UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Head Office: Università degli Studi di Padova

Department Department of Biomedical Sciences

Ph.D. COURSE IN BIOMEDICAL SCIENCES
SERIES XXXV cycle

SARS-COV-2 AND IMMUNITY: MODULATION OF TMPRSS2 EXPRESSION BY PRO-INFLAMMATORY CYTOKINES AND IMPACT OF COVID-19 VACCINATION ON SYSTEMIC DANGER SIGNALS IN SARS-COV-2 INFECTED PATIENTS

Thesis written with the financial contribution of Fondazione Istituto di Ricerca Pediatrica Città della Speranza (IRP) and Fondazione Cassa di Risparmio di Padova e Rovigo (CARIPARO)

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SUMMARY

List of abbreviations

+ssRNA	positive-sense single-stranded RNA
3CLpro	3C-like protease
ACE2	angiotensin-converting enzyme 2
ALT	alanine aminotransferase
APCs	antigen presenting cells
ARDS	acute respiratory distress syndrome
ASC	apoptosis-associated speck-like protein containing a caspase recruitment domain
AST	aspartate transaminase
AT1	alveolar type I cells
AT2	alveolar type II cells
BCA	bicinchoninic acid
BSA	bovine serum albumin
CBC	complete blood count
CD	connector domain
CFR	case fatality rate
CH	central helix
CMP	comprehensive metabolic panel
COPD	chronic obstructive pulmonary disease
COVID-19	coronavirus disease 2019
CoVs	Coronaviruses
CRP	C-reactive protein
cryo-EM	cryogenic electron microscopy
CSS	cytokine storm syndrome
CT	computed tomography
CT	cytoplasmic tail
CTCF	corrected total cell fluorescence
CTD	C-terminal domains
CTLs	cytotoxic T lymphocytes
CVS	cardiovascular system
CXCL2	C-X-C MOTIF CHEMOKINE LIGAND 2
DAMPs	damage-associated molecular patterns
DEPC	Diethyl pyrocarbonate

DIC	disseminated intravascular coagulation
DNA	deoxyribonucleic acid,
E protein	Envelope protein
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EMA	European Medicines Agency
ER	endoplasmic reticulum
ERG	erythroblast-specific-related gene
ESR	erythrocyte sedimentation rate
FDA	Food and Drug Administration
FP	fusion peptide
FPPR	fusion-peptide proximal region
GDH	Glutamate dehydrogenase
GISAID	Global Initiative on Sharing Avian Influenza Data
GM-CSF	granulocyte-macrophage colony-stimulating factor
GSDMD	gasdermin-D
HCoV	human Coronaviruses
HGF	hepatocyte growth factor
HIV	human immunodeficiency virus
HMGB1	High Mobility Group Protein B1
HMPV	human metapneumovirus
HPIV	human parainfluenza viruses
HR	heptad repeat
ADAM17	Disintegrin And Metalloproteinase Domain-Containing Protein
ICU	intensive care unit
IFITM2	Interferon Induced Transmembrane Protein 2
IFN	interferon
IFNGR1	Interferon Gamma Receptor 1
IFR	infection fatality rate
IL	interleukin
IL18R1	interleukin 18 receptor 1
IL18RAP	interleukin 18 receptor accessory protein
IRF1	INTERFERON REGULATORY FACTOR 1
IRF9	INTERFERON REGULATORY FACTOR 9

ISG15	ISG15 UBIQUITIN LIKE MODIFIER
ISGs	interferon-stimulated genes
JAK	Janus kinase
LDH	lactate dehydrogenase
LGP2	Laboratory of genetics and physiology 2 protein
M protein	Membrane protein
MDA5	melanoma differentiation-associated protein 5
MERS	Middle-East Respiratory Syndrome
MHC I and II	major histocompatibility complex I and II
mTNF	transmembrane TNFα protein
N protein	Nucleocapsid protein
NAAT	Nucleic Acid Amplification Test
NF-kappa-B	Nuclear Factor Kappa B
NISBD1	neonatal inflammatory skin and bowel disease 1
NK	natural killer
NLRP3	NLR family pyrin domain containing 3
NLRs	nucleotide oligomerization domain (NOD)-like receptors
NoVax	non-vaccinated
NSPs	non structural proteins
NTD	N-terminal domain
ORF1a	open reading frame 1a
ORF1b	open reading frame 1b
PAMPs	pathogen-associated molecular patterns
PAR2	protease activated receptor-2
PB	peripheral blood
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline solution
PFA	paraformaldehyde
PGE2	prostaglandin E2
PLpro	papain-like protease
PRRs	pattern recognition receptors
PT	prothrombin time
PVDF	polyvinylidene fluoride
RAAS	renin-angiotensin-aldosterone system
RAS	renin-angiotensin system

RBD	receptor binding domain
RDT	antigenic-rapid-detection test
RDW	red blood cells distribution width
RIG-I	retinoic acid-inducible gene I
RLRs	retinoic acid-inducible gene I (RIG-I)-like receptors
RNA	ribonucleic acid
RNF20	RING FINGER PROTEIN 20
RNPs	ribonucleoprotein complexes
RT	room temperature
RT-qPCR	reverse transcription quantitative real-time polymerase chain reaction
RTC	replication and transcription complexes
S protein	Spike
SARS	Severe Acute Respiratory Syndrome
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
SEM	standard error of mean
SeV	Sendai virus
SLC6A19	Solute Carrier Family 6 Member A19
STAT	signal transducer of activators of transcription
sTNF	soluble TNFα protein
Th1	T helper 1
Th17	T helper 17
TLRs	Toll-Like Receptors
TM	transmembrane segment
TMB	3,3′,5,5′-Tetramethylbenzidine
TMPRSS2	transmembrane protease serine 2
TNFR1	tumor necrosis factor receptor 1
TNFR2	tumor necrosis factor receptor 2
TNFα	tumor necrosis factor α
U.S.A.	United States of America
Vax	vaccinated
VOCs	variants of concern
WHO	World Health Organization

ABSTRACT

After the outburst of the COVID-19 pandemic, a global research effort has been made to unveil many aspects of COVID-19 pathogenesis, among which the outstanding role played by inflammatory cytokine milieu in the disease progression. At the time of our study indeed the molecular mechanisms orchestrating SARS-CoV-2 infection and disease pathogenesis were largely unknown.

Yet, we investigated whether the host cytokine milieu could modulate SARS-CoV-2 receptors ACE2 and TMPRSS2 expression, hence influencing cell susceptibility to the virus infection. Also, we analyzed if this hyperinflammatory signature varies between vaccinated and non-vaccinated COVID-19 patients. Our results clearly indicated that the host inflammatory milieu, and in particular the pro-inflammatory cytokine IL1β, can favor SARS-CoV-2 infection by inducing TMPRSS2 overexpression. We shed light on the molecular mechanism behind this modulation, which may be therapeutically targeted.

Moreover, in line with the net efficacy of COVID-19 vaccination in preventing a severe clinical manifestation of the disease, we revealed a significant reduction in the levels of IL1β and DAMPs molecules, as S100A8 and HMGB1, in vaccinated patients as compared to non-vaccinated ones.

1. INTRODUCTION

1.1. COVID-19

Coronavirus disease 2019 (COVID-19) is a highly contagious disease caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). Up to date (December 2022) this illness has resulted in more than 650 millions of infections and 6 million deaths worldwide (*WHO Coronavirus (COVID-19) Dashboard*).

The first cases of COVID-19 were reported in the city of Wuhan, Hubei Province, in China, in late December 2019. Since then, SARS-CoV-2 quickly spread around the world, leading to the consequent declaration of global pandemic by the World Health Organization (WHO) on March 11, 2020 (Ganesh *et al.*, 2021; Koelle *et al.*, 2022). The disease had a dramatic impact on the healthcare systems and the economy worldwide.

Until now, the scientific community has been devoted to the understanding of dominant pathogenetic mechanisms of the disease, and to the development of effective therapies to counteract the spreading of COVID-19 and its severity.

In this regard, a remarkable achievement has been the rapid maturing of the first European Medicines Agency (EMA) approved vaccine on the 21st of December, 2020: Comirnaty (developed by BioNTech and Pfizer) (Polack *et al.*, 2020).

However, an urgent need to enlarge the current knowledge on this pathology and mechanism of infections still remains, in particular due to the rapid spread of novel SARS-CoV-2 variants of concern (VOCs).

1.1.1. Epidemiology

Since the beginning of the pandemic, SARS-CoV-2 has spread globally with more than 650 millions of infected patients and more than 6 million of reported deaths. WHO recently reported that SARS-CoV-2 variants of concern are

almost worldwide spread, with the Omicron VOC, first identified in November 2021, being the currently dominant circulating variant, accounting for more than 98% of the viral sequences reported on GISAID (Global Initiative on Sharing Avian Influenza Data) (*Tracking SARS-CoV-2 variants*). Up to date, the U.S.A. have experienced the highest number of reported SARS-CoV-2 infections and COVID-19 related deaths - with this disease being the third leading cause of death in the U.S.A. in 2020 after heart disease and cancer - followed by India and Brazil (Ahmad *et al.*, 2021). The WHO currently estimates a global case fatality rate for COVID-19 of 2.2%, that significantly varies between countries, yet this number is clearly conditioned by several factors including patient's age, gender and underlying pre-existing conditions (Cascella *et al.*, 2022).

From the very beginning, epidemiological studies indeed indicated that age, gender and comorbidities such as obesity, diabetes, hypertension, respiratory or cardiovascular diseases, chronic kidney disease, cancer, along with patient ethnicity are linked to the development of a more severe form of COVID-19 (Treppiedi *et al.*, 2022).

In particular, these studies indicated that patients older than 60 years with underlying medical comorbidities are at increased risk of developing severe COVID-19. The percentage of COVID-19 patients requiring hospitalization is six times higher in people experiencing comorbidities as compared to those without pre-existing medical conditions; remarkably, the percentage of deaths is 12 times higher in patients with comorbidities (Stokes *et al.*, 2020).

Although the incidence of COVID-19 has been reported to be equal among men and women, data regarding the gender-based differences showed that male patients have an higher risk of developing severe illness and have increased mortality due to COVID-19 compared to female patients: according to a recent epidemiological study, the infection fatality rate (IFR) was 3.17% in male patients, and 2.26% in female patients (Ramírez-Soto, Ortega-Cáceres and Arroyo-Hernández, 2021).

The severity of the disease and the mortality rate also vary between different ethnic groups. Indeed, meta-analysis reported that people of Black, Hispanic, and Asian ethnic minority groups are at increased risk of contracting and dying

from Sars-CoV-2 infection, with COVID-19 related death rates being the highest among Hispanic people (Sze *et al.*, 2020; Ahmad *et al.*, 2021).

1.1.2. Etiology

SARS-CoV-2 is the etiologic agent of coronavirus disease 2019 (COVID-19). It belongs to the Coronaviridae family of viruses (CoVs): as such it is characterized by positive-single stranded RNA and by a crown-like appearance when visualized at the electron microscope because of the presence of Spike glycoproteins on the virus envelope (Figure 1) (Tyrrell *et al.*, 1975; Weiss and Navas-Martin, 2005). The Coronaviridae family includes viruses that can cause respiratory, hepatic, neurological and enteric pathologies in a variety of animal species and comprises the Orthocoronavirinae subfamily, which includes four genera of CoVs: Alphacoronavirus, Betacoronavirus, Gammacoronavirus, Deltacoronavirus (Weiss and Navas-Martin, 2005; Chan *et al.*, 2013).
In humans, the viruses belonging to this family can cause diseases that vary from a common cold to severe respiratory conditions such as SARS (Severe Acute Respiratory Syndrome) and MERS (Middle-East Respiratory Syndrome); seven CoVs that are able to infect humans (HCoVs) have been identified up to date: HCoV-HKU1 and HCoV-OC43 (betaCoVs), HCoV-NL63 and HCoV-229E (alphaCoVs) can cause common cold and upper airways infections; SARS-CoV and MERS-CoV (betaCoVs), as SARS-CoV-2, can cause more severe diseases with respiratory and extra-respiratory symptoms (Kesheh *et al.*, 2022).

SARS-CoV-2 belongs to the subgenus of SARS-CoV and MERS-CoV, which have caused previous epidemics characterized by a mortality rate of, respectively, 10% and 35% (Chan *et al.*, 2020): SARS-CoV broke out in Foshan municipality, Guangdong Province, China, in 2002 and caused 8.098 infections and 774 deaths, while MERS-CoV emerged in 2012 in Saudi Arabia and infected 2.494 patients, causing the death of 858 of them (Walls *et al.*, 2020). As such, SARS-CoV-2 represents the third documented case, from the beginning of the 21st century, of a coronavirus that has been able to cross the species barrier and to provoke deadly respiratory infections.

The new HCoV genomic characterization has revealed that it shares 82% of nucleotide identity with SARS-CoV and has therefore been named SARS-CoV-2 by the International Committee on Taxonomy of Viruses. It also shares 96% of homology with the betaCoV RaTG13 found in bats (Rhinolophus affinis), reason why it is believed, although still not proven, to have originated from a zoonotic transmission (Andersen *et al.*, 2020; P. Zhou *et al.*, 2020).

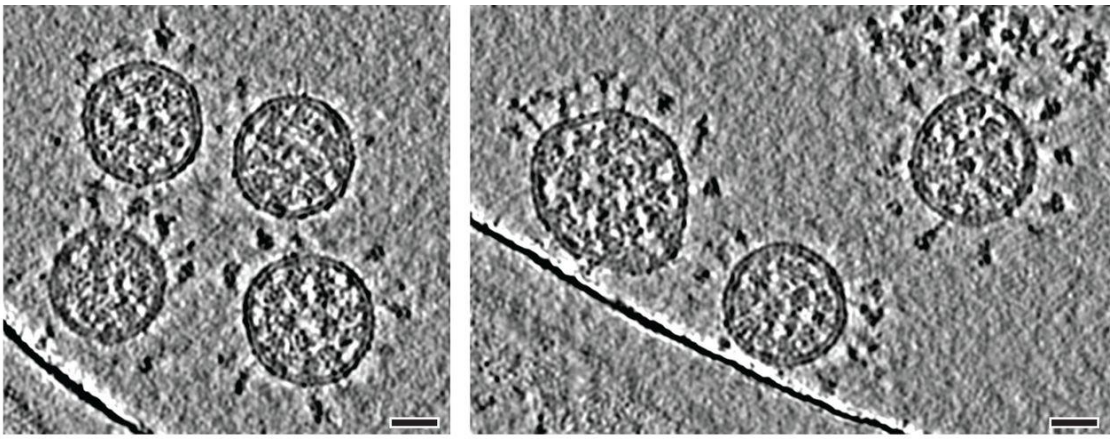


Figure 1. Cryo–electron tomography of SARS-CoV-2 virions. Scale bars, 30 nm (source: Turoňová *et al.*, 2020).

1.1.3. Transmission routes

The primary mode of transmission of SARS-CoV-2 is *via* direct exposure to respiratory droplets that carry the virus, mainly by symptomatic individuals (Meyerowitz *et al.*, 2020). This transmission, which can occur during the incubation period, meaning 1-3 days before the clinical onset of the disease (Wei *et al.*, 2020), occurs when the droplets are inhaled or deposited on the nasal, oral or conjunctival mucosa (Hui *et al.*, 2020). Paucisymptomatic individuals, i.e. patients with mild symptoms, are characterized by large amounts of SARS-CoV-2 in the upper respiratory tract; this condition may contribute to the easy and fast spread of the virus (Cevik *et al.*, 2021). Asymptomatic individuals can also contribute to the spread of the virus, even though in a milder way (Wilmes *et al.*, 2021). The majority of transmissions occur by means of close prolonged direct contact, such as 15 min face to face exposure within 2 meters, between an infectious individual and a healthy one, with a particular efficiency of spread in indoor environments due to the poor

ventilation (Cevik *et al.*, 2020; Morawska *et al.*, 2020). Apart from this main airborne transmission, other routes of SARS-CoV-2 have been investigated: the virus can survive on surfaces of different materials, although it can rarely spread through contaminated surfaces and consequent touch of the mouth, nasal or eye mucosas (Lewis, 2021); oral-fecal transmission route has been reported to be possible, but likely rare (Meyerowitz *et al.*, 2020; Yeo, Kaushal and Yeo, 2020); no transmission through other body fluids (blood, semen, urine etc.) has been reported (Meyerowitz *et al.*, 2020), while vertical transmission (mother-to-newborn) of the virus is possible but has been verified in a minority of cases (Kotlyar *et al.*, 2021).

1.1.4. Clinical manifestations and pathophysiology

COVID-19 is defined by a wide spectrum of symptoms. In the majority of affected individuals, the clinical signs of disease develop after an incubation period that lasts, in median, ca. 5 days; the 97,5% of the symptomatic patients will develop clinically evident signs of COVID-19 within ca. 11 days of infection (Lauer *et al.*, 2020).

Based on the severity of the disease, patients can be classified into asymptomatic (will not develop symptoms), paucisymptomatic (will develop few, mild symptoms) and symptomatic (will develop proper symptoms).
An estimated 20-80%, depending on SARS-CoV-2 variant, of infected individuals will not develop symptoms (asymptomatic patients) (Mizumoto *et al.*, 2020; Nishiura *et al.*, 2020; Alene *et al.*, 2021).
The most common symptoms among symptomatic patients include fever, cough and dyspnea, while sore throat, anosmia and ageusia, myalgia, chest pain, fatigue, headache, nausea, and diarrhea are less common (Stokes *et al.*, 2020; Dixon *et al.*, 2021; Larsen *et al.*, 2021).
According to the WHO classification of COVID-19 severity reported in Table 1, the mild patients are symptomatic but they have no signs of pneumonia or hypoxia, moderate ones have mild pneumonia, severe and critical have

respiratory failure and require intensive care unit support and mechanical ventilation (*Clinical management of COVID-19: Living guideline, 23 June 2022*).

Mild disease	Symptomatic patients meeting the case definition for COVID-19 without evidence of viral pneumonia or hypoxia.
Moderate disease	Clinical signs of pneumonia (fever, cough, dyspnoea, fast breathing) but no signs of severe pneumonia, including SpO2 ≥ 90% on room air.
Severe disease	Clinical signs of pneumonia (fever, cough, dyspnoea) plus one of the following: respiratory rate > 30 breaths/min, severe respiratory distress, or SpO2 < 90% on room air.
Critical disease	Presence of acute respiratory distress syndrome (ARDS), sepsis, septic shock, acute thrombosis.

Table 1. WHO classification of COVID-19 severity in the adolescent and adult (source: *Clinical management of COVID-19: Living guideline, 23 June 2022*).

Among the symptomatic patients, most individuals develop mild (40%) or moderate (40%) disease, while around 15% develop severe disease and 5% develop critical disease, the staging of the disease is of primary importance to define the proper clinical management of patients. The 80% of COVID-19 patients recovers within a month from the beginning of the infection, while 5% of infected individuals develop the so called “long COVID”, which refers to the persistence of SARS-CoV-2 infection symptoms for more than 12 weeks (Liu *et al.*, 2021).

The case fatality rate (CFR, number of reported deaths divided by the number of reported confirmed cases) is extremely variable among different countries, going from <0,1% to around 25% according to the most recent WHO esteems, percentages that increases with aging and in presence of comorbidities (*Estimating mortality from COVID-19: Scientific brief, 4 August 2020*). Thanks to the gradual progresses in the clinical management of COVID-19 patients and to the diffusion of vaccination, this mortality rate has significantly decreased from the beginning of the pandemic (de la Calle *et al.*, 2021).

As mentioned, COVID-19 comprises a wide range of symptoms that are indicative of the infection and dysfunction of different organs and, in particular, of the respiratory system, where SARS-CoV-2 can induce pneumonia. In the early phase of the disease (the first week post symptoms onset), lung damage is driven by the replication of the virus in the host cells, which can induce the death of the lung parenchyma cells via apoptosis, necroptosis, pyroptosis and autophagy (Yapasert, Khaw-on and Banjerdpongchai, 2021); viral replication is then followed by a second phase of host immune response in which the viral infection triggers the immune response, characterized by the recruitment of neutrophils, monocytes and T lymphocytes at the infected sites and by the release of multiple cytokines including interferon (IFN) γ, interleukin 1β (IL1β), IL6, IL8, IL12, tumor necrosis factor α (TNFα) and granulocyte-macrophage colony-stimulating factor (GM-CSF). Hence the viral load decreases about one week post-infection, in particular due to T cell activity and neutralizing antibodies. However, in severe and critical cases, a later phase of endothelial insult, with subsequent coagulopathy, and of hyperinflammation follows, as the exacerbated activation of the host inflammatory response can lead to the condition known as “cytokine storm”, which consists in a deregulated and excessive release of cytokines, especially IL1β, IL6 and TNFα (Azkur et al., 2020; J. Wang et al., 2020; Osuchowski et al., 2021).

In this hyperinflammatory context, pneumonia can develop into acute respiratory distress syndrome (ARDS). In fact, ARDS is defined as a life-threatening respiratory insufficiency characterized by the rapid onset of an inflammatory condition in the lungs and, on the symptomatology profile, it includes dyspnea (shortness of breath), tachypnea (fast breathing) and cyanosis (Fanelli *et al.*, 2013; Matthay *et al.*, 2019). ARDS progression is impressively rapid and it normally develops within a 10 days after disease onset in severe-critical patients: the median time from symptoms onset to hospitalization is of 7 days, to dyspnea is of 8 days, to ARDS is of 9 days, to intensive care unit (ICU) is of 10,5 days (Huang *et al.*, 2020).

The damage caused by multiple mechanisms triggered by SARS-CoV-2 infection leads to the impairment of lungs ability to exchange oxygen: clinically,

ARDS is classified by the Berlin Definition into 3 levels (mild, moderate and severe) based on the degree of hypoxemia (The ARDS Definition Task Force*, 2012).

Pulmonary edema can arise as a consequence of increased vascular permeability due to viral-induced damage of the endothelium and inflammation. The cardiovascular system (CVS) can also be affected in COVID-19 patients: since angiotensin-converting enzyme 2 (ACE2), the receptor for the virus, is expressed also in myocardiocytes, a direct viral damage could lead to myocarditis (Vukusic *et al.*, 2022). Myocardium inflammation, together with vascular inflammation and cardiac arrhythmias can also be mediated by pro-inflammatory cytokines like IL6 (Huang *et al.*, 2020). Another clinical COVID-19 manifestation at the CVS level is the acute coronary syndrome, which can be linked to the virus-associated release of pro-inflammatory cytokines, alteration of coagulation and cardiomyopathy (Guo *et al.*, 2020; Hua *et al.*, 2020).

The involvement of the hematological system in COVID-19 is significant, although the exact mechanisms of such alteration are still to be investigated. Leukopenia, that commonly figures among laboratory abnormalities, has been hypothesized to be caused by direct viral invasion in leukocytes or by the excess of pro-inflammatory cytokines (L. Tan *et al.*, 2020). Neutrophilia and thrombocytopenia are also markers of severe COVID-19 (Coopersmith *et al.*, 2021). The least has been postulated to be caused by the activation of the coagulation cascade, which leads to a rapid platelet consumption (Abou-Ismail *et al.*, 2020). This COVID-19 associated hypercoagulability has been suggested to be the result of the viral- or cytokine- induced endothelial damage that drives the coagulation cascade all through the formation of the fibrin clot (Abou-Ismail *et al.*, 2020; Amgalan and Othman, 2020; Alam, 2021).

The gastrointestinal system symptoms are likely to be multifactorial: direct ACE2-mediated viral cytotoxicity of the enterocytes, pro-inflammatory cytokine release, gut microbiota alterations and vascular dysfunction (Patel *et al.*, 2020).

COVID-19 can also involve the renal system: SARS-CoV-2 can cause kidney injury *via* direct viral cytotoxicity, cytokine-induced hyperinflammation, alteration of the renin-angiotensin-aldosterone system (RAAS), in which the virus-exploited host ACE2 receptor is involved, and vascular injury (Gabarre *et al.*, 2020).

Therefore, in general sight, SARS-CoV-2-induced multi-organ dysfunction can be explained by direct viral damage, vasculitis-caused ischemic injury, thrombosis, deregulation of the immune system and/or of the RAAS (Coopersmith *et al.*, 2021).

The clinical-pathological features of COVID-19 patients reflect this kind of multi-organ dysfunction: lymphopenia, elevated C-reactive protein levels and elevated cardiac enzymes have been reported to be rather common in COVID-19 patients, but also abnormal liver function tests, leukopenia, leukocytosis, elevated D-dimer, elevated erythrocyte sedimentation rate, and abnormal renal function characterize a relevant percentage of SARS-CoV-2 infected individuals (Zhu *et al.*, 2020).

1.1.5. Immune response in COVID-19

The immune system is a fundamental player in COVID-19 pathogenesis and a strong determinant of the severity of the disease. The immune system reaction towards SARS-CoV-2 infection consists in two main phases.

The first phase is marked by the activation of the innate immune system, which represents the aspecific first line response to the virus. Innate immune cells, such as monocytes, macrophages, neutrophils, dendritic cells, and natural killer (NK) cells, are provided with pattern recognition receptors (PRRs) that can sense pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). The activation of these receptors leads to the stimulation of inflammatory signaling pathways and immune responses, in particular to the production of inflammatory cytokines and chemokines and

eventually to the induction of cell death to eliminate infected cells (Kanneganti, 2020).

Recent studies have proven that SARS-CoV-2 is able to activate several PRRs, in particular Toll-like Receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and nucleotide oligomerization domain (NOD)-like receptors (NLRs) and inflammasomes (Diamond and Kanneganti, 2022). In particular, when SARS-CoV-2 infects a cell, the viral RNA is released into the host cell cytoplasm, triggering the activation of several pathways, such as TLR 3, 7, 8 and 9 pathway and melanoma differentiation-associated protein 5 (MDA5), retinoic acid-inducible gene I (RIG-I) and Laboratory of genetics and physiology 2 protein (LGP2) pathway (D.-M. Yang *et al.*, 2021; Rebendenne *et al.*, 2021; Thorne *et al.*, 2021; Yin *et al.*, 2021). This activation results in the transcription of inflammatory genes, in particular of type I interferon (IFN) genes. The secreted type I IFN stimulates the transcription of interferon-stimulated genes (ISGs), which induce cell resistance to viral infection by activating endoribonucleases to degrade the viral RNA and by inhibiting the protein biosynthesis. Type I IFN also upregulates the expression of major histocompatibility complex I (MHC-I) on all the cell types, improves the capability to process antigens in antigen presenting cells (APCs) and induces NK cells to kill virus-infected cells (Schultze and Aschenbrenner, 2021).

Therefore, this PRR signaling in innate immune cells leads to a consistent release of both IFNs and other cytokines and chemokines, which in turn, initiate the adaptive immune response, for example by recruiting leukocytes (Schultze and Aschenbrenner, 2021).

Of note, an improper innate immune response is one of the factors by which the immune system can affect the severity of COVID-19: both its overactivation or insufficient activation are deleterious to the host (Diamond and Kanneganti, 2022). For example, recent studies have proven that an impaired IFN response, e.g. in the elderly, can lead to an increased recruitment of innate immunity cells, which then drive to an exacerbated inflammation (Mishra, Singh and Singh, 2020).

This activation of the adaptive immune system delineates the second phase of the immune response to SARS-CoV-2 infection. In this specific type of immune response, $CD8^+$ cytotoxic T cells directly target the infected cells, whereas $CD4^+$ helper T cells induce B cells to produce antibodies which specifically target the virus (Sette and Crotty, 2021). $CD4^+$ T cell response appears to be more prominent compared to $CD8^+$ T cell response: circulating $CD8^+$ T cells that are specific to SAR-CoV-2 have been detected in 70% of convalescent patients, while circulating $CD4^+$ T cells have been found in 100% of patients (Wong, 2021). Interestingly, recent studies demonstrated that an early induction of T cell immunity against SARS-CoV-2 is associated with a more efficient viral clearance and with a mild disease (Moderbacher *et al.*, 2020; Peng *et al.*, 2020; Tan *et al.*, 2021).

For what concerns B cell immunity, the majority of SARS-CoV-2 infected patients get seroconverted within 5 to 15 days post symptom onset and about 90% seroconversion is detected by the 10^{th} day post symptom onset (Long *et al.*, 2020; Moderbacher *et al.*, 2020; Premkumar *et al.*, 2020; Suthar *et al.*, 2020; Sette and Crotty, 2021). B cell-produced neutralizing antibodies mainly target the Spike protein of SARS-CoV-2, with the receptor binding domain (RBD) of Spike being the target of >90% of neutralizing antibodies in COVID-19 patients (Sette and Crotty, 2021).

Overall, an improper immune response, with an increase in innate immune cells and a parallel reduction in lymphocytes (lymphopenia), is associated with more severe disease (Lucas *et al.*, 2020; M. Tan *et al.*, 2020; Qin *et al.*, 2020). A delayed type I IFN response during the innate response phase may lead to an unperturbed initial viral replication and delayed induction of the adaptive response; this could lead to an unbearable viral load and a more severe form of COVID-19, with higher risk of ICU requirement and death (Magleby *et al.*, 2021; Wong, 2021).

1.1.5.1. Immune cell alteration

Several studies have highlighted a common immune pattern in SARS-CoV-2 infected patients, which are characterized by lymphocytopenia and emergency

myelopoiesis, an inflammation-induced haematopoiesis aimed to replace the immune cells that have migrated in the periphery. Importantly, the degree of such alteration correlates with COVID-19 severity (Schulte-Schrepping *et al.*, 2020; Z.-L. Zhang *et al.*, 2020; O'Driscoll, 2022). In the following paragraphs, the alteration of immune cell populations in COVID-19 patients will be briefly discussed.

The monocytes-macrophages compartment is profoundly affected in COVID-19 patients: an early activation of HLA-DR^{++}CD11c^{++} monocytes with a robust antiviral IFN-signature is a hallmark of mild COVID-19 (Schulte-Schrepping *et al.*, 2020). Parallelly, a loss of circulating CD14^{+}CD16^{++} non-classical monocytes has been reported (Kuri-Cervantes *et al.*, 2020; Merad and Martin, 2020; Schulte-Schrepping *et al.*, 2020). A reduced number of blood monocytes correlated with increased inflammatory markers, procalcitonin, and CRP levels (Sánchez-Cerrillo *et al.*, 2020). Macrophages have not been detected in the bronchoalveolar lavage fluid (BALF) of most patients with mild disease; on the other side, patients with severe-critical COVID-19 present an enrichment of monocytes and macrophages in the BALF and a decreased surface expression of HLA-DR on circulating monocytes (D'Alessio and Heller, 2020). In these severe patients, BALF macrophages show an elevated expression of the ISGs, which promote the production of inflammatory cytokines and reactive oxygen and nitrogen species (ROS and RNS), as well as a tissue-repair phenotype. The strong antiviral and fibrotic response exerted by these macrophages is likely to contribute to the tissue damage and fibrosis that characterize the later stages of this disease in severe patients (Z. Zhou *et al.*, 2020).

Neutrophils show alterations in their relative abundance, phenotype and functionality in COVID-19 patients: an increase of circulating neutrophils, characterized by an activation signature, has been reported especially in severe COVID-19 patients (Reusch *et al.*, 2021). Notably, the neutrophil-to-lymphocyte ratio (NLR), which represents a clinical biomarker of inflammation, is increased in COVID-19 patients and is able to predict severe disease in the early stage of SARS-CoV-2 infection. In the severe COVID-19 cases, neutrophil signature includes an upregulation of markers of immaturity and of exhaustion. These

PD-L1-enriched neutrophils that emerge prematurely from the bone marrow show an anti-inflammatory and suppressive phenotype (Schulte-Schrepping *et al.*, 2020).

Myeloid-derived suppressor cells (MDSCs), in particular circulating monocytic-MDSCs (M-MDSCs), are highly functional and strongly suppressive towards T cell proliferation in COVID-19 patients (Agrati *et al.*, 2020). MDSCs expand in the acute phase of the COVID-19, when they potentially counteract dangerous T cell hyperactivation. In severe COVID-19 patients, the emergency myelopoiesis, to which high levels of IL6 and IL10 contribute, promotes the expansion of M-MDSC cells. Hence, the hyperactivation of the immune system with an excessive production of MDSC-generating inflammatory mediators can contribute to an ineffective T cell response in these later stages of the disease (Agrati *et al.*, 2020; Schulte-Schrepping *et al.*, 2020).

NK cells have been reported to be reduced in the peripheral blood of COVID-19 patients, and this reduction correlates with an increased severity of the disease. In the mild form of COVID-19, most of the NK cells present in the BALF are characterized by a mature $CD16^{+}CD56^{dim}$ phenotype and are functionally able to induce cell cytotoxicity. Indeed, $CD16^{+}CD56^{dim}$ NK cell activation can be induced - through Fc receptor recognition - by IgG antibodies either bound to surface SARS-CoV-2 antigens expressed by infected cells or to extracellular virions as immune complexes (Xu *et al.*, 2020; Di Vito *et al.*, 2022). This NK activation triggers cytokine production and lysis of infected cells through antibody-dependent cellular cytotoxicity (ADCC) (Vabret *et al.*, 2020). On the contrary, in the severe COVID-19 patients, NK cells show a functionally exhausted phenotype, marked by increased levels of the immune checkpoint NKG2A, a protein involved in the inhibition of NK cytotoxicity; this phenotype can contribute to viral escape (Zheng *et al.*, 2020).

T cells are typically present in normal or slightly higher counts in mild-moderate COVID-19 patients. An increase in Tregs percentage, that positively correlates to increased plasma levels of the anti-inflammatory cytokine IL10, has also been reported in mild cases (Vabret *et al.*, 2020). As mentioned, severe

COVID-19 patients display lymphopenia: the reduction in numbers of both $CD4^+$ and CD8+ T cells has been reported to correlate with disease severity and with mortality (De Biasi *et al.*, 2020; J.-Y. Zhang *et al.*, 2020; Vabret *et al.*, 2020; Wilk *et al.*, 2020; Hanna *et al.*, 2021). T cells also display an altered phenotype in severe patients, characterized by markers of activation and proliferation but also of exhaustion and of deficient cytokine production, with the latter being linked to an increased disease severity (Vabret *et al.*, 2020; Hanna *et al.*, 2021). Particularly, an increased frequency of activated HLA-DR^+ $CD38^+$ T cells has been reported in patients who will later progress to severe COVID-19. PD-1, Tim-3, CTLA4, LAG-3, BTLA and NKG2A, which represent markers of chronic activation and exhaustion in both $CD4^+$ and $CD8^+$ T cells, increase with the progression of the disease and correlate with a poor prognosis (Hanna *et al.*, 2021). Moreover, in severe-critical COVID-19 patients, $CD4^+$ and $CD8^+$ T cells showed impaired functionality, with reduced frequencies of polyfunctional (producing more than one cytokine) T cells and a diminished production of the antiviral cytokine IFNγ (Vabret *et al.*, 2020). Also, a reduction in the frequencies of Treg cells has been reported in severe COVID-19 patients (G. Chen *et al.*, 2020; Qin *et al.*, 2020). These cells promote the resolution of ARDS in mouse models, hence a decrease in Treg numbers might contribute to the development of COVID-19 lung hyperinflammatory damage (De Biasi *et al.*, 2020; Hanna *et al.*, 2021).

B cell response to SARS-CoV-2 infection has been shown to be robust, with a generally diffused detection of virus-specific IgM, IgG and IgA, and neutralizing IgG antibodies (nAbs) in the days following infection (Vabret *et al.*, 2020). COVID-19 patients exhibit no significant differences in circulating $CD19^+$ B cell absolute numbers, yet the proportions of some B cell subsets are altered (Kuri-Cervantes *et al.*, 2020; Sosa-Hernández *et al.*, 2020). In particular, transitional B cells, a subset which increases in infectious diseases, has been reported to increase in percentage in mild-moderate cases, while it decreases in more severe cases. Transitional B cells then differentiate into the terminally differentiated plasmablasts, which show an expansion in COVID-19 patients. It has also been reported that higher memory B cell counts correlate with lower clinical risk and shorter hospitalization time. On the contrary, these cell levels

are lower in more severe COVID-19 cases (Cox and Brokstad, 2020; Sosa-Hernández *et al.*, 2020; Vabret *et al.*, 2020).

1.1.5.2. Cytokine storm

As mentioned, severe and critical COVID-19 patients can develop the condition known as cytokine storm syndrome (CSS). This clinical status is defined as an acute overproduction and release of pro-inflammatory cytokines (Montazersaheb *et al.*, 2022); in particular, it is characterized by an elevated level of circulating cytokines, systemic acute inflammation symptoms and secondary organ dysfunction caused by the excessive inflammatory state (Fajgenbaum and June, 2020).

In a physiological context, cytokine production and release are extremely controlled in dosing and timing; indeed, cytokines have a reduced half-life, which is functional to prevent their effect outside the site of inflammation and lymphoid organs (Turner *et al.*, 2014). In peculiar pathological conditions, such as inappropriate danger signaling (e.g. autoimmune diseases), overwhelming pathogen burden (e.g. sepsis) or failure to resolve the immune response, the immune system hyperactivation can result in a cytokine storm (Fajgenbaum and June, 2020). This status is characterized by fever, headache, fatigue, anorexia, rash, myalgia and it can rapidly progress to disseminated intravascular coagulation (DIC) with eventually vascular occlusion, hemorrhages, hypoxemia, hypotension vasodilatory shock and ultimately to death. Respiratory symptoms might also include cough, tachypnea and, in the most severe cases, acute respiratory distress syndrome (ARDS) (Figure 2) (Fajgenbaum and June, 2020). The cytokines that are mainly found to be elevated in the CSS are IFNγ, IL1β, IL6, TNFα, and IL18 (Figure 3) (Fajgenbaum and June, 2020).

In the following paragraphs, a concise description of the main biological and clinical features of such mediators in the SARS-CoV-2 pathological background will be provided.

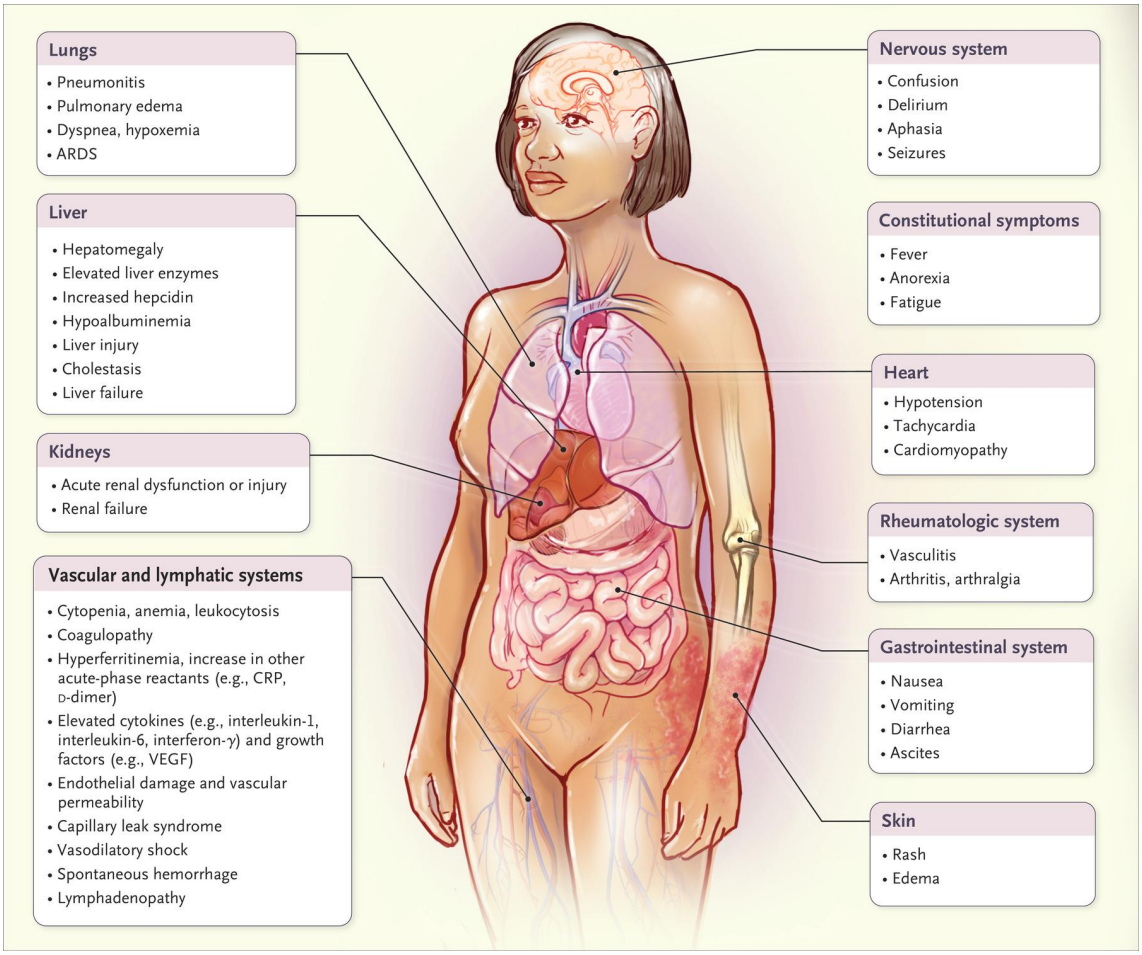


Figure 2. Clinical presentations of cytokine storm syndrome. Details of the clinicopathological manifestation of SARS-CoV-2 induced cytokine syndrome (source: Fajgenbaum and June, 2020).

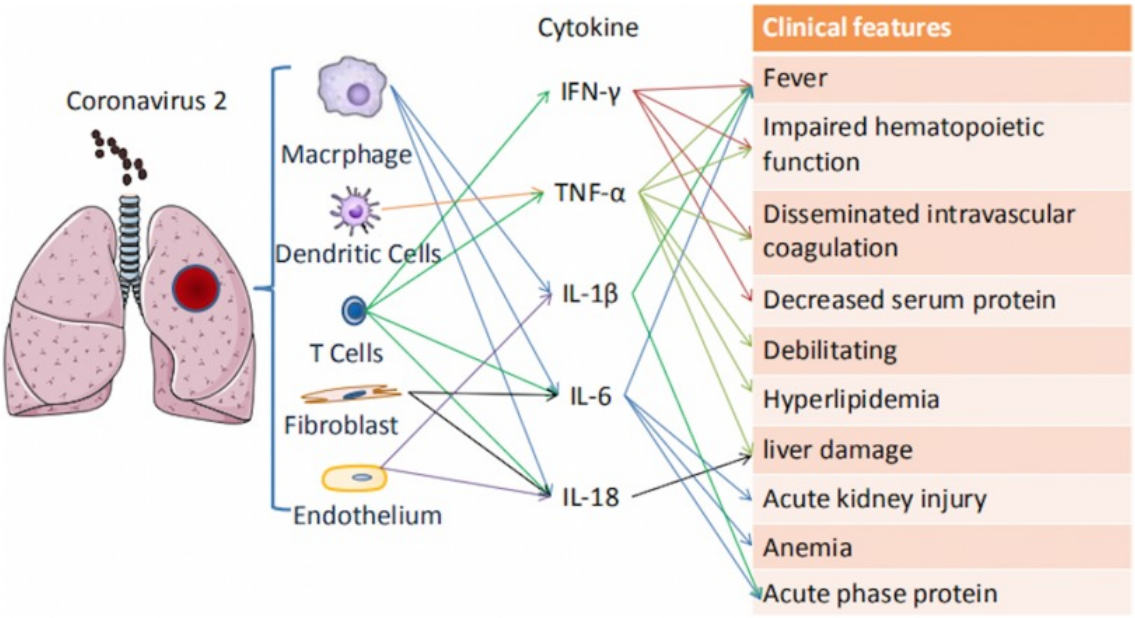


Figure 3. Schematic representation of clinical features caused by pathogenic inflammatory cytokine response in COVID-19. The main cells and produced cytokines involved in the CSS are reported (source: Sun *et al.*, 2020).

a) IFNγ

IFNγ is secreted by cells of both the innate and adaptive immune systems, such as T helper 1 (Th1) cells, cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells following an infection and it is a strong activator of macrophages; after the binding to its receptor Interferon Gamma Receptor 1 (IFNGR1), it activates the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling and triggers the response to viral and microbial infections (Hammarén et al., 2019; Fajgenbaum and June, 2020). It also enhances the antigen presentation through both MHC I and MHC II (Akiyama et al., 1994; Laha et al., 1995).

From a pathological point of view, a great release of IFNγ can cause headache, dizziness, fatigue and fever (Fajgenbaum and June, 2020).

Fever, which is a clinical hallmark of the CSS, can also be elicited by IL1β, IL6 and TNFα.

b) IL1β

IL1β is a potent pro-inflammatory cytokine, mainly produced by macrophages and epithelial cells, but it is also a specific soluble mediator produced by pyroptotic cells (Fajgenbaum and June, 2020). Indeed, IL1β can be released by cells that undergo this type of inflammatory programmed cell death which mostly occurs following intracellular-pathogen infection and relies on inflammasome to take place. The inflammasome is a multiprotein complex which comprises sensor protein (NLR, which is activated by pathogens or alarmins), inflammatory caspases, and an adapter protein connecting the two (apoptosis-associated speck-like protein containing a caspase recruitment domain, ASC). After sensing pathogen- or damage- associated molecular patterns (PAMPs, DAMPs), the NLR activates caspase-1, the enzyme that subsequently mediates the maturation and secretion of IL1β and IL18 (Fernandes-Alnemri *et al.*, 2007; Yap, Moriyama and Iwasaki, 2020; Zheng, Liwinski and Elinav, 2020).

Notably, SARS-CoV-2 is known to trigger the NLR family pyrin domain containing 3 (NLRP3) inflammasome activation and therefore to increase the release of IL1β, which in turn activates other pro-inflammatory cytokines, including IL6 and TNFα and hence can contribute to the phenomenon of the cytokine storm (Figure 4) (Costela-Ruiz *et al.*, 2020; Yap, Moriyama and Iwasaki, 2020); higher levels of serum IL1β have been reported to be associated with an higher risk to develop the severe form of COVID-19, in particular with ARDS, hypercoagulation and DIC, and to have a fatal outcome (Swartz *et al.*, 2020; W. Zhang *et al.*, 2020).

As mentioned, IL1β is a proinflammatory alarmin cytokine which acts on macrophage and T helper 17 (Th17) cell activation, of whom it promotes the the differentiation, and possesses pyrogenic function: along with IL6, TNFα and other cytokines, it induces the release of prostaglandin E2 (PGE2), a potent inflammatory mediator that acts on the hypothalamus to raise the set point of body temperature (Luheshi and Rothwell, 1996; Kita *et al.*, 2015; He, Hara and Núñez, 2016).

It also contributes to neutrophil influx in the inflamed tissue and their activation, along with T-cell and B-cell activation, cytokine and antibody production,

fibroblast proliferation and collagen production and to Th1 cells induction of IFNγ production, together with IL12 (Van Damme *et al.*, 1985).

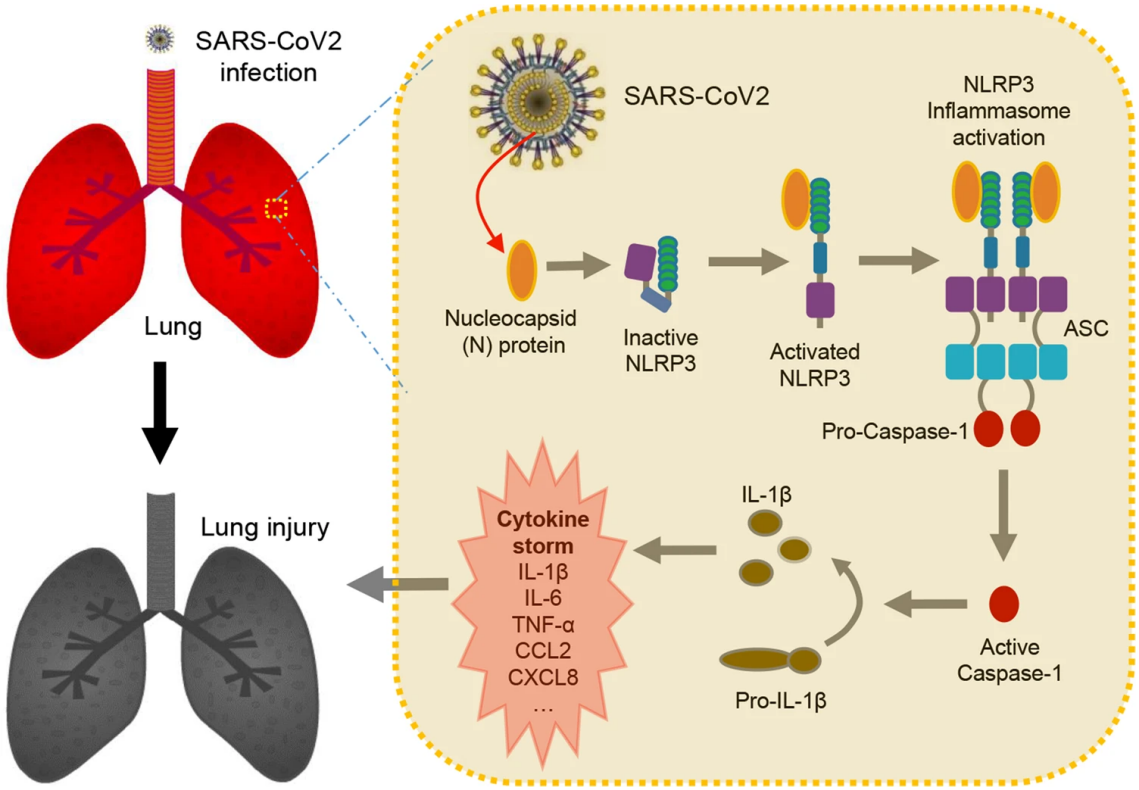


Figure 4. SARS-CoV-2 can activate NLRP3 inflammasome. In SARS-CoV-2 infection, the Nucleocapsid protein (N) directly interacts with NLRP3 to promote the assembly of NLRP3 inflammasome, thus leading to the activation of caspase-1 and to the production of a large number of inflammatory factors (such as IL1β, IL6, TNF, CCL2) which can subsequently lead to lung injury (source: Pan *et al.*, 2021).

c) IL6

IL6 is a cytokine that plays a pivotal role in a variety of biological processes in the contexts of immunity, tissue regeneration and metabolism. For what concerns immunity, in the innate immune response it is produced by myeloid cells as macrophages and dendritic cells after the pathogen recognition through TLRs; in the adaptive immune response it is produced by T cells and it is necessary for the maturation of B cells into antibody-secreting plasma cells, for the differentiation of CD4+ T cells subsets, in particular of naive CD4+ into Th17 cells (Kang *et al.*, 2019; Fajgenbaum and June, 2020). This protein is synthesized at the sites of acute and chronic inflammation after TLR recognition

or IL1β induction and is then secreted into the blood to induce the transcription of inflammation-associated genes through its binding to and consequent signaling of the IL6 receptor. This response comprises fever, since IL6 is an endogenous pyrogen, increase in antibody production and induction of acute-phase molecules (Fajgenbaum and June, 2020; Rose-John, 2021). IL6 synthesis contributes to the host defense against the infecting pathogen and in tissue injury, yet an excessive production of this cytokine is involved in several inflammation-associated pathological states, such as systemic juvenile rheumatoid arthritis, along with viral infections including COVID-19 (Kang *et al.*, 2019; Zhou *et al.*, 2021). High levels of IL6 have been reported in SARS-CoV-2 infected patients and have been associated with more severe disease, with an higher risk of developing ARDS and with an increased mortality (Santa Cruz *et al.*, 2021; Zhou *et al.*, 2021).

d) TNFα

The multifunctional pro-inflammatory cytokine TNFα is mainly produced by macrophages upon TLR stimulation, but is also synthetized by T cells, NK cells and mast cells and has been related to pro-inflammatory responses mediated by IL1β and IL6 (Fajgenbaum and June, 2020). It is synthetized as a transmembrane protein (mTNF), which can signal in cell-cell contacts and which can then be proteolytically cleaved by Disintegrin And Metalloproteinase Domain-Containing Protein 17 (ADAM17) to produce the soluble form (sTNF) (Black *et al.*, 1997).

TNFα receptors are tumor necrosis factor receptor 1 (TNFR1), which is constitutively expressed ubiquitously and can be activated by both mTNF and sTNF, and tumor necrosis factor receptor 2 (TNFR2), whose expression is specific for determined cell types, such as immune cells, endothelial cells and neurons, and which can only bind mTNF. The signaling that originates from the receptor-ligand interaction mainly promotes inflammation and tissue degeneration, but also proliferation and apoptosis, for what concerns TNFR1 signaling, while opposite homeostatic processes such as cell survival and tissue regeneration are mediated by anti-inflammatory TNFR2 signaling (Kalliolias and Ivashkiv, 2016; Heir and Stellwagen, 2020).

From the pathological point of view, it increases vascular permeability therefore contributing to COVID-19 pulmonary edema and its pyrogenic function adds to the ones of the aforementioned IL1β and IL6 (Fajgenbaum and June, 2020).
As IL1β and IL6, also TNFα serum levels are elevated in COVID-19 patients and in particular are significantly higher in more severe patients (Costela-Ruiz *et al.*, 2020).

e) IL18

IL18 is a pro-inflammatory cytokine that belongs to the IL1 family. It is constitutively present in a precursor form in the cytoplasm of a variety of cells, including monocytes, macrophages, dendritic cells (Fajgenbaum and June, 2020). Upon proper stimulus, the inflammasome caspase-1 processes it to its active form, which is subsequently released through the gasdermin-D (GSDMD) pore (Xia *et al.*, 2021).
It synergizes with IL12 to stimulate the IFNγ production in Th1 and NK cells. In particular, in these cells, upon binding to interleukin 18 receptor 1 (IL18R1) and interleukin 18 receptor accessory protein (IL18RAP) and subsequent activation of nuclear factor kappa B (NF-kappa-B), it induces the transcription of inflammatory mediators (Tominaga *et al.*, 2000; Kato *et al.*, 2003; Tsutsumi *et al.*, 2014).
Moreover, together with IL1β, it is a potent inducer of IL6 secretion by macrophages (Netea *et al.*, 2000).

1.1.6. Diagnosis

The standard diagnostic methods for COVID-19 are the SARS-CoV-2 nucleic acid amplification test (NAAT) or antigenic-rapid-detection test (RDT). The first test analyzes a nasopharyngeal swab with the aim of detecting SARS-CoV-2 nucleic acids *via* reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) assay. Oropharyngeal, anterior/mid-turbinate nasal swabs, nasopharyngeal aspirates, bronchoalveolar lavage and saliva can also be utilized as specimens for the detection of the virus *via* RT-qPCR. The second modality, SARS-CoV-2 antigen tests, is a rapid chromatographic immunoassay

for the qualitative detection of specific antigens of SARS-CoV-2. The latest diagnostic test is less sensitive compared to NAAT, but it has the advantage of a rapidly available result (Gandhi, Lynch and del Rio, 2020; Cascella *et al.*, 2022; *Antigen-detection in the diagnosis of SARS-CoV-2 infection*).

Laboratory assessments can also support the diagnosis and the evaluation of the severity of COVID-19. In particular, in COVID-19 patients, the complete blood count (CBC) test usually reveals an elevated red blood cells distribution width (RDW), leukocytosis, lymphopenia and thrombocytopenia whereas a comprehensive metabolic panel (CMP) normally shows elevated liver enzymes (aspartate transaminase AST, alanine aminotransferase ALT, bilirubin) as well as impaired renal function. The coagulation screen reveals elevated D-dimer levels, prolonged prothrombin time (PT) and elevated fibrinogen. The testing for other inflammatory markers such as erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), ferritin, serum lactate dehydrogenase (LDH) and procalcitonin often shows elevated levels in severe patients (Z.-L. Zhang *et al.*, 2020).

Imaging analyses play a fundamental role in the diagnosis and management of the disease, in particular for the assessment of lung involvement and damage. They may consist of chest x-ray, lung ultrasound or chest computed tomography (CT).

The chest X-ray examination can be completely normal in the early stages, while it commonly highlights bilateral multifocal alveolar opacities, which tend to progress to the complete opacity of the lung in severe and critical patients (Huang *et al.*, 2020).

CT scan of the chest is recommended in those patients who manifest severe COVID-19 symptoms despite having a normal x-ray. This technique is characterized by a high sensitivity (90%) for COVID-19 diagnosis in symptomatic adults, yet the specificity is lower, as most of the findings can also be observed in other lung infections. The most common CT findings in COVID-19 are multifocal bilateral “ground-glass opacity”, with a distribution pattern typically peripheral/subpleural, with a lower lobe of the posterior region predominance (Ojha *et al.*, 2020).

Ultrasonographic examination of the lung can also be used as an examination to evaluate the progression of the disease, from the initial stage of focal

interstitial pattern up to the severe stage of a "white lung". It is useful to determine the need of mechanical ventilation and prone positioning, even more so due to the non-invasive and radiation-free nature of this technique.
Finally, since the early symptoms are nonspecific, differential diagnosis should discriminate COVID-19 from other infectious (e.g. adenovirus, influenza, parainfluenza, rhinovirus infections and tuberculosis) and non-infectious respiratory disorders (e.g. aspiration pneumonia, vasculitis, dermatomyositis (Cascella *et al.*, 2022).

1.1.7. Treatment / clinical management

In the early phases of the pandemic, the clinical management of COVID-19 patients suffered from the lack of knowledge on the disease and of proper therapies, fostering the need for new medications and drug repurposing. Since then, significant progress has been made thanks to the immense effort of clinical research, which has allowed the development of novel therapeutic strategies and vaccines at unprecedented speed.
Up to date, a variety of pharmacological therapeutic options are available to treat COVID-19 patients, including antiviral drugs, anti-SARS-CoV-2 monoclonal antibodies, anti-inflammatory drugs and immunomodulator agents (Coopersmith *et al.*, 2021).
Of note, the choice of the proper therapeutic option based on the severity of the disease is of paramount importance: indeed, the clinical utility and efficacy of these treatments widely varies in mild, moderate, severe or critical COVID-19 patients; patient risk factors also have to be taken into consideration in the definition of the therapeutic path.
COVID-19 clinical course can be distinguished in 2 phases: in the early phase, before or immediately after the symptoms onset, there is a copious replication of SARS-CoV-2; in this time window antiviral medications, such as Molnupiravir, Paxlovid, Remdesivir, and anti-SARS-CoV-2 neutralizing antibody-based treatments, e.g. REGN-COV2 (Casirivimab and Imdevimab), Bamlanivimab and Etesevimab, Sotrovimab, Bebtelovimab, Tixagevimab and Cilgavimab, are likely to be more effective. In the later phase, the disease is mainly caused by an

hyperinflammatory and prothrombotic state, induced respectively by the dysregulated release of cytokines and by the activation of the coagulation system. In this stage, anti-inflammatory drugs like corticosteroids, immunomodulating therapies, like anti-IL6 receptor monoclonal antibodies (Tocilizumab, Sarilumab, Siltuximab) and Janus kinase (JAK) inhibitors, or a combination of these therapeutic options can help controlling this hyperinflammatory state more than antiviral therapies (Gandhi, Lynch and del Rio, 2020; Cascella *et al.*, 2022).

1.1.8. Prevention: vaccines

Along with infection control measures such as social distancing, keeping indoor spaces well ventilated, cleaning hands regularly and using masks to prevent the spread of SARS-CoV-2, the turning point in the containment of the COVID-19 pandemic has been the development of vaccines against this virus. The tremendous worldwide research effort to urgently find an effective way to contrast the infection has led to the development of the first Food and Drug Administration (FDA) approved vaccine, the Pfizer-BioNTech COVID-19 Vaccine, at unprecedented speed: the first COVID-19 case was diagnosed in December 2019, the vaccine was approved on December 11th, 2020 (Commissioner, 2021). These vaccines have shown to provide powerful protection against serious disease, hospitalization and death from COVID-19 (L. Chen et al., 2020; Andrews et al., 2022).

Different vaccine platforms against SARS-CoV-2 have been developed, which include recombinant viral vector, inactivated and attenuated viruses, DNA and RNA based vaccines and protein subunits (Hu *et al.*, 2021). As of 8 April 2022, WHO has declared that 10 examined vaccines have met the criteria for safety and efficacy: AstraZeneca/Oxford vaccine, Johnson and Johnson, Moderna, Pfizer/BionTech, Sinopharm, Sinovac, Covaxin, Covovax, Nuvaxovid, CanSino (*COVID-19 Vaccines Advice*). In addition to these, other vaccines, including protein-based and inactivated vaccines, have been developed independently in India (Covaxin), Russia (Sputnik V), and China (CoronaVac) and have been

approved or granted emergency use authorization in many countries around the world to prevent COVID-19 (Cascella *et al.*, 2022).

Up to date (23 December 2022), the public health effort has successfully administered 13.073.712.554 vaccine doses, with 4.856.585.086 people who received at least 1 dose of vaccine and 4.101.203.911 completed vaccination cycles (*COVID-19 vaccines*; *WHO Coronavirus (COVID-19) Dashboard*). The vaccination rate is hugely variable across different global areas, but also among states of the same area: it shifts from the 60-80% of European, Asian, American countries, to around 50% of the middle-east countries, to around 30% of the African countries, with an average of 60% of vaccinated population in WHO Member States.
The vaccination campaign is estimated to have prevented 19,8 million deaths in 2021 (Watson *et al.*, 2022) and a study has recently reported that vaccination with AstraZeneca/Oxford or Pfizer/BionTech vaccines is 90% effective in preventing people from dying (Christie, 2021).

1.2. SARS-CoV-2

As mentioned, the etiologic agent of COVID-19 is the virus SARS-CoV-2.
SARS-CoV-2 genome is composed of a positive-sense single-stranded RNA molecule (+ssRNA) that has a 5'-cap structure and a 3'-poly-A tail and that accounts for about 30 kb, encoding for ca. 9.000 amino acids and 29 proteins. Of these, 4 are structural proteins and 25 are putative non-structural and accessory proteins (P. Zhou *et al.*, 2020; Wu *et al.*, 2022).

The 5' terminus encodes for pp1a and pp1b, two polyproteins that derive from a -1 shift between open reading frame 1a (ORF1a) and ORF1b and that are then digested into 16 non structural proteins (NSPs) by two viral proteases (papain-like protease (PL^{pro}) and a 3C-like protease ($3CL^{pro}$)). These NSPs are necessary for the viral transcription and replication (Chen, Liu and Guo, 2020). At the 3' terminus, 4 ORFs encode for the structural proteins: Spike (S) protein, Envelope (E) protein, Membrane (M) protein and Nucleocapsid (N) protein. Genes encoding for the accessory proteins ORF3a, ORF3b, ORF6, ORF7a, ORF7b, ORF8b, ORF9b and ORF14 are interspaced between these structural-protein coding genes (Figure 5) (Yang and Rao, 2021).

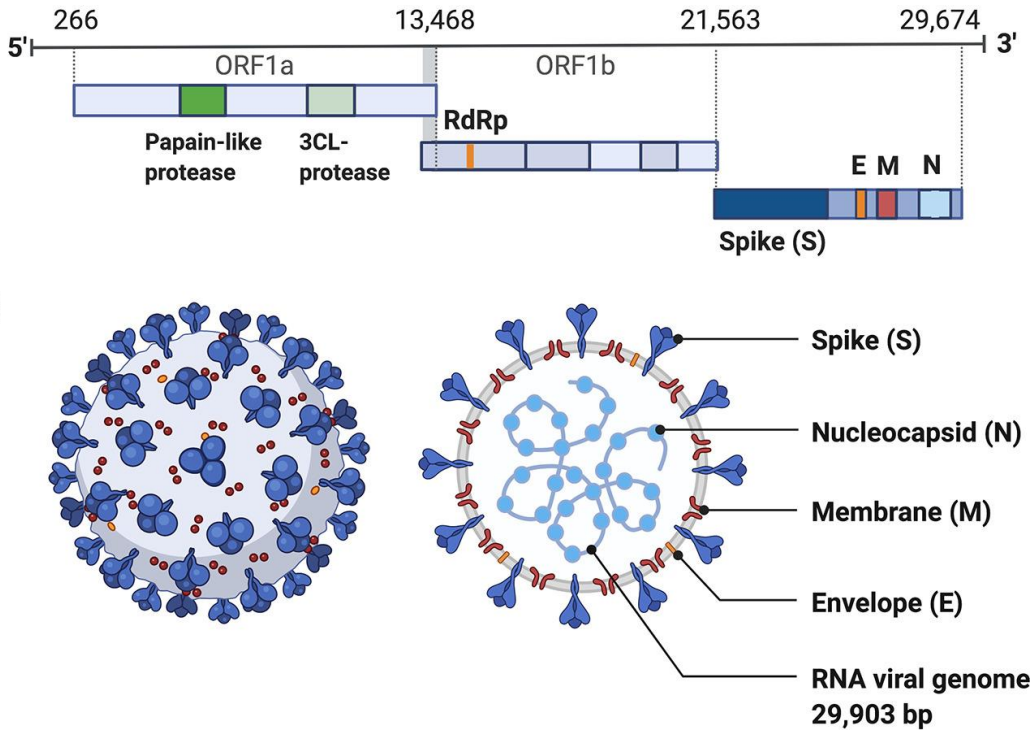


Figure 5. SARS-CoV-2 genome organization and virion structure (modified from Rando *et al.*, 2021).

1.2.1. Replication cycle

In the first step of the replication cycle of SARS-CoV2, the Spike protein located on the surface of the virion binds the angiotensin-converting enzyme 2 (ACE2) receptor on the host cell membrane through its receptor binding domain (RBD). S1/S2 and S2' cleavage sites are then proteolytically cleaved by the host transmembrane protease serine 2 (TMPRSS2), which facilitates facilitates the SARS-CoV-2 entry at the cell membrane (Hoffmann *et al.*, 2020; P. Zhou *et al.*, 2020; Wu *et al.*, 2020). In alternative, cathepsins, mainly cathepsin L, can activate the S protein in endosomes therefore allowing the viral entry into TMPRSS2-lacking cells, yet cathepsin plays a minor role compared to TMPRSS2, so its inhibitor hydroxychloroquine does not represent a valid treatment option (Figure 6) (Hoffmann *et al.*, 2020; Tanni *et al.*, 2021; Jackson *et al.*, 2022; Schwartz, Boulware and Lee, 2022).

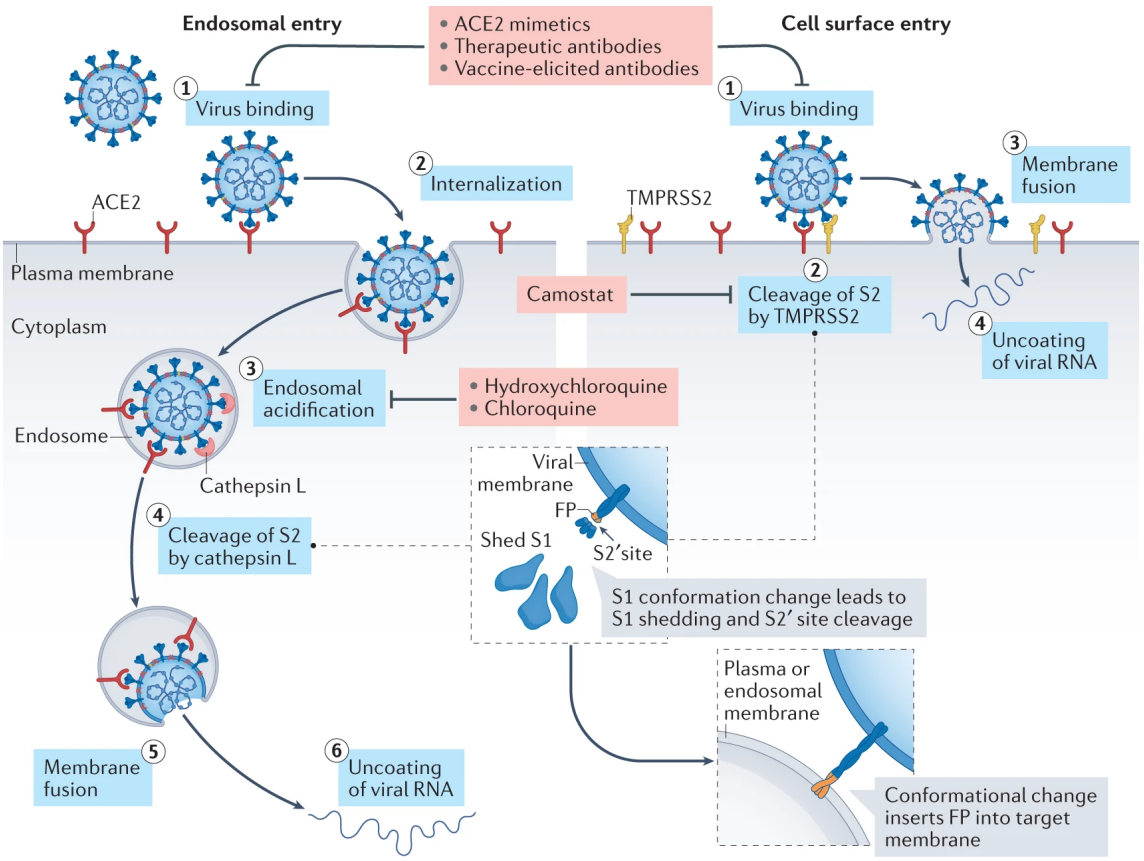


Figure 6. SARS-CoV-2 cell entry pathways (source: Jackson *et al.*, 2022).

After the TMPRSS2 cleavage, the S protein shifts from a pre-fusion conformation to a post-fusion with the ACE2 receptor and the SARS-CoV-2 genome is released into the cell. With the viral RNA now in the host cell cytoplasm, the ORF1a and ORF1b are translated into the two replicase polyproteins pp1a and pp1b by the host ribosomes and these are subsequently cleaved into 16 NSPs, which, by assembling themselves, generate the replication and transcription complexes (RTC), which translate the subgenomic viral mRNAs into proteins. The newly synthesized structural proteins and genomic RNA are then assembled into virions in the endoplasmic reticulum and in the Golgi apparatus and finally the progeny virions are released *via* exocytosis (Figure 7) (Snijder *et al.*, 2006; Wu and Brian, 2010; Harrison, Lin and Wang, 2020; Yang and Rao, 2021).

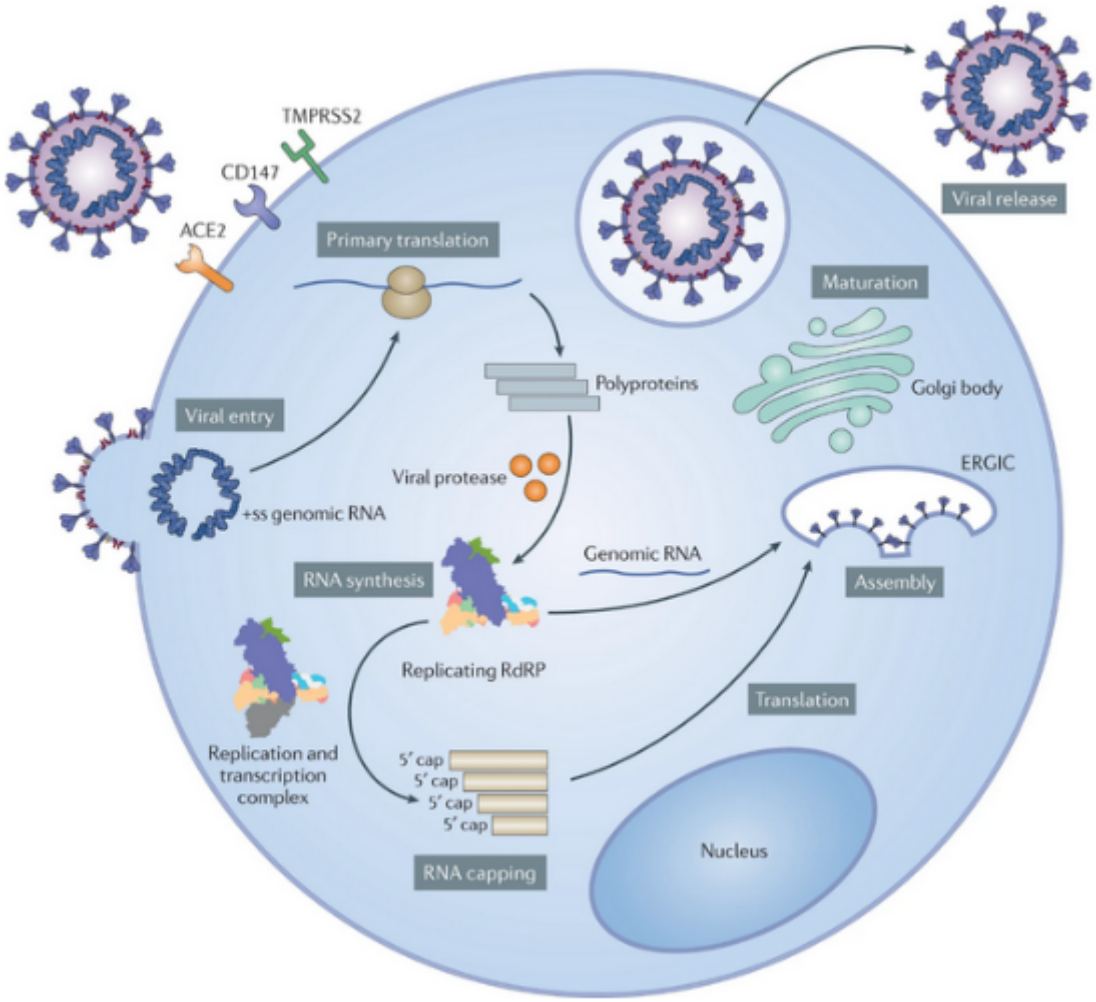


Figure 7. SARS-CoV-2 replication cycle. SARS-CoV-2 life cycle, including viral entry, replication and transcription, assembly and release. SARS-CoV-2 enters host cells through S protein-ACE2-TMPRSS2 interactions. In the cytoplasm, viral RNA is translated into ORF1a or ORF1b by the host ribosome. The viral polyproteins obtained from ORFs are subsequently cleaved into NSPs and assembled into the replication and transcription complexes (RdRp, RNA-dependent RNA polymerase). After capping, subgenomic viral mRNAs act as templates for viral protein translation. Progeny virions are assembled in the endoplasmic reticulum–Golgi intermediate compartment (ERGIC) and then exocytosed (modified from Yang and Rao, 2021).

1.2.2. SARS-CoV-2 proteins

As mentioned, SARS-CoV-2 genome codifies for 29 proteins: the NSPs play crucial roles in the replication of the viral genome and in the evasion of the host immune response, while accessory proteins improve the viral infection and contribute to dampening the host immune response (Redondo *et al.*, 2021; Thomas, 2021; Wu *et al.*, 2022).

The 4 structural proteins (Spike protein, Envelope protein, Membrane protein and Nucleocapsid protein) are essential for the organization of the SARS-CoV-2 virions, which are characterized by a spherical/ellipsoidal shape with an average diameter around 100 nm (Yao *et al.*, 2020; Hardenbrook and Zhang, 2022). The external surface of the virion is covered by Spike proteins; the outer membrane also contains the Membrane protein and the Envelope protein. Inside the virion lumen, the Nucleocapsid protein assembles with the viral RNA genome and packages it into the ribonucleoprotein complexes (RNPs), which amount to 30-35 per virion (M.-Y. Wang *et al.*, 2020; Yao *et al.*, 2020).

In the next paragraph, the characteristics and roles of the Spike protein will be discussed.

1.2.2.1. Spike protein

The Spike (S) protein is a structural protein which plays a fundamental role in the viral entry into the host cell: it facilitates the anchorage of the virion to the surface of the host cell and mediates the fusion of the virus with the host plasma membrane, allowing the viral entry into the cell (Tortorici and Veesler, 2019). This type I membrane protein decorates the surface of the virus, giving it a solar crown appearance, hence the name Coronavirus (Zhang *et al.*, 2021). It is a homotrimer that is anchored to and protrudes from the virion membrane (Belouzard *et al.*, 2012) and it is characterized by an abundant glycosylation: indeed, each monomer of the S protein accounts for 22 glycosylated sites (Watanabe *et al.*, 2020); this glycan coverage, together with the characteristic protein flexibility, allows it to scan the host cell surface by moving and sticking, until it can finally interact and bind the host ACE2 receptor. In addition, this

glycan coat protects the protein from the neutralizing antibodies (Turoňová *et al.*, 2020; Yao *et al.*, 2020), yet some neutralizing antibodies have been proven to be able to still bind the glycosylated epitopes, therefore allowing an immune response against the virus (Pinto *et al.*, 2020; Du *et al.*, 2021; Rapp *et al.*, 2021). In general, the S protein elicits a strong immune response being highly immunogenic, reason why it has been considered the main target in the development of SARS-CoV-2 vaccines (Du *et al.*, 2020; Letko, Marzi and Munster, 2020; Robbiani *et al.*, 2020; Zost *et al.*, 2020).

The S protein is composed by ca. 1.200 residues and it presents two functional subunits: S1, the receptor-binding subunit, which is responsible for the binding to the host cell surface, and S2, the fusion subunit, that mediates the viral-host membrane fusion (Walls *et al.*, 2020). These two subunits are composed of several functional domains (Figure 8): from the N-terminus to che C-terminus, the N-terminal domain (NTD), the receptor-binding domain (RBD) and C-terminal domains (CTD1 and CTD2) form the S1 subunit, while the fusion peptide (FP), the fusion-peptide proximal region (FPPR), the heptad repeat 1 (HR1), the central helix (CH), the connector domain (CD), the heptad repeat 2 (HR2), transmembrane segment (TM) and the cytoplasmic tail (CT) are the domains of the S2 subunit (Figure 8) (Zhang *et al.*, 2021).

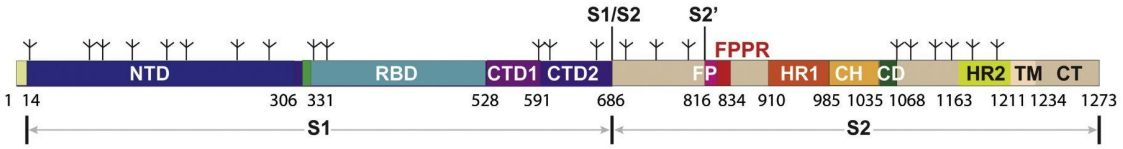


Figure 8. SARS-CoV-2 functional domains. The S1 subunit includes N-terminal domain (NTD), the receptor-binding domain (RBD) and C-terminal domains (CTD1 and CTD2), while the S2 subunit is composed of the fusion peptide (FP), the fusion-peptide proximal region (FPPR), the heptad repeat 1 (HR1), the central helix (CH), the connector domain (CD), the heptad repeat 2 (HR2), transmembrane segment (TM) and the cytoplasmic tail (CT) (modified from Zhang *et al.*, 2021).

In particular, the RBD, which consists of a β-sheet core with 5 antiparallel sheets flanked by a short helix, can assume two conformational states in its prefusion form: the “RBD up” and the “RBD down” conformations; in the “down” state, the receptor-binding regions of this domain are shielded, therefore it

represents the receptor-inaccessible state, while in the “up” state, the conformational movement of the RDB exposes the regions that interact with the host ACE2 receptor, making it receptor-accessible. Only this last conformation has been shown to be able to bind the receptor (Figure 9) (Xu *et al.*, 2021; Zhu *et al.*, 2021).

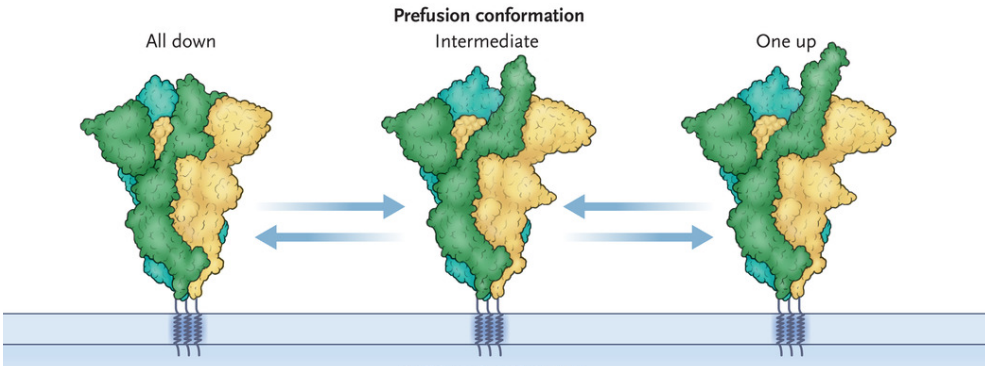


Figure 9. “RBD up” and the “RBD down” conformations of the SARS-CoV-2 Spike protein (source: Koenig and Schmidt, 2021).

After the binding of the S protein to the host ACE2 receptor, the rearrangement from the prefusion to the postfusion form of the S protein allows it to carry out its role: the fusion of the viral coat and the host membranes starts with the RBD domain of the Spike S1 subunit binding the host ACE2 through 14 hydrogen bonds and 1 salt bridge (Figure 10) (Lan *et al.*, 2020). This interaction triggers the following priming by the cellular protease TMPRSS2, which cleaves the S protein at the S1/S2 and the S2’ cleavage sites (Hoffmann *et al.*, 2020; Zhang *et al.*, 2021). This cleavage site comprises 4 residues, P681, R682, R683, and A684, which constitute the basic serine proteases cleavage site, RXXR, with X being a positively charged lysine or arginine. If deprived of this site, the SARS-CoV-2 has a reduced S protein priming, hence the relevance of this site in the SARS-CoV-2 replication and pathogenicity (Johnson *et al.*, 2020).

After the cleavage, the S1 and S2 subunits remain associated thanks to non-covalent bindings, as elucidated by cryogenic electron microscopy (cryo-EM) (Wrapp *et al.*, 2020). Thus, HR1 and HR2 helices of the S2 subunit interact to form a 6 helix bundle, which brings the virion and host membranes into proximity, therefore facilitating the following membrane fusion. This process

is in turn mediated by the fusion peptide, which consists of a short hydrophobic proteic segment (Xia *et al.*, 2020). After the virus-host cell membrane fusion, the RNA genome is released into the host cell and the replication cycle can start.

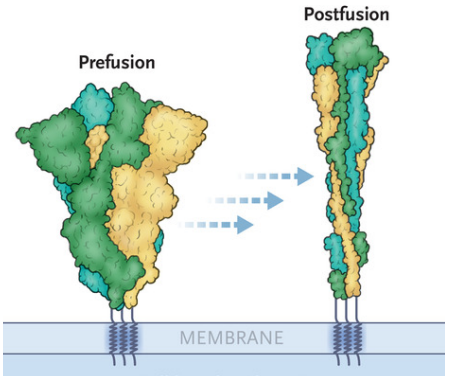


Figure 10. Perfusion and postfusion conformations of SARS-CoV-2 Spike protein (source: Koenig and Schmidt, 2021).

1.2.3. SARS-CoV-2 receptors: ACE2, TMPRSS2 and others

1.2.3.1. ACE2

ACE2 is the human receptor that binds the S1 subunit of the S protein of SARS-CoV-2 and mediates its entry into the host cell.

i) Coding

It is encoded by the *ANGIOTENSIN CONVERTING ENZYME 2* gene, that is located on the X chromosome of the human genome (Xp22.2) (Culebras and Hernández, 2020).

Besides the full length isoform, a second isoform has been identified, the so called deltaACE2 (dACE2), which is a truncated isoform of ACE2 lacking 356 amino-terminal amino acids and that is unable to bind the SARS-CoV-2 S protein and is functionless as a carboxypeptidase (Onabajo *et al.*, 2020).

Polymorphisms in *ACE2* gene have been associated with different susceptibility to and severity of COVID-19 (Bakhshandeh *et al.*, 2021; Chen *et al.*, 2021).

ii) Structure

ACE2 protein has a molecular weight of 92kDa but it migrates to 120kDa due to N-glycosylation (Warner *et al.*, 2005). It composed of several domains: the extracellular region of the protein consists of a zinc metallopeptidase domain and, at the C-terminus, of a domain which is 48% identical to human collectrin, a homologue of ACE2, while the cytoplasmic tail comprises several linear motifs like LIR, PDZ-binding, PTB and endocytic sorting signal motifs, which enable the interaction with other proteins involved in endocytic trafficking and autophagy (Towler *et al.*, 2004; Kliche *et al.*, 2021; Mészáros *et al.*, 2021).

iii) Function

It belongs to the angiotensin-converting enzyme family of dipeptidyl carboxypeptidase and it has a remarkable homology with the human angiotensin converting enzyme 1 (ACE).

Physiologically, it is an essential negative regulator of the renin-angiotensin system (RAS), which plays a fundamental role in the regulation of cardiovascular and renal function: this protein is critical for the regulation of the blood volume/pressure, the systemic vascular resistance and therefore the global cardiovascular homeostasis (Wang *et al.*, 2016). In particular, ACE2 is responsible for the cleavage of angiotensin I into angiotensin 1-9, a small peptide that exerts anti-hypertrophic effects on cardiomyocytes, and of angiotensin II into angiotensin 1-7, which acts as vasodilator and anti-proliferation agent, counteracting the effects of the vasoconstrictor angiotensin II, overall lowering the blood pressure (Donoghue *et al.*, 2000; Tipnis *et al.*, 2000; Vickers *et al.*, 2002; Zisman *et al.*, 2003; Rushworth, Guy and Turner, 2008).

ACE2 is also involved in the removal of residues at the C-terminus from three other peptides with vasoactive properties: neurotensin, kinetensin, and des-Arg bradykinin (Donoghue *et al.*, 2000; Vickers *et al.*, 2002).

Moreover, it cleaves additional peptides such as apelins (peptides that mediate beneficial effects on the cardiovascular system), casomorphins and dynorphin A (two endogenous opioid peptides involved in inhibitory neurotransmission in the central nervous system) (Vickers et al., 2002; Wang et al., 2016; Yang et al., 2017). Also, ACE2 C-terminus takes part in the trafficking of the neutral amino

acid transporter Solute Carrier Family 6 Member A19 (SLC6A19) to its functional allocation on the plasma membrane of intestinal epithelial cells, thus regulating its surface expression and its catalytic activity (Kowalczuk et al., 2008; Camargo et al., 2009). Furthermore, ACE2 is a regulator of the female and male fertility, with its role being mediated by angiotensin 1-7, which regulates the blood flow to the reproductive organs (Mitsube et al., 2003; dos Reis et al., 2009; Ferreira et al., 2011).

As previously mentioned, ACE2 is involved in the COVID-19 infection as it binds the SARS-CoV-2 S protein with high affinity. It is also the functional receptor for the Spike protein of other human coronaviruses, such as CoV-NL63 and SARS-CoV-1 (Li *et al.*, 2003, 2004, p. 2, 2005; Hofmann *et al.*, 2005; Wu *et al.*, 2009).

iv) Tissue expression

ACE2 is expressed in several human organs, including heart, arteries, kidneys, testis, gastrointestinal system. In the lung, it is expressed in the alveolar type II cells, with levels of expression that vary among individuals (Donoghue *et al.*, 2000; Harmer *et al.*, 2002; Douglas *et al.*, 2004; Hamming *et al.*, 2004; Burrell *et al.*, 2005; Hikmet *et al.*, 2020; Smith *et al.*, 2020; Zou *et al.*, 2020; Blume *et al.*, 2021). It is also expressed in the nasal epithelial cells (Jackson *et al.*, 2020; Blume *et al.*, 2021).

Notably, its expression can be induced by IFN alpha and gamma (IFNα and γ), an aspect that generates a link between ACE2 expression and the immune system (Ziegler *et al.*, 2020).

The expression of a novel short ACE2 isoform has been recently proven to be increased in airway epithelial cells, upon the infection with influenza viruses and rhinovirus, but not SARS-CoV-2, and upon interferon response (Blume *et al.*, 2021).

Moreover, ACE2 can be upregulated in heart failure (Zisman *et al.*, 2003; Goulter *et al.*, 2004; Burrell *et al.*, 2005) and by the exposure of lungs to cigarette smoke (Smith *et al.*, 2020). On the contrary, it is down-regulated in the nasal and bronchial epithelium of allergic individuals after the allergen challenge (Jackson *et al.*, 2020).

The expression of this gene can also be regulated by several factors such as gender, comorbidities, medications (anti-hypertensives), environmental factors, and the interaction with other RAS genes (Medina-Enríquez *et al.*, 2020).

v) Processing

ACE2 is proteolytically cleaved by the aforementioned TMPRSS2. It can be also cleaved by other proteases such as Disintegrin And Metalloproteinase Domain-Containing Protein 17 (ADAM17, also known as TACE), whose cleavage produces a secreted soluble form of the protein (Lambert *et al.*, 2005; Yeung *et al.*, 2021), and Furin (also known as Paired Basic Amino Acid Cleaving Enzyme, PACE) (Zipeto *et al.*, 2020; Fuentes-Prior, 2021).

1.2.3.2. TMPRSS2

The Transmembrane Serine Protease 2 (TMPRSS2) is the serine protease responsible for the S protein cleavage; as mentioned, this priming exposes the S2 subunit of the S protein, allowing the fusion of the virion and the host plasma membrane.

i) Coding

TMPRSS2 is encoded by the *TRANSMEMBRANE SERINE PROTEASE 2* gene, which is located on the chromosome 21 of the human genome (21q22.3) (Paoloni-Giacobino *et al.*, 1997). This gene has two isoforms, both of which shift from the inactive zymogen form to the active form *via* autocatalysis (Figure 11). Mutations in *TMPRSS2* gene have not been associated with inherited diseases; *TMPRSS2* knockout mice showed no abnormal phenotype, thus suggesting a functional redundancy in the serine protease family or, alternatively, a specific but not vital function that might be manifest only in specific contexts such as stress or disease backgrounds (Kim *et al.*, 2006).

ii) Structure

This 54 kDa protein is composed by a type II transmembrane domain, a class A receptor domain, a scavenger receptor cysteine-rich domain and a protease domain (Thunders and Delahunt, 2020).

iii) Function

It belongs to the serine protease family, which are involved in several physiological and pathological processes. Indeed, TMPRSS2 is involved in a variety of processes, such as digestion, tissue remodeling, fertility, along with tumor cell invasion and activation of pain neurons (Wilson *et al.*, 2005; Lucas *et al.*, 2014; Ko *et al.*, 2015; Lam *et al.*, 2015; Thunders and Delahunt, 2020).

In particular, the TMPRSS2 expression is elevated in the luminal epithelium of the prostate gland and it is regulated by androgenic hormones: indeed it presents androgen-responsive elements at the 5' UTR, which are bound by testosterone and dihydrotestosterone (Lin *et al.*, 1999; Chen *et al.*, 2010; Mollica, Rizzo and Massari, 2020). In this organ, this protein is involved in the proteolytic cascades that are fundamental for prostate homeostasis and it is released into the seminal fluid as a component of prostasomes, vesicles involved in facilitating the sperm function (Chen *et al.*, 2010; Lucas *et al.*, 2014).

Regarding the pathological functions, it has been proven to be overexpressed in prostate cancer cells upon androgenic hormone stimulation and down-regulated in the prostate cancer tissue in androgen-independent tumors. In particular, the fusion gene resulting from *TMPRSS2* gene binding with *ERG* gene (erythroblast-specific-related gene), an oncogenic transcription factor with a role in cell proliferation, differentiation, apoptosis and inflammation, is the most diffuse chromosomal aberration in prostate cancer and it regulates cancer cell invasion and metastasis (Lucas *et al.*, 2014; Mollica, Rizzo and Massari, 2020).

Moreover, TMPRSS2 activates various substrates that include the pro-hepatocyte growth factor (HGF), the protease activated receptor-2 (PAR2 or F2RL1) and the ST14 transmembrane serine protease matriptase, leading to the disruption of the extracellular matrix and favoring the metastasis of prostate cancer cells (Wilson *et al.*, 2005; Lucas *et al.*, 2014; Ko *et al.*, 2015).

This protein is also used, alongside SARS-CoV-2, by the influenza A viruses (strains H1N1, H3N2 and H7N9), by the human parainfluenza 1, 2, 3, 4a and 4b

viruses (HPIV), by Sendai virus (SeV), by the human coronaviruses HCoV-229E (that causes common cold), HCoV-EMC, MERS-CoV, SARS-CoV and by the human metapneumovirus (HMPV) to enter the host cell *via* the proteolytic cleavage and activation of viral envelope glycoproteins (Figure 11) (Shirogane *et al.*, 2008; Shulla *et al.*, 2011; Abe *et al.*, 2013; Bertram *et al.*, 2013; Shirato, Kawase and Matsuyama, 2013; Limburg *et al.*, 2019).

TMPRSS2 contributes to facilitating SARS-CoV-2 infection also through the proteolytic cleavage of the ACE2 receptor, a process that promotes the virion uptake (Heurich *et al.*, 2014). Moreover, this protease increases the rate of syncytia formation by enhancing the fusion process (Buchrieser *et al.*, 2020; Koch *et al.*, 2021).

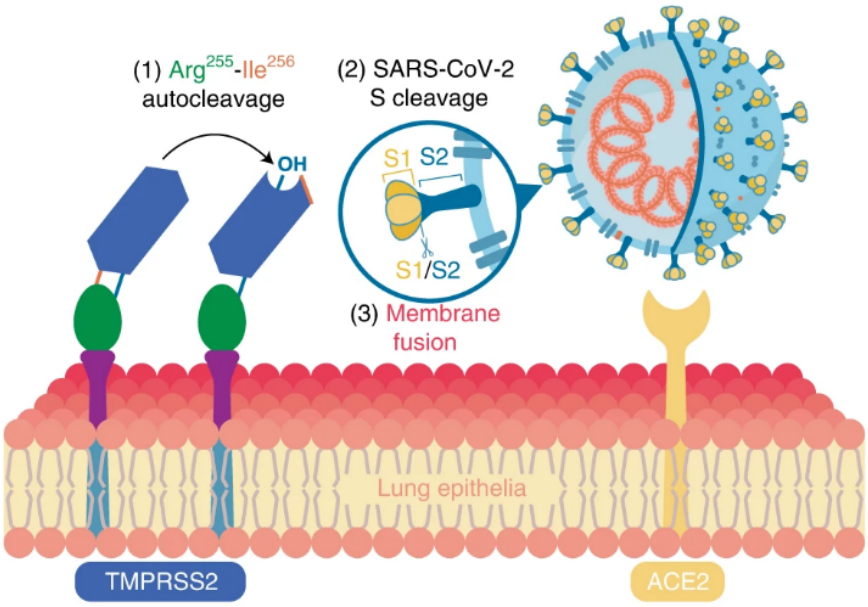


Figure 11. TMPRSS2 schematic representation. TMPRSS2 zymogen undergoes autocleavage activation and the matured enzyme performs the proteolysis of SARS-CoV-2 Spike protein bound to the ACE2 receptor, process which drives membrane fusion (modified from Fraser *et al.*, 2022).

iv) Tissue expression

TMPRSS2 is highly expressed in the epithelial cells of human lungs, and, in particular, it shows an higher expression in alveolar type II cells (AT2) compared

to type I (AT1) (Glowacka *et al.*, 2011; Schuler *et al.*, 2021); it is also expressed in the prostate and the gastrointestinal system, particularly in the small intestine, although it is also present in the heart, liver, kidney and other regions of the respiratory tract, such as bronchus, larynx, trachea, nasal mucosa, and respiratory sinuses, and of the gastrointestinal tract such as colon, stomach and salivary glands. TMPRSS2 is co-expressed with ACE2 in the aforementioned type II pneumocytes, in the nasal goblet secretory cells, in the intestinal epithelial cells, in the gallbladder and in the cornea (Chen *et al.*, 2010; Bertram *et al.*, 2012; Thunders and Delahunt, 2020; Zang *et al.*, 2020; Schuler *et al.*, 2021).

The distribution of TMPRSS2 expression, together with that of the ACE2 receptor, has been correlated with SARS-CoV-2 infection in the lungs (Tomris *et al.*, 2022). Also, TMPRSS2 and ACE2 have been found to be co-expressed in the human corneal epithelium and in the conjunctival tissue, proposing that cells of the ocular surface could be an entry site for the virus (L. Zhou *et al.*, 2020).

Interestingly, the expression of TMPRSS2 increases during aging, with a lower expression in the fetal lung epithelial tissue compared to the adult one, therefore suggesting that the TMPRSS2 developmental regulation of expression may underlie the age-related severity of SARS-CoV-2 infection (Schuler *et al.*, 2021).

1.2.3.2.1. TMPRSS2 inhibitors in COVID-19 treatment

Camostat mesylate is a TMPRSS2 inhibitor that has been clinically approved for the treatment of pancreatitis. Since COVID-19 outbreak, it has been repositioned for the treatment of this disease (Breining *et al.*, 2021).

It has been proven to inhibit SARS-CoV-2 infection in lung cells *via* the blockage of the TMPRSS2 performed cleavage (Hoffmann *et al.*, 2020). Yet a double-blinded randomized placebo-controlled multicenter trial on SARS-CoV-2 hospitalized patients that were treated with 200 mg of camostat mesylate for 3 times/day for 5 days reported that the treatment was not significantly associated with an amelioration in COVID-19 symptoms or mortality (Gunst *et al.*, 2021). Another recent study confirmed these results (Tobback *et al.*, 2022).

Recently, a TMPRSS2-inhibitor small-molecule compound named N-0385 has shown to inhibit SARS-CoV-2 infection in human lung cells and to be therapeutically beneficial in the severe COVID-19 K18-human ACE2 transgenic mouse model (Shapira *et al.*, 2022).

1.2.3.3. ADAM17 and Furin

In addition to TMPRSS2, other 2 proteases have been reported to perform the cleavage that primes the SARS-CoV-2 Spike protein: ADAM17 and Furin.

ADAM17 is a 90 kDa protein that is encoded by the *A DISINTEGRIN AND METALLOPROTEINASE DOMAIN-CONTAINING PROTEIN 17* gene, which maps on the chromosome 2 of the human genome (2p25.1) (Hirohata, Seldin and Apte, 1998; Cerretti *et al.*, 1999).

This membrane-anchored protein with structural affinity to snake venom disintegrins has been proven to contribute in a variety of biologic processes that involve cell-cell and cell-matrix interactions. It plays an important role in the immune and nervous systems (Zunke and Rose-John, 2017). In particular, this protease is responsible for the cleavage of tumor necrosis factor alpha (TNFα), allowing the release of the soluble TNFα from its membrane-bound precursor (Moss *et al.*, 1997).
ADAM17 is also involved in the shedding of various other substrates, including cell adhesion proteins, cytokine and growth factor receptors (epidermal growth factor, EGF) and EGF ligands, along with its role in the activation of the Notch signaling pathway (Thathiah, Blobel and Carson, 2003; Rabquer *et al.*, 2010; Boskovski *et al.*, 2013; Lokau *et al.*, 2016; Riethmueller *et al.*, 2017; Zunke and Rose-John, 2017).

Elevated expression levels of this protease has been reported in multiple types of cells from rheumatoid arthritis, psoriasis, Crohn's disease and multiple sclerosis patients, supporting the active involvement of ADAM17 in different autoimmune diseases (Calligaris *et al.*, 2021). Mutations of this gene cause the neonatal inflammatory skin and bowel disease 1 (NISBD1), an inflammatory

disorder that involves the gut, skin and hair (Blaydon *et al.*, 2011). Knock-out mice for *ADAM17* are not viable, highlighting the essential role of this protease in mammalian development (Peschon *et al.*, 1998).
This protein is ubiquitously expressed, with higher levels in adult heart, skeletal muscle, spleen, pancreas, thymus, placenta, ovary, testes, prostate and small intestine (*ADAM17 protein expression summary - The Human Protein Atlas*).

As previously mentioned, ADAM17 can perform the cleavage of ACE2, generating its soluble form. This cleavage activity is similar to the one shown by TMPRSS2, even though the two proteases have different ACE2 shedding sites. Yet, on the contrary of the TMPRSS2 processing, ADAM17 cleavage of ACE2 does not agument SARS-CoV-1 cell entry (Heurich *et al.*, 2014). Moreover, the recombinant human soluble ACE2, which mimics the shed ACE2 variant, has shown to prevent SARS-CoV-2 entry and to inhibit infection in human blood vessel and kidney organoids, therefore proving that ADAM17 could have a beneficial role in blocking early stages of SARS-CoV-2 infections by shedding ACE2 and hence preventing the virus entry (Monteil *et al.*, 2020). However, other studies have suggested a detrimental role of this protease in COVID-19 development: high levels of plasmatic ACE2, which might be due to an enhanced ADAM17 activity, correlate with an increased severity of the disease (Sharif-Askari *et al.*, 2020). This effect might be linked to the decrease of cellular levels of functional receptor and to the consequent deregulation of the renin-angiotensin-aldosterone system (Gheblawi *et al.*, 2020). Moreover, by shedding and releasing TNFα, ADAM17 might contribute to the cytokine storm syndrome (Jose and Manuel, 2020). Overall, additional *in vivo* studies are required to determine whether ADAM17 role is detrimental or beneficial in COVID-19 development.

The other mentioned protease, Furin (Furin, Paired Basic Amino Acid Cleaving Enzyme) is a 86 kDa protein encoded by the *FURIN* gene on the 15q26.1 locus in the human genome (Zhao *et al.*, 2018).
Furin is a type 1 membrane bound endoprotease that processes protein precursors that traffick through the secretory pathways. The precursor of the

active Furin is processed in the endoplasmic reticulum (ER) and Golgi network in order to acquire its catalytic activity, that consists in cleaving substrates at the RX(K/R)R consensus motif; in particular, among these substrates, there are transforming growth factor beta 1 precursor, membrane type-1 matrix metalloproteinase, beta subunit of pro-nerve growth factor, von Willebrand factor, proalbumin and proparathyroid hormone (Wise *et al.*, 1990; Oda *et al.*, 1992; Takahashi *et al.*, 1993, 1995; Dubois *et al.*, 1995; Hendy *et al.*, 1995; Anderson *et al.*, 1997, 2002; Yana and Weiss, 2000; Urban *et al.*, 2013; Dahms *et al.*, 2014). Notably, ADAM17 itself is a substrate of Furin (Wong *et al.*, 2015).

This protein expression is ubiquitous (Barr *et al.*, 1991) and *FURIN* knockout mice show early embryonic lethality, underlining the fundamental role of this protein during development (Roebroek *et al.*, 2004).

In addition to processing cellular precursor proteins, Furin is exploited by pathogens in different types of viral and bacterial infection: indeed, it cleaves and activates the diphtheria toxin (Tsuneoka et al., 1993), the anthrax toxin (Klimpel et al., 1992; Molloy et al., 1992), the S.pneumoniae serine-rich repeat protein (Schulte et al., 2016), the hemagglutinin of H7N1 and H5N1 influenza viruses (Hardes et al., 2015), the viral fusion F protein of mumps virus (Ueo et al., 2020) and the gp160 envelope protein of human immunodeficiency virus (HIV) (Hallenberger et al., 1992).
For what concerns COVID-19, Furin is able to facilitate the SARS-CoV-2 infection by cleaving the S protein at the S1/S2 cleavage site, which is essential for the S protein to mediate the virion fusion to the host cell, but also for the cell-cell fusion that is mediated by the S protein and leads to syncytia formation (Hoffmann, Kleine-Weber and Pöhlmann, 2020).

1.2.4. SARS-CoV-2 variants

Like other Coronaviruses, SARS-CoV-2 is prone to adapt to the human host environment showing a high genetic recombination rate, hence it develops mutations that result in mutant variants of the virus, characterized by properties that differ from those of the ancestral viral strain. (Yang and Rao, 2021).
Several SARS-CoV-2 have been up to date identified and genotyped, some of which have been declared variants of concern (VOCs) by the World Health Organization (WHO), due to their repercussions on human health and on the global healthcare system. Indeed, VOCs can be characterized by an increased transmissibility or virulence, a reduced neutralization by antibodies, both obtained *via* natural infection or *via* vaccination, a marked ability to evade diagnostic detection, and a reduction in the therapeutic efficacy (Aleem, Akbar Samad and Slenker, 2022). In table 2, a brief recapitulation of the variants identified up to December 2022 (*Tracking SARS-CoV-2 variants*).

WHO label	**Pango lineage**	**Earliest documented samples**
Alpha	B.1.1.7	United Kingdom, Sep-2020
Beta	B.1.351	South Africa, May-2020
Gamma	P.1	Brazil, Nov-2020
Delta	B.1.617.2	India, Oct-2020
Omicron	B.1.1.529	Multiple countries, Nov-2021

Table 2. SARS-CoV-2 variants of concern (source: *Tracking SARS-CoV-2 variants*).

The emergence of these new variants constitute a considerable obstacle to the eradication of COVID-19 as the new strains might limit the beneficial effect of the developed and globally diffused vaccines.

In particular, the Omicron variant was designated as a VOC by WHO on November 26th, 2021 and within two months it replaced the previously

dominating Delta variant, becoming the the only currently circulating VOC (*Tracking SARS-CoV-2 variants*) accounting for the 99,7% of the global SARS-CoV-2 infections up to date (December 2022), according to the last WHO data (Guo *et al.*, 2022; *Weekly epidemiological update on COVID-19 - 21 December 2022*).

Omicron variant has been reported to present multiple mutations in the S protein (Cele *et al.*, 2022). These mutations confer to the S protein an higher affinity for ACE2, even though also a lower efficiency in being cleaved by TMPRSS2 compared to Delta variant, and an increased evasion towards the neutralizing antibodies that are elicited by the double dose of mRNA vaccines, as well as towards the therapeutic monoclonal antibodies. This reduction of the protective effect of neutralizing antibodies is likely to have contributed, together with the Omicron increased transmissibility, to the rapid spread of this variant, possibly leading to reinfection cases (Meng *et al.*, 2022).

Fortunately, the booster vaccination dose has been reported to significantly improve the role of neutralizing antibodies. Moreover, this variant has been associated with a lower risk of hospitalization and mortality; also, the antiviral drugs Remdesivir and Molnupiravir appear to be equally effective in the treatment of this variant as they were for the previous ones (Guo *et al.*, 2022).

2. AIM OF THE STUDY

COVID-19 pandemic has represented and still represents a significant global risk to health and economy.

SARS-CoV-2 infection can lead to a spectrum of clinical manifestations, that can range from the lack of symptoms in asymptomatic individuals that are positive to COVID-19 test, to a critical disease that can lead to ARDS and, eventually, death (*Clinical management of COVID-19: Living guideline, 23 June 2022*). Up to date, an increasing amount of studies has suggested that the host immunity is a crucial determinant for the disease severity and outcome, with a dysregulated immune response as possible responsible for fatal COVID-19 complications (Diamond and Kanneganti, 2022, Shi et al., 2020).

In this regard, a recent study from our laboratory has highlighted interesting differences in the immune response of mild, moderate, severe and critical COVID-19 patients (Angioni *et al.*, 2020). In particular, an immune signature was identified to be associated with the disease severity: CXCL8, IL10, IL15, IL27, and TNFα positively correlated with older age, longer hospitalization time and a more severe form of the disease in a cohort of 44 hospitalized COVID-19 patients (Angioni *et al.*, 2020). Many other studies have reported the importance of the deregulation of patients immunity, in particular cytokine dysregulation due to an excessive immune response, in determining the severity and the outcome of the disease (Giamarellos-Bourboulis, 2021; Kaklamanos *et al.*, 2021).

<u>Aim 1</u>

On these bases, we considered of primary importance to understand the molecular basis of the association between dysregulated cytokine release and severity of SARS-CoV-2 infection.

At the time of our study, the molecular mechanisms of regulation of SARS-CoV-2 pathogenesis were still almost unidentified. The first aim of this study was therefore to investigate whether this cytokine dysregulation, and in particular the pro-inflammatory milieu of the host, could affect the susceptibility to SARS-CoV-2 infection by modulating the expression of the two main

receptors of the virus that are present on the surface of the host cell: ACE2 and TMPRSS2.

To this purpose, the A549 human lung carcinoma cell line and the TT1 primary immortalized human alveolar epithelial cells have been exposed to different concentrations of 3 cytokines that are reported to be particularly involved in the dysregulation and associated with poor outcomes (IL1β, IL6, TNFα). Cells were particularly responsive to IL1β, which was also increased in the blood of COVID-19 patients. We therefore tested whether this cytokine could increase the expression of the receptors that are responsible for SARS-CoV-2 entry into the cell and sought for the molecular mechanism beyond this phenomenon.

Aim 2

On the same bases of correlation among cytokine aberrant production and disease severity, we wondered if the SARS-CoV-2 vaccination, which is well known to not only determine a consistent reduction in the risk of SARS-CoV-2 infection, but also to contribute to disease attenuation in infected people, could reduce this immune dysregulation and the associated tissue damage. In this regard, hyperinflammation and damage-associated molecular patterns (DAMPs) have been reported to be associated with the severe form of COVID-19 and with poor prognosis in SARS-CoV-2 infected patients (L. Chen *et al.*, 2020; R. Chen *et al.*, 2020).

To test this hypothesis, we analyzed a panel of 46 cytokines in the plasma of a cohort of 45 COVID-19 patients, along with the levels of two DAMPs that are reported in literature to increase in the plasma of more severe patients compared to milder ones (High Mobility Group Protein B1 - HMGB1 - and S100A8/9) (L. Chen *et al.*, 2020; R. Chen *et al.*, 2020).

3. MATERIALS AND METHODS

3.1. Participants, study design, and data collection

Peripheral Blood (PB) was collected at the admission time in EDTA tubes from enrolled healthy controls and COVID-19 patients (aged > 18 years) that were admitted to the Infectious and Tropical Disease Unit of the University Hospital of Padua.

All patients were clinically diagnosed with COVID-19 with positive laboratory RT-PCR test on nasopharyngeal swabs for SARS-CoV-2 infection. All demographics and clinical characteristics were retrieved from medical health records. All patients were classified into mild, moderate, severe, and critical cases according to WHO guidelines, based on results from chest imaging, clinical examination, and symptoms (briefly, asymptomatic: individuals who test positive for SARS-CoV-2 but who have no symptoms that are consistent with COVID-19; mild: individuals who have any of the various signs and symptoms of COVID-19 but who do not have shortness of breath, dyspnea, or abnormal chest imaging; moderate: individuals who show evidence of lower respiratory disease during clinical assessment or imaging and who have an oxygen saturation (SpO_2) ≥ 94% on room air at sea level; severe: individuals who have SpO_2 < 94% on room air at sea level, a ratio of arterial partial pressure of oxygen to fraction of inspired oxygen (PaO_2/FiO_2) < 300 mm Hg, a respiratory rate > 30 breaths/min, or lung infiltrates > 50%; critical: individuals who have respiratory failure, septic shock, and/or multiple organ dysfunction) (*Clinical management of COVID-19: Living guideline, 23 June 2022*).

Plasma was obtained after peripheral blood mononuclear cells (PBMC) isolation by density-gradient sedimentation using Ficoll–Paque PLUS (GE Healthcare, Germany) according to the manufacturer's protocol. Briefly, Ficoll-Paque media PLUS was added to the centrifuge tube and PB was carefully layered onto the Ficoll-Paque media solution. After centrifugation, plasma was removed from the top layer using a sterile serological pipette, avoiding to disrupt the mononuclear cell interphase and stored at -80 °C until use.

The cohort studied for Aim 1 included 44 COVID-19 patients and 4 healthy controls who tested positive between 09/04/2020 and 05/05/2020. The median age of the patients was 59.3 years (98-25).
The cohort studied for Aim 2 included 47 COVID-19 patients who tested positive between 12/08/2021 and 02/09/2021, either treated as inpatients or outpatients. The median age of the patients was 61 years (98-23).
The study was performed according to the ethical guidelines of the Declaration of Helsinki (7th revision). The study was approved by the Ethics Committee and the general authorization issued by the Data Protection Authority. Cod CESC n. 4933/AO/20. All the patients gave their written informed consent and all analyses were carried out on anonymized data as required by the Italian Data Protection Code (Legislative Decree 196/2003) and the general authorization issued by the Data Protection Authority.

3.2. Luminex assay

In the cohort studied for Aim 1, IL1β was quantified in the plasma from 4 controls and 44 COVID-19 patients by Luminex assay (Merck Millipore, Burlington, MA, USA). In the cohort studied for Aim 2, 46 analytes were measured by multiplex biomarker assays, based on Luminex xMAP technology (Merck Millipore, Burlington, MA, USA) following manufacturer's instructions.
Briefly, the plate was washed and the diluted standard, quality control and samples were prepared according to the manufacturer's instructions and added to the appropriate wells. Analyte-specific dyed beads were then added to each well and the plate was incubated overnight in shaking. Wells were emptied and washed, then detection antibodies were added to each well. After an incubation of 1 hour at room temperature (RT), Streptavidin-Phycoerythrin was added and the plate was incubated for 30 minutes at RT. Wells were then washed, Sheath Fluid PLUS was added and beads were resuspended by shaking. The plate was read on Luminex 200™. Analysis was performed using xPONENT 3.1 software. Statistical analysis was carried out with GraphPad Prism 6.0.

3.3. ELISA Assay

Plasma DAMPs (S100A8 and HMGB1) were evaluated by enzyme-linked immunosorbent assay (ELISA) (antibodies-online GmbH, Aachen, Germany) according to the manufacturer's instructions.

In brief, in this sandwich enzyme immunoassay, the provided microtiter plate has been pre-coated with an antibody specific to target. Standards and samples were added to the appropriate wells with a target-specific biotin-conjugated antibody and incubated for 1 hour at 37°C. Then, wells were emptied and Avidin conjugated to Horseradish Peroxidase was added to each well and incubated for 1 hour at 37°C. Wells were washed and TMB (3,3′,5,5′-Tetramethylbenzidine) substrate solution was added as a developer agent. The wells that contain the target, biotin-conjugated antibody and enzyme-conjugated Avidin exhibited a color change. The enzyme-substrate reaction was stopped by adding sulphuric acid solution and the absorbance was measured with the Spark-Tecan spectrophotometer (Lifesciences) at 450 nm. The concentration of the target in the samples was then determined by comparing the optical density (O.D.) of the samples to the O.D. of the standard curve.

3.4. Glutamate dehydrogenase (GDH) activity assay

GDH activity was determined by a coupled enzyme assay (Sigma-Aldrich) according to the manufacturer's instructions.

In this assay glutamate is catabolized by GDH generating NADH, which reacts with a probe generating a change in absorbance at 450 nm that is proportional to the GDH activity. Samples and NADH standards were prepared as indicated and added to the appropriate wells of a 96-well plate. A mix containing GDH Assay Buffer, GDH Developer and glutamate was assembled as indicated and then added to each well; the plate was then incubated for 3 minutes. From 3 minutes to 15 minutes, the absorbance was measured every 30 seconds with the Spark-Tecan spectrophotometer (Lifesciences) at 450 nm. The obtained measurements were then used to calculate GDH activity following the

manufacturer's instructions. The quantification was performed applying the formula:

GDH Activity = (B × Sample Dilution Factor) / (Reaction Time × V),

where B = Amount (nmole) of NADH generated between $T_{initial}$ and T_{final}; Reaction Time = T_{final}– $T_{initial}$ (minutes); V = sample volume (mL) added to well.

3.5. Cell culture and treatments

Human A549 cell line was donated from the ECSIN lab, Padua. The cells were maintained in DMEM high glucose medium supplemented with 1% Na-pyruvate, 1% L-glutamine, 10% Gibco FBS, and 1% Penicillin-Streptomycin at 37°C under 5% CO2, while human alveolar epithelial cell line (TT1) was donated by Montagner lab, Padua and was maintained as reported by Montagner et al. (Montagner *et al.*, 2020).

Cells were plated in 24 well tissue culture plates at the concentration of 100'000 cells per well. After 24 hours, cells were starved for 1 hour in the starvation medium (DMEM high glucose medium supplemented with 1% Penicillin-Streptomycin) and then stimulated in the treatment medium (DMEM high glucose medium supplemented with 2% Gibco FBS and 1% Penicillin-Streptomycin) with recombinant human IL1β, IL6 or TNFα (PeproTech) at the concentration of 1, 15 or 50 ng/mL either in combination or not with the inhibitors Birb796 (100 nM) or K7174 (10 μM) (Selleck Chemicals). Vehicle (DMSO, dimethyl sulfoxide) treated and non-stimulated (NS) cells were used as a control condition.

RNA extraction was performed after 4 hours of treatment, whereas protein extraction was performed at 24 hours of treatment.

For the evaluation of cell susceptibility to SARS-CoV-2 infection, cells were either stimulated or not with IL1β (50 ng/mL) and the selected inhibitors (Birb796, 100 nM or K7174, 10 μM) as described above for 8 hours. Cells were then exposed to the Heat-inactivated SARS-CoV-2 (VR-1986HK, ATCC) at 4 TCID50/mL. RNA extraction was performed after 2, 24 and 48 hours of treatment, whereas cells were processed for immunofluorescence assay as described below after 72 hours.

3.6. Silencing

A549 cells were transfected with GATA2 Stealth siRNA or scramble Silencer Select Negative Control (Thermo Fisher Scientific), according to the manufacturer's protocol.

Briefly, one day before transfection, cells were plated in 24 well tissue culture plates at the concentration of 30'000 cells per well, in 400 µL of culture medium without antibiotics, in triplicate. After 24 hours, cells were starved in 400 µL DMEM high glucose for 1 hour. Meanwhile, Stealth siRNA - Lipofectamin2000 (Invitrogen) complexes were prepared as described: a Stealth RNAi (GATA2 siRNA or scramble siRNA) solution containing 50 µL of Opti-MEM with 75 pmol of Stealth RNAi (GATA2 siRNA or scramble siRNA) was prepared per well; a Lipofectamin2000 solution containing 50 µL of Opti-MEM with 1 µL Lipofectamin2000 was prepared per well, mixed and incubated for 15 minutes at RT. The two solutions were combined, gently mixed and incubated for 15 minutes at RT to allow complexes formation. 100 µL of Stealth RNAi-Lipofectamine complexes solution was added to each well containing cells and medium. Cells were incubated at 37°C for 5 hours, then the medium was changed to the normal growth medium and cells were incubated for 24 hours. Cells were then stimulated with recombinant human IL1β as previously described. RNA extraction was performed after 4 hours of treatment, whereas protein extraction was performed at 24 hours of treatment.

3.7. RNA extraction, reverse transcription, and RT-qPCR

3.7.1. RNA extraction

Total RNA was extracted for RT-qPCR analysis from unstimulated and stimulated cells with TRIzol reagent (Thermo Fisher Scientific) following manufacturer's instructions, working on ice and cold centrifuge.

Briefly, medium was removed from wells and cells were lysed with Trizol. The solution was transferred to RNAse and DNAse-free tubes, where chloroform was added to solubilize nucleic acids. After centrifugation, the clear fraction was collected in a new tube and 2-Propanol was added to precipitate RNA. Samples

were mixed and centrifuged, then the supernatant was removed and the pellet was washed twice with Ethanol 70% in Diethyl pyrocarbonate (DEPC) water. After centrifugation and supernatant removal, the pellet was dried at RT. Samples were then rehydrated in DEPC water and RNA concentration was quantified by NanoDrop (Thermo Scientific).

For the evaluation of cell susceptibility to SARS-CoV-2 infection, concerning the RNA extraction, we followed the protocol by Vogels *et al.* (Vogels *et al.*, 2021). which substitutes nucleic acid extraction with proteinase K treatment and heat inactivation. Briefly, at the end of the treatment/infection (described above), cells were washed and lysed by adding 50 µL of DEPC water per well. Samples were collected in PCR tubes and 6.5 µL of 20 mg/mL Proteinase K was added to each sample. Samples were vortexed for 1 min at 3'200 RPM and then heat-inactivated at 95°C for 5 minutes. 5 µL of processed sample were used to perform reverse transcription and RT-qPCR as described below.

3.7.2. Reverse transcription

500 ng of RNA were retrotranscribed using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.
Briefly, the retrotranscription mix was prepared as described in Table 3.

Component	**Volume**
10X RT Buffer	2.0 µL
25X dNTP Mix (100 mM)	0.8 µL
10X RT Random Primers	2.0 µL
MultiScribe™ Reverse Transcriptase	1.0 µL
Nuclease-free H2O	4.2 µL
Total per reaction	10.0 µL

Table 3. High-Capacity cDNA Reverse Transcription mix per sample.

10µL of mix were added to 10µL of the sample and retrotranscription was performed with T100™ Thermal cycler (Bio-Rad) following the cycle:

- 25°C for 10 minutes
- 37°C for 2 hours
- 85°C for 5 minutes
- 4°C infinite

3.7.3 RT-qPCR

The obtained cDNA was diluted 1:10 and amplified with specific primers for the genes listed in Table 4.

Gene	**Forward primer**	**Reverse primer**
GAPDH	AACAGCCTCAAGATCATCAGC	GGATGATGTTCTGGAGAGCC
TMPRSS2	CCTCTAACTGGTGTGATGGCGT	TGCCAGGACTTCCTCTGAGATG
ACE2	TCCATTGGTCTTCTGTCACCCG	AGACCATCCACCTCCACTTCTC
FURIN	GCCACATGACTACTCCGCAGAT	TACGAGGGTGAACTTGGTCAGC
ADAM17	AACAGCGACTGCACGTTGAAGG	CTGTGCAGTAGGACACGCCTTT
-13 kb *TMPRSS2*	GCTGACCTTTAATGAAGTTTG	CCTAGTGAATTTGGCCTCCTC
IL1B promoter	TCGCACCCACTTCCTTCTCTT	TGCCAGAGGAAATGGTGACC
IL8 promoter	GCTGAACCAGAGTTGGAACCC	GGTGCACTGGAGCTGCTTG
nCoV_IP2	ATGAGCTTAGTCCTGTTG	CTCCCTTTGTTGTGTTGT
IRF1	TTGGCCTTCCACGTCTTG	GAGCTGGGCCATTCACAC

IRF9	AACTGCCCACTCTCCACTTG	AGCCTGGACAGCAACTCAG
ISG15	GGCTTGAGGCCGTACTCC	CTGTTCTGGCTGACCTTCG
IFITM2	ATCCCGGTAACCCGATCAC	CTTCCTGTCCCTAGACTTCAC
RNF20	AAAGCATCGCACCATGTCTC	ATCCCACTGCAGGTCATCAA
GATA2	GGTCAAGCTTTACTGTGGCTGTC	TGGTCACTACATCAGCACAATCCTC
E-CADHERIN	TCTTCACCCAGACCAAGTACA	GGCTCATGTATCGGAGGTCG

Table 4. Primers used in this study.

Briefly, the reaction mix was prepared with Power SYBR Green Master Mix (Thermo Fisher Scientific) to a final volume of 8µL as described in Table 5.

Component	**Volume**
Power Sybr green PCR Master mix	4 µL
Forward Primer (100µM)	0,075 µL
Reverse Primer (100µM)	0,075 µL
cDNA	4µL

Table 5. Power SYBR Green Master Mix per sample.

The reaction was performed in a 384 wells plate and run on a QuantStudio™ 5 Real-Time PCR System, 384-well (Applied Biosystems), according to the following protocol:

- 50°C for 2 min.
- 95°C for 10 min.
- repeated for 45 cycles:
    - 95°C for 15 s.

    - 60°C for 1 min.
- 95°C for 15 s.
- 60°C for 1 min.
- 95°C for 15 s.

The analysis of the obtained results was performed by QuantStudio Design and analysis software v1.5 (Applied Biosystems). To evaluate differential gene expression against the housekeeping gene (GAPDH) ΔΔCt method was applied. Statistical analysis was carried out with GraphPad Prism 6.0.

3.8. Chromatin Immunoprecipitation

Chromatin immunoprecipitation was performed with MAGnify™ Chromatin Immunoprecipitation System (Thermo Fisher Scientific) according to the manufacturer's protocol.

Briefly, A549 cells were plated in Petri dishes at a concentration of 10 millions per dish, in 10 mL of culture medium. After 24 hours, they were starved for 1 hour and then they were stimulated or not with recombinant IL1β (50 ng/mL) and either treated or not with the indicated inhibitors Birb796 (100 nM) or K7174 (10 μM) (Selleck Chemicals) in the presence of IL1β (50 ng/mL). After 4 hours of treatment, the treatment medium was removed and 1% formaldehyde (Sigma Aldrich) in phosphate-buffered saline solution (PBS) was added for 15 min at RT in soft shaking to perform the DNA-protein crosslink. The reaction was stopped with glycine (Sigma) 125 mM. After 5 minutes, cells were washed with PBS, scraped in 1,5 mL PBS, centrifuged and resuspended in 100 μL of the kit-provided lysis buffer.

Each sample was then sonicated 20 times at 60 Hz for 20 seconds each time. In order to enrich GATA2 DNA binding sites, the sonicated chromatin was incubated overnight with 1 μg of GATA2 polyclonal antibody (GeneTex, cat#GTX113441), which was previously coupled to magnetic beads (Dynabeads). Rabbit IgG was used as an antibody specificity control in parallel. The chromatin-antibody-Dynabeads complexes were then washed to remove the unbound product. DNA was then purified by reversing the formaldehyde

crosslinking of the chromatin with the kit-provided buffer and subsequently using kit-provided magnetic beads. qPCR amplification was performed on the obtained DNA with Power SYBR Green Master Mix (Thermo Fisher Scientific) on a QuantStudio™ 5 Real-Time PCR System (Applied Biosystems) as described above, using the reported specific primers (Table 4). Fold enrichment was calculated following the manufacturer's instructions:

$$DDCt = (Ct\ IP) - (Ct\ mock)$$

where Ct IP is the average of the replicate measurements for each IP reaction and Ct mock is the average of the replicate measurements for the mock condition (IgG);

$$Fold\ enrichment = (2^{-DDCt})$$

3.9. Protein extraction and western blot analysis

Protein lysates from A549 or TT1 cells that were treated as described above were prepared as follows. The medium was removed, cells were washed and FASP lysis buffer (10 mM Tris-HCl pH 9, 4% SDS, 1 mM DTT) was added to each well. Cells were scraped and collected into new tubes, then an insulin syringe was used to ease the cell lysis by mechanical action. Proteins were denatured at 95° C for 5 minutes and protein concentration was determined using a bicinchoninic acid (BCA) assay.

5 µg of total protein extracts were loaded in Bolt™ 4-12% Bis-Tris Plus gels (Thermo Fisher Scientific) and blotted onto a methanol-activated polyvinylidene fluoride (PVDF) membrane (Bio-Rad). Membranes were blocked by incubating them with 3% bovine serum albumin (BSA) (Sigma Aldrich) for 1 hour at RT in shaking. Then, they were incubated with the primary antibodies anti-TMPRSS2 antibody (EPR3862, Abcam), anti-GATA2 (AF2046, R&D) or anti-GRP75 (D-9, Santa Cruz Biotechnology) overnight at 4°C in shaking. Subsequently, membranes were washed to remove the excess of primary antibody and incubated with the secondary antibody goat α-rabbit IgG (H + L)-HRP Conjugate (Bio-Rad) for 1 hour at RT in shaking. Membranes were then washed and ECL Prime Western Blotting Detection Reagent (GE Healthcare) was added. Chemiluminescence was detected with iBright 1500 (Invitrogen). Images

were analyzed with ImageJ software (Fiji) and the protein was quantified and normalized on the housekeeping protein (GRP75) and compared to the non-stimulated condition.

3.10. Immunofluorescence analysis

For TMPRSS2 protein immunofluorescence, A549 and TT1 cells were plated on 8 well chamber slides at a concentration of 25’000 cells per well. Cells were either stimulated or not with IL1β (50 ng/mL) as described above, for 24 hours. By contrast, for the evaluation of cell susceptibility to SARS-CoV-2 infection, cells were plated in the same way and either stimulated or not with IL1β and the selected inhibitors for 8 hours and then infected using Heat-inactivated SARS-CoV-2 (VR-1986HK, ATCC) at 4 TCID50/mL for 72 hours.

After the end of the treatment/infection, the treatment medium was removed and cells were fixed with paraformaldehyde PFA 4% (Sigma) in PBS. Cells were then blocked using a blocking solution (PBS, 1% BSA, 0,02% NP40) and incubated with either an anti-TMPRSS2 antibody (H-4, Santa Cruz Biotechnology) or an anti-SARS-CoV-2 Spike antibody (polyclonal, GeneTex), respectively followed by the secondary fluorophore-conjugated antibodies goat anti-mouse Alexa Fluor 488 antibody (polyclonal, Invitrogen) and goat anti-rabbit Alexa Fluor 488 antibody (polyclonal, Invitrogen). Nuclei were counterstained with Hoechst 33342 (Invitrogen) and actin was stained with Phalloidin TexasRed (Invitrogen).

Fluorescence images were acquired using the Zeiss LSM 800 confocal laser scanning microscope (Carl Zeiss). The fluorescent signal per cell was quantified using ImageJ software (Fiji) and the corrected total cell fluorescence (CTCF) was calculated. 3D reconstruction was performed using ZEN 3.2 Blue edition software (Carl Zeiss).

3.11. Statistical Analysis

Data are reported as the mean ± standard error of mean (SEM) of at least 3 independent experiments. Statistical analysis was performed using GraphPad

Prism 6.0 (GraphPad Software, CA, USA). Data were analyzed for Gaussian distribution using D'Agostino-Pearson and Shapiro-Wilk normality test. Statistical comparisons between two groups were performed using unpaired nonparametric Mann–Whitney U test or Kruskal-Wallis test for multiple comparisons with Dunn's *post hoc* test. Differences were considered statistically significant with *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

4. RESULTS

4.1. Modulation of SARS-CoV-2 receptor expression through pro-inflammatory cytokines

Patients with SARS-CoV-2 infection can experience a range of clinical manifestations that, according to the World Health Organization, can be classified into 4 severity levels: mild, moderate, severe and critical. In the early stage of the disease, the activation of the host immune system is crucial to counteract the spreading of the viral infection; however, in severe and critical cases the immune response of the host can be dysregulated and uncontrolled leading to the so called hyperinflammatory damage instead of having a beneficial role (Shi *et al.*, 2020).
Indeed, severe and critical patients frequently suffer from severe pneumonia and acute respiratory distress syndrome (ARDS), conditions that can be triggered by an excessive activation of the host immune response, and in particular by the cytokine storm syndrome (J. Wang *et al.*, 2020).

Of note, the inflammatory cytokine milieu of the patient is a crucial determinant for COVID-19 outcome. Indeed, worse severity, longer hospitalization time, and patient's age have been associated with specific circulating cytokines (Angioni *et al.*, 2020).

Multiple disorders, such as diabetes and obesity, as well as aging, representing risk factors for developing a severe disease after SARS-CoV-2 infection, are characterized by chronic inflammation and elevated levels of pro-inflammatory cytokines (Y. Zhou *et al.*, 2020).

Among the cytokines that have been associated to COVID-19 severity, IL1β, IL6 and TNFα are higher in the plasma of severe and critical patients as compared with the mild/moderate ones (Azkur *et al.*, 2020; Huang *et al.*, 2020; McElvaney *et al.*, 2020; Teuwen *et al.*, 2020).

As already discussed, to enter the host, SARS-CoV-2 first needs to dock the target cell through the binding of the viral structural Spike protein (S), in particular the receptor-binding domain (RBD) of the S1 subunit, to the host angiotensin I converting enzyme 2 (ACE2) receptor. Subsequently, the S protein needs to be proteolytically processed by the host transmembrane protease/serine subfamily 2 (TMPRSS2), which cleaves the S protein to facilitate membranes fusion by allowing the exposition of the fusion subunit and therefore the infection of the cell (Hoffmann et al., 2020).

ACE2 and TMPRSS2 expression and co-expression are crucial in defining the susceptibility of target organs to SARS-CoV-2 infection (Sungnak et al., 2020). Moreover, it has been proven that the expression of ACE2 can be regulated in response to interferon alpha and gamma (IFNα and γ) signaling (Ziegler et al., 2020); on this basis, it is reasonable to hypothesize a putative link between the immune response and the modulation of the cell receptors that are involved in SARS-CoV-2 entry into the host cell.

4.1.1. TMPRSS2 and ACE2 expression in response to cytokine stimulation

On this ground, we moved to investigate whether the pro-inflammatory milieu of the host can affect the expression of TMPRSS2 and ACE2 in SARS-CoV-2 target cells.
TMPRSS2 is expressed in several tissues including lungs, gastrointestinal tract, liver and kidney so we first treated *in vitro* different cell lines with different concentrations (15 or 50 ng/mL) of IL1β, IL6, and TNFα inflammatory cytokines. In particular, for this purpose we selected various human cell lines that do express TMPRSS2 and ACE2 and are permissive and susceptible to the infection: A549, a lung epithelial cell line, CaCo2, a colon epithelial cell line, HUH-7, a hepatocyte cell line, and HEK293, a kidney epithelial cell line.
By RT-qPCR, we then analyzed the expression levels of *TMPRSS2* and *ACE2* mRNA in the indicated cell lines after 4 hours of stimulation with IL1β, IL6, and TNFα cytokines at two different concentrations (15 or 50 ng/mL).

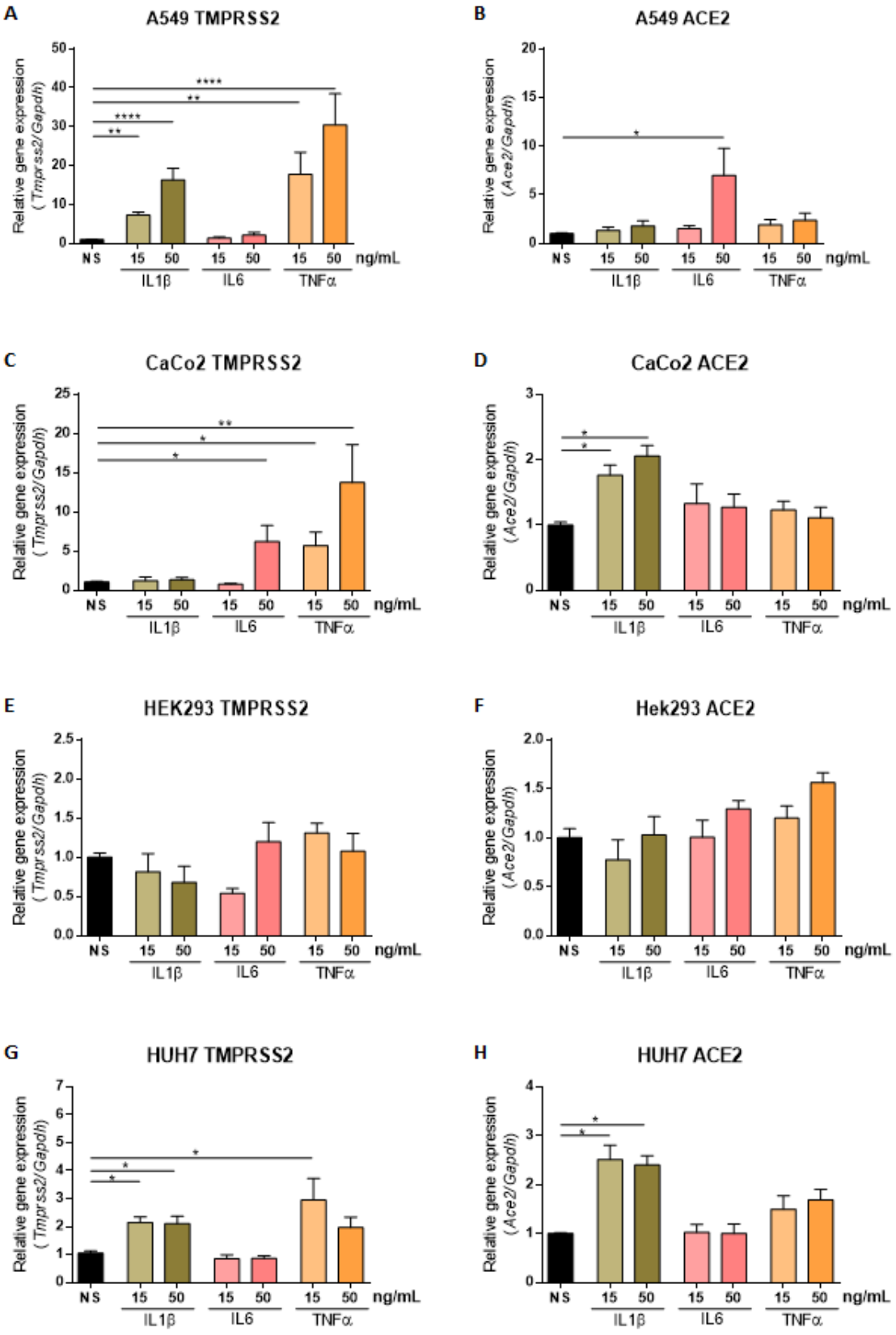

A
A549 TMPRSS2
Relative gene expression (Tmprss2/Gapdh)
B
A549 ACE2
Relative gene expression (Ace2/Gapdh)
C
CaCo2 TMPRSS2
Relative gene expression (Tmprss2/Gapdh)
D
CaCo2 ACE2
Relative gene expression (Ace2/Gapdh)
E
HEK293 TMPRSS2
Relative gene expression (Tmprss2/Gapdh)
F
Hek293 ACE2
Relative gene expression (Ace2/Gapdh)
G
HUH7 TMPRSS2
Relative gene expression (Tmprss2/Gapdh)
H
HUH7 ACE2
Relative gene expression (Ace2/Gapdh)
NS
15
50
ng/mL
IL1β
IL6
TNFα

Figure 12. Relative gene expression of *TMPRSS2* **(A, C, E, G)** and *ACE2* **(B, D, F, H)** in A549 **(A, B)**, CaCo2 **(C, D)**, HEK293 **(E, F)**, HUH7 **(G, H)** cell lines by RT-qPCR analysis. Cells were either stimulated or not (NS) for 4 hours with IL1β, IL6 or TNFα (15 or 50 ng/mL); n=3 independent experiments. Data are presented as means ± SEM. Kruskal-Wallis test for multiple comparisons with Dunn's *post hoc.* *P < 0.05, **P < 0.01, ****P < 0.0001.

As shown in Figure 12, the expression of *TMPRSS2* was significantly upregulated in the A549 cell line upon IL1β and TNFα stimulation; in contrast, *ACE2* expression was upregulated only in presence of IL6. The expression of *TMPRSS2* was upregulated in the CaCo2 cell line upon TNFα and IL6 stimulation, whereas *ACE2* was upregulated when cells were treated with IL1β. On the other hand, no significant changes in the expression of these two receptors was observed in the HEK293 cell line; the expression of *TMPRSS2* was upregulated upon IL1β and TNFα stimulation in the HUH7 cell line, whereas *ACE2* was upregulated in this cell line upon IL1β stimulation.
This different response of the various cell lines to cytokine stimulation can be attributed to the tissue-specific role of the cytokines, which can exert different effects in different tissues. Also, different basal levels of both ACE2 and TMPRSS2 in the examined cell lines, prior cytokine stimulation, could be responsible for the different magnitude of alteration of transcription upon cytokine stimulation.

Remarkably, among all the cell lines that were tested, the A549 lung cell line was particularly responsive to the cytokine stimulation. Thus, considering our results and the major involvement of the lung in the disease (Harrison, Lin and Wang, 2020), we focused on the A549 cell line for the following experiments.

Alongside TMPRSS2, two other proteases have been reported to be involved in the cleavage of SARS-CoV-2 Spike protein and in the promotion of the infection: Furin and ADAM17 (Zipeto *et al.*, 2020). Hence, we tested *via* RT-qPCR whether the transcription of these genes was also affected by IL1β stimulation and we found that neither *ADAM17* or *FURIN* expression was

controlled by the pro-inflammatory cytokine stimulation in the A549 cell line (Figure 13).

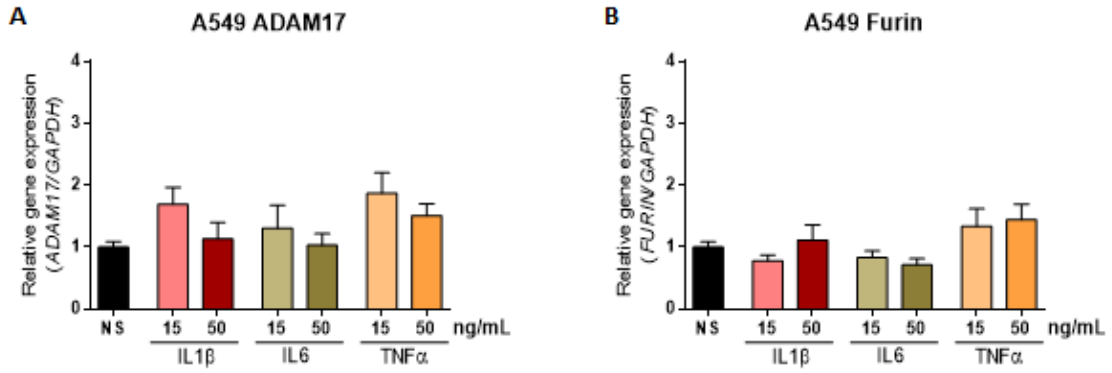


Figure 13. Relative gene expression of **(A)** *ADAM17* and **(B)** *FURIN* in the A549 cell line by RT-qPCR analysis. Cells were either stimulated or not (NS) for 4 hours with IL1β, IL6 or TNFα (15 or 50 ng/mL); n=3 independent experiments. Data are presented as means ± SEM.

In line with the literature (Yap, Moriyama and Iwasaki, 2020); our data confirmed that COVID-19 patients showed higher levels of circulating IL1β as compared to healthy subjects (Figure 14 A). Of note, the concentration of IL1β was significantly higher in severe and critical patients compared with the mild and moderate ones (Figure 14 B).

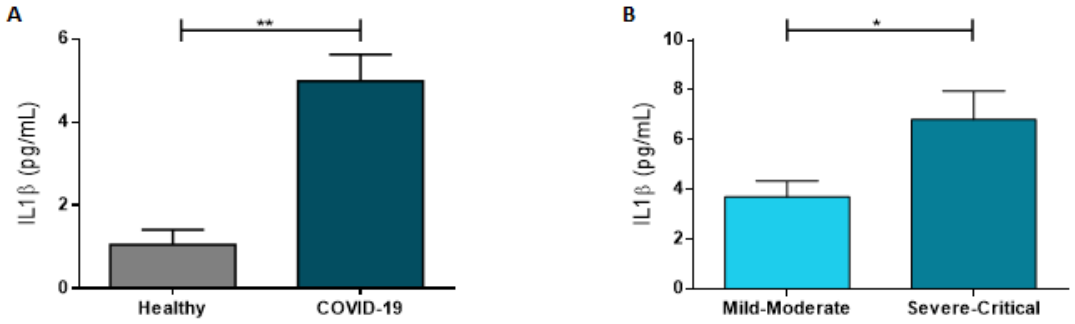


Figure 14. (A) Circulating IL1β concentration (pg/mL) in COVID-19 patients and healthy, age-matched controls. **(B)** IL1β plasma concentration (pg/mL) in COVID-19 patients grouped by disease severity (mild-moderate versus severe-critical); n=44 patients. Data are presented as means ± SEM. Nonparametric Mann–Whitney U test. *P < 0.05, **P < 0.01.

Moreover, SARS-CoV-2 infection has been reported to trigger the NLRP3 inflammasome activation in several cell types and the activation of this pathway, that leads to a strong release of IL1β, might exacerbate ARDS and systemic inflammation (Yap, Moriyama and Iwasaki, 2020).

Therefore, we focused our analysis on IL1β-induced transcriptional regulation of *TMPRSS2* expression.

We first validated the previous result obtained upon stimulation with IL1β using the lower and more physiological concentration of 1 ng/mL. The expression level of *TMPRSS2* mRNA was evaluated in the A549 cell line.

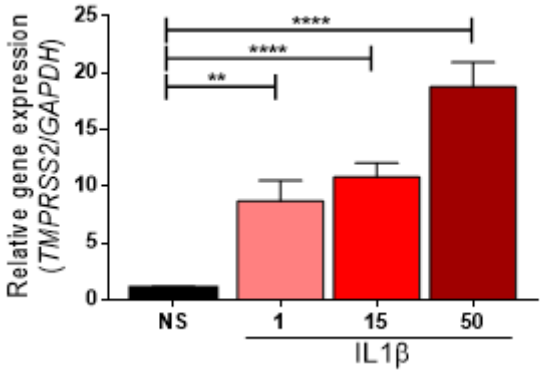


Figure 15. Relative gene expression of *TMPRSS2* in the A549 by RT-qPCR analysis. Cells were either stimulated or not (NS) for 4 hours with IL1β (1, 15 or 50 ng/mL); n=5 independent experiments. Data are presented as means ± SEM. Kruskal-Wallis test for multiple comparisons with Dunn's *post hoc.* **P < 0.01, ****P < 0.0001.

As shown in Figure 15, the 1 ng/mL concentration of IL1β was able to induce the overexpression of *TMPRSS2* upon 4 hours treatment in the A549 cell line.

Moreover, to further corroborate our data in a more physiological model, we repeated the indicated experiments on an immortalized primary human alveolar epithelial cell line (TT1). TT1 cells were treated with IL1β at the three different concentrations of 1, 15 and 50 ng/mL; TMPRSS2 expression was significantly increased upon IL1β stimulation at all the indicated concentrations (Figure 16)

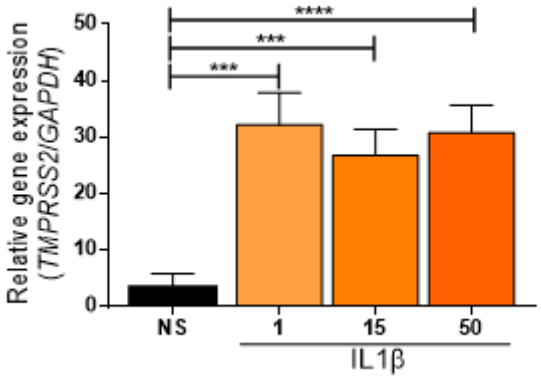


Figure 16. Relative gene expression of *TMPRSS2* in TT1 cells by RT-qPCR analysis. Cells were either stimulated or not (NS) for 4 hours with IL1β (1, 15 or 50 ng/mL); n=5 independent experiments. Data are presented as means ± SEM. Kruskal-Wallis test for multiple comparisons with Dunn's post hoc. ***P < 0.001, ****P < 0.0001.

We also evaluated the effect of this cytokine on the expression of *ACE2*. As reported in Figure 17, the stimulation with IL1β had no effect on this gene expression, both in the A549 cell line (Figure 17 A) and in the TT1 cell line (Figure 17 B).

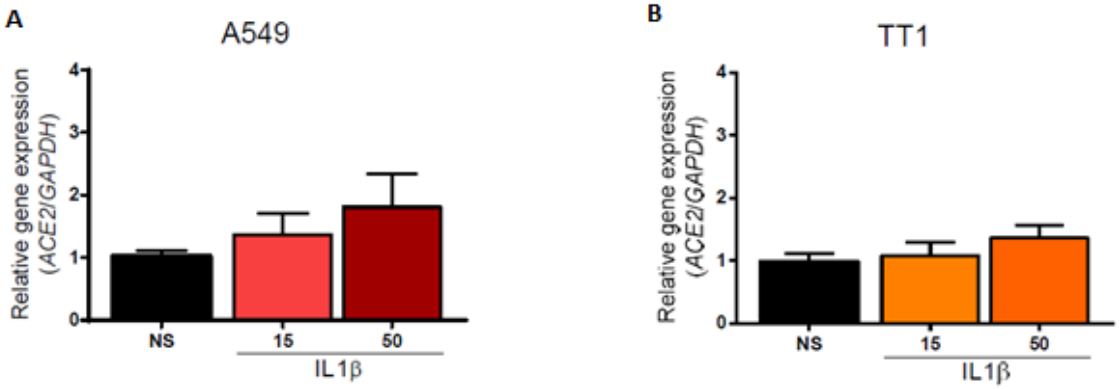


Figure 17. Relative gene expression of *ACE2* in **(A)** A549 or **(B)** TT1 cells by RT-qPCR analysis. Cells were either stimulated or not (NS) for 4 hours with IL1β (15 or 50 ng/mL). Data are presented as means ± SEM. Nonparametric Mann–Whitney U test.

Due to several regulatory mechanisms occurring in the translation process, the increase in mRNA transcription does not always positively correlate with a concomitant increase in the protein level. Hence, we addressed whether the IL1β-induced *TMPRSS2* mRNA upregulation also leads to an increase at the protein level. To this aim, we evaluated TMPRSS2 protein level by performing

immunofluorescence and western blot analyses in both the A549 and TT1 cells upon IL1β stimulation. Both A549 and TT1 cells showed a significant upregulation in TMPRSS2 protein levels upon cytokine stimulation (Figure 18 and Figure 19).

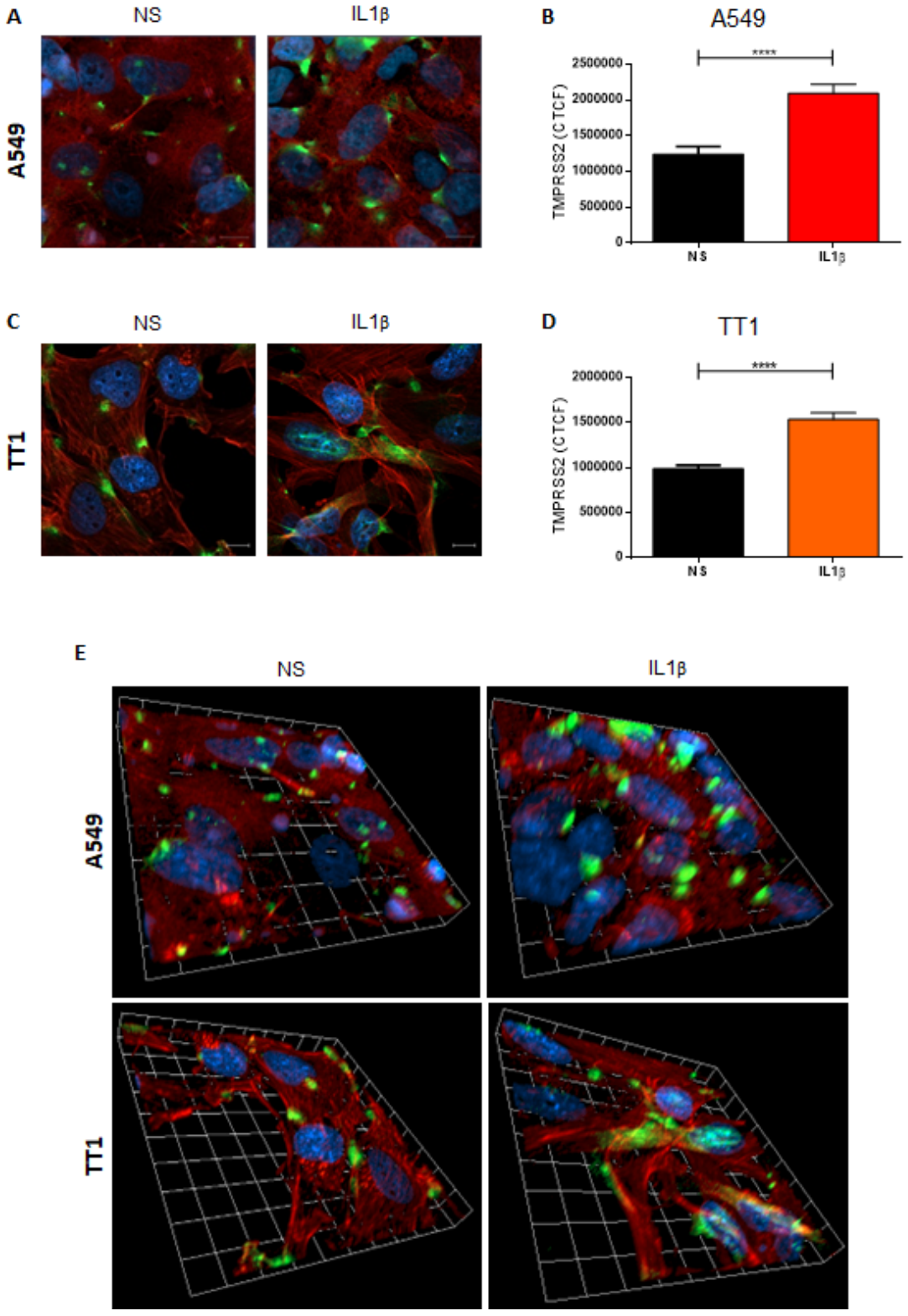


Figure 18. (A, C) Representative images of immunofluorescence analysis for TMPRSS2 (green) and **(B, D)** quantification of the fluorescence intensity of TMPRSS2 in the A549 **(A, B)** and TT11 **(C, D)** cell lines. Cells were either stimulated or not (NS) with IL1β (50 ng/mL) for 24 hours. Nuclei were counterstained with Hoechst 33342 (blue) and actin was stained with Phalloidin TexasRed (Red). CTCF: corrected total cell fluorescence. Scale bar: 10 µm; n=3 independent experiments. **(E)** 3D reconstruction of TMPRSS2 (green) immunofluorescent confocal images in A549 and TT1 cells either stimulated or not (NS) with IL1β (50 ng/mL). Nuclei were counterstained with Hoechst 33342 (blue) and actin was stained with Phalloidin TexasRed (Red), n=3 independent experiments. Data are presented as means ± SEM. Nonparametric Mann–Whitney U test. ****P < 0.0001.

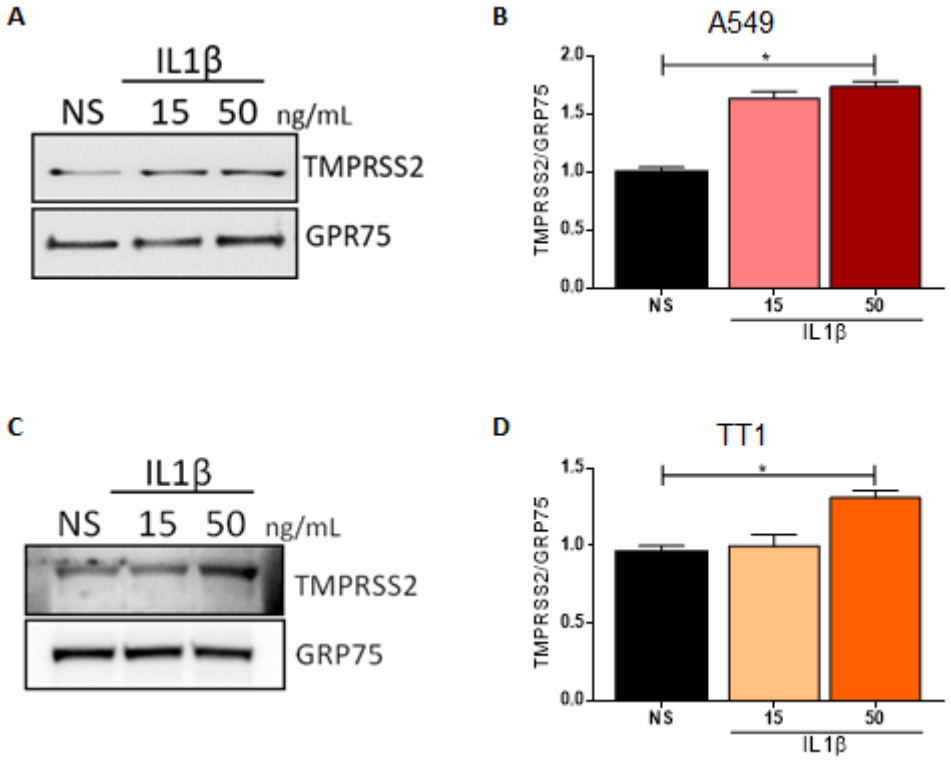


Figure 19. Protein quantification TMPRSS2 in total protein lysates from A549 cells **(A, B)** and TT1 cells **(C, D)**. **(A, C)** Western blot analysis (representative of 3 independent experiments) and **(B, D)** quantification of western blots. Cells were either stimulated or not (NS) with IL1β (15 or 50 ng/mL) for 24 hours; GRP75 was used as a loading control; n=3 independent experiments. Data are presented as means ± SEM. Nonparametric Mann–Whitney U test. *P < 0.05.

As mentioned, upon S protein binding to the host cell, viral and host membranes can fuse thus enabling the release of the viral RNA directly into the cytoplasm (Hoffmann *et al.*, 2020). Alternatively, the virus can be internalized *via* endocytosis, and, after cathepsin-mediated cleavage, viral membranes fuse

with the endosomal membrane and allow the viral genome to enter the cytoplasm (Bayati *et al.*, 2021; Diamond and Kanneganti, 2022).

Hence, we also evaluated whether IL1β could modulate other infection-related processes, such as the entry via endosome and the SARS-CoV-2 replication. To this aim, we assessed by RT-qPCR the transcription levels of *INTERFERON INDUCED TRANSMEMBRANE PROTEIN 2* (*IFITM2*) and *RING FINGER PROTEIN 20* (*RNF20*), two endosome and replication related-genes (Prelli Bozzo *et al.*, 2021) upon IL1β stimulation in both A549 and TT1 lung cell lines. No significant transcriptional changes were observed for these genes in the analyzed cell lines (Figure 20).

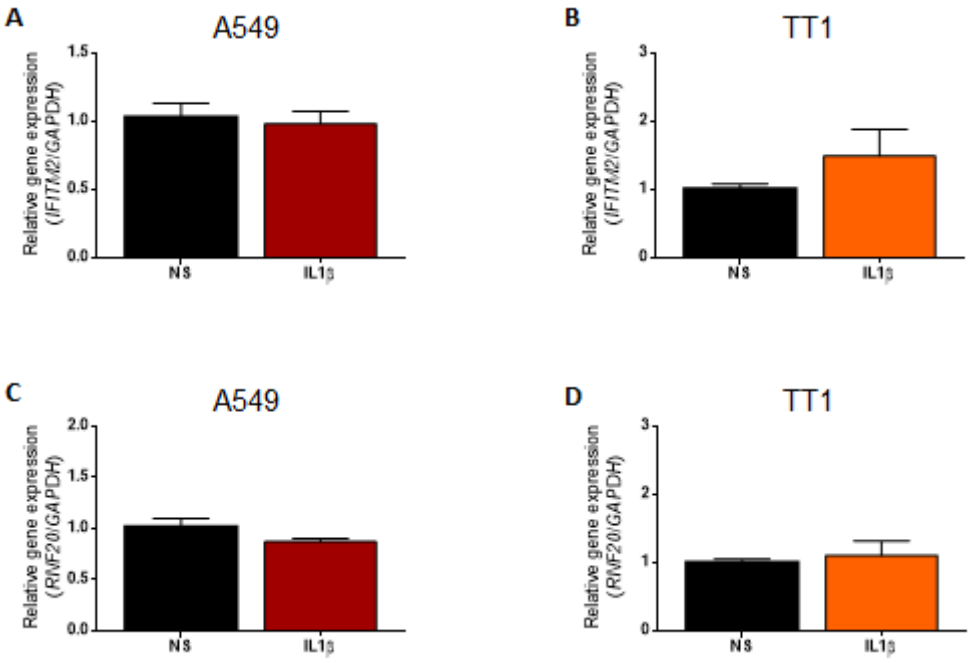


Figure 20. Relative gene expression of **(A, B)** *IFITM2* and **(C, D)** *RNF20* in the A549 and TT1 cell lines by RT-qPCR analysis. Cells were either stimulated or not (NS) with IL1β (50 ng/mL) for 4 hours; n=3 independent experiments. Data are presented as means ± SEM.

From this first set of experiments, we concluded that the stimulation of both the A549 and TT1 lung epithelial cell lines with the pro-inflammatory cytokine IL1β

promoted an increase in the expression of TMPRSS2, at both the transcriptional and the protein level, and that other viral-related processes appear not to be influenced by this cytokine stimulation.

Therefore, we sought to unveil the molecular mechanism underpinning TMPRSS2 overexpression upon IL1β stimulation.

4.1.2. Mechanism of IL1β-induced TMPRSS2 overexpression

Previous studies reported that *TMPRSS2* expression is regulated by the master regulator transcription factor GATA2. Indeed, GATA2 has a binding site in the -13 kb DNA enhancer region of the gene (-13 kb upstream the promotor) (Clinckemalie *et al.*, 2013). GATA2 has also been reported to be activated by its phosphorylation through the p38 MAPK pathway (Katsumura *et al.*, 2016).
Moreover, GATA2 is known to be involved in the transcriptional regulation of various pro-inflammatory cytokines, including *IL1B* and *C-X-C MOTIF CHEMOKINE LIGAND 2* (*CXCL2)* and a positive feedback between IL1β signaling and GATA2 activation has been reported in Acute Myeloid Leukemia (Katsumura *et al.*, 2016).

Therefore, we reasoned that the GATA2 transcription factor might be the regulator of TMPRSS2 transcription upon cytokine stimulation and, more precisely, we hypothesized that IL1β might promote *TMPRSS2* expression through the p38 MAPK-GATA2 axis.

To test this hypothesis, we stimulated the lung cell lines, A549 and TT1, with IL1β in the presence of a specific p38 MAPK or GATA2 inhibitor, named Birb796 and K7174 respectively. Cells either treated or not with IL1β were used as negative and positive controls respectively. *TMPRSS2* transcription was analyzed *via* RT-qPCR. As previously shown, IL1β significantly increased the transcription of *TMPRSS2,* but interestingly *TMPRSS2* overexpression was significantly blunted in cells treated with IL1β in the presence of either Birb796 or K7174 inhibitors (Figure 21 A-B). We also analyzed *ACE2* expression in these conditions: as previously reported IL1β stimulation did not alter its

expression levels and neither did the treatment with the selected inhibitors (Fig. 21 D).

These results suggested that both p38 MAPK and GATA2 may be involved in the IL1β-induced *TMPRSS2* overexpression.

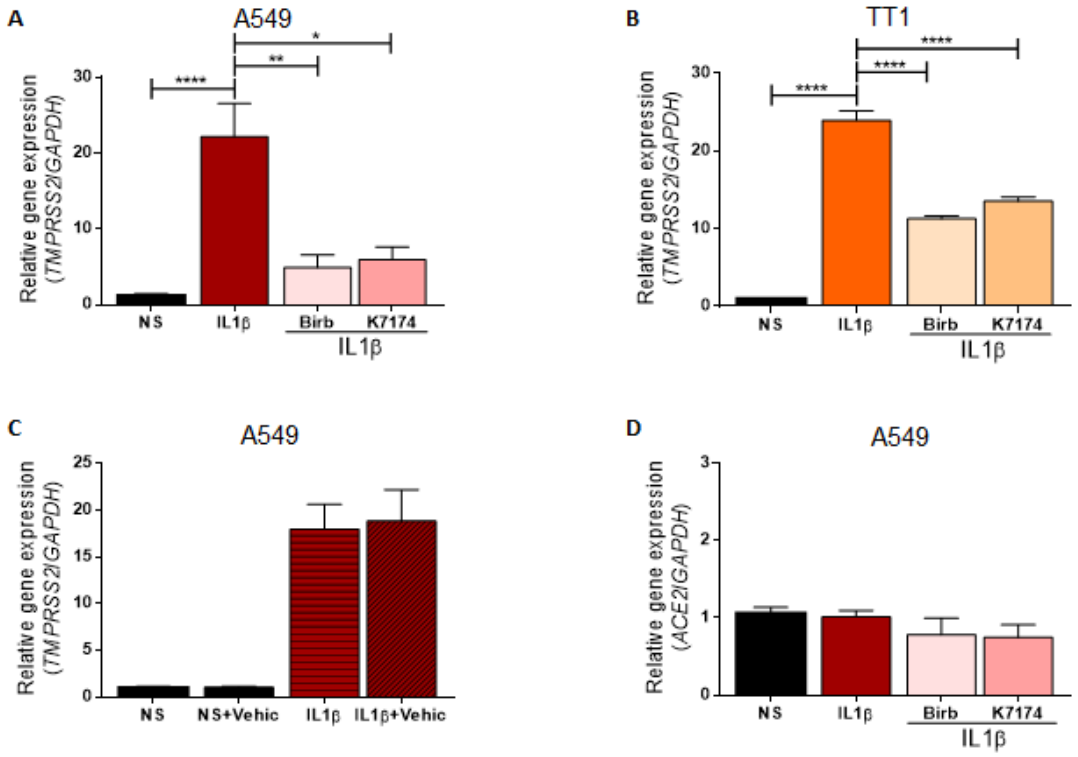


Figure 21. Relative gene expression of *TMPRSS2* in **(A)** A549 and **(B)** TT1 cells by RT-qPCR analysis Cells were stimulated with IL1β (50 ng/mL) and either treated or not with Birb796 (p38 MAPK inhibitor, 100 nM) or K7174 (GATA2 inhibitor, 10 μM). NS: Not Stimulated; n=5 independent experiments for 4 hours. **(C)** Relative gene expression of *TMPRSS2* in the A549 cell line by RT-qPCR analysis. Cells were either stimulated or not (NS) with IL1β (50 ng/mL) for 4 hours in presence or absence of the inhibitor vehicle (DMSO). **(D)** Relative gene expression of *ACE2* in the A549 cell line by RT-qPCR analysis. Cells were either stimulated or not (NS) with IL1β (50 ng/mL) and treated with Birb796 (100 nM) or K7174 (10 μM) for 4 hours, NS: Not Stimulated; n=4 independent experiments. Data are presented as means ± SEM. Kruskal-Wallis test for multiple comparisons with Dunn's *post hoc.* *P < 0.05, **P < 0.01, ****P < 0.0001.

In order to confirm the role of GATA2 on the IL1β-induced transcriptional regulation of TMPRSS2, we performed GATA2 chromatin immunoprecipitation. Our data indicated that upon IL1β stimulation the chromatin enrichment in the *TMPRSS2* -13 kb enhancer region was increased compared to the non-stimulated (NS) condition (Figure 22).

In addition, we performed the chromatin immunoprecipitation on the cells stimulated with IL1β and treated with p38 MAPK and GATA2 inhibitors. Notably, Birb796 and K7174 were able to significantly reduce the GATA2 binding to the enhancer region to levels which were comparable to the non-stimulated condition.

The binding of GATA2 to the *IL1B* and *IL8* promoter regions with and without IL1β stimulation was used as positive and negative control, respectively.

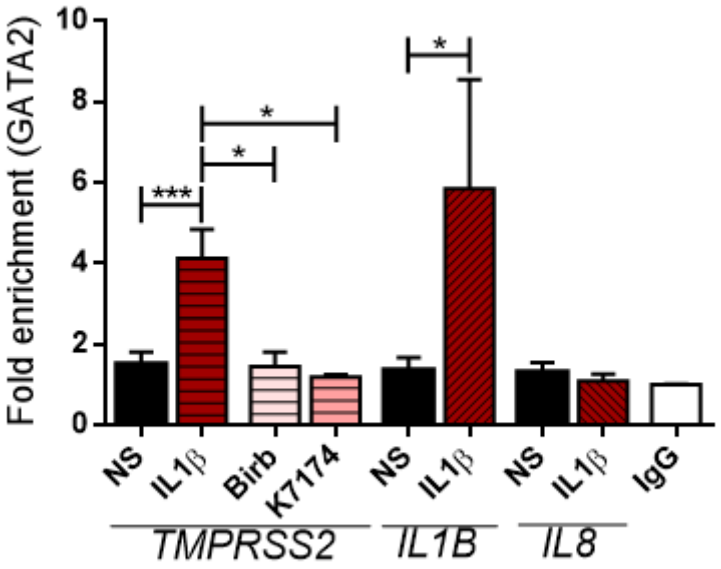


Figure 22. Fold enrichment of GATA2 binding to the *TMPRSS2* enhancer promotor region, in A549 cells by ChIP-RTqPCR analysis. Cells were either treated or not with Birb796 (p38 MAPK inhibitor, 100 nM) or K7174 (GATA2 inhibitor, 10 μM) and stimulated or not (NS) with IL1β (50 ng/mL) for 4 hours. Data were adjusted by input for each sample and normalized with IgG (Mock). The fold enrichment of GATA2 binding to the *IL1B* and to the *IL8* promotors was used as positive and negative control, respectively. IgG was used as antibody specificity control. N=3 independent experiments. Data are presented as means ± SEM. Kruskal-Wallis test for multiple comparisons with Dunn's *post hoc.* *P < 0.05, ***P < 0.001.

To investigate whether the observed effect relies on GATA2 activity or on a modulation of its expression level, we performed a RT-qPCR on A549 and TT1 cells either stimulated or not with the pro-inflammatory cytokine in two concentration (15 and 50 ng/mL). As reported in Figure 23, the IL1β stimulation did not significantly affect *GATA2* transcription levels in these lung cell lines.

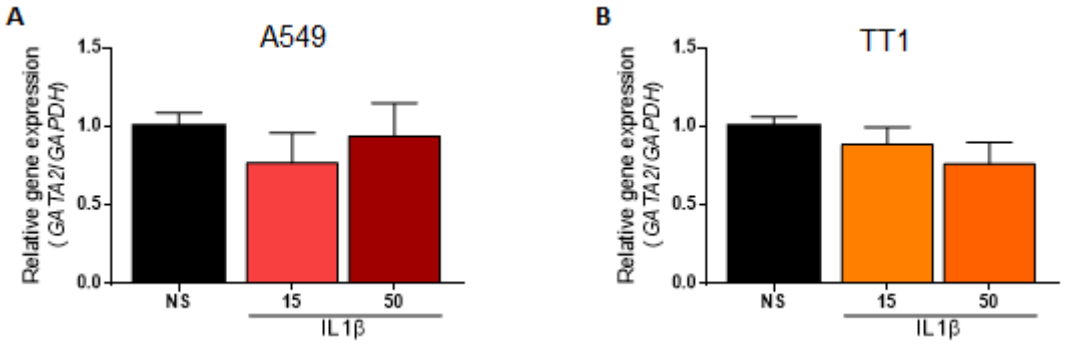


Figure 23. Relative gene expression of *GATA2* in **(A)** A549 or **(B)** TT1 cells by RT-qPCR analysis. Cells were either stimulated or not (NS) with IL1β (15 or 50 ng/mL) for 4 hours; n=3 independent experiments. Data are presented as means ± SEM.

GATA2 significantly increased its binding to the TMPRSS2 enhancer region upon IL1β stimulation, thus supporting the hypothesis that this transcription factor could be responsible for the IL1β-induced TMPRSS2 overexpression. Since IL1β did not increase *GATA2* transcription levels, the outlined effect seems to rely on GATA2 activity rather than on a modulation of its expression level. Moreover, the p38 MAPK and GATA2 inhibitors were able to decrease the GATA2 binding to the enhancer region, remarking the role of these two proteins in the IL1β-induced TMPRSS2 overexpression.

To definitely corroborate the role of GATA2 in the increased *TMPRSS2* transcription upon IL1β stimulation, we performed GATA2 silencing in A549 cells (Figure 24 A). The downregulation of GATA2 expression blocked the enhancement of *TMPRSS2* transcription in response to IL1β exposure. To check the effectiveness of the silencing, we tested the *GATA2* transcription levels (Figure 24 B). Moreover, we verified the *E-CADHERIN* transcription levels, as positive control since its expression is regulated by GATA2, to check if this decrease in mRNA could correspond to an effective reduction in the protein levels: we reported a significant reduction in *E-CADHERIN* expression in both non-stimulated and IL1β stimulated cells (Figure 24 C). To definitely prove the downregulation of GATA2 at the protein level, we performed a western-blot. The blot and its quantification (Figure 24 D-E) demonstrated a significant reduction in GATA2 protein levels upon GATA2 silencing, in both non-stimulated and IL1β-exposed cells.

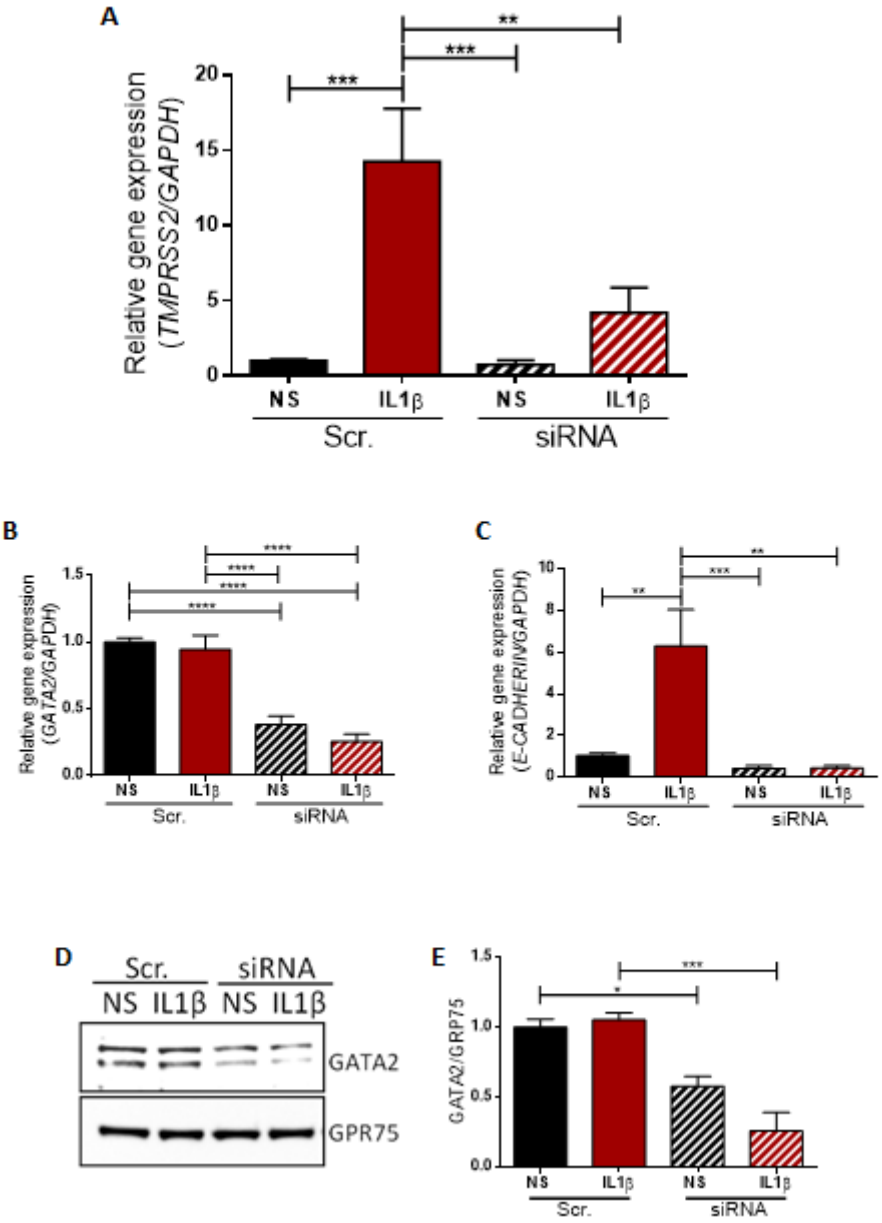


Figure 24. (A) Relative gene expression of *TMPRSS2* in A549 cells upon GATA2 silencing by RT-qPCR analysis. GATA2 silencing was performed and cells were then either stimulated or not (NS) with IL1β (50 ng/mL) for 4 hours. Scramble siRNA (Scr.) was used as a negative control; n=4 independent experiments. Relative gene expression of **(B)** *GATA2* and **(C)** *E-CADHERIN* upon GATA2 silencing in A549 cell line. **(D)** Representative western blot for GATA2 and **(E)** protein quantification upon GATA2 silencing in A549 cell line. Scramble siRNA was used as a control. n=3 independent experiments. Data are presented as means ± SEM. Kruskal-Wallis test for multiple comparisons with Dunn's *post hoc.* **P < 0.01, ***P < 0.001, ****P < 0.0001.

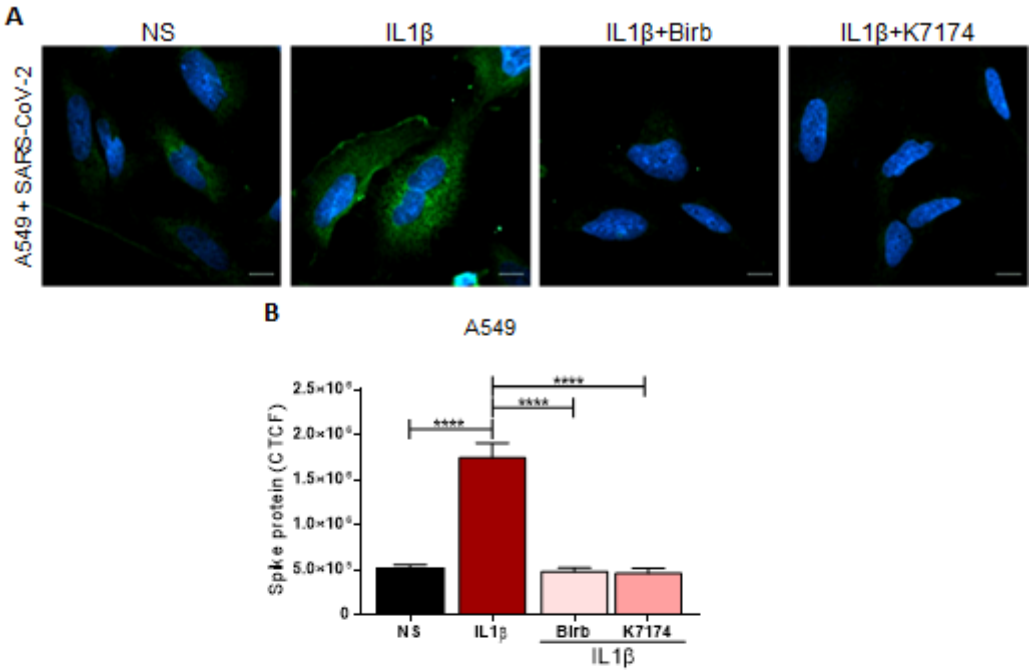
These results confirmed that the downregulation of GATA2 expression impairs the increase of TMPRSS2 transcription in response to IL1β stimulation.

Overall, our data indicated that the inhibition of the p38 - GATA2 axis *via* inhibitors or *via* silencing reduces the IL1β-induced TMPRSS2 overexpression, finally proving the role of this pathway in the examined process.

4.1.3. Effect of IL1β-induced TMPRSS2 overexpression on cell susceptibility to SARS-CoV-2 infection

Finally, we moved to evaluate whether the IL1β-induced TMPRSS2 overexpression could influence the cell susceptibility to SARS-CoV-2 infection.

To this aim, we exploited the non-replicative heat-inactivated SARS-CoV-2. We treated A549 and TT1 cells with IL1β in presence or absence of the selected inhibitors of p38 MAPK and of GATA2 and then we exposed them to the heat-inactivated SARS-CoV-2. We observed that IL1β stimulation significantly increased the virus entry compared to the non-stimulated condition; the pharmacological block of the p38 MAPK-GATA2 axis through Birb796 or K7174 significantly blocked the increased viral entry; the viral entry in this condition was comparable to the non-stimulated condition levels (Figure 25 A-D). As negative control, we considered A549 and TT1 cells which were not exposed to the heat-inactivated SARS-CoV-2 (Figure 25 E).


A
NS
IL1β
IL1β+Birb
IL1β+K7174
A549 + SARS-CoV-2
B
A549
Spike protein (CTCF)
2.5×10⁶
2.0×10⁶
1.5×10⁶
1.0×10⁶
5.0×10⁵
0
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****
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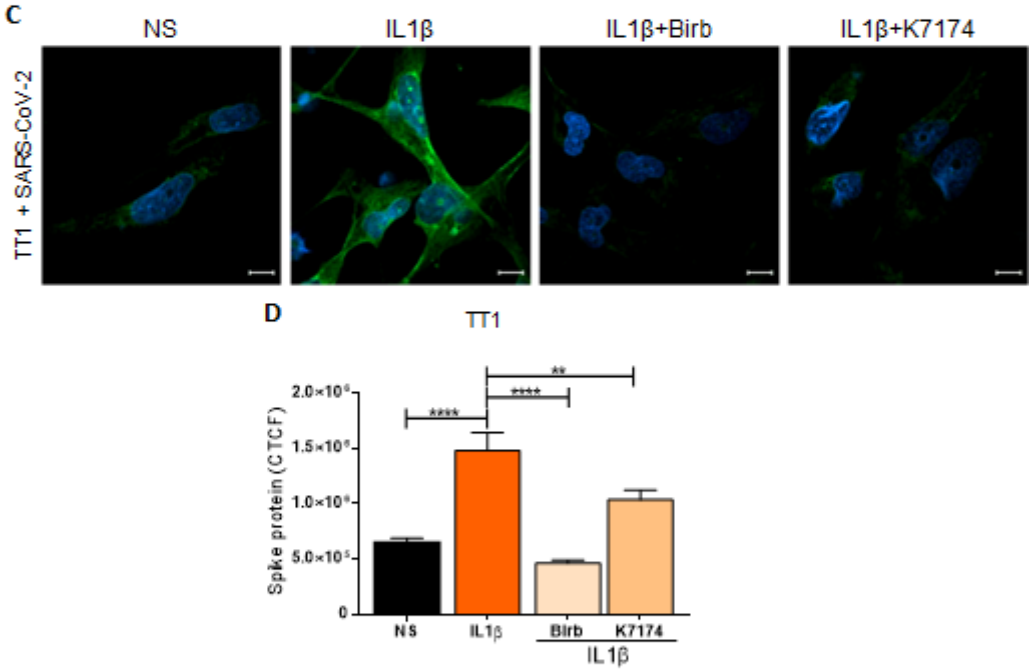
NS
IL1β
Birb
K7174
IL1β


C
NS
IL1β
IL1β+Birb
IL1β+K7174
TT1 + SARS-CoV-2
D
TT1
Spike protein (CTCF)
2.0×10⁶
1.5×10⁶
1.0×10⁶
5.0×10⁵
0
**
****
****
NS
IL1β
Birb
K7174
IL1β

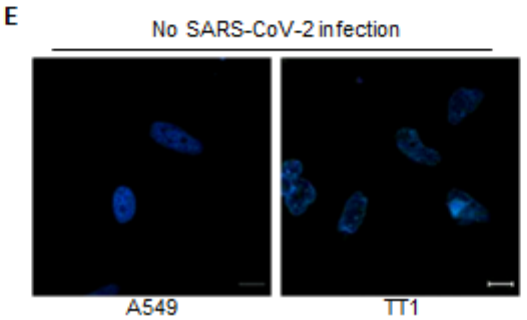

E
No SARS-CoV-2 infection
A549
TT1

Figure 25. (A, C) Representative confocal images and **(B, D)** quantification of fluorescence intensity of SARS-CoV-2 Spike protein (green) in A549 **(A, B)** and TT1 **(C, D)** cells. Cells were stimulated or not (NS) with IL1β (50 ng/mL) for 8 hours and either treated or not with Birb796 (p38 MAPK inhibitor, 100 nM) or K7174 (GATA2 inhibitor, 10 μM); cells were then exposed to heat-inactivated SARS-CoV-2 virus. **(E)** Representative confocal images of A549 and TT1 cells not exposed to heat-inactivated SARS-CoV-2 virus. Nuclei were counterstained in blue (Hoechst 33342). Scale bar: 10 μm; n=3 independent experiments. Data are presented as means ± SEM. Kruskal-Wallis test for multiple comparisons with Dunn's *post hoc.* **P < 0.01, ****P < 0.0001.

To corroborate our findings we treated the A549 cell line with IL1β and p38 MAPK / GATA2 inhibitors, and then exposed them to heat-inactivated SARS-CoV-2. We then performed a RT-qPCR on the viral RdRp gene (*nCoV_IP2*) to detect the presence of SARS-CoV-2; this gene was the one selected by WHO for the RT-qPCR protocol for the diagnosis of SARS-CoV-2 infection in suspectedly infected patients.

Results confirmed our previous data: the exposure to IL1β significantly increased the viral entry into A549 cells compared to the non-stimulated condition. Moreover, the treatment with p38 MAPK and GATA2 inhibitors significantly reduced this increased viral entry, at all the analyzed timepoints (Figure 26).

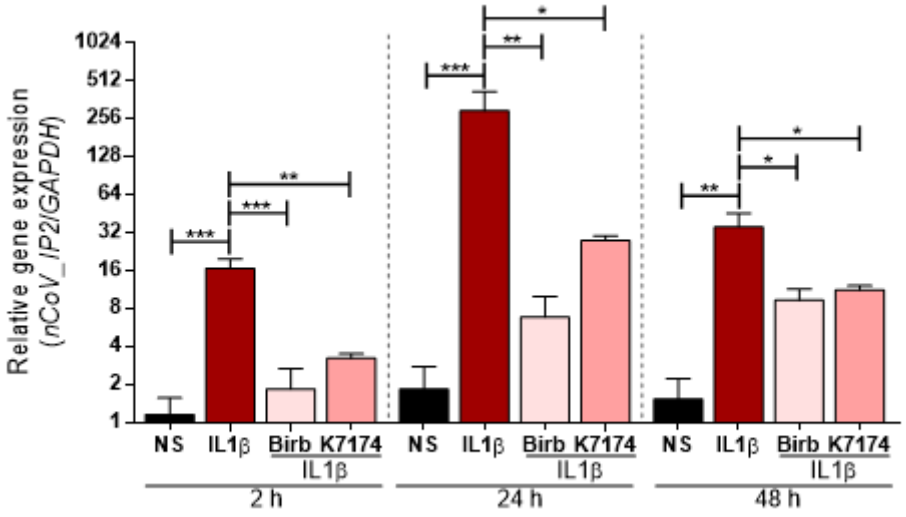


Figure 26. Relative gene expression of *nCoV_IP2* upon exposure to heat-inactivated SARS-CoV-2 at different time points (2, 24, and 48 hours). Before infection, cells were stimulated or not (NS) with IL1β (50 ng/mL) and either treated or not with Birb796 (p38 MAPK inhibitor, 100 nM) or K7174 (GATA2 inhibitor, 10 μM) for 8 hours. Data are presented as means ± SEM. Kruskal-Wallis test for multiple comparisons with Dunn's post hoc. *P < 0.05 **P < 0.01 ***P < 0.001.

IL1β is known to promote the expression of interferon response genes (IRF) in epithelial and myeloid cells (Aarreberg *et al.*, 2019). Therefore, we tested whether IL1β stimulation could regulate other host innate immune responsive pathways, which could ultimately influence host cell susceptibility to SARS-CoV-2 infection.

By RT-qPCR, we observed that the stimulation with the pro-inflammatory cytokine promoted an overexpression of *INTERFERON REGULATORY FACTOR 1* (*IRF1*) and of its target *ISG15 UBIQUITIN LIKE MODIFIER* (*ISG15*), but not of *INTERFERON REGULATORY FACTOR 9* (*IRF9*) (Figure 27). Interestingly, the expression of these genes has been reported to be associated with cytokine storm syndrome in COVID-19 patients (Cao, 2021; Munnur *et al.*, 2021). Future high throughput studies could be performed to shed light on the link between IL1β and innate immune response in SARS-CoV-2 infection.

Overall, the last set of experiments indicated that SARS-CoV-2 entry in the target cell can be increased by IL1β exposure and can be counteracted by the pharmacological blocking of the p38 - GATA2 pathway.

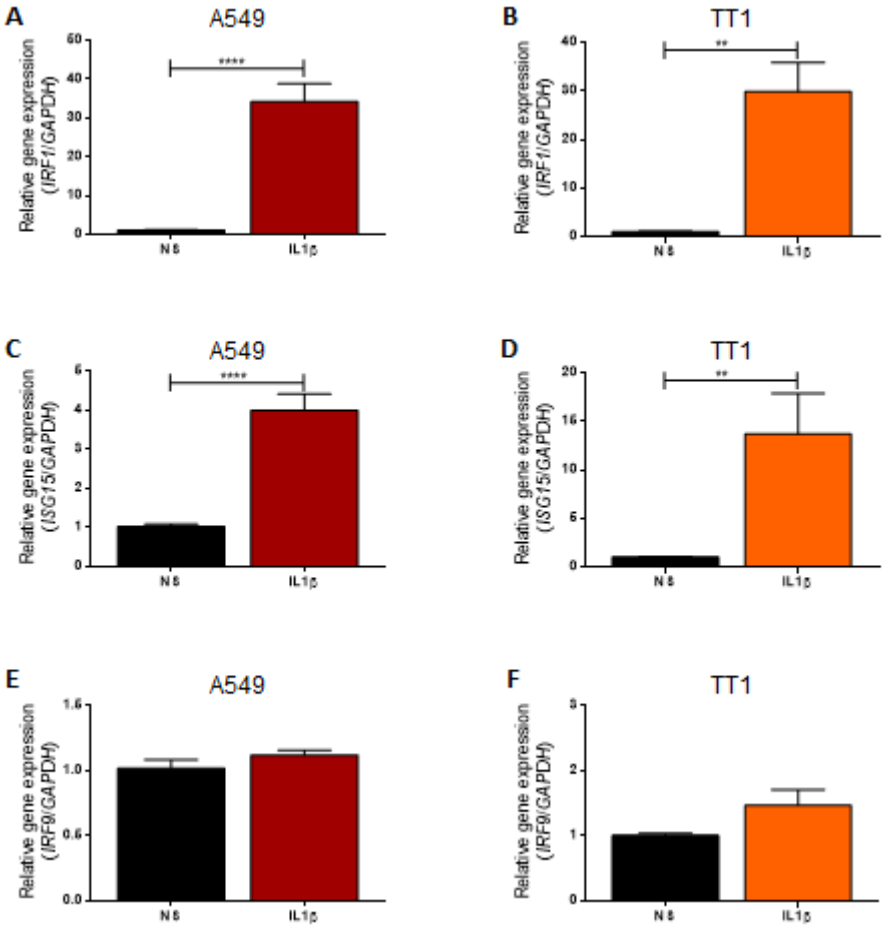


Figure 27. Relative gene expression of *IRF1* **(A, B)**, *ISG15* **(C, D)** and *IRF9* **(E, F)** in A549 **(A, C, E)** and TT1 **(B, D, F)** cell line either stimulated or not (NS) with IL1β (50 ng/mL) for 4 hours; n=3 independent experiments. Data are presented as means ± SEM. Nonparametric Mann–Whitney U test. **P < 0.01, ****P < 0.0001.

4.2. Effects of vaccination on hyperinflammation and damage-associated molecular patterns

As mentioned, the immune response to SARS-CoV-2 infection has come out as a critical determinant of the disease progression and outcome (F. Zhou *et al.*, 2020; Zhou and Ye, 2021) and even though an antiviral immune response is necessary to eradicate the virus, a strong and persistent response can eventually cause a significant production of pro-inflammatory cytokines, possibly leading to the development of cytokine storm syndrome and to the damage of the host tissues and organs (Merad and Martin, 2020; L. Yang *et al.*, 2021). Indeed, the cytokine storm syndrome can lead to apoptosis of epithelial and endothelial cells, a diffuse vascular leakage and can cause ARDS and eventually patient death (Sinha, Matthay and Calfee, 2020; Bonaventura *et al.*, 2021).

Vaccination represents the most effective way to prevent COVID-19 infection, along with daily good practices such as wearing masks and frequently sanitizing hands (Vitiello *et al.*, 2021). The available SARS-CoV-2 vaccines have demonstrated to have a good efficacy and safety profile (Polack *et al.*, 2020; Knoll and Wonodi, 2021; Vitiello *et al.*, 2021): they not only determine a consistent reduction in the risk of SARS-CoV-2 transmission and infection, but they also contribute to disease attenuation in infected people.
Yet, the precise effect of COVID-19 vaccination on the immune system in terms of long-term immunity generation and protection from cytokine storm syndrome need to be further examined. Indeed, up to date, systematic studies on the cytokine profile in SARS-CoV-2 vaccinated patients who subsequently acquired the infection are lacking.

4.2.1. Plasma cytokines in COVID-19 patients

We examined the SARS-CoV-2 cytokine response in two groups of SARS-CoV-2 infected patients, one vaccinated (Vax) and one non-vaccinated (NoVax), with the final aim of identifying the features of the inflammatory

response that significantly differ between the two groups and their association with disease severity.

In particular, we analyzed a cohort of 47 patients who tested positive in the period from 12 August to 2 September 2021. Of these, two patients were excluded from the study, one for an ongoing pulmonary tuberculosis and one for long COVID-19. The demographic and clinical data of the studied population composed of the remaining 45 patients are reported in Table 6, with patients grouped by vaccination status (vaccinated = Vax and non-vaccinated = NoVax). We first compared the two groups for demographic and comorbidity characteristics; P values for the corresponding trait are reported in the right column. We observed no significant differences for the considered parameters, with the exception of the median age, which was significantly lower in the NoVax group compared with that in the Vax group (57 years, IQR: 41.5–62 vs. 79 years, IQR: 49.5–87, $p < 0.05$).

	Vax (n = 22)	NoVax (n = 23)	p Value
Demographics			
Age, median	79	57	0.002
Male (%)	15 (68.2%)	14 (60.9%)	0.421
Comorbidities			
None (%)	9 (40.9)	13 (56.5)	0.179
1–3 (%)	13 (49.1)	10 (43.5)	0.566
>3 (%)	2 (9.09)	6 (26.1)	0.107
Severity of COVID-19			
Mild	6 (27.3%)	0	0.009
Moderate	1 (4.5%)	0	0.489
Severe	13 (59.1%)	20 (87%)	0.037
Critical	2 (9.1%)	3 (13%)	0.522
Supplemental oxygen therapy			
None	8 (36.4%)	0 (0.0%)	0.001
Low flow	11 (50%)	14 (60.9%)	0.333
High flow	1 (4.5%)	6 (26.1%)	0.054
Mechanical ventilation	2 (9.1%)	3 (13%)	0.522

Table 6. Demographic and clinical data of the studied population, subdivided in vaccinated (Vax) and non-vaccinated (NoVax) groups.

In the whole cohort, males represented 63% (29/45) and the median age was 61 years. Most of the subjects (86.6%, 39/45) were admitted and hospitalized

because of the severity of COVID-19, whereas 6/45 were managed as outpatients.

More than half of the subjects (51.1%, 23/45) had not received any vaccine for the prevention of SARS-CoV-2 infection (NoVax), on the contrary 49.9% of the patients had received at least one dose before the infection (Vax) (Figure 28 A).

Analyzing the cohort by grouping Vax and NoVax, we noticed that the median age was significantly lower in the NoVax group compared with the Vax group (57 years *vs.* 79 years, $p < 0.05$) (Figure 28 B). The male gender was equally represented in both groups (68,2% *vs.* 60,9%). For what concerns comorbidities, the most common overall were: malignancies (11/45, 24.4%), diabetes (10/45, 22.2%), ischemic heart disease (6/45, 13.3%), chronic obstructive pulmonary disease (COPD) (4/45, 8.9%). Notably, the two groups were not significantly different in terms of comorbidities (Table 6).

As for the disease severity, patients who were managed as outpatients had mild disease, while among subjects who were admitted to the hospital (39 in total): 1 (2.5%) had moderate disease, 33 (84.7%) severe disease and 5 (12.8%) critical disease requiring intensive care unit admission (Figure 28 C). One patient, a 92-year-old woman with multiple comorbidities, died.

Most of the hospitalized patients (80.4%, 37/46) received oxygen support. The percentage of subjects who required oxygen support was significantly different between the two groups. Overall, 25/45 patients needed low flow oxygen; among them, 14 (60.9%) were in the NoVax group and 11 (50%) in the Vax group (*p* value 0.333). Moreover, 7/45 patients needed high flow oxygen; among them, 6 (26,1%) were in the NoVax group and 1 (4.5%) in the Vax group; *p* value 0.054). Finally, 5/45 required mechanical ventilation, 2 (9.1%) in the NoVax group and 3 (13%) in theVax group (*p* value 0.522) (Table 6).

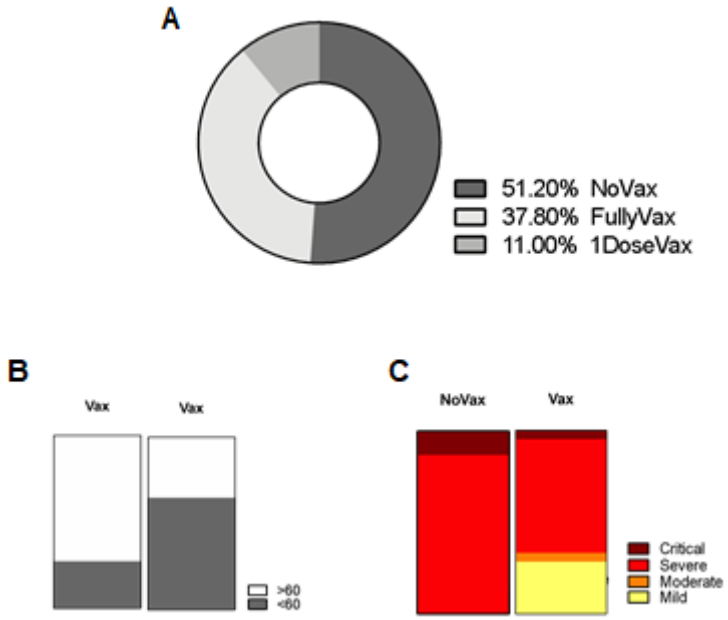


Figure 28. (A) Percentage of non-vaccinated (NoVax), fully vaccinated (Vax) and one dose vaccinated patients in the analyzed cohort. Distribution of NoVax and Vax patients by age **(B)** and disease severity **(C)**.

Cytokine storm is a relevant determinant for the severe COVID-19 outcomes, like ARDS, systemic inflammatory response syndrome and multiple organ failure. Current evidence indicates that the cytokine storm plays a crucial role in determining COVID-19 severe outcomes (Land, 2021). We moved to analyze the cytokine profile in our patient cohort by measuring the circulating levels of 46 cytokines in the plasma specimens of Vax and NoVax infected patients (Figures 29-34). To evaluate the influence of age - whose median was, as stated, significantly different between the two groups - on the cytokine profile, we also performed a comparison among cytokines levels by stratifying patients according to their age, with the threshold set at 60 years of age.

Among all the examined cytokines, we observed that the IL1β plasma levels were significantly lower in the Vax group compared to the NoVax group (Figure 29 A). Interestingly, circulating IL1β has been reported to positively correlate with higher hospitalization time, age and disease severity, hence underlying relevance in the COVID-19 progression (Angioni *et al.*, 2020).

Notably, the comparison between the groups stratified by age pointed out a significant difference only in the <60 years old Vax *versus* NoVax groups (Figure 29 B).

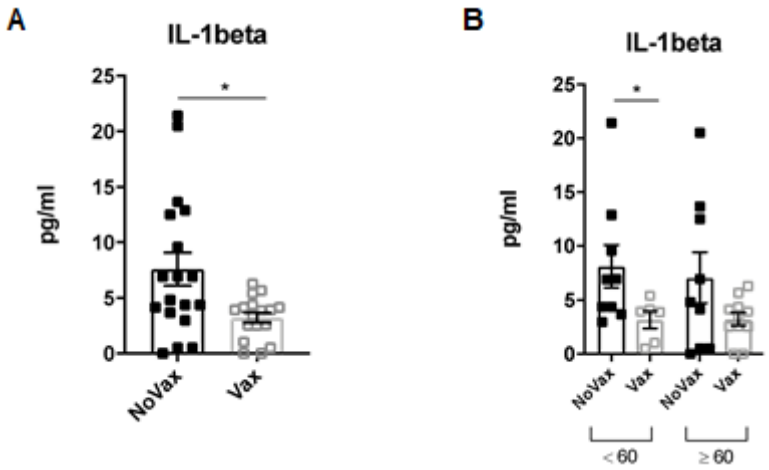


Figure 29. (A) IL1β plasma concentration (pg/mL) in NoVax and Vax patients. **(B)** IL1β plasma concentration (pg/mL) in NoVax and Vax patients stratified by age (under and over 60 years old). Data are presented as means ± SEM. Nonparametric Mann–Whitney U test. *P < 0.05.

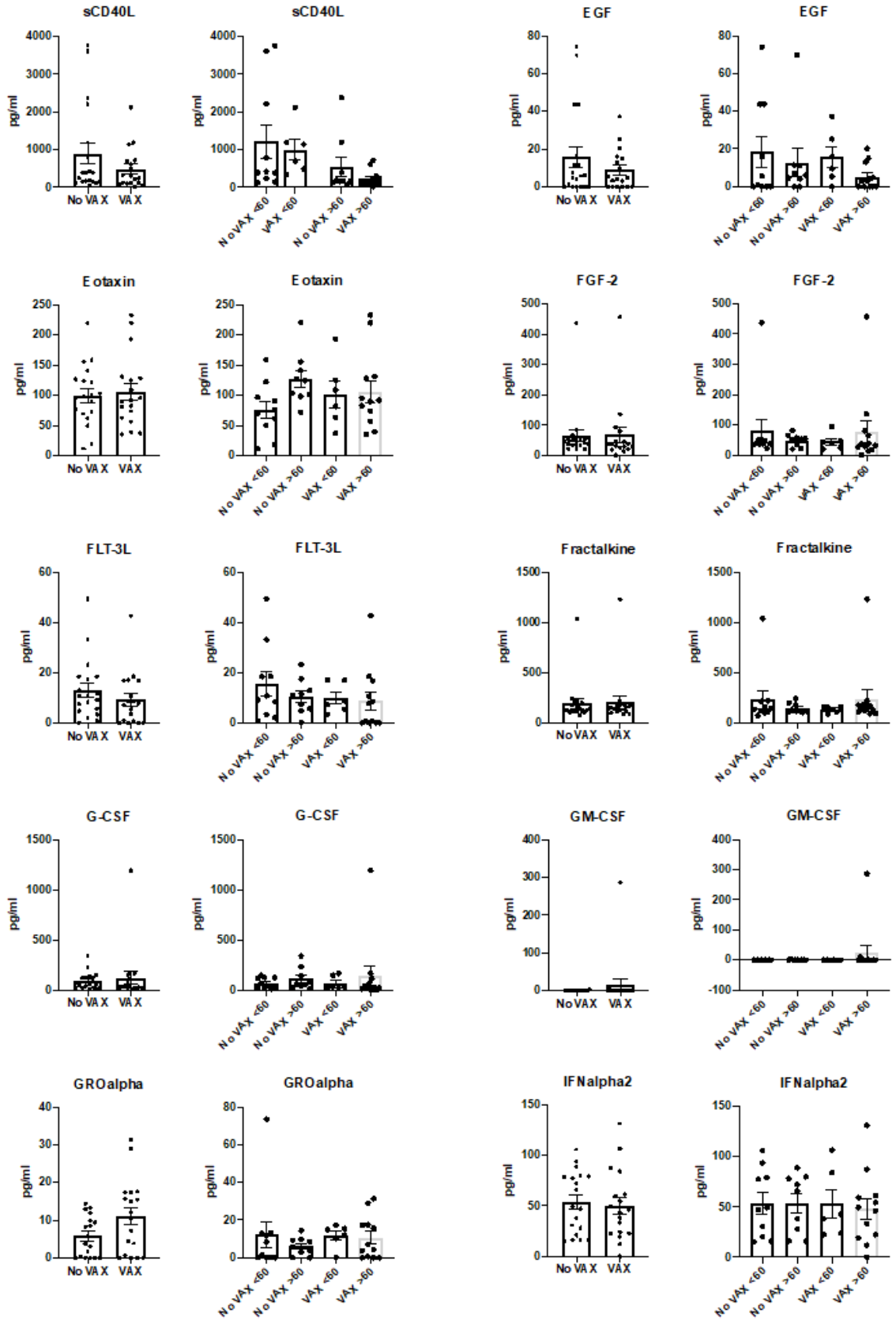


Figure 30. Plasma concentration (pg/mL) of the indicated analytes in NoVax and Vax patients.

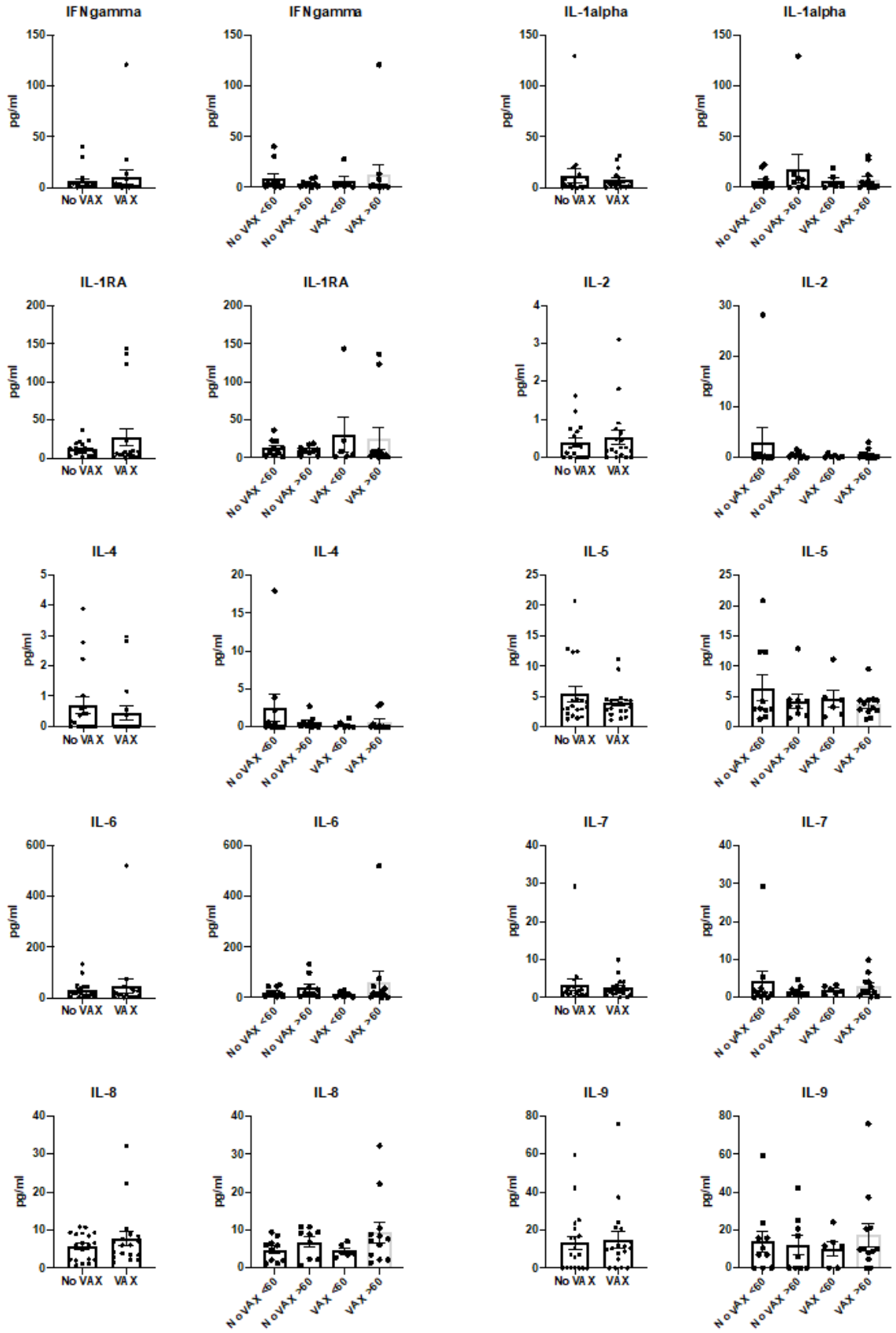


Figure 31. Plasma concentration (pg/mL) of the indicated analytes in NoVax and Vax patients.

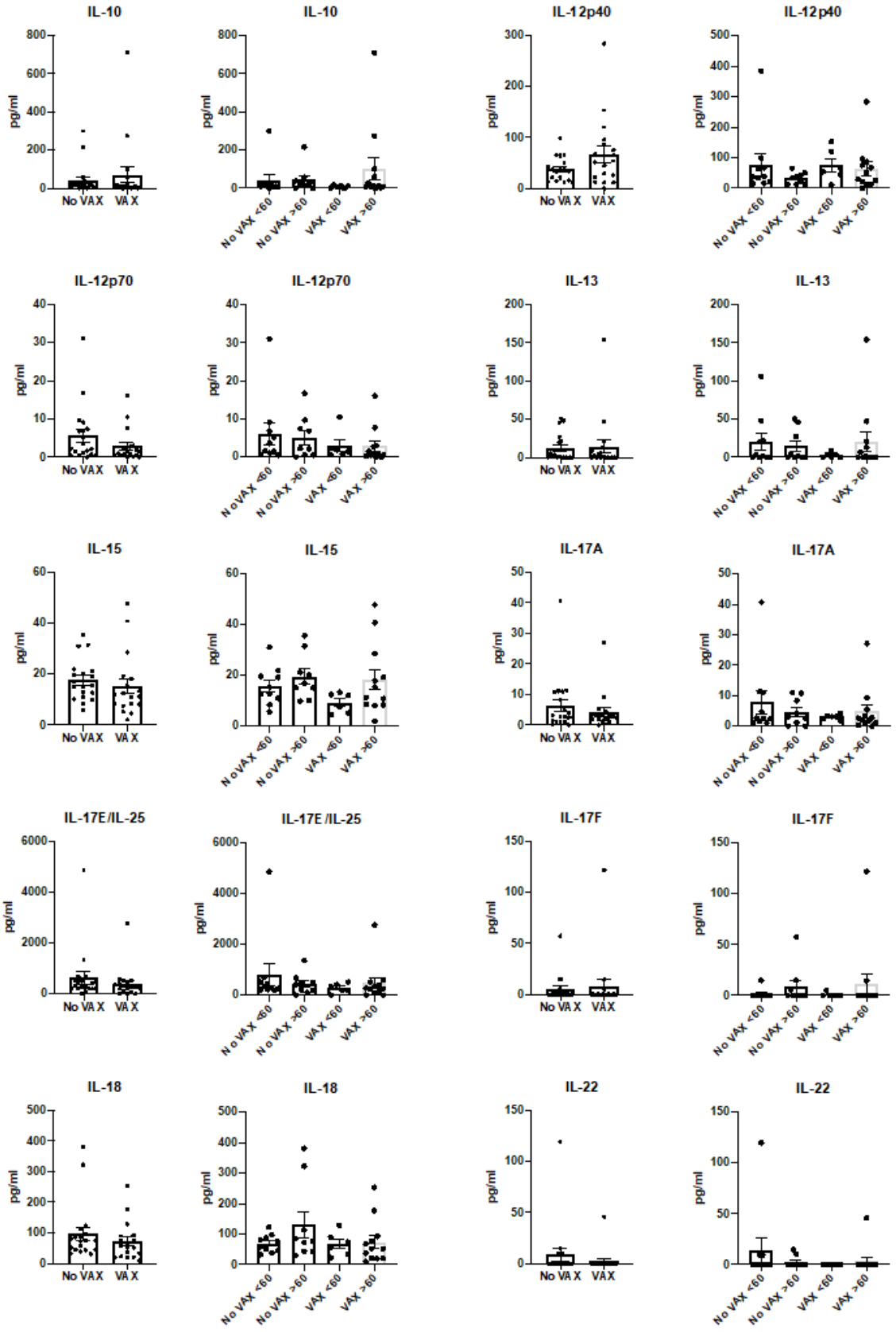


Figure 32. Plasma concentration (pg/mL) of the indicated analytes in NoVax and Vax patients.

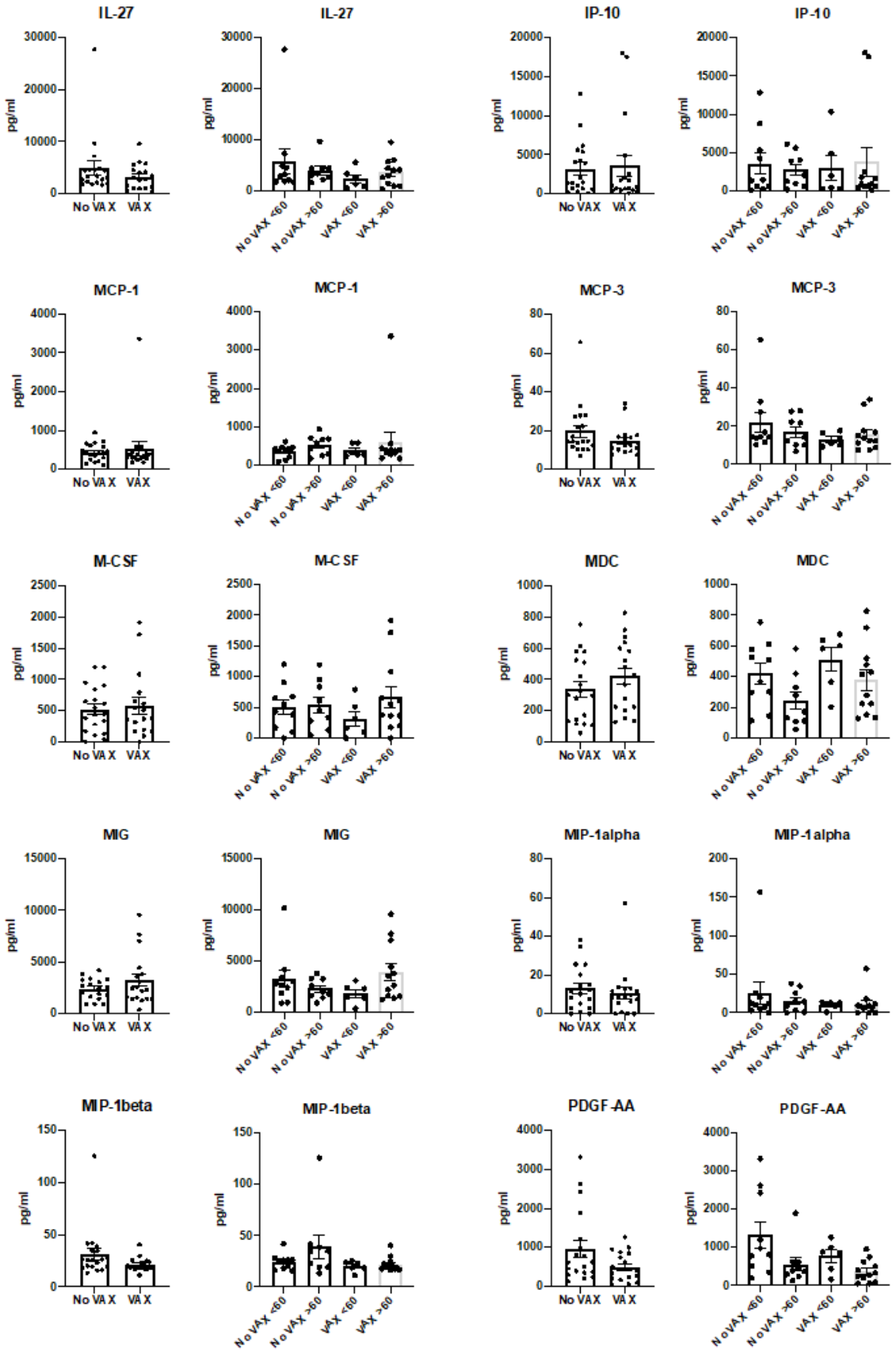


Figure 33. Plasma concentration (pg/mL) of the indicated analytes in NoVax and Vax patients.

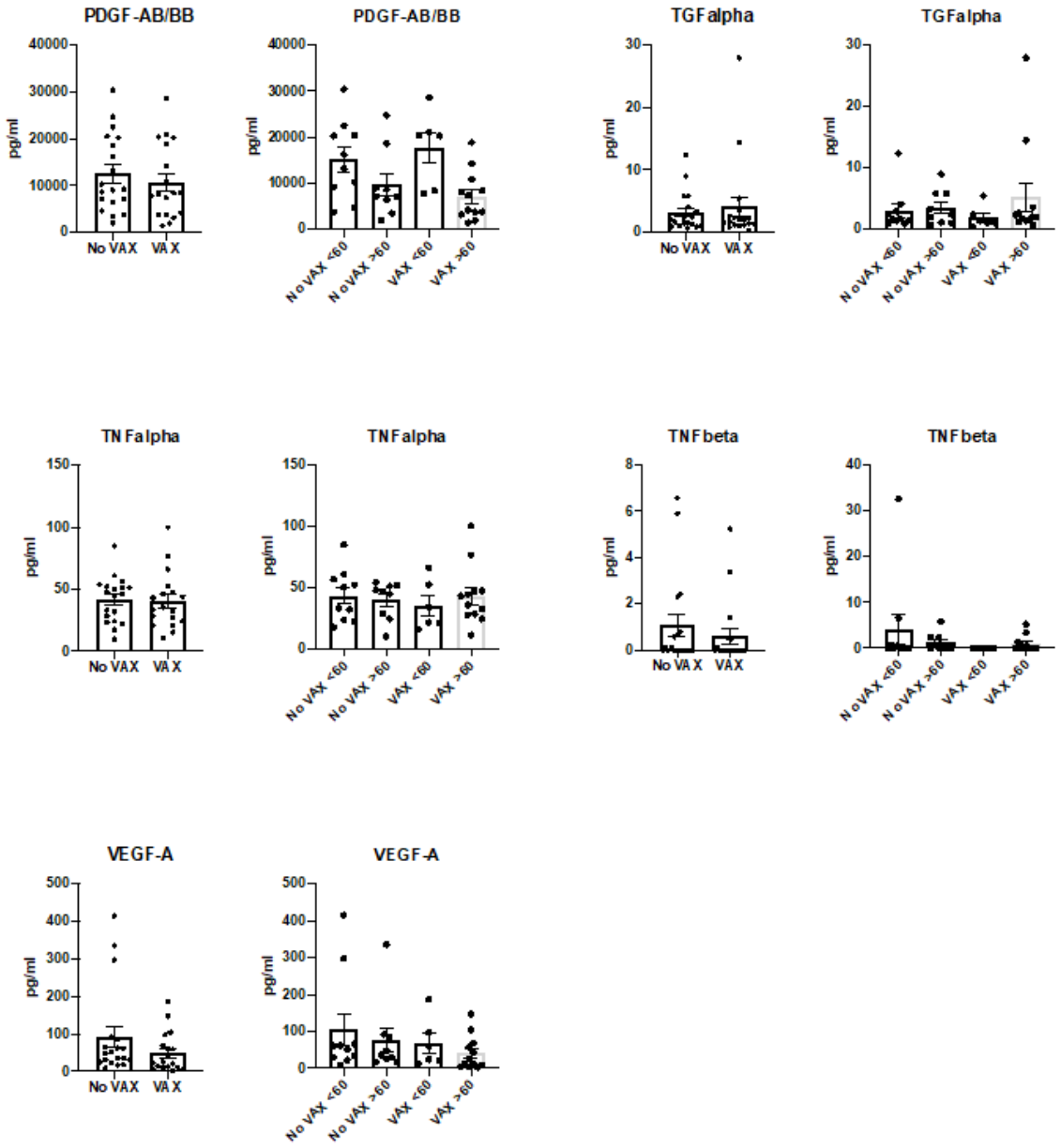


Figure 34. Plasma concentration (pg/mL) of the indicated analytes in NoVax and Vax patients.

4.2.2. Plasma DAMPs in COVID-19 patients

IL1β is a potent pro-inflammatory cytokine which is synthetized by the cell in an inactive form (pro-IL1β); in order to be converted into the active form, it requires the activation of the inflammasome, a multiprotein complex which is composed of a sensor protein (NOD-like receptor, NLR), caspases, and an adapter protein linking the two (apoptosis-associated speck-like protein containing a caspase recruitment domain, ASC). The activation of the inflammasome can be triggered by pathogen-associated (PAMPs) and/or damage-associated (DAMPs) molecular patterns (Zheng, Liwinski and Elinav, 2020). Notably, SARS-CoV-2 has been reported to be able to trigger the NLRP3 inflammasome activation and therefore to increase the release of IL1β (Costela-Ruiz *et al.*, 2020; Yap, Moriyama and Iwasaki, 2020). Moreover, a strong correlation between DAMP release and poor clinical outcomes (higher risk of ICU admission and death) in COVID-19 patients has recently been reported, in particular for the HMGB1 and S100A8/9 DAMPs (L. Chen *et al.*, 2020; R. Chen *et al.*, 2020; Land, 2021).

Hence, we proceeded to quantify the levels of these two alarmins in the plasma of Vax and NoVax patients *via* ELISA. Our results showed that HMGB1 was significantly lower in Vax patients (Figure 35 A), in particular the age-stratified analysis pointed out that this reduction was significant for the >60 years old groups, even though a non-significant reduction was also present in the <60 years old groups (Figure 35 C). Moreover, a trend of decrease (although not significant) was also observed for S100A8, (Figure 35 B); the age-stratified comparison revealed a significant reduction in S100A8 plasmic levels in the <60 years old groups (Figure 35 D).

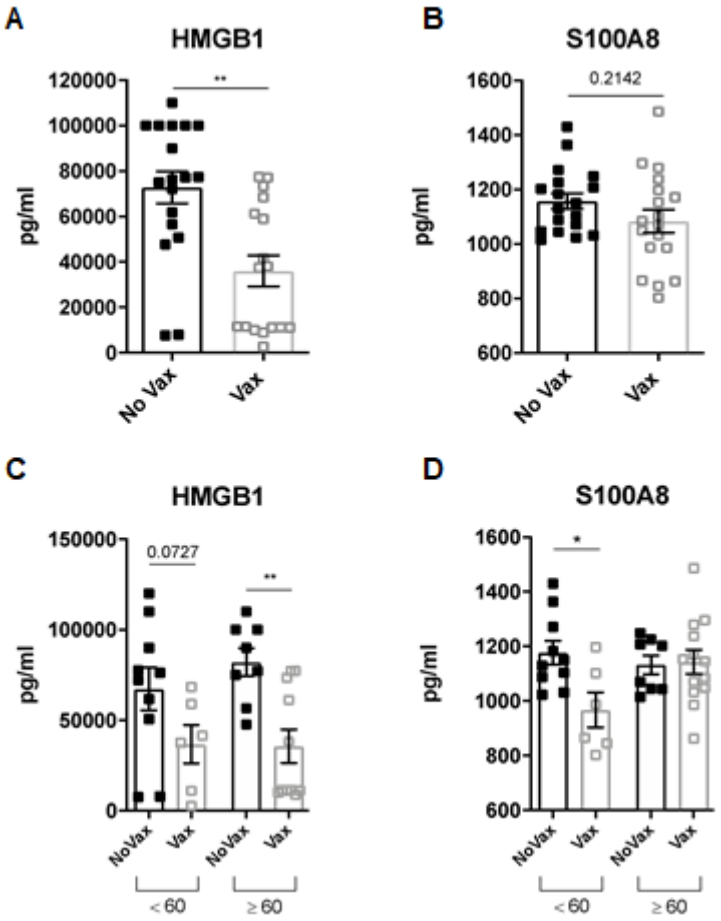


Figure 35. HMGB1 **(A)** and S100A8 **(B)** plasma concentration (pg/mL) in NoVax and Vax patients. HMGB1 **(C)** and S100A8 **(D)** plasma concentration (pg/mL) in NoVax and Vax patients stratified by age (under and over 60 years old). Data are presented as means ± SEM. Nonparametric Mann–Whitney U test. *P < 0.05, **P < 0.01.

Considering the fact that the systemic inflammatory status of COVID-19 patients can lead to liver dysfunction and, eventually, failure (Ali *et al.*, 2021; McConnell *et al.*, 2022), we tested the liver functionality in our cohort. To this aim, the plasma levels of alanine aminotransferases (ALT) and aspartate aminotransferases (AST), which are the most widely used markers for liver functionality (Kariyawasam *et al.*, 2022), were obtained from hospital routine analysis.

Although data on COVID-19-related liver abnormalities in patients remain limited, liver injury in patients is associated with prolonged hospitalization (Fierro, 2020).

So, we next analyzed biomarkers of liver function in our patient cohort. Interestingly, ALT and AST levels were comparable between Vax and NoVax groups (Figure 36 A-B), hence showing a comparable liver alteration in the two groups, if present. A comparison with a healthy control group could further clarify the dynamics of these biomarkers in COVID-19.

Parallelly, we also analyzed plasma glutamate dehydrogenase activity (GDH or GLDH), which constitutes a biomarker of hepatocellular injury (Schomaker *et al.*, 2020): levels of circulating GDH activity were higher in the NoVax group compared to the Vax group (Figure 36 C). Interestingly, GDH is a mitochondrial enzyme, therefore an increase in GDH plasma levels indicates liver mitochondrial damage. In line with this, high levels of circulating mitochondrial DNA have been already identified as a potential early indicator for poor COVID-19 prognosis (Scozzi *et al.*, 2021).

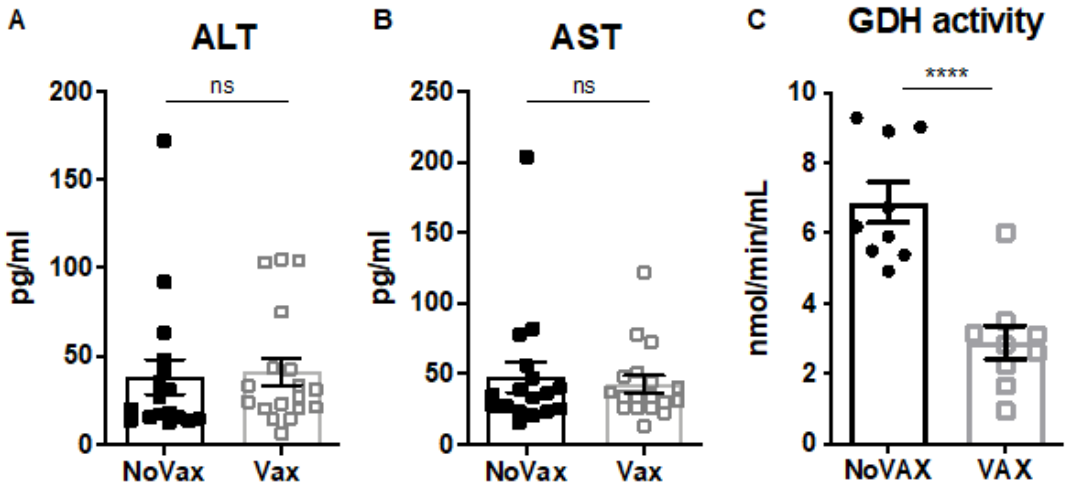


Figure 36. (A) ALT and **(B)** AST liver transaminases and **(C)** GDH activity levels in NoVax and Vax patients. Data are presented as means ± SEM. Nonparametric Mann–Whitney U test. ****P < 0.0001.

Overall, our results suggest that COVID-19 vaccination can prevent the release of the pro-inflammatory cytokine IL1β and systemic danger signals HMGB1 and S100A8, along with GDH, in SARS-CoV-2 infected patients. Hence, we confirm that vaccination represents a great strategy to prevent potential side-effects caused by the hyperinflammation associated to SARS-CoV-2 infection.

5. DISCUSSION AND CONCLUSIONS

COVID-19 is a highly contagious disease whose etiological factor is the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Up to date, this illness has caused more than 650 millions of infections and 6 million deaths worldwide as reported by WHO (*WHO Coronavirus (COVID-19) Dashboard*).
From the very beginning, consistent research work has been made to shed light on the mechanisms of infection and disease pathogenesis to develop effective therapies and to limit the virus spreading. Yet, keeping updating the current knowledge is of primary importance, considering that the spread of SARS-CoV-2 in its variants has become a matter of increasing concern.

COVID is characterized by a wide spectrum of symptoms, including fever, cough, dyspnea, sore throat, anosmia and ageusia in mild and moderate patients; in addition, pneumonia, ARDS, sepsis, septic shock, acute thrombosis characterize the severe and critical forms of the disease (*Clinical management of COVID-19: Living guideline, 23 June 2022*).

Importantly, hyperinflammation has come out as a peculiar feature of severe and critical cases: in particular, the cytokine storm syndrome, namely the acute overproduction and release of pro-inflammatory cytokines, and CSS-related clinical manifestations, have been associated to poor clinical outcome (Ramatillah *et al.*, 2022). In the early stage of the disease, the activation of the immune system protects the host from the viral infection, but in severe and critical cases the host immune response becomes excessive and uncontrolled, thus causing an hyperinflammatory damage instead of having a protective role; the massive release of cytokines can contribute to the development of the acute respiratory distress syndrome and of respiratory failure, along with multiorgan failure and eventually death (Fajgenbaum and June, 2020; Shi *et al.*, 2020).

Hence, the focus of our research, that started early during the pandemic, was represented by the investigation of the role of the host inflammatory response in COVID-19 pathogenesis. In particular, we first addressed whether pro-inflammatory cytokines might contribute to SARS-CoV-2 infectivity by

altering the expression of the virus receptors in the target cell; secondly, we studied the effects of vaccination on hyperinflammation and damage-associated molecular patterns.

SARS-CoV-2 is known to be a virus whose genome is composed of a single strand of positive RNA. It has an envelope that contains several proteins, among which there is the Spike protein, which is composed of 2 subunits: S1 which allows the binding to the host cell receptor, and S2, which contains the fusion peptide that allows the entry of the virus in the host cell. In order to enter the cell, the virus Spike proteins are required first to bind the ACE2 receptor on the surface of the host epithelial cell and secondly to be cleaved by a protease, TMPRSS2. This processing allows the exposure of the fusion subunit and the infection of the cell (Harrison, Lin and Wang, 2020; Hoffmann *et al.*, 2020). There are two other proteases that have been reported to be involved in this cleavage: Furin and ADAM17 (Zipeto *et al.*, 2020).

Among the cytokines that are abundantly reported in literature to increase in the plasma of COVID-19 patients there are IL1β, IL6 and TNFα (Azkur *et al.*, 2020; Huang *et al.*, 2020; McElvaney *et al.*, 2020; Teuwen *et al.*, 2020).

Of note, ACE2 expression levels have been reported to be associated to the immune response, in particular they can be regulated through IFNα and ɣ signaling, and this strengthens our hypothesis that also other cytokines could have a role in the regulation of expression of these receptors (Ziegler *et al.*, 2020).
So we wondered if the cytokine milieu of the host can modulate the expression of the proteins that the virus requires in order to enter the cell.

In order to evaluate our hypothesis, we initially performed a screening of the effect of these cytokines on different epithelial cell lines of organs (lungs, gut, liver, kidney) that are targets of SARS-CoV-2 infection (Shah *et al.*, 2021). We stimulated the cells with different concentrations of IL1β, IL6 and TNFα and we then analyzed *TMPRSS2* and *ACE2* expression by RT-qPCR. Among the tested lines, the pulmonary epithelial cell line A549 was particularly responsive,

showing a significant increase in the expression of *TMPRSS2* upon IL1β and TNFα stimulation. Considering these results and the huge involvement of the lung in COVID-19 (Harrison, Lin and Wang, 2020), we focused on the A549 cell line for the following experiments.
Of note, neither *ADAM17* nor *Furin* showed any relevant difference in their expression upon IL1β and TNFα stimulation in this cell line.

Among all the tested cytokines we focused on IL1β; indeed, as we and others (Yap, Moriyama and Iwasaki, 2020) observed, IL1β is critically increased in COVID-19 patients plasma and, in particular, higher levels of IL1β characterize the severe and critical cases compared to mild-moderate ones. Moreover, SARS-CoV-2 has been reported to promote IL1β release by activating the NLRP3 inflammasome (Yap, Moriyama and Iwasaki, 2020).
We therefore further corroborated the data obtained on IL1β in the A549, a carcinoma cell line, in a more physiological setting. Hence, we tested an immortalized primary human alveolar epithelial cell line named TT1 with the same IL1β stimulation and the effect of this cytokine on *TMPRSS2* expression was confirmed: IL1β increased the transcription of *TMPRSS2*.
Results on mRNA expression were further validated in both the lung cell lines by the protein analysis that was performed both by immunofluorescence and by western blot analysis.
We concluded that the stimulation of both the A549 and TT1 lung epithelial cell lines with the pro-inflammatory cytokine IL1β promoted an increase in the expression of TMPRSS2, at both the transcriptional and the protein level, and that other viral-related processes appear not to be influenced by this cytokine stimulation.

Therefore, we moved to investigate the molecular mechanism beyond TMPRSS2 overexpression upon IL1β stimulation.
TMPRSS2 expression has been reported to be regulated by the master regulator transcription factor GATA2, which has a binding site in the -13 kb DNA enhancer region of the gene (Clinckemalie *et al.*, 2013). This transcription factor has also been reported to be activated by its phosphorylation through the p38 MAPK pathway (Katsumura *et al.*, 2016) and is known to be involved in the

transcriptional regulation of several pro-inflammatory cytokines, including *IL1B* and *CXCL2*; also, a positive feedback between IL1β signaling and GATA2 activation has been reported in Acute Myeloid Leukemia (Katsumura *et al.*, 2016).
Considering this, we reasoned that IL1β might be responsible for the induction of TMPRSS2 overexpression *via* the p38 MAPK-GATA2 axis.
To test this hypothesis, we took advantage of 2 inhibitors: Birb796 that inhibits p38 MAPK and K-7174, an inhibitor of GATA2. In particular, we stimulated the A549 and TT1 cells with IL1β and treated them with these 2 inhibitors separately. Our results showed that *TMPRSS2* was, as before, overexpressed upon IL1β simulation and interestingly this overexpression was significantly reduced after the inhibition of p38 MAPK or GATA2 in both the cell lines.

To further confirm the role of GATA2 in the IL1β-induced TMPRSS2 transcriptional upregulation, we performed the GATA2 chromatin immunoprecipitation by repeating the stimulation of the previous experiment. The binding of GATA2 in the *TMPRSS2* enhancer region was increased upon IL1β stimulation. The use of p38 and GATA2 inhibitors was able to inhibit the increased GATA2 binding to the *TMPRSS2* enhancer region.
The fact that the inhibitors were able to block the IL1β-induced TMPRSS2 overexpression indicated that both GATA2 and p38 have a role in the IL1β downstream signaling that leads to this *TMPRSS2* overexpression. Moreover, the fact that GATA2 binding to the *TMPRSS2* enhancer promotor region increased upon IL1β stimulation and returned to unstimulated levels upon p38-GATA2 inhibition contributed to support our hypothesis that, upon IL1β stimulation, GATA2 may be activated by p38 pathway, resulting in an increased GATA2 transcriptional activity.

To investigate whether the observed effect relies on GATA2 activity or on a modulation of its expression level, we treated A549 and TT1 cells with IL1β and analyzed the relative gene expression of *GATA2*. The stimulation did not significantly affect GATA2 transcription levels in these lung cell lines, hence the outlined effect seems to rely on GATA2 activity rather than on a modulation of its expression level.

To definitively corroborate the role of GATA2 in this process, we performed a GATA2 silencing in A549 cells. The obtained results showed that, with GATA2 downregulation, the IL1β-induced overexpression of TMPRSS2 is reduced.

Overall, these data confirmed the role of the p38 - GATA2 axis in the IL1β downstream signaling that leads to TMPRSS2 overexpression.

Finally, we wanted to assess whether the IL1β-induced TMPRSS2 overexpression could increase the cell susceptibility to SARS-CoV-2 infection.
To test this hypothesis, we treated the lung cell lines with IL1β and p38/GATA2 inhibitors as previously done and then exposed them to heat-inactivated SARS-CoV-2. The immunofluorescence analysis showed that IL1β exposure was able to increase the virus entry into the host cells and that interestingly this entry was significantly decreased by the pharmacological inhibition of p38 and GATA2. Of note, studies that have been published later showed that A549 cell line is poorly permissive to SARS-CoV-2 infection (Wang *et al.*, 2021); however, the A549 cell line appeared to become permissive to infection upon cytokine treatment, that indeed increased the expression level of TMPRSS2 in stimulated cells. In line with this, the genetically-engineered overexpression of both ACE2 and TMPRSS2 also favor SARS-CoV-2 infection in this cell line, hence supporting the key role of these proteins and the relevance of the cytokine milieu in the infection process (Wang *et al.*, 2021; Chang *et al.*, 2022).

Overall, our data indicated that the host inflammatory milieu might favor SARS-CoV-2 infection by increasing TMPRSS2 expression. Also, we identified the molecular process that is responsible for this effect and that could be targeted in COVID-19 patients therapy. In detail, we unveiled that IL1β is capable of inducing an overexpression of TMPRSS2 and that this overexpression is induced through the p38 - GATA2 axis. On support of our data, a recent paper has described the co-downregulation of *IL1B* and *TMPRSS2* upon azithromycin treatment, in basal nasal epithelial cells, further providing a link between inflammation and protease expression in SARS-CoV-2 infection (Renteria *et al.*, 2020).

Moreover, our results elucidated a novel pathogenetic mechanism, which associates the pro-inflammatory cytokine IL1β with an increased susceptibility to SARS-CoV-2 infection: as shown by our data, SARS-CoV-2 entry can be increased by IL1β exposure and is inhibited by the pharmacological blocking of the p38 - GATA2 axis.

All in all, our results indicated that IL1β might favor SARS-CoV-2 infection by directly increasing TMPRSS2 expression through the p38 - GATA2 axis. Hence, targeting IL1β could be helpful both in reducing exacerbated inflammatory responses in severe-critical COVID-19 patients and in limiting the SARS-CoV-2 entry in lung epithelial cells.

Of note, *in vitro* data obtained by stimulating the cells prior the infection with the heat-inactivated SARS-CoV-2 could be clinically translatable to a condition of inflammation due to underlying pathologies present in a SARS-CoV-2 infected patient prior to infection. Interestingly, as mentioned several disorders characterized by chronic inflammation and elevated levels of pro-inflammatory cytokines, such as obesity and diabetes, as well as aging, constitute risk factors for developing a severe disease after SARS-CoV-2 infection (Y. Zhou et al., 2020), hence our *in vitro* model could be considered to mimic these clinical conditions that are characterized by chronic inflammation .

At the time of our study, there was a lack of effective therapies for severe and critical COVID-19 patients. Our findings proved that the p38 - GATA2 axis could represent a potential therapeutic target in the management of COVID-19. Blocking this axis indeed could potentially ameliorate the critical conditions dictated by the cytokine storm syndrome, by at least partially taming its effects. Since, as showed, the use of both p38 MAPK and GATA2 inhibitors was able to reduce virus entry into the host cell to levels comparable with the non-stimulated condition, inhibiting the IL1β-induced TMPRSS2 overexpression by acting on this pathway could possibly reduce the spread of the virus among cells in infected patients.

Our results highlighted the relevant role of IL1β on SARS-CoV-2 infection and disease outcome.

The recent SAVE-MORE trial has shown that early treatment with anakinra, a recombinant human IL1 receptor antagonist that blocks IL1 activity, has significantly reduced hospital time and improved survival in patients with moderate or severe COVID-19 (Kyriazopoulou *et al.*, 2021; Potere *et al.*, 2022). In December 2021, Kineret (anakinra) was approved by EMA for COVID-19 treatment in adult patients with pneumonia who require supplemental oxygen (low or high flow) and are at risk of developing severe respiratory failure (EMA, 2021).

Hence, targeting IL1β, by repurposing a drug that is already in use for the treatment of inflammatory diseases (e.g. rheumatoid arthritis), was one of the first treatment choices. The inhibitors we tested were not clinically available: Birb796 was in clinical trial phase II for the treatment of rheumatoid arthritis, Chrone's disease and psoriasis, whereas K7174 was still in preclinical phase. In the future, the targeting of this pathway by acting on the intermediate molecules, that we unveiled and that will in future studies be discovered, might be useful to treat the first stages of the disease to limit virus spreading without affecting a beneficial pro-inflammatory immune response.

It is however important to remember that timing after infection must be taken into consideration to choose the adequate therapeutic strategy the clinical management of COVID-19 patients: as mentioned, the first week of infection is characterized by the phase of viral replication and the curves of the viral loads decrease after one week post-infection, thanks to the activity of neutralizing antibodies and T cell, which begin to limit the spread of the virus. Hence, after the first week, the pathology is not driven by viral replication, but rather by a dysregulated immune response: indeed, a second phase of consistent cytokine production follows this first phase (Blanco-Melo et al., 2020; T. Zhou et al., 2020). A third phase, characterized by coagulopathy and late hyperinflammation, can eventually tail, possibly leading to the severe-critical features of COVID-19, such as ARDS and DIC (Fajgenbaum and June, 2020). Therefore in the first virus-induced phase of the disease, antiviral medications, such as Molnupiravir, Remdesivir and Paxlovid, and anti-SARS-CoV-2

neutralizing antibody-based treatments, e.g. REGN-COV2 (Casirivimab and Imdevimab) are likely to be more effective. The targeting of the pathway that we unveiled would be effective in this phase. In the later phases, in which the disease is mainly caused by an hyperinflammatory and prothrombotic status, anti-inflammatory drugs like corticosteroids, immunomodulating agents like anti-IL6 receptor monoclonal antibodies, or a combination of these therapeutic options can contribute to ameliorate the disease (Gandhi, Lynch and del Rio, 2020; Cascella et al., 2022). The use of a therapeutic agent out of its optimal effectiveness window could lead to important adverse effects, besides not being beneficial.

Future high throughput studies could be performed to better elucidate the examined pathway, shedding light on other molecules involved in the IL1β downstream signaling that leads to the overexpression of TMPRSS2. Also, they could provide more information on other pathways that might be influenced in this signaling and that might have been influenced by the selected inhibitors; in this regard, the p38 MAPK inhibitor that we used is a pan p38 inhibitor; since p38 isoforms are involved in a variety of processes, the use of more specific inhibitors might attenuate potential off-target effects. Deeper transcriptome studies might also elucidate the link between IL1β and viral innate immune response in SARS-CoV-2 infection.

In a second study, we investigated the effects of vaccination on hyperinflammation and damage-associated molecular patterns.

Vaccination has come out as the best way to prevent COVID-19 infection, along with daily good practices such as social distancing, keeping indoor spaces well ventilated, cleaning hands regularly and using masks to prevent the spread of SARS-CoV-2. It can not only determine a consistent reduction in the risk of SARS-CoV-2 infection but also contributes to disease attenuation in infected people. In particular, vaccination against SARS-CoV-2 has shown to provide powerful protection against serious disease, hospitalization and death from COVID-19 (L. Chen *et al.*, 2020; Andrews *et al.*, 2022). A recent study has

reported that a third dose of Pfizer/BionTech or Moderna vaccines is able to guarantee an estimated protection against Omicron - the currently dominant SARS-CoV-2 variant - of 61% against symptomatic infection and 95% against severe outcomes, further highlighting the importance of this way of prevention (Buchan *et al.*, 2022).
As mentioned before, the immune response to SARS-CoV-2 infection has come out as a critical determinant of the disease progression and outcome (Y. Zhou *et al.*, 2020) and even though an antiviral immune response is necessary to eradicate the virus, a strong and persistent response can eventually cause a significant production of pro-inflammatory cytokines, possibly leading to the development of cytokine storm syndrome.

So we wondered if vaccination can also impinge on the development of an hyperinflammatory state; in particular, we analyzed whether the prevailing cytokine milieu is different between vaccinated (Vax) and non-vaccinated (NoVax) COVID-19 patients. To this aim, we analyzed the cytokine profile (46 analytes) in the plasma of Vax and NoVax COVID-19 patients, in a cohort of 45 patients. Among all the analyzed cytokines, IL1β was significantly higher in NoVax patients compared to Vax patients and was higher in NoVax patients under 60 years old, compared to age-matched Vax patients.

As mentioned, the severe patients of COVID-19 can develop cytokine storm syndrome, which leads to the damage of several tissues, and thus to the release of damage associated molecular patterns (DAMPs), endogenous danger signals that are released in the extracellular environment by damaged or dying cells in response to trauma or pathogen infection. A strong correlation between DAMPs release and poor clinical outcome in COVID-19 patients has recently been reported, in particular with HMGB1 and S100A8/A9 (L. Chen *et al.*, 2020; R. Chen *et al.*, 2020).
In order to understand if the tissue damage is different between Vax and NoVax, we moved to quantify indicated DAMPs in the plasma of Vax and NoVax COVID-19 patients. We found that vaccination is associated with a significant reduction of the levels of HMGB1. This result is in line with literature on NoVax patients and it suggests a reduction of tissue damage in Vax patients. Our

results also highlighted a relevant, even though not significant, reduction of S100A8 levels, in Vax patients.

Considering the fact that the systemic inflammatory status of COVID-19 patients can lead to liver dysfunction and, eventually, failure (Ali *et al.*, 2021; McConnell *et al.*, 2022), we tested the liver functionality in our cohort, by measuring plasma AST, ALT and GDH activity. AST and ALT levels were comparable between Vax and NoVax groups, whereas GDH activity was increased in NoVax compared to Vax patients. This result indicated a liver mitochondrial damage and in line with this, high levels of circulating mitochondrial DNA have been already identified as a potential early indicator for poor COVID-19 prognosis (Scozzi *et al.*, 2021).

In conclusion, our results indicated that COVID-19 vaccination decreased the release of IL1β and DAMPs in SARS-CoV-2 infected patients when compared with unvaccinated patients, confirming that vaccination represents the best strategy to prevent SARS-CoV-2 severe clinical manifestations. Future studies performed on a larger cohort could eventually strengthen these results.

Also, plasma GDH activity can be used to differentiate between liver diseases caused by liver inflammation, which do not show elevated plasma GDH activity, and liver diseases that result in hepatocyte necrosis, which show elevated plasma GDH activity (Waes and Lieber, 1977; Contreras-Zentella and Hernández-Muñoz, 2015). Hence, the diminished plasmatic GDH activity in vaccinated patients compared with unvaccinated ones, along with comparable AST and ALT among the two groups, showed that even if the vaccine may not be fully able to prevent liver damage due to inflammation, it is capable of preventing liver necrosis.

All in all, we confirmed the advantages of vaccination in preventing SARS-CoV-2 related inflammation and associated damage. Moreover, our data can contribute to defining an adequate therapy specifically for non-vaccinated patients, like the direct targeting of IL1β .

6. ACKNOWLEDGMENTS

My acknowledgements are directed to Professor Barbara Molon and Professor Antonella Viola (Department of Biomedical Sciences, University of Padova) for giving me the opportunity to pursue my PhD in their group and for their guidance. I would also like to thank all the lab members for sharing their scientific experience and for offering me technical assistance and precise supervision. I would especially like to express my sincere gratitude to Dr. Ricardo Sánchez-Rodríguez (Department of Biomedical Sciences, University of Padova) for his fundamental role in my practical education and scientific growth during this PhD and for his guidance and coordination of the experiments presented here.

Moreover, I would like to thank Fondazione Istituto di Ricerca Pediatrica (IRP) Città della Speranza for funding my PhD and Fondazione Cassa di Risparmio di Padova e Rovigo (CARIPARO) for funding the presented projects.

I would like to thank ECSIN lab and Professor Montagner's lab for kindly sharing A549 and TT1 cell lines, respectively.

Finally, I would like to thank my family and friends for supporting me through this journey and for encouraging me to give my best.

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The reported results have been included in:

Cioccarelli C, Sánchez-Rodríguez R, Angioni R, Venegas FC, Bertoldi N, Munari F, Cattelan A, Molon B, Viola A. IL1β Promotes TMPRSS2 Expression and SARS-CoV-2 Cell Entry Through the p38 MAPK-GATA2 Axis. Front Immunol. 2021 Dec 7;12:781352. doi: 10.3389/fimmu.2021.781352. PMID: 34950146; PMCID: PMC8691651.

Angioni R, Sasset L, Cioccarelli C, Sánchez-Rodríguez R, Bertoldi N, Putaggio CC, Viola A, Cattelan A, Molon B. COVID-19 Vaccination Limits Systemic Danger Signals in SARS-CoV-2 Infected Patients. Viruses. 2022 Mar 9;14(3):565. doi: 10.3390/v14030565. PMID: 35336973; PMCID: PMC8949226.

frontiers in Immunology

ORIGINAL RESEARCH
published: 07 December 2021
doi: 10.3389/fimmu.2021.781352

IL1β Promotes TMPRSS2 Expression and SARS-CoV-2 Cell Entry Through the p38 MAPK-GATA2 Axis

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Edited by:
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Specialty section:
This article was submitted to Cytokines and Soluble Mediators in Immunity, a section of the journal Frontiers in Immunology

Received: 22 September 2021
Accepted: 16 November 2021
Published: 07 December 2021

Citation:
Cioccarelli C, Sánchez-Rodríguez R, Angioni R, Venegas FC, Bertoldi N, Munari F, Cattelan A, Molon B and Viola A (2021) IL1β Promotes TMPRSS2 Expression and SARS-CoV-2 Cell Entry Through the p38 MAPK-GATA2 Axis. Front. Immunol. 12:781352. doi: 10.3389/fimmu.2021.781352

After the outburst of the SARS-CoV-2 pandemic, a worldwide research effort has led to the uncovering of many aspects of the COVID-19, among which we can count the outstanding role played by inflammatory cytokine milieu in the disease progression. Despite that, molecular mechanisms that regulate SARS-CoV-2 pathogenesis are still almost unidentified. In this study, we investigated whether the pro-inflammatory milieu of the host affects the susceptibility of SARS-CoV-2 infection by modulating *ACE2* and *TMPRSS2* expression. Our results indicated that the host inflammatory milieu favors SARS-CoV-2 infection by directly increasing TMPRSS2 expression. We unveiled the molecular mechanism that regulates this process and that can be therapeutically advantageously targeted.

Keywords: GATA2, SARS-CoV-2, IL1β, p38, TMPRSS2

INTRODUCTION

At more than one year after the Coronavirus disease 19 (COVID-19) has been declared a global pandemic, the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infection is still threatening the healthcare systems around the world. The critical and severe forms of COVID-19 are associated with pneumonia and Acute Respiratory Distress Syndrome (ARDS), conditions that are triggered by deregulated immunity and uncontrolled inflammation. In particular, the inflammatory cytokine milieu of the patient plays a crucial role in COVID-19 progression. Indeed, specific cytokines have been linked to worse severity, longer hospitalization time, or to patient's age (1). Accordingly, multiple diseases such as diabetes and obesity, as well as aging, that are characterized by chronic inflammation and elevated levels of pro-inflammatory cytokines, represent high risk factors for developing a severe illness after SARS-CoV-2 infection (2). Among the cytokines that have been associated to COVID-19 severity, several reports identified higher levels of IL1β in severe and critical patients compared with mild and moderate ones (3). Actually, SARS-CoV-2 infection is known to trigger the NLRP3 inflammasome activation in various cell types and the activation of this pathway, that leads to a strong increase in IL1β release, might exacerbate ARDS and systemic inflammation (4).

SARS-CoV-2 entry into the target cells involves the binding of the viral spike protein (S) to the host Angiotensin I Converting Enzyme 2 (ACE2) receptor and its proteolytical activation by proteases. Following the binding of the S1 subunit to ACE2 through its receptor-binding domains (RBD), the host transmembrane protease/serine subfamily 2 (TMPRSS2) cleaves the S protein to facilitate membranes fusion (5). The expression levels and the co-expression of *ACE2* and *TMPRSS2* seem to be key factors in determining the susceptibility of target organs to SARS-CoV-2 infection (6). Recently, it has been proved that *ACE2* expression can be regulated in response to interferon alpha and gamma signaling (7), linking the immune response to the modulation of the cell receptors that mediate SARS-CoV-2 entry. TMPRSS2 is expressed in several tissues including lungs, gastrointestinal tract and kidney. Notably, the *TMPRSS2* gene is upregulated in response to androgenic hormones (8), suggesting that the expression of this protease might be involved in the observed higher severity of COVID-19 in men compared to women.

RESULTS AND DISCUSSION

In this study, we investigated whether the pro-inflammatory milieu of the host affects *TMPRSS2* and *ACE2* expression in SARS-CoV-2 target cells. To this aim, the A549 human lung cell line was exposed to various concentrations of IL1β, Il6, and TNFα inflammatory cytokines and the expression level of *TMPRSS2* and *ACE2* mRNA was evaluated. Interestingly, we found that IL1β and TNFα stimulation enhanced the transcription of *TMPRSS2* (**Figure 1A** and **Supplementary Figure 1A**). On the other side, TNFα and IL1β treatment did not affect *ACE2* expression that was rather increased by Il6 (**Supplementary Figures 1B, C**). The effect of IL1β over *TMPRSS2* and *ACE2* expression was also confirmed in primary immortalized human alveolar epithelial cells (TT1) (**Figure 1B** and **Supplementary Figure 1D**).

In addition to *TMPRSS2*, other proteases, such as *ADAM17* and *FURIN*, have been reported to promote SARS-CoV-2 infection (9) but none of them appeared to be transcriptionally regulated by pro-inflammatory cytokine stimulation in the A549 cells (**Supplementary Figure 2**).

In line with other reports (4), our data confirmed that COVID-19 patients showed an increased level of circulating IL1β (**Figure 1C**) compared to healthy subjects. Notably, IL1β concentration was significantly higher in severe and critical patients compared with mild and moderate ones (**Figure 1D**). Therefore, we focused our analysis on IL1β-induced transcriptional regulation of TMPRSS2 expression. We first validated the induction of TMPRSS2 expression by IL1β stimulation at the protein level by performing immunofluorescence and western blot analyses on both A549 (**Figures 1E–G** and **Supplementary Figure 3**) and TT1 cells (**Figures 1H–J** and **Supplementary Figure 3**). Furthermore, we evaluated whether IL1β stimulation can modulate SARS-CoV-2 endosome and replication related-genes such as IFITM2 (10) and *RNF20* (11). No transcriptional changes were observed for these genes upon IL1β stimulation in lung cells (**Supplementary Figure 4**).

Previous studies showed that *TMPRSS2* expression is controlled by the master regulator transcription factor GATA2 through its binding to the -13 kb DNA enhancer region of the gene (-13 kb upstream the promotor) (8). Furthermore, GATA2 activation has been reported to be mediated by its phosphorylation *via* p38 MAPK pathway (12). GATA2 is also involved in the transcriptional regulation of several pro-inflammatory cytokines, including *IL1B* and *CXCL2* (12). In addition, a positive feedback between IL1β signaling and GATA2 activation has been previously described in Acute Myeloid Leukemia (12). Thus, we speculated that IL1β may induce *TMPRSS2* expression through the p38 MAPK-GATA2 axis. To validate our hypothesis, we first analyzed the effect of IL1β stimulation in A549 and TT1 cells in the presence or absence of specific inhibitors that selectively block p38 MAPK and GATA2 activity. Remarkably, we observed that *TMPRSS2* transcription was significantly decreased by the treatment with p38 MAPK (Birb796) or GATA2 (K7174) inhibitors in IL1β-stimulated A549 and TT1 cells (**Figures 1K, L** and **Supplementary Figure 5A**). *ACE2* transcription was not affected by the inhibitors (**Supplementary Figure 5B**).

In order to confirm the role of GATA2 on the IL1β-induced transcriptional regulation of TMPRSS2, GATA2 chromatin immunoprecipitation was performed. Our results showed that the chromatin enrichment in the TMPRSS2 -13 kb enhancer region was increased upon IL1β stimulation (**Figure 1M**). Importantly, the p38 MAPK and GATA2 inhibitors, that blocked TMPRSS2 overexpression induced by IL1β, were also able to inhibit the GATA2 binding to the enhancer region (**Figure 1M**). Moreover, GATA2 transcription was not affected by pro-inflammatory cytokine exposure (**Supplementary Figure 6**), suggesting that the outlined effect relies on GATA2 activity rather than on a modulation of its expression level. To further corroborate the role of GATA2 on *TMPRSS2* transcription upon IL1β stimulation, we performed GATA2 silencing in A549 cells. Our results confirmed that the downregulation of GATA2 expression blocked the enhancement of TMPRSS2 transcription in response to IL1β exposure. (**Figure 1N** and **Supplementary Figures 7A–C**).

Finally, we evaluated whether the IL1β-induced TMPRSS2 overexpression could increase cell susceptibility to SARS-CoV-2 infection. A549 and TT1 cells were exposed to the non-replicative heat-inactivated SARS-CoV-2 virus, in presence or absence of IL1β and of the selected inhibitors. We observed that IL1β exposure increased the virus entry (**Figures 1O–R** and **Supplementary Figures 7D, E**), which was significantly inhibited by the pharmacological block of the p38 MAPK-GATA2 axis. IL1β has been reported to promote the expression of interferon response genes in epithelial and myeloid cells (13). We found that IL1β stimulation promoted the overexpression of *IRF1* (**Supplementary Figures 8A, B**) and its target (14) *ISG15* (**Supplementary Figures 8C, D**) but not of *IRF9* (**Supplementary Figures 8E, F**) in lung cells. Of note, the expression of these targets has been associated with cytokine

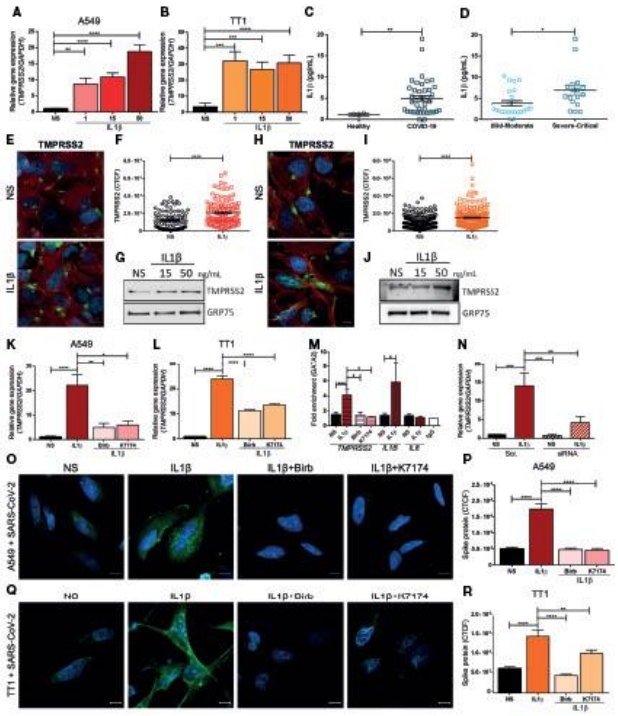

FIGURE 1 | TMPRSS2 expression is up-regulated by inflammatory cytokines enhancing SARS-CoV-2 entry in human lung cells. RT-qPCR analysis of the relative gene expression of *TMPRSS2* in **(A)** A549 and **(B)** TT1 cells either stimulated or not (NS) for 4 hours with IL1β (1, 15 or 50 ng/mL); n=5 independent experiments. IL1β plasma concentration (pg/mL) **(C)** in healthy, age-matched controls and COVID-19 patients, and **(D)** in COVID-19 patients grouped by disease severity (mild-moderate versus severe-critical); n=44 patients. **(E)** Representative immunofluorescence for TMPRSS2 and **(F)** quantification of fluorescence intensity of TMPRSS2 (green) in A549 cell line either stimulated or not (NS) for 24 hrs with IL1β (50 ng/mL). Nuclei were counterstained with Hoechst 33342 (blue) and actin was stained with Phalloidin TexasRed (Red). CTCF: corrected total cell fluorescence. Scale bar: 10 μm; n=3 independent experiments. **(G)** Western blot analysis of TMPRSS2 in total protein lysates from A549 cells stimulated or not (NS) for 24 hours with IL1β (15 or 50 ng/mL); GRP75 was used as a loading control (representative of 3 independent experiments). **(H)** Representative immunofluorescence for TMPRSS2 and **(I)** quantification of fluorescence intensity of TMPRSS2 (green) in TT1 cell line either stimulated or not (NS) for 24 hrs with IL1β (50 ng/mL). Nuclei were counterstained with Hoechst 33342 (blue) and actin was stained with Phalloidin TexasRed (Red). CTCF, corrected total cell fluorescence. Scale bar: 10 μm; n=3 independent experiments. **(J)** Western blot analysis of TMPRSS2 in total protein lysates from TT1 cells stimulated or not (NS) for 24 hours with IL1β (15 or 50 ng/mL); GRP75 was used as a loading control (representative of 3 independent experiments). RT-qPCR analysis of the relative gene expression of *TMPRSS2* in **(K)** A549 or **(L)** TT1 cells stimulated for 4 hours with IL1β (50 ng/mL) and either treated or untreated with Birb796 (p38 MAPK inhibitor, 100 nM) or K7174 (GATA2 inhibitor, 10 μM). NS, Not Stimulated; n=5 independent experiments. **(M)** ChIP-RTqPCR analysis of the fold enrichment of GATA2 binding to the *TMPRSS2* enhancer promotor region, in A549 cells either treated or untreated with Birb796 (p38 MAPK inhibitor, 100 nM) or K7174 (GATA2 inhibitor, 10 μM) and stimulated or not (NS) for 4 hours with IL1β (50 ng/mL). Data were adjusted by input for each sample and normalized with IgG (Mock). The fold enrichment of GATA2 binding to the *IL1B* and to the *IL8* promotors was respectively used as positive and negative control. IgG was used as antibody specificity control. N=3 independent experiments. **(N)** RT-qPCR analysis of the relative gene expression of *TMPRSS2* in A549 cells upon GATA2 silencing with or without stimulation with IL1β (50 ng/mL) for 4 hours. Scramble siRNA (Scr.) was used as a negative control; n=4 independent experiments. **(O)** Representative confocal images and **(P)** quantification of fluorescence intensity of SARS-CoV-2 Spike protein (green) in A549 cells upon infection with heat-inactivated SARS-CoV-2 virus. Cells were stimulated or not (NS) for 8 hours with IL1β (50 ng/mL) and either treated or not with Birb796 (p38 MAPK inhibitor, 100 nM) or K7174 (GATA2 inhibitor, 10 μM). Nuclei were counterstained in blue (Hoechst 33342). Scale bar: 10 μm; n=3 independent experiments. **(Q)** Representative confocal images and **(R)** quantification of fluorescence intensity of SARS-CoV-2 Spike protein (green) in TT1 cells upon infection with heat-inactivated SARS-CoV-2 virus. Cells were stimulated or not (NS) for 8 hours with IL1β (50 ng/mL) and either treated or not with Birb796 (p38 MAPK inhibitor, 100 nM) or K7174 (GATA2 inhibitor, 10 μM). Nuclei were counterstained in blue (Hoechst 33342). Scale bar: 10 μm; n=3 independent experiments. Data are presented as means ± SEM. nonparametric Mann–Whitney U test **(C, D, F, I)** or Kruskal-Wallis test for multiple comparisons with Dunn's *post hoc* **(A, B, K, L, M, N, P, R)** *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

storm syndrome in COVID-19 patients (15, 16). Future high throughput studies should be performed to better understand the interconnection between IL1β and innate immune response in SARS-CoV-2 infection.

Collectively, our results indicated that the host inflammatory milieu might favor SARS-CoV-2 infection by increasing TMPRSS2 expression. In addition, we identified the molecular pathway that regulates this process that can be therapeutically targeted. Our findings unveiled a novel pathogenetic mechanism that associates the pro-inflammatory cytokine IL1β with an increased susceptibility to SARS-CoV-2 infection. Importantly, co-downregulation of *IL1B* and *TMPRSS2* upon azithromycin treatment was recently described in basal nasal epithelial cells, further suggesting a link between inflammation and protease expression in SARS-CoV-2 infection (17). Thus, targeting IL1β may not only reduce high inflammatory responses in severe COVID-19 patients, but also limit the viral entry in lung epithelial cells.

METHODS

Participants, Study Design, and Data Collection

Peripheral Blood (PB) was collected in EDTA tubes from enrolled controls and 44 COVID-19 patients that were admitted to the Infectious and Tropical Disease Unit of the University Hospital of Padua. Plasma was obtained after peripheral blood mononuclear cells (PBMC) isolation by density-gradient sedimentation using Ficoll–Paque PLUS (GE Healthcare, Germany) according to the manufacturer's protocol. Then, plasma was carefully removed from the 2/3 of the top layer using a sterile serological pipette until the mononuclear cell interphase and stored at −80 °C until the analysis. The median age of the patients was 59.3 years (98-25). All patients were clinically diagnosed with COVID-19 (at least one positive laboratory PCR test for SARS-CoV-2 infection). All patients were classified into mild, moderate, severe, and critical cases based on results from chest imaging, clinical examination, and symptoms (WHO guidelines). The study was performed according to the ethical guidelines of the Declaration of Helsinki (7th revision). The study was approved by the Ethics Committee and the general authorization issued by the Data Protection Authority. Cod CESC n. 4933/AO/20.

IL1β Quantification

IL1β level was analyzed in the plasma from 4 controls and 44 patients by Luminex assay (Millipore, Billerica, USA). The diluted standard and quality control were used according to the manufacturer's instructions. The plate was read on Luminex 200™. Analysis was performed using xPONENT 3.1 software.

Cell Culture and Treatments

Human A549 cell line was donated from ECSIN lab, Padua. The cells were maintained in DMEM high glucose medium supplemented with 1% Na-pyruvate, 1% L-glutamine, 10% Gibco FBS, 1% Penicillin-Streptomycin at 37°C under 5% CO2, while human alveolar epithelial cell line (TT1) were maintained as Montagner et al. reported (18). Cells were stimulated with recombinant human IL1β, IL6 or TNFα (PeproTech) at the concentration of 1, 15 or 50 ng/mL either in combination or not with the inhibitors BIRB 796 (100 nM) or K-7174 (10 μM) (Selleck Chemicals). Vehicle treated and non-stimulated cells were used as a control condition. The treatment medium was composed of DMEM high glucose supplemented with Penicillin-Streptomycin and 2% FBS Gibco. RNA extraction was performed after 4 hours of treatment, whereas protein extraction was performed after 24 hours of treatment.

Silencing

Cells were transfected with GATA2 Stealth siRNA or scramble Silencer Select Negative Control (Thermo Fisher Scientific), according to the manufacturer's protocol. Briefly, cells were plated 24h hours before transfection in a growth medium without antibiotics. Stealth siRNA - Lipofectamin2000 (Invitrogen) complexes were prepared according to the manufacturer's instructions. Both siRNA and scramble were used at the concentration of 75 pmol. 24 hours after transfection cells were stimulated with recombinant human IL1β as previously described. RNA extraction was performed after 4 hours of treatment.

RNA Extraction, Reverse Transcription, and RT-qPCR

Total RNA was extracted for RT-qPCR analysis from unstimulated and stimulated cells with TRIzol reagent (Thermo Fisher Scientific) following manufacturer instructions. 500 ng of RNA were retrotranscribed using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacturer instructions. cDNA was diluted 1:10 and amplified with specific primers for the human genes *TMPRSS2, ACE2, GATA2, FURIN, ADAM17* and *GAPDH* (**Table 1**) using Power SYBR Green Master Mix (Thermo Fisher Scientific) on a QuantStudio™ 5 Real-Time PCR System, 384-well (Applied Biosystems). The analysis of the obtained results was performed by the ΔΔCt method.

For the evaluation of cell susceptibility to SARS-CoV-2 infection, concerning the RNA extraction, we followed the protocol by Vogels et al. (19). We then performed reverse transcription as described above and RT-qPCR as described by WHO (20).

Chromatin Immunoprecipitation

Chromatin immunoprecipitation was performed with MAGnify™ Chromatin Immunoprecipitation System (Thermo Fisher Scientific) according to the manufacturer's protocol. Briefly, cells were plated and after 24 hours they were starved for 1 hour; then they were stimulated or not with recombinant IL1β (50 ng/mL) and either treated or not with the indicated inhibitors in presence of IL1β (50 ng/mL). After 4 hours of treatment, the DNA-transcription factor crosslink was performed with 1% formaldehyde (Sigma Aldrich) in PBS for 15 min at RT in soft shaking; the reaction was stopped with

TABLE 1 | Primers used in this study.

	Forward	Reverse
GAPDH	AACAGCCTCAAGATCATCAGC	GGATGATGTTCTGGAGAGCC
TMPRSS2	CCTCTAACTGGTGTGATGGCGT	TGCCAGGACTTCCTCTGAGATG
ACE2	TCCATTGGTCTTCTGTCACCCG	AGACCATCCACCTCCACTTCTC
FURIN	GCCACATGACTACTCCGCAGAT	TACGAGGGTGAACTTGGTCAGC
ADAM17	AACAGCGACTGCACGTTGAAGG	CTGTGCAGTAGGACACGCCTTT
-13 kb *TMPRSS2*	GCTGACCTTTAATGAAGTTTG	CCTAGTGAATTTGGCCTCCTC
IL1B promoter	TCGCACCCACTTCCTTCTCTT	TGCCAGAGGAAATGGTGACC
IL8 promoter	GCTGAACCAGAGTTGGAACCC	GGTGCACTGGAGCTGCTTG
nCoV_IP2	ATGAGCTTAGTCCTGTTG	CTCCCTTTGTTGTGTTGT
IRF1	TTGGCCTTCCAAGTCTTG	GAGCTGGGCCATTCACAC
IRF9	AACTGCCCACTCTCCACTTG	AGCCTGGACAGCAACTCAG
ISG15	GGCTTGAGGCCGTACTCC	CTGTTCTGGCTGACCTTCG
IFITM2	ATCCCGGTAACCCGATCAC	CTTCCTGTCCCTAGACTTCAC
RNF20	AAAGCATCGCACCATGTCTC	ATCCCACTGCAGGTCATCAA

glycine (Sigma) 125 mM (5 min). Cells were then washed with PBS, scraped in 1,5 mL PBS and resuspended in 100uL of lysis buffer. Cells were then sonicated 20 times at 60 Hz for 20 seconds. To enrich GATA2 DNA binding sites, chromatin was incubated overnight with 1 μg of GATA2 polyclonal antibody (GeneTex, cat#GTX113441). Rabbit IgG was used as an antibody specificity control. DNA was purified using kit provided-magnetic beads. Fold enrichment was calculated following the manufacturer's instructions after qPCR amplification with Power SYBR Green Master Mix (Thermo Fisher Scientific) on a QuantStudio™ 5 Real-Time PCR System. Reported specific primers for -13kb *TMPRSS2*, *IL1B*, *IL8*, *nCoV_IP2*, *IRF1*, *IRF9*, *ISG15*, *IFITM2 and RNF20* were used (**Table 1**) (20–24).

Protein Extraction and Western Blot

Protein lysates from A549 or TT1 cells were prepared using a lysis buffer (10 mM Tris-HCl pH 9, 4% SDS, 1 mM DTT). 5 μg of total protein extracts were loaded for western blot in Bolt™ 4-12% Bis-Tris Plus gels (Thermo Fisher Scientific) and blotted onto a PVDF membrane (Bio-Rad). Membranes were blocked with 3% bovine serum albumin (Sigma Aldrich) for 1 hour and then incubated with the primary antibodies α-TMPRSS2 antibody (EPR3862, Abcam) or α-GRP75 (D-9, Santa Cruz Biotechnology) at 4°C. After that, membranes were incubated with the secondary antibody goat α-rabbit IgG (H + L)-HRP Conjugate (Bio-Rad) for 1 hour. Chemiluminescence was detected with iBright 1500 (Invitrogen). Images were analyzed with ImageJ software (Fiji) and the protein was quantified and normalized on the housekeeping protein (GRP75) and on the non-stimulated condition.

Immunofluorescence Analysis

For TMPRSS2 protein immunofluorescence, A549 and TT1 cells were either stimulated or not with IL1β for 24 hours. By contrast, for the evaluation of cell susceptibility to SARS-CoV-2 infection, cells were either stimulated or not with IL1β and the selected inhibitors for 8 hours as previously described. The infection was then performed, using Heat-inactivated SARS-CoV-2 (VR-1986HK, ATCC) at 4 TCID50/mL. 72 hours after the infection, cells were fixed with PFA 4% (Sigma) in PBS, blocked with blocking solution (PBS, 1% BSA, 0,02% NP40) and incubated with SARS-CoV-2 spike antibody (polyclonal, GeneTex) followed by anti-rabbit Alexa Fluor 488 antibody (polyclonal, Invitrogen). Nuclei were counterstained with Hoechst 33342 (Invitrogen) and actin was stained with Phalloidin TexasRed (Invitrogen). Fluorescence images were acquired using the Zeiss LSM 800 confocal laser scanning microscope. The fluorescent signal per cell was quantified using ImageJ software (Fiji) and the corrected total cell fluorescence (CTCF) was calculated. TMPRSS2 staining in A549 and TT1 cells was performed by using H-4 (Santa Cruz Biotechnology) antibody according to the indicated protocol. 3D reconstruction was performed using ZEN 3.2 Blue edition software (Carl Zeiss).

Statistical Analysis

Data are reported as the mean ± SEM of at least 3 independent experiments. Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software). Comparisons between two groups were performed by the nonparametric Mann–Whitney U test or Kruskal-Wallis test for multiple comparisons with Dunn's *post hoc* test *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee and the general authorization issued by the Data Protection Authority. Cod CESC n. 4933/AO/20. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization, AV, RS-R and BM. Investigation CC, FCV, RA, NB, FM, AC and RS-R. Writing- original draft, RS-R, CC and BM. Critical data discussion, AV, BM and RS-R. Funding acquisition AV. All authors contributed to the article and approved the submitted version.

FUNDING

The study was funded by Fondazione Città della Speranza, grant number 20/02CoV, and Fondazione Cassa di Risparmio di Padova e Rovigo (CARIPARO, Bando Progetti di Ricerca Covid 2019, n° 55784) to AV.

ACKNOWLEDGMENTS

We thank all the patients who participated to the study and the entire clinical staff of the Infectious Disease Unit of the Padova University Hospital. We thank Diletta Arcidiacono, Alice Zaramella and Stefano Realdon for LUMINEX assay.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.781352/full#supplementary-material

Supplementary Figure 1 | Role of IL1β, IL6, and TNFα in SARS-CoV-2 receptors expression. RT-qPCR analysis of the relative gene expression of **(A)** *TMPRSS2* in A549 cells either stimulated or not (NS) for 4 hours with IL6 or TNFα (15 or 50 ng/mL); n=5 independent experiments; **(B)** *ACE2* in A549 cell line either stimulated or not (NS) for 4 hours with IL6 or TNFα (15 or 50 ng/mL); n=3 independent experiments. *ACE2* expression in **(C)** A549 or **(D)** TT1 cells either stimulated or not (NS) for 4 hours with IL1β. Data are presented as means ± SEM. Nonparametric Mann–Whitney U test. ****P < 0.0001, **P < 0.01.

Supplementary Figure 2 | ADAM17 and FURIN expression was not transcriptionally modulated by inflammatory cytokines. RT-qPCR analysis of the relative gene expression of **(A)** *ADAM17* and **(B)** *FURIN* in A549 cell line either stimulated or not (NS) for 4 hours with IL1β, IL6 or TNFα (15 or 50 ng/mL); n=3 independent experiments. Data are presented as means ± SEM.

Supplementary Figure 3 | TMPRSS2 expression in response to IL1β stimulation in A549 cells. Quantification of western blots for TMPRSS2 protein in **(A)** A549 or **(B)** TT1 cells stimulated or not with IL1β (15 or 50 ng/mL) for 24 hours. Data are presented as means ± SEM. Nonparametric Mann–Whitney U test. *P < 0.05. **(C)** 3D reconstruction of TMPRSS2 (green) immunofluorescent confocal images in A549 and TT1 cells either stimulated or not (NS) with IL1β (50 ng/mL). Nuclei were counterstained with Hoechst 33342 (blue) and actin was stained with Phalloidin TexasRed (Red), n=3 independent experiments.

Supplementary Figure 4 | IL1β did not affect transcriptionally endosomal and replication genes for SARS-CoV-2. RT-qPCR analysis of the relative gene expression of **(A, B)** *IFITM2* and **(C, D)** *RNF20* in A549 and TT1 cell line either stimulated or not (NS) for 4 hours with IL1β (50 ng/mL); n=3 independent experiments. Data are presented as means ± SEM.

Supplementary Figure 5 | IL1β and selected inhibitors did not affect *ACE2* expression in A549 cell line and the inhibitors vehicle did not alter the observed results. **(A)** RT-qPCR analysis of the relative gene expression of TMPRSS2 in A549 cell line either stimulated or not (NS) with IL1β (50 ng/mL) for 4 hours in presence or not of DMSO (Vehic. of the inhibitors). **(B)** RT-qPCR analysis of the relative gene expression of ACE2 in A549 cell line either stimulated or not (NS) with IL1β (50 ng/mL) and with Birb796 (100 nM) or K7 174 (10 μM) for 4 hours, NS: Not Stimulated; n=4 independent experiments. Data are presented as means ± SEM.

Supplementary Figure 6 | *GATA2* expression is not affected by inflammatory cytokine stimulation. RT-qPCR analysis of the relative gene expression of GATA2 in **(A)** A549 or **(B)** TT1 cells are either stimulated or not for 4 hours with IL1β (15 or 50 ng/mL); NS: Not Stimulated; n=3 independent experiments. Data are presented as means ± SEM.

Supplementary Figure 7 | GATA2 silencing controls and Spike immunofluorescence negative controls. Relative gene expression of **(A)** *GATA2* and **(B)** *E-Cadherin* after GATA2 silencing in A549 cell line. **(C)** Representative western blot for GATA2 and quantification after silencing in A549 cell line. Scramble siRNA was used as a control. n=3 independent experiments. **(D)** Representative confocal images of A549 and TT1 cells without infection with heat-inactivated SARS-CoV-2 virus. **(E)** Relative gene expression of nCoV_IP2 upon infection with heat-inactivated SARS-CoV-2 virus at different time points (2, 24, and 48 hours). Before infection, cells were stimulated or not (NS) for 8 hours with IL1β (50 ng/mL) and either treated or not with Birb796 (p38 MAPK inhibitor, 100 nM) or K7174 (GATA2 inhibitor, 10 μM). Data are presented as means ± SEM. Nonparametric Kruskal-Wallis test for multiple comparisons with Dunn's post hoc. *P < 0.05 **P < 0.01 ***P < 0.001.

Supplementary Figure 8 | Effect of IL1β over interferon response genes. Relative gene expression for **(A, B)** *IRF1* and **(C, D)** ISG15 and **(E, F)** *IRF9* in A549 and TT1 cell line either stimulated or not (NS) for 4 hours with IL1β (50 ng/mL); n=3 independent experiments. Data are presented as means ± SEM. Nonparametric Mann–Whitney U test. **P < 0.01, ****P < 0.0001.

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

viruses

MDPI

Brief Report

COVID-19 Vaccination Limits Systemic Danger Signals in SARS-CoV-2 Infected Patients

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Abstract: Vaccination with an mRNA COVID-19 vaccine determines not only a consistent reduction in the risk of SARS-CoV-2 infection but also contributes to disease attenuation in infected people. Of note, hyperinflammation and damage-associated molecular patterns (DAMPs) have been clearly associated with severe illness and poor prognosis in COVID-19 patients. In this report, we revealed a significant reduction in the levels of IL-1ß and DAMPs molecules, as S100A8 and High Mobility Group Protein B1 (HMGB1), in vaccinated patients as compared to non-vaccinated ones. COVID-19 vaccination indeed prevents severe clinical manifestations in patients and limits the release of systemic danger signals in SARS-CoV-2 infected people.

Keywords: COVID-19; vaccination; DAMPs; personalized therapy

Citation: Angioni, R.; Sasset, L.; Cioccarelli, C.; Sánchez-Rodríguez, R.; Bertoldi, N.; Putaggio, C.C.; Viola, A.; Cattelan, A.; Molon, B. COVID-19 Vaccination Limits Systemic Danger Signals in SARS-CoV-2 Infected Patients. *Viruses* 2022, *14*, 565. https://doi.org/10.3390/v14030565

Academic Editors: Célia F. Rodrigues and Natália Cruz-Martins

Received: 2 February 2022
Accepted: 7 March 2022
Published: 9 March 2022

1. Introduction

Since the beginning of the SARS-CoV-2 pandemic, 359,561,272 cases have been confirmed as of 25 January 2021 and 5,635,677 deaths have occurred worldwide.

COVID-19 patients present with a variety of clinical manifestations, ranging from asymptomatic or mild respiratory illness to fulminant severe acute respiratory distress syndrome (ARDS) with extra-pulmonary complications [1].

Exploring this remarkable variability has been a main focus of COVID-19 research. Current evidence indicates that the immune response to the viral infection—depending on age, sex, viral load, genetics, and other known and unknown variables—largely defines the course of the disease [2]. Although the antiviral immune response is crucial for eliminating SARS-CoV-2, a robust and persistent antiviral immune response can also cause a massive production of inflammatory cytokines and damage to the host [3,4]. In addition, the overproduction of cytokines caused by an aberrant immune activation (termed a cytokine storm) may be a major cause of tissue damage [5,6]. Indeed, the cytokine storm can lead to apoptosis of epithelial cells and endothelial cells, and vascular leakage and, finally, result in ARDS, other severe syndromes, and even death [7,8]. Many studies have also demonstrated that T lymphocytes (T cell) (CD3+ CD4+ T cell and CD3+ CD8+ T cells) are reduced in COVID-19 and are significantly lower in SARS-CoV-2 severely ill patients in which high levels of C reactive protein (CRP) and IL-6 have been reported [9]. Vaccines represent the most effective means to prevent infectious diseases, and the SARS-CoV-2 vaccines in use, using different methods, mRNA, viral vector, show a good efficacy and safety profile [10–12]. While SARS-CoV-2 vaccines remain very effective on preventing severe

disease and death they do not fully prevent transmission and infection. In addition, specific challenges in COVID-19 vaccination such as long-term immunity and avoiding cytokine storms need to be further explored. To date, there are poor data regarding the cytokine profile in SARS-CoV-2 fully vaccinated patients who subsequently acquired the infection.

To address this issue, we measured the SARS-CoV-2-specific cytokine response in two groups of SARS-CoV-2 vaccinated and non-vaccinated patients who developed the disease in order to define the differentiating features of the inflammatory response and their association with severe disease.

2. Materials and Methods

2.1. Participants, Study Design, and Data Collection

All patients (aged > 18 years) who consecutively tested positive for SARS-CoV-2 at the Infectious and Tropical Diseases Institute of Padua, either as inpatient or outpatients, were included. All demographics and clinical characteristics were retrieved from medical health records. Testing for SARS-CoV-2 was performed by using real-time reverse transcriptase-polymerase chain reaction (RT-PCR) assay on nasopharyngeal swabs. Severity of COVID-19 was defined according to NIH definition (Asymptomatic: Individuals who test positive for SARS-CoV-2 but who have no symptoms that are consistent with COVID-19. Mild: individuals who have any of the various signs and symptoms of COVID-19 but who do not have shortness of breath, dyspnea, or abnormal chest imaging. Moderate: individuals who show evidence of lower respiratory disease during clinical assessment or imaging and who have an oxygen saturation (SpO_2) $\geq$ 94% on room air at sea level [13]. Severe: Individuals who have SpO_2 < 94% on room air at sea level, a ratio of arterial partial pressure of oxygen to fraction of inspired oxygen (PaO_2/FiO_2) < 300 mm Hg, a respiratory rate > 30 breaths/min, or lung infiltrates > 50%. Critical Illness: Individuals who have respiratory failure, septic shock, and/or multiple organ dysfunction). For each patient who agreed to participate by consenting, blood samples were collected and stored to dose immunological parameters as per our objectives. Time from both positive results for SARS-CoV-2 and symptom onset and blood sampling was recorded. The local ethics committee was notified about the study protocol. The study was performed according to the ethical guidelines of the Declaration of Helsinki (7th revision). All the patients gave their written informed consent and all analyses were carried out on anonymized data as required by the Italian Data Protection Code (Legislative Decree 196/2003) and the general authorization issued by the Data Protection Authority.

2.2. Luminex and ELISA Assay

Peripheral blood from enrolled COVID-19 inpatients was collected in EDTA tubes and stored at 4 °C prior to processing. Plasma was isolated by Ficoll procedure and stored at −80 °C until the analysis. Some 48 analytes were measured by multiplex biomarker assays, based on Luminex xMAP technology (Merck Millipore, Burlington, MA, USA) following manufacturer's instructions. Plasma DAMPs (S100A8 and HMGB1) were evaluated by ELISA (antibodies-online GmbH, Aachen, Germany) according to the manufacturer's instructions.

2.3. Statistical Analysis

Data were analyzed using the Prism Software (GraphPad, La Jolla, CA, USA). Statistical comparison between the two groups was carried out using unpaired nonparametric Mann–Whitney. Differences were considered statistically significant at confidence levels * $p < 0.05$ or ** $p < 0.01$. Data plotted were expressed as mean with standard error of mean (SEM).

3. Results

Between 12 August and 2 September 2021, 47 patients tested positive in our setting. The baseline characteristics of the studied population, by vaccination status (non-vaccinated = group A and vaccinated = group B) are depicted in Table 1. Two patients were excluded: one for an ongoing pulmonary tuberculosis and one for long COVID-19, probably due to an

overlapping hematological disease. Therefore, 45 patients were considered in our analysis (Table 1). Overall, 29/45 (63%) were males and the median age was 61 (IQR: 48–79) years. Most of the subjects (39/45, 86.6%) were admitted due to severity of COVID-19, while six were managed as outpatients. More than 50% of the patients (23/45, 51.1%) had not received any vaccine against SARS-CoV-2 (NoVax), while 49.9% of the subjects had received at least one dose before the infection (Vax) (Figure 1A). The median age in the NoVax group was significantly lower than that in the Vax group (57 years, IQR: 41.5–62 vs. 79 years, IQR: 49.5–87, $p < 0.05$). The male gender was equally represented in both groups (Figure 1B).

Table 1. Demographic and clinical data of enrolled COVID-19 patients.

	Vax ($n = 22$)	NoVax ($n = 23$)	p Value
Demographics			
Age, year, median	79	57	0.002
Male (%)	15 (68.2%)	14 (60.9%)	0.421
Comorbidities			
None (%)	9 (40.9)	13 (56.5)	0.179
1–3 (%)	13 (49.1)	10 (43.5)	0.566
>3 (%)	2 (9.09)	6 (26.1)	0.107
Severity of COVID-19			
Mild	6 (27.3%)	0	0.009
Moderate	1 (4.5%)	0	0.489
Severe	13 (59.1%)	20 (87%)	0.037
Critical	2 (9.1%)	3 (13%)	0.522
Supplemental oxygen therapy			
None	8 (36.4%)	0 (0.0%)	0.001
Low flow	11 (50%)	14 (60.9%)	0.333
High flow	1 (4.5%)	6 (26.1%)	0.054
Mechanical ventilation	2 (9.1%)	3 (13%)	0.522

In terms of comorbidities, the most common overall were: malignancies (11/45, 24.4%), diabetes (10/45, 22.2%), ischemic heart disease (6/45, 13.3%), chronic obstructive pulmonary disease (COPD) (4/45, 8.9%). The two groups were not significantly different in terms of comorbidities (Table 1). Patients who were managed in the outpatient's setting all had mild disease. Among those who were admitted (39): 1 (2.5%) had moderate disease, 33 (84.7%) severe disease and 5 (12.8%) critical disease requiring intensive care unit admission (Figure 1C). One patient, a 92-year-old woman with multiple comorbidities, died.

Most of the patients 37/46 (80.4%) who were hospitalized received oxygen support. The proportion of patients who required oxygen support was significantly different between the two groups. A total of 25/45 patients needed low flow oxygen; among them, 14 (60.9%) were in the NoVax group and 11 (50%) in the Vax group (p value 0.333), 7/45 patients needed high flow oxygen; among them, 6 (26,1%) were in the NoVax group and 1 (4.5%) in the Vax group; p value 0.054). Of the patients, 5/45 required mechanical ventilation, 2 (9.1%) in the NoVax group and 3 (13%) in theVax group (p value 0.522) (Table 1).

Current evidence indicates that the cytokine storm plays a crucial role in determining COVID-19 severe outcomes [14]. To analyze the cytokine profile of our patients, we measured the circulating levels of 48 cytokines in the plasma of both Vax and NoVax SARS-CoV-2 infected patients, stratified according to patient age (Figures S1–S5). Among all the analytes, we pointed out a significant difference in the levels of IL-1ß between the two groups (Figure 1D). Intriguingly, the plasmatic IL-1ß content positively correlated with higher hospitalization time, age and disease severity [15,16].

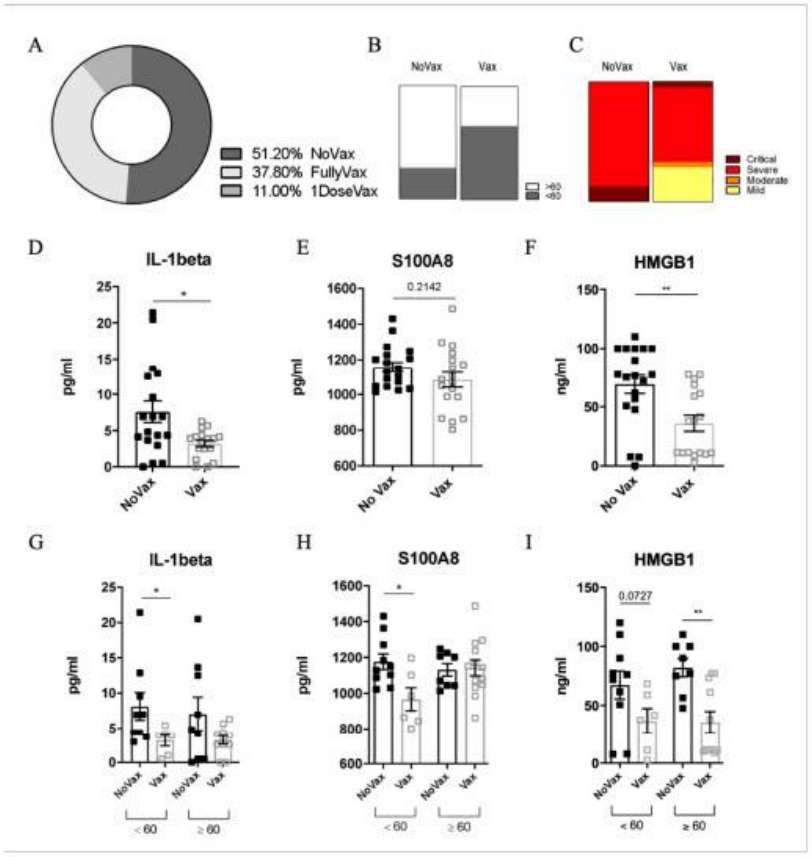

Figure 1. (**A**) Percentage of non-vaccinated (NoVax), vaccinated (Vax) and 1 dose vaccinated patients in our cohort. Age (**B**) and disease severity (**C**) distribution in NoVax and Vax patients. IL-1ß (**D**), S100A8 (**E**), and HMGB1 (**F**) plasma concentration (pg/mL) in NoVax and Vax patients. IL-1ß (**G**), S100A8 (**H**), and HMGB1 (**I**) plasma concentration (pg/mL) in NoVax and Vax patients stratified by age (younger or older than 60 years old). Differences were considered statistically significant at confidence levels * $p < 0.05$ or ** $p < 0.01$.

IL-1ß is a cytokine released through the activation of the inflammasome, a multimeric complex triggered by pathogen-associated (PAMPs) and/or damage-associated (DAMPs) molecular patterns. Of note, recent reports clearly indicated a strong correlation between DAMP release and poor clinical outcomes for COVID-19 patients [17,18]. We found that vaccination is associated to a relevant reduction of the levels of S100A8 (Figure 1E) and HMGB1 (Figure 1F). Although the difference was significant for HMGB1, we only observed a trend of decrease for S100A8 that can be explained by a different pattern of expression of the DAMPS in our cohort. These DAMPs have been already detected in the serum of COVID-19 patients, where they strongly correlate with higher risk of ICU admission and death [14].

Given the systemic inflammatory status of COVID-19 patients, we evaluated liver functionality in our cohort. The level of both alanine (ALT) and aspartate (AST) were

comparable in the two groups. On the other hand, we found high levels of circulating glutamate dehydrogenase activity (GDH) in the NoVax group (Figure S6), possibly suggesting mitochondrial damage in these patients. In this line, high circulating mitochondrial DNA has been already defined as a potential early indicator for poor COVID-19 prognosis [19].

4. Discussion

Overall, our results indicate that COVID-19 vaccination prevents the release of systemic danger signals and IL-1ß in SARS-CoV-2 infected patients. Moreover, our data provide a new insight into the definition of the proper therapeutic paths for non-vaccinated patients as the direct targeting of IL-1ß.

Collectively, we confirm that vaccination represents the best strategy to prevent potential long-term side-effects caused by the SARS-CoV-2 related inflammation.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/v14030565/s1, Figure S1: circulating levels of the indicated analytes (18 cytokines) in NoVax and Vax patients, Figure S2: circulating levels of the indicated analytes (18 cytokines) in NoVax and Vax patients, Figure S3: circulating levels of the indicated analytes (10 cytokines) in NoVax and Vax patients, Figure S4: circulating levels of the indicated analytes (10 cytokines) in NoVax and Vax patients, Figure S5: circulating levels of the indicated analytes (10 cytokines) in NoVax and Vax patients, Figure S6: ALT and AST liver transaminases and GDH levels in NoVax and Vax patients.

Author Contributions: R.A., C.C., R.S.-R. and N.B. performed the experiments. L.S. and C.C.P. collected clinical samples and data. R.A., L.S., A.V., A.C. and B.M. conceived the study and write the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: The study was funded by Fondazione Città della Speranza, grant number 20/02CoV, and Fondazione Cassa di Risparmio di Padova e Rovigo (CARIPARO, Bando Progetti di Ricerca COVID 2019, n° 55784) to AV.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by local Ethics Committee (CESC, code: 4923/AO/20, date: 24/09/2020).

Informed Consent Statement: Informed Consent was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable.

Acknowledgments: We thank all the patients who participated in the study and the entire clinical staff of the Infectious Disease Unit of the Padova University Hospital.

Conflicts of Interest: The authors declare no conflict of interest.

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