. Clopidogrel, cangrelor, and ticagrelor were analysed for COVID-19 treatment in NCT04518735, a retrospective study that enrolled 1707 participants. However, there are no publicly available results. NCT02735707 (the REMAP-CAP trial) enrolled more than 10,000 participants and compared several drugs for treatment of community-acquired pneumonia, including COVID-19. Among the multiple arms of the study, medications including clopidogrel, prasugrel, and ticagrelor were analysed. These medications did not improve the number of organ support-free days up to 21 days [37]. In vitro studies have shown that ticagrelor reduced the risk of secondary pulmonary infections and sepsis, possibly due to the influence on the immune system by decreasing IL-6 as well as neutrophil infiltration into the lungs [38].

Glucocorticoids have anti-inflammatory and immunosuppressive effects that can help reduce the severity of the cytokine storm, which is a key driver of severe COVID-19. Recently, omics-driven studies have shown the potential pleiotropic actions of synthetic glucocorticoids [38]. In addition, dexamethasone use in hospitalized COVID-19 patients without intensive respiratory support (IRS) did not show significant benefit and may even have potential harm, meaning that glucocorticoid therapy, such as dexamethasone, should be reserved for patients with severe or critical COVID-19 who require IRS [39]. Unlike glucocorticoids, P2Y12 receptor antagonists are more targeted and therefore they may have fewer off-target effects.

Taken together, there has been relatively little evidence that pharmacological modulation of the P2Y12 receptor reduces the severity of SARS-CoV-2 infection. Although the drugs are safe and have shown positive results in reducing prothrombotic complications and mortality, larger studies with more patients are needed to definitively determine their potential to treat COVID-19.

P2Y14

Although there are no records of clinical trials that have targeted P2Y14 to treat COVID-19, in vitro studies have shown that pharmacological modulation of the P2Y14 and A3 receptors as well as the deletion of the P2Y14R and P2X7R genes reduce cytokine levels and neutrophilia in a mouse model [37]. Both platelets and neutrophils are highly activated in COVID-19; neutrophil activation and neutrophil extracellular trap (NET) formation causes more thrombotic complications than platelet activation [40]. NET infiltration has a crucial role in infection due to the production of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α, monocyte chemoattractant protein-1 [MCP-1], and granulocyte-macrophage colony-stimulating factor [GM-CSF]), resulting in severe tissue damage, thrombus formation, vascular leakage, and potential necrosis. In SARS-CoV-2 infection, there is an increase in the levels of neutrophils and NET markers in patients with severe COVID-19 and those who die. These findings indicate a correlation between NETs and COVID-19 severity [41]. P2Y14 receptor antagonism may reduce neutrophil recruitment, minimising lung infiltration and the attenuating cytokine storm at the primary site of infection [42]. It is also worth mentioning that P2Y14 is an important proinflammatory receptor in other pathologies, such as ischaemic acute kidney injury [43], eosinophilic airway inflammation [44], and even glucose and oxygen deprivation in brain microvascular endothelial cells [45]. It also appears to have a great influence in diabetes, obesity, and even stem cell senescence [46–48]. Hence, therapies targeted to the P2Y14 receptor alongside pharmacological management of NET formation might represent a future approach to treat COVID-19.

3.1.2. P2X Receptors

P2X receptors have been considered in the pathophysiology and pharmacological treatment of COVID-19. Unlike the P2Y12 receptor, there are no approved medications that directly act on P2X receptors. Our systematic review identified two preclinical studies that analysed P2X receptors. (Table 1).

P2X3 Receptor

Edwards et al. [17,18] showed the indirect effect of dexamethasone on the P2X3 receptor. Dexamethasone is a mineralocorticoid receptor inhibitor that suppresses cortisol secretion, thus preventing ATP release and subsequent P2X3 receptor activation. Activation of this purinergic receptor is linked to cough symptoms during COVID-19 and attenuation of this signalling cascade may result in reduction of this symptom [17,18]. While there is no clinical trial registered at clinicaltrials.gov testing this treatment alternative, a randomised clinical trial evaluating the P2X3 antagonist sivopixant in refractory chronic cough, not related to COVID-19, reported a symptom reduction [49]. Thus, this treatment alternative could also be investigated in COVID-19 with the aim of reducing symptoms and improving health-related quality of life among patients.

P2X7 Receptor

The role of the P2X7 receptor has been studied in the pathophysiology of many diseases, including COVID-19 [50]. Found in different cells and tissues, the P2X7 receptor has an important pro-inflammatory influence on immune cells and it facilitates the cytokine storm in COVID-19, which can persist for months even after recovery [50,51]. Recently, García-Villalba et al. [52] showed that the soluble P2X7 receptor concentration increases in blood plasma of patients with COVID-19, and this increase is positively correlated with disease severity and C-reactive protein levels, suggesting that this receptor could be a prognosis biomarker.

P2X7 receptor activation may lead to an influx of Ca^{2+} that stimulates the NLRP3 inflammasome, which is responsible for enhancing the release of pro-inflammatory cytokines, such as 1L-1β and IL-18, as well as caspase-1 activation that cleaves these cytokines into their mature and biologically active forms [51]. Other cytokines released through P2X7 receptor activation include IL-6, TNF-α, CCL2, IL-8, CCL3, and CXCL2 (Figure 3) [53,54].

Thus, researchers have suggested that P2X7 receptor activation can worsen COVID-19 [53], and the use of antagonists for this receptor could represent a strategy to attenuate the effects of its activation [50]. In this view, researchers have demonstrated that P2X7 receptor blockade or P2X7R gene deletion has direct effects on reducing the inflammatory state during the infectious process in an animal model [13].

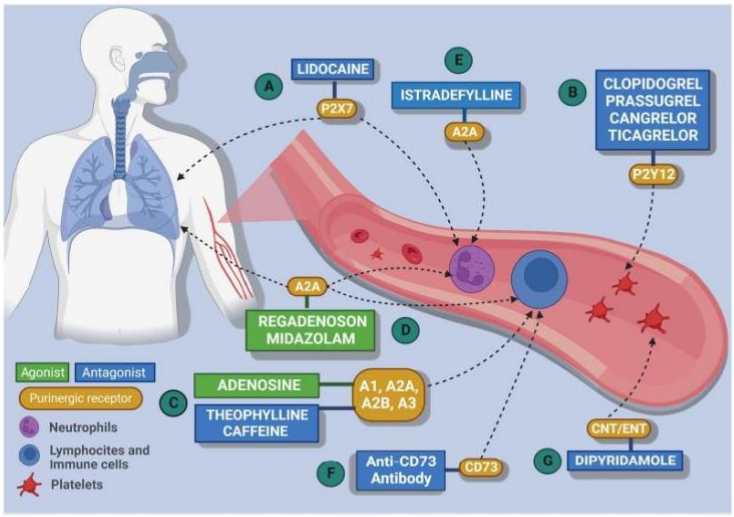

Figure 3. Schematic representation of the main targets of the drugs found in the systematic review. A. Receptor. The P2X7 antagonist, lidocaine act in the pulmonary environment, neutrophils, and immune cells. B. The P2Y antagonist (Clopidogrel, Prasugrel, Cangrelor, and Ticagrelor) act in the coagulation cascade by the P2Y12 receptors in platelets. C. Adenosine (ADO) is the main agonist of the A1, A2A, A2B, and A3 receptors, and, in contrast, theophylline and caffeine acts as an antagonist of these same receptors in immune cells. D. Regadenoson acts in the A2A receptor as an antagonist in immune cells and in the lung, where midazolam only acts as a potentiator of A2A receptor. E. Istradefylline acts as a antagonist in A2A receptors in neutrophils F. Anti-CD73 act as an enzymatic inhibitor in immune cells G. Dipyridamole acts as an antagonist of CNT and ENT transporters mostly in the platelets, culminating in the excess of ADO, which acts as an agonist in the P1 receptors (A1, A2A, A2B, A3) in the platelets, leading to vasodilation and antiplatelet aggregation effects. Figure constructed using Biorender.

Considering the importance of the P2X7 receptor in inflammation, we searched for medications that act on this receptor. We found that lidocaine shows partial modulation of the P2X7 receptor beyond its primary mechanism of action. There have been two clinical trials registered at clinitrials.gov that have evaluated the use of lidocaine for COVID-19 treatment. NCT04609865 (LidoCovid) is a phase III randomised clinical trial aiming to evaluate the effect of intravenous lidocaine 2% on gas exchange and inflammation in patients with ARDS related or not related to COVID-19 [55]. The other phase I randomised clinical trial, NCT04979923, will evaluate the efficacy of lidocaine nebulisation in cough suppression and amelioration of hypoxia in comparison with two other medications, salbutamol and beclomethasone. Although there are no results from these clinical trials, there is a case report from a 71-year-old man with COVID-19 and severe respiratory distress who received lidocaine intravenously. This treatment improved his inflammatory condition. As lidocaine was the only medication he received, this attenuation could be linked to

modulation of the P2X7 receptor, reinforcing the potential of targeting this purinergic element directly or indirectly [56].

There are other medications with indirect effects on the P2X family of receptors. For example, ivermectin indirectly modulates the P2X4 receptor. However, the use of this medication as a prophylactic or therapeutic alternative for COVID-19 is controversial. Indeed, large randomised clinical trials and detailed reviews have already shown that this anti-helminthic medication has no benefit in the COVID-19 context [57–61].

3.2. P1 Receptors in COVID-19

In recent years, the P1 receptors have been extensively investigated as targets for drug development, mainly due to their immunosuppressive potential when activated by their main agonist ADO. These G protein–coupled receptors are divided into four isoforms—A1, A2A, A2B, and A3—each one with different affinities to ADO. The A1 and A2A receptors have high affinity to ADO (from 10 nm to 1 µm), while the A2B and A3 receptors have a low affinity to ADO (>10 µm). Once activated, it may inhibit (A1 and A3) or stimulate (A2A and A2B) adenylate cyclase (AC), leading to changes in cyclic AMP (cAMP) levels [8] (Figure 1).

The A1, A2A, A2B and A3 Receptors

Our search resulted in three preclinical and four clinical trials using therapies targeting P1 receptors. Among P1 receptors, the A2A receptor has been the most investigated as a therapeutic target for COVID-19. Indeed, this receptor is widely expressed in the pulmonary epithelium and modulates resident macrophages. It also prevents adherence and activation of neutrophils to the pulmonary epithelium [62,63]. The A2A receptor has a high affinity to ADO and stimulates AC, consequently increasing the intracellular cAMP levels and leading to complementary activation of anti-inflammatory mechanism. This mechanism is involved in the suppression of cytokine production and oxidising molecules, modulating neutrophils, macrophages, lymphocytes, and platelet aggregation [63]. Therefore, molecules acting on the A2A receptor may be a good alternative treatment for COVID-19.

Tokano et al. [14] analysed the modulation of the A2A receptor with istradefylline in two preclinical studies (Table 1). Istradefylline is a selective A2A receptor antagonist indicated as an adjunct to levodopa and carbidopa for the treatment of Parkinson's disease. In a recent study, the authors evaluated the effects of this drug on PBMCs. They showed suppression of IL-17A and IL-8 production and attenuation of consequent neutrophilic inflammation. As mentioned above, neutrophil activation and NET formation have a very strong influence on inflammation and severity in COVID-19. Therefore, targeting the A2A receptor with an antagonist could provide effective pharmacological modulation of COVID-19.

Another medication acting through the A2A receptor and evaluated as a treatment for COVID-19 is regadenoson. This molecule is a partial agonist of P1 receptors with affinity for the A2A receptor. It dilates coronary vessels and has been used as a diagnostic tool for cardiac pathologies [25]. This medication increases blood-flow in vessels and the myocardium and mimics the effects of ADO. NCT04606069 is currently recruiting participants to evaluate regadenoson in COVID-19; the results are expected in 2023.

In clinical practice, ADO is used to convert paroxysmal supraventricular tachycardia to sinus rhythm [25]. Because ADO acts on all four P1 receptors, it is not clear which receptors are responsible for this effect. For example, A1 receptor activation is related to monocyte phagocytosis, dendritic cell chemotaxis, and mucus promotion, increasing inflammation. In contrast, the A3 receptor has been touted to inhibit degranulation in eosinophils and neutrophils [64].

From this view, even with controversial results, clinical trials evaluating ADO in COVID-19 have been initiated and registered at clinicaltrials.gov (Table 2). NCT04588441 (the ARTIC trial) aims to measure the efficacy of aerosolised inhaled ADO against lung inflammation in patients with ARDS caused by COVID-19. This trial is not yet recruiting participants, and, therefore, no results have been released. On the other hand, a case-control

study with the same treatment protocol was conducted in Italy. It showed that SARS-CoV-2-positive patients who received aerosolised ADO had an improved PaO2/FIO2 ratio, a reduced hospitalisation time and decreased SARS-CoV-2-positive days after diagnosis [65].

Moreover, as a possible role of the ADO receptors in COVID-19, cAMP regulation could be involved in the development of hyposmia and hypogeusia [66]. Lower cAMP levels are correlated with worsening of hyposmia and hypogeusia [67]. So, it seems that cAMP levels dictate the direction of the prognosis, because low levels worsen and high levels improve the ability to taste and smell [68].

Theophylline is a medication that can regulate cAMP concentration [69] and could increase the levels of this secondary messenger through ADO receptors and lead to olfactory neuroepithelium recovery in patients with COVID-19 [70]. To investigate this hypothesis, NCT04789499 evaluated the efficacy of theophylline in patients with symptoms of hyposmia and hypogeusia after SARS-CoV-2 infection. Even though 59% of the patients reported at least a slight improvement in their senses of smell and taste, there were no significant differences between the groups [71]. Another study had similar results in the recovery of smell and taste, in which patients treated with theophylline reported an improvement in smell compared with the placebo group [72]. Beyond the effects on hyposmia and hypogeusia, another pilot study showed that the administration of pentoxifylline and theophylline increased the efficiency of pulmonary gas exchange (PaO2/FiO2) and decrease C-reactive protein levels and mortality compared with the control group [73].

In addition to theophylline, other methylxanthines such as caffeine (1,3,7-trimethylxanthine) are natural compounds that act as P1 receptor antagonists. In silico analysis indicated that theophylline is a potential inhibitor of SARS-CoV-2 replication [74]. So, a molecule with a similar structure to caffeine could also have some beneficial effects on the pathology of COVID-19 by diminishing the cytokine storm and protecting the lungs from exacerbated inflammation [64]. Indeed, preclinical research has already demonstrated the potential of caffeine to inhibit the entry of SARS-CoV-2 into host cells by blocking the spike protein–ACE2 interaction [75]. Moreover, caffeine has been shown to improve oxygenation through relaxation in the pulmonary vascular muscles in chronic lung disease in premature cases, and improve lung function in asthma patients and patients with exercise-induced bronchoconstriction [64]. Clinical trials have been designed to prove the benefits of caffeine: there are 22 registered at clinicaltrials.gov with different outcome measures, from behavioural changes to molecular effects. NCT05594615 is a phase I trial that will evaluate drug–drug interaction between EDP-235 (SARS-CoV-2 antiviral), midazolam, caffeine, and rosuvastatin in healthy subjects. This trial will hopefully clarify whether caffeine can decrease the systemic inflammation manifested in COVID-19.

3.3. Purinergic Enzymes in COVID-19

Considering the importance of the ectoenzymes CD39 and CD73 in controlling extracellular nucleotides and nucleosides, several researchers have shown the correlation of their expression and function with COVID-19 development and severity [9,15,76,77]. Some metabolites derived from nucleotide metabolism are increased in blood samples of patients with COVID-19. Higher ADO levels are positively correlated with higher platelet counts [9]. Moreover, platelets from patients with COVID-19 show greater ATP, ADP, and AMP hydrolysis, and higher ADA activity, which could lead to higher INO blood concentrations [15]. Interestingly, the increase in INO is negatively correlated with lower white blood cell (WBC) and platelet counts, suggesting that high ADO levels could reduce WBC counts [9]. Therefore, the use of P2Y12 inhibitors could attenuate platelet activation and aggregation, reducing thrombus formation and the negative effects of the excessive nucleotide metabolism.

3.3.1. CD39 and CD73

Although we did not include prognosis papers in this review, most of the papers we found from our search evaluated the prognostic value of CD39 and CD73 in COVID-19

(Table 1). For example, Da Silva et al. [15] analysed blood samples from patients with moderate and severe COVID-19, and confirmed the increased expression of both in total leucocytes based on the nucleotide hydrolysis activity. Indeed, Dorneles et al. [77] observed higher expression of CD73 (encoded by NT5E) and CD39 (encoded by ENTPD1) in T cells; the levels correlated with lower concentrations of ATP in blood plasma. However, another study observed lower expression of NT5E in the cytotoxic lymphocyte population of CD8 and natural killer T cells from patients with COVID-19, which secreted higher amounts of pro-inflammatory cytokines [12].

When searching clinicaltrials.gov, we did not find any clinical trials evaluating the modulation of CD39. However, two trials have been registered to evaluate monoclonal antibodies against CD73 in COVID-19, aiming to minimise ADO production [78]. NCT04516564, a phase I, randomised, double-blind, placebo-controlled trial is evaluating the safety, tolerability, pharmacokinetics, and immunogenicity of AK119 (monoclonal anti-CD73) in 29 healthy subjects. No results have been posted. The other phase I, non-randomised and open-label trial (NCT04464395) is testing mupadolimab (CPI-006), an anti-CD73 monoclonal antibody, in hospitalised patients with mild and moderate COVID-19. In this trial, the researchers have measured the efficacy, duration of COVID-19-related symptoms, hospitalisation time, rate of medical interventions during hospitalization, and changes in anti-SARS-CoV-2 immunoglobulin levels. The status of this study is listed as completed, but the results have not yet been released. Although there are no results yet, if anti-CD73 proves to be safe and tolerable, its use in patients with COVID-19 can be analysed in future clinical trials.

Due the multiple functions that CD73 has in inflammation, it is unclear what benefits CD73 inhibition would provide for COVID-19 treatment. Indeed, no publication has presented a clear explanation for this hypothesis. The only possible function we could assume so far is the modulation of cytotoxic lymphocytes described by Dorneles et al. [77], which could diminish the cytokine storm and inflammatory process of COVID-19. Even then, CD73 inhibition could cause the opposite effect because this enzyme is mainly anti-inflammatory (via the production of ADO). Therefore, more in vitro, in vivo, and clinical trials are needed to understand the real application of CD73 in COVID-19.

3.3.2. PDEs and ADA

Researchers have also observed the pharmacological potential of modulating the intracellular enzymes of purinergic signalling. Zlamal et al. [79] showed that upregulation of intracellular cAMP levels could prevent the worsening of the prognosis and disease progression by attenuating immunoglobulin G–induced formation of procoagulant platelets. Targeting this pathway could minimise blood coagulation dysregulation. For example, dipyridamole is an inhibitor of nucleoside transporters, of PDE4A, PDE5A and PDE10A, and ADA, increasing the intracellular cAMP levels [80]. So, clinical trials using dipyridamole for COVID-19 treatment have been initiated. NCT04391179 and NCT04410328 have shown positive results based on the reduction of some inflammatory markers [81]. Therefore, targeting intracellular PDEs may be a good therapeutic choice for COVID-19 to avoid coagulation dysregulation because it prevents platelet aggregation and clot formation.

4. Conclusions

Purinergic signalling is directly linked to several physiological and pathological processes, such as inflammation, blood coagulation, and cellular signalling, which are affected in moderate and severe COVID-19. Although there is a limited amount of preclinical and clinical data evaluating the mechanism of action of medications targeting the purinergic system directly, there have been numerous indirect benefits for patients with COVID-19. These benefits indicate that there could be an advantage to using them mainly as a complementary treatment for COVID-19. We highlight the following:

- P2Y12 modulators such as cangrelor and ticagrelor could be the most promising medications due to their mechanism of action and reversible platelet blocking action, avoiding haemorrhagic events with excessive bleeding.
- P2Y14 is involved in neutrophil recruitment in COVID-19, and targeting this receptor may attenuate blood clot formation by minimising NET formation. However, no medication has been approved so far.
- Targeting the P2X3 receptor could relieve cough symptoms and perhaps improve quality of life.
- The P2X7 receptor is a promising target for inflammation reduction. Because this receptor is linked to NLRP3 inflammasome activation, blocking this element would reduce the release of pro-inflammatory cytokines such as IL-1 and IL-18.
- Targeting the ectoenzymes CD39 and CD73 does not seem to represent the best COVID-19 treatment strategy. If the CD39 enzyme is blocked and inactivated, ATP released by dying cells could concentrate in the extracellular space and chemoattract immune cells to the infection site, causing a loop of cytokine release resulting in tissue damage. On the other hand, blocking the enzyme CD73 would prevent the production of ADO, which could result in clinical improvements via activation of P1 receptors. However, using these enzymes in the same way as for prognostic biomarkers seems to be a good choice because measuring their expression and the levels of nucleotides can indicate the extent of tissue damage and the course of the disease.
- Targeting PDE and ADA intracellular enzymes could be an alternative treatment to avoid coagulation dysregulation and clot formation.
- Modulation of the A2A receptor with istradefylline and regadenoson represents a possible COVID-19 treatment because this receptor modulates neutrophils and the inflammatory process.
- The use of methylxanthines such as theophylline and caffeine could also be a good strategy in COVID-19 treatment due to their potential to help smell and taste recovery and to improve blood oxygen saturation.

Larger and well-designed studies are required to definitively assess whether medications targeting the purinergic system should serve as first-line or complementary treatment for COVID-19. Preclinical studies are necessary to confirm whether their mechanisms of action remain the same as the one originally approved, and clinical studies are crucial to evaluate their efficacy and safety in patients with COVID-19.

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