Knowledge from human relevant cell, tissue and mathematics-based methods as key tools for understanding COVID-19 dynamics, kinetics, symptoms, risk factors and non-conventional treatments

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Abstract

Over the last two decades, the world experienced several outbreaks of coronaviruses with elevated morbidity rates. The Covid-19 disease caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2 virus) belonging to Beta coronavirus emerged in humans in late 2019 and has spread via human-to-human transmission to most countries in the world. Many people infected with this virus experience a mild to moderate respiratory illness and recover without requiring special treatment. Complications leading to death may include respiratory failure, acute respiratory distress syndrome (ARDS), sepsis and septic shock, thromboembolism, and/or multi-organ failure.

Although knowledge from previous SARS infection has been instrumental, not all specific SARS-CoV2 features have been clarified nor are the underlying molecular and cellular mechanisms fully understood. It is therefore necessary to understand in detail the dynamics and kinetics of the SARS-CoV-2 virus, which includes the means of entry into the organism via the external body barriers (e.g., nose, lung, eye, intestine), its distribution and passage through internal body barriers (e.g., placenta barrier, blood-brain barrier) into the various human body organs, and its subsequent viral dynamics. It is important to understand the effects the virus triggers in the whole organism, mapping its cellular entrance via the external body barriers (e.g., respiratory system and digestive system) by binding to cellular receptors of these barriers, its replication and the subsequent mechanism of action (e.g., inflammation) SARS-CoV-2 has on the cells and tissues of its human target organ systems. The cellular immune response and specific cellular responses to the pathogen, but not the pathogen itself, can contribute to multi-organ dysfunction. The mechanistic understanding on modulators of the immune response and cell homeostasis balance and specific risk factors is critical to understand the systems biological processes underlying the multi-organ systems effect and will improve diagnosis, prevention and therapeutic strategies.

Knowledge from human relevant cell, tissue and mathematics-based methods are key tools for understanding COVID-19 dynamics, kinetics, symptoms, risk factors and treatments. Cross-community research on SARS-CoV-2 is essential to understand its detailed pathophysiology and mechanisms of infection, host-virus interactions, replication kinetics and targets for therapeutic screening. Greater investment and innovative methodological approaches are needed to accelerate knowledge gathering on SARS-CoV-2 in all the aspects of the disease. Due to the seriousness of the global health situation with this extraordinary crisis of the human race caused by COVID-19, medical researchers, cell biologists, life science experts, mathematical modelers and bioengineers across the world are actively collaborating to accelerate the rapid development of the relevant cell, tissue and mathematical methods to gain detailed mechanistic knowledge of this new disease. This global collaborative knowledge sharing effort will assist in the unravelling of mechanistic understanding of SARS-CoV-2 kinetics and dynamics. Consequently, almost in real time innovative solutions for developing diagnostic tools and providing preventive and curative strategies will be possible using knowledge from the new generation of in vitro and in silico methods and related technologies.

XX.1 Relationship of COVID-19's clinical and cellular features

Over the last two decades, the world experienced several outbreaks of coronaviruses with elevated morbidity rates. The COVID-19 disease caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2 virus) belonging to Beta coronavirus emerged in the human population in late 2019 and has spread via human-to-human transmission to most countries in the world.

The clinical spectrum of SARS-CoV-2 infection ranges from asymptomatic infection to critical illness (Fig. 1). Many people infected with this virus experience a mild to moderate respiratory illness and recover without requiring special treatment. Some patients who have mild symptoms initially will subsequently have precipitous clinical deterioration that occurs approximately 1 week after symptom onset. [1]

Many factors that influence the course of the COVID-19 disease. Although knowledge from previous SARS infection has been instrumental, not all specific SARS-CoV-2 features have been clarified, and the underlying molecular and cellular mechanisms are not fully understood. Older people and those with underlying medical problems like cardiovascular disease, diabetes, chronic respiratory disease, and cancer are more likely to develop serious COVID-19 symptoms. But there are also predisposed individuals who can develop life-threatening pneumonia, cytokine storm, and multiorgan failure. [2]

Among those who develop symptoms, most (about 80%) recover from the disease without needing specialized recovery treatments. About 15% become seriously ill and require oxygen and 5% become critical ill and need intensive care. [3]

Complications leading to death may include respiratory failure, acute respiratory distress syndrome (ARDS), sepsis and septic shock, thromboembolism, and/or multi-organ failure, including injury of the heart, liver or kidneys. [3]

A better understanding of each pathophysiological step will help in establishing effective diagnostic tests and subsequent therapeutic interventions.

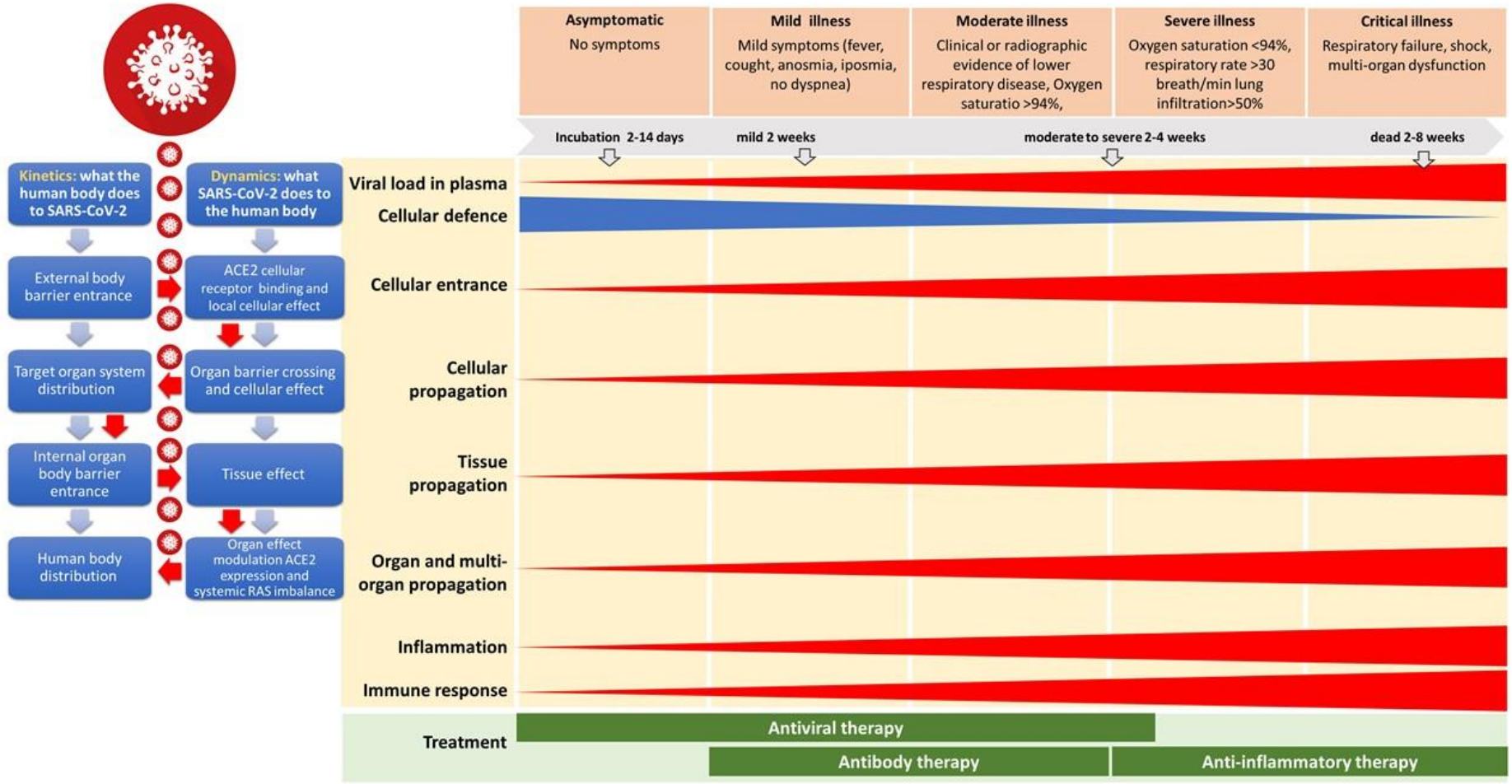


Figure 1 : Relationship between cellular kinetics, dynamics and variability of symptoms of COVID-19 disease [1]

It is therefore necessary to understand in detail the dynamics and kinetics of SARS-CoV-2, which includes the ways of entry into the organism via the external body barriers (e.g., nose, lung, eye, intestine), its distribution and passage through internal body barriers (e.g., placenta barrier, blood-brain barrier) into the various human body organs and its subsequent viral dynamics (Figure 1).

There are inconsistent findings for the association between disease severity (and/or ICU admission), and the duration of virus detection, with studies reporting either a positive association, or no association. [4] SARS-CoV-2 RNAemia and viral RNA load in plasma are associated with critical illness in COVID-19. Viral RNA load in plasma correlates with key signatures of dysregulated host responses, suggesting a major role of uncontrolled viral replication in the pathogenesis of this disease [5] (Figure 1).

Furthermore, the cellular and immunological response towards the pathogen, but not the pathogen itself, can contribute to the multi-organ dysfunction and will improve diagnosis [6], [7], prevention and therapeutic strategies such as using e.g., immunomodulators and cytokine-directed therapies.

High-throughput sequencing demonstrates that SARS-CoV-2, belonging to the Nidovirales order, highly resembles the severe acute respiratory syndrome (SARS-CoV) and the Middle East respiratory syndrome (MERS-CoV), both classified as beta coronaviruses identified in bats (Figure 2).
Its genome sequence is 96.2% identical to the bat CoV RaTG13. SARS-CoV-2 is mainly composed of four compartments with distinct roles in the viral replication: the membrane spike glycoprotein (S), membrane (M), envelope (E) and nucleocapsid (N). Additionally, SARS-CoV-2 presents biological features that resemble other β-coronaviruses class members, especially SARS-CoV, such as genome, protein structure, human infection mechanisms involving most likely mainly receptor interaction with angiotensin-converting enzyme 2 (ACE2) and the enzyme transmembrane protease, serine 2 (TMPRSS2) and tissue and organ tropism indicating the ability of SARS-CoV-2 to infect a specific organ or sets of organs (Figure 2). [8] [9]

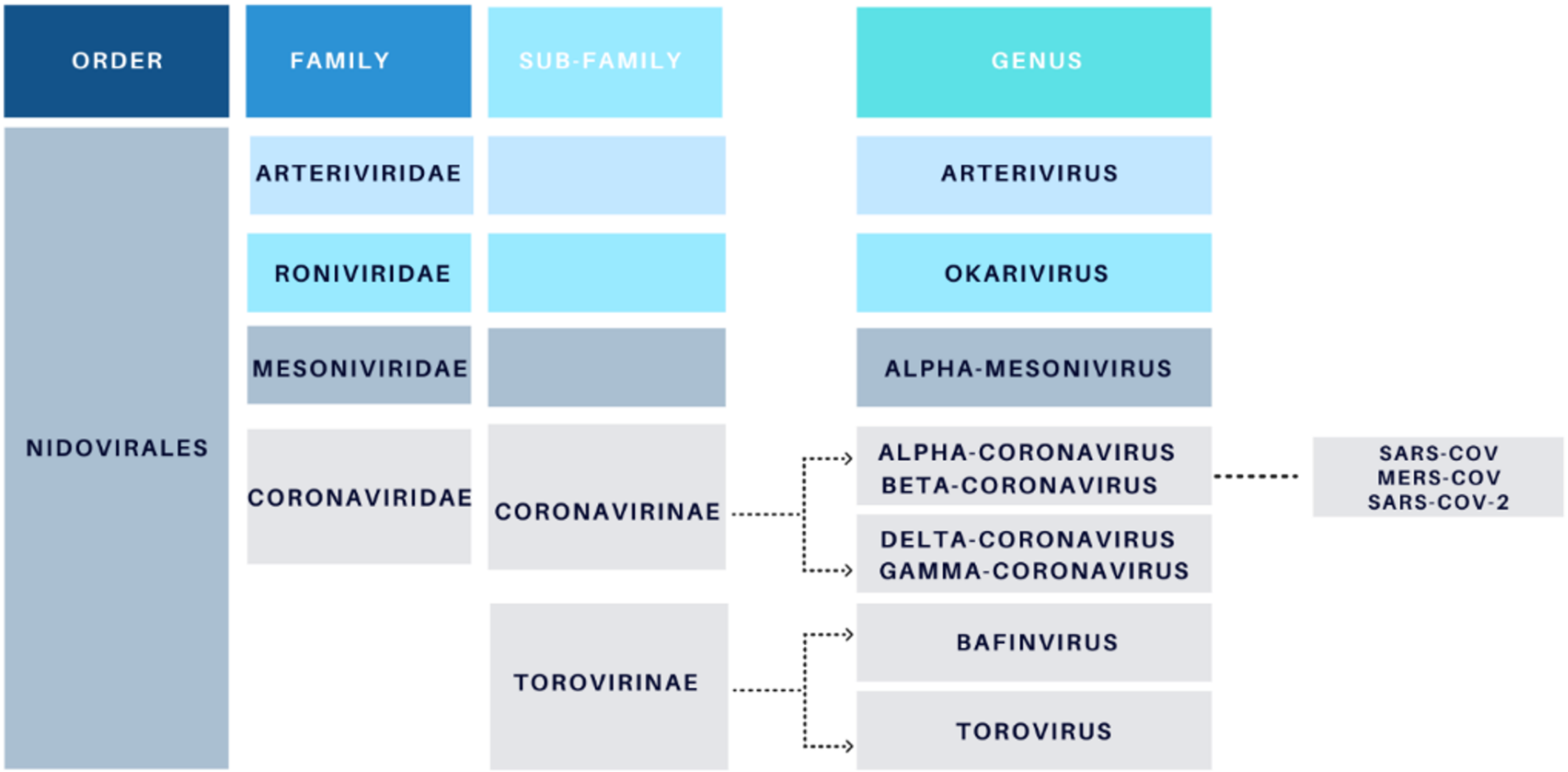


Figure 2. Classification of Coronaviruses and SARS-CoV-2 compartments, their distinct roles in the viral replication, cellular binding to the host cells receptor (e.g., ACE2 and TMPRSS2) and cellular and extracellular dynamics.

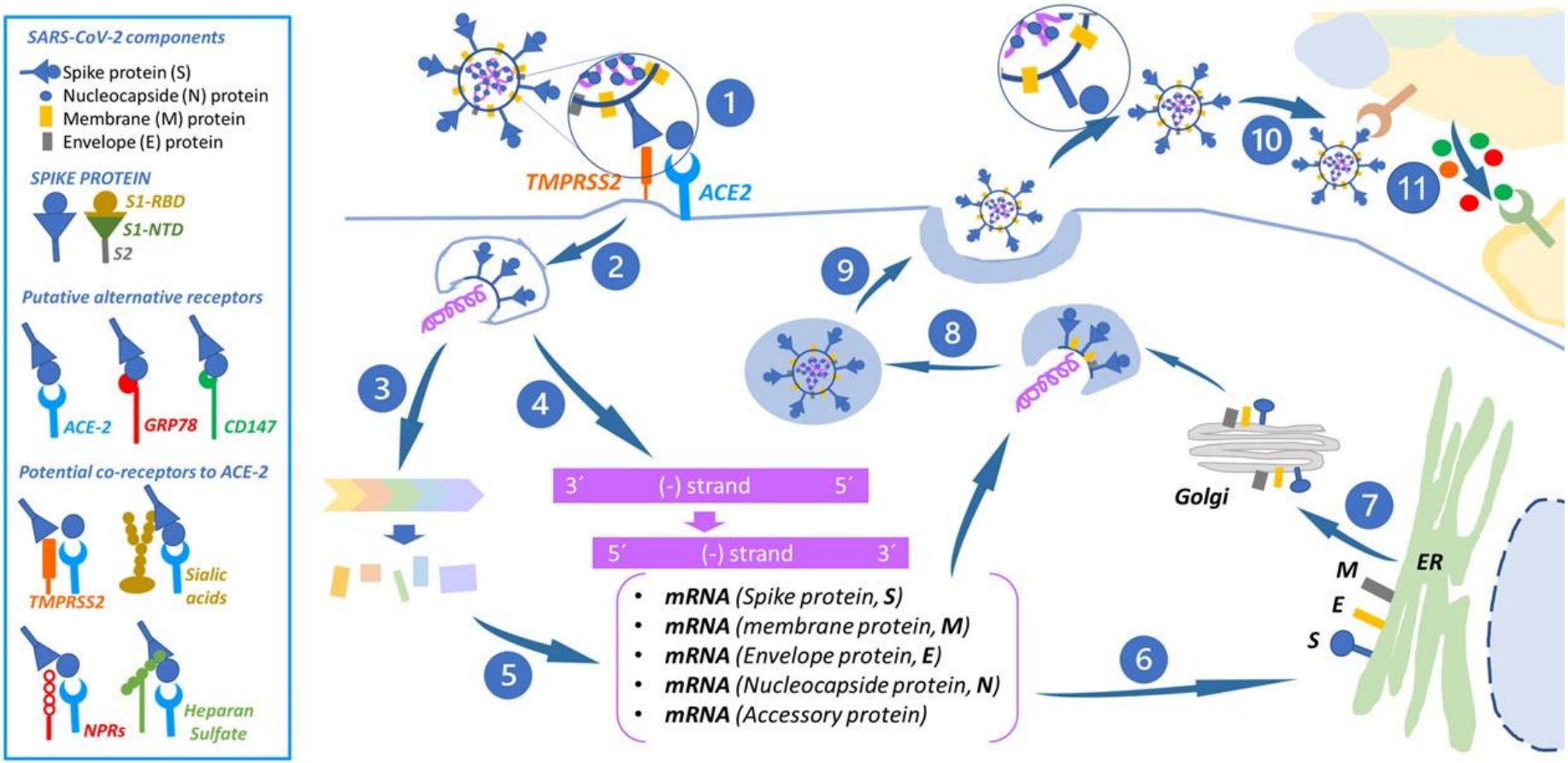


Figure 3. Adapted from [10]. Access, multiplication and release of COVID-19 particles in human cells. The process is divided in the following steps: *1. Attachment to the host cell; 2. Viral-host membrane fusion and release of viral RNA; 3. Translation and cleavage of polyproteins; 4. Translation of full-length (-) strand by replication complex; 5. Transcription and replication of viral RNA genome; 6. Translation of structural*

and accessory proteins; 7. Trafficking of newly synthesized proteins from ER to the Golgi; 8. Assembly of mature virion in building vesicle; 9. Mature virion release via endocytosis; 10. Presentation of SARS-CoV-2 antigens to APCs; 11. Release of cytokines (enhanced pro-inflammatory response)

The infection cycle of the SARS-CoV-2 virus begins with its cellular entry. Using the S glycoprotein, it attaches itself to a surface receptor of the host cell. The place of entry via the external body barriers (Figure 6) and the host cell receptor(s) and its (their) distribution determine which type of cells, tissues and organ systems get infected. The specificity of the S protein to a particular receptor influences viral tropism. SARS-CoVs uses the human ACE2 receptor as points of entry. The ACE2 enzyme is a pivotal component of the renin-angiotensin system and exerts its physiological functions by modulating the levels of angiotensin II (Ang II) and Ang-(1-7). [11]

When the spike protein attaches to its target cell receptor ACE2, it is cleaved into two parts (S1 and S2) by extracellular proteases. S protein is subdivided into S1 and S2 subunits. S1 is formed by two domains, an N-terminal domain (NTD) and a C-terminal receptor-binding domain (RBD) that binds specifically the host receptor ACE2 on the host cell surface (Figure 2). While S1 remains attached to its target, the S2 subunit (also multi-domains) induces viral and host membranes fusion and is further cleaved by the host cell enzyme, the transmembrane serine protease 2 (TMPRSS2). Upon fusion, the contents of the virus particle are released into the host cell's cytoplasm. The virus's genomic positive-sense RNA, which comprises two overlapping open reading frames (ORFs), ORF1a and ORF1b, is quickly translated into two polyproteins, pp1a and pp1ab. These proteins are the so-called replicase-transcriptase-complex, because of their role in replication and further transcription. The newly formed polyproteins are immediately autocatalytically proteolyzed into smaller proteins by two viral proteases, 3C-like protease ($3CL^{pro}$), otherwise known as main protease (M^{pro}), and papain-like protease (PL^{pro}).

The cleavage products include 16 non-structural proteins (nsp) like the RNA-dependent RNA polymerase (RdRP) that facilitates the production of antisense RNA, as well as 4 structural proteins like the S glycoprotein, envelope (E) proteins, membrane proteins (M), and nucleocapsid (N) proteins. Newly generated antisense RNA is used as a template for new copies of viral positive-sense RNA as well as for the production of differently sized subgenomic mRNAs, which can be translated into new viral proteins at the endoplasmic reticulum. Finally, proteins and genomic RNA are assembled, packed into vesicles in the Golgi apparatus and exocytosed to the outside to repeat the process in surrounding cells.

This process does not pass unnoticed by the host organism, as infected cells present viral structures on their surface. As a response, many defensive pathways are initiated, such as the production of different cytokines and chemokines like interleukin 1 (IL-1), IL-6, IL-8, IL-21, TNF-β, and MCP-1. The release of these mediators and their effector cells activate inflammatory mechanisms to destroy the SARS-CoV-2 virus. [12]

The Coronaviridae family consists of four genera (α-, β-, γ- and δ-coronavirus), among of which only α-coronaviruses and β-coronaviruses are capable of infecting mammals [8]. Over the last two decades, the world experienced three outbreaks of coronaviruses with elevated morbidity rates. Currently, the global community is facing emerging virus SARS-CoV-2 belonging to Betacoronavirus, which appears to be more transmissible but less deadly than SARS-CoV. The tracking the evolutionary ancestors and different evolutionary strategies genetically adapted by SARS-CoV-2 reveals from the whole-genome analysis, that SARS-CoV-2 was the descendant of Bat SARS/SARS-like CoVs and that they served as a natural reservoir. SARS-CoV-2 used mutations and recombination as crucial strategies in different genomic regions including the envelop, membrane, nucleocapsid, and spike glycoproteins to become a novel infectious agent. Mutations in different genomic regions of SARS-CoV-2 have specific influence on virus reproductive adaptability, allowing for genotype adjustment and adaptations in rapidly changing environments. Nine putative recombination patterns in SARS-CoV-2, which encompass spike glycoprotein, RdRp, helicase and ORF3a and six recombination regions spotted in the S gene are important for evolutionary survival, meanwhile this permitted the virus to modify superficial antigenicity to find a way from immune reconnaissance in animals and adapt to a human host. With these combined natural selected strategies, SARS-CoV-2 emerged as a novel virus in human society. [13]

SARS-CoV-2 is primarily spread from person to person through respiratory particles, probably of varying sizes, which are released when an infected person coughs, sneezes, or speaks. Because both smaller particles (aerosols) and larger particles (droplets) are concentrated within a few meters, the likelihood of transmission decreases with physical distancing and increased ventilation. Most SARS-CoV-2 infections are spread by respiratory-particle transmission within a short distance (when a person is <2 m from an infected person). [1]

The incubation period of SARS-CoV-2 is around 1–14 days. Viral interaction with the cells of the external body barriers (Figure 1 and Figure 8) are critical for SARS-CoV-2 infection. Figure 4 illustrates the main routes and external cellular barriers or cellular body fluids critical for human viral transmission. Important routes of transmission are via body fluids (tears, respiratory droplets, aerosols, secretions), air in confined spaces and contaminated particles from e.g., contaminated hands. The respiratory tract is responsible for droplet transmission (sneezing or cough), while contact transmission can occur via e.g., hand wiping eyes. Other transmission routes are fecal-eye transmission, nasal-eye transmission, mouth-eye transmission (through contaminated hands or objects) and the transmission of eye secretions and tears.

As indicated in Figure 3 evidencing all these possible routes of SARS-CoV-2 transmission can range from a single external cellular body barrier entrance port to parallel transmission due to continued viral exposure sources, self-transmission and co-transmission in the same patient which may impacts the course, duration, and severity of the disease. [14]

Apart from respiratory droplets and tears, SARS-CoV-2 has also been detected in other human body fluids, such as saliva, blood, urine, feces, sputum, and semen, indicating potential risk factors for invasion of virus into the body. [15]

Reports indicated that possibility apart from droplets and contact transmission, the possibility of mother-to-child and sexual transmission via semen also exists. [11] [15]

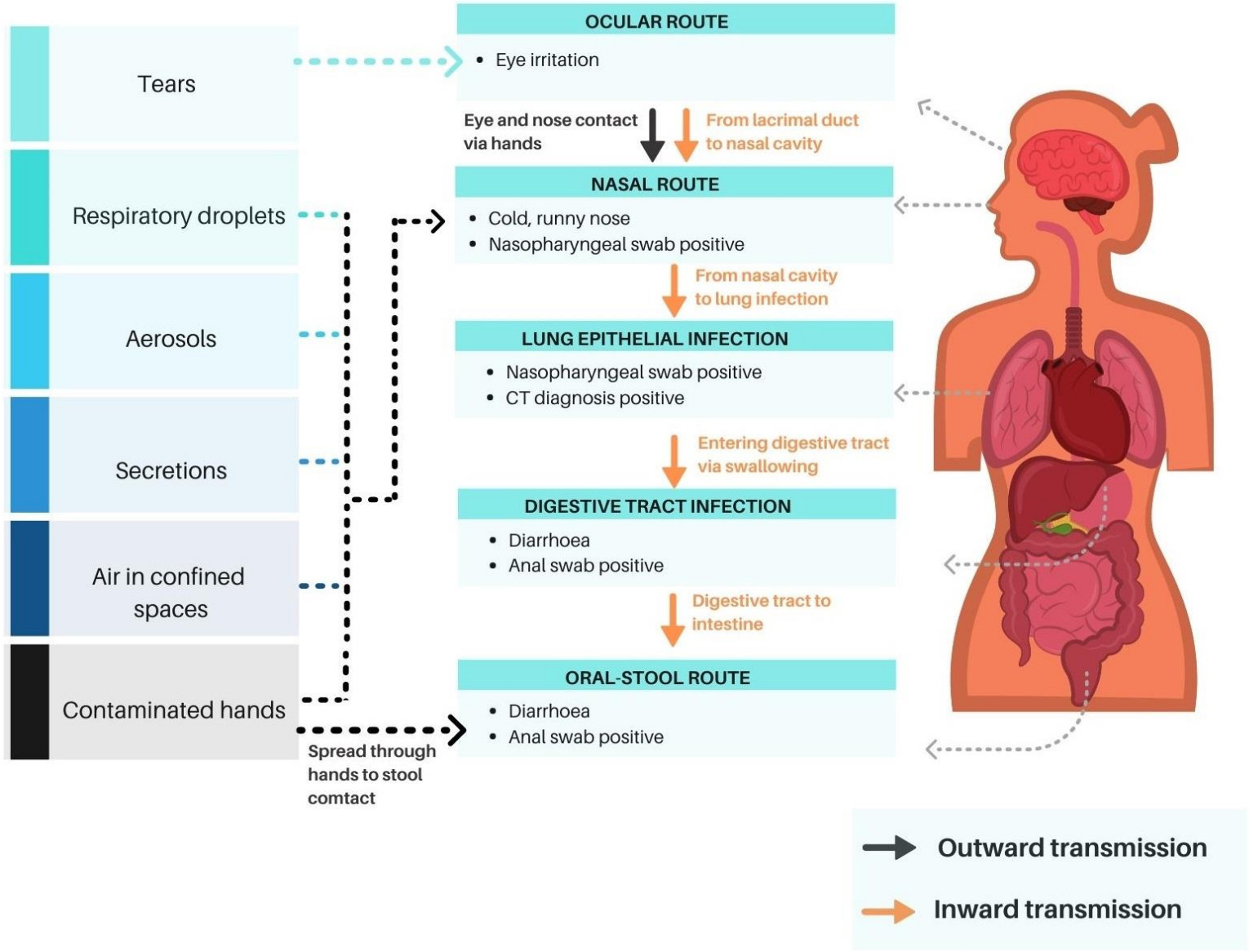

Tears
Respiratory droplets
Aerosols
Secretions
Air in confined spaces
Contaminated hands
OCULAR ROUTE
• Eye irritation
Eye and nose contact via hands
From lacrimal duct to nasal cavity
NASAL ROUTE
• Cold, runny nose
• Nasopharyngeal swab positive
From nasal cavity to lung infection
LUNG EPITHELIAL INFECTION
• Nasopharyngeal swab positive
• CT diagnosis positive
Entering digestive tract via swallowing
DIGESTIVE TRACT INFECTION
• Diarrhoea
• Anal swab positive
Digestive tract to intestine
ORAL-STOOL ROUTE
• Diarrhoea
• Anal swab positive
Spread through hands to stool comtact
Outward transmission
Inward transmission

Figure 4: Routes of human SARS-CoV-2 virus inward and outward transmission, self-transmission and co-transmission via parallel transmission routes when continued viral exposure sources in different human body organ systems are present.

XX.2 Cell, tissue and mathematic based methods used for COVID-19 research.

The often-recurrent paradigm for biomedical research and drug testing postulates that cell and tissue-based methods (*in vitro*, Latin for "within the glass") and mathematical based methods (in silico, pseudo- Latin for "in silicon", relating to the use of silicon material for computer chips) inform animal studies that will subsequently inform human studies.

In vitro studies use components of a whole organism that have been isolated from their usual biological surroundings, such as tissue or organ fragment, organ explant, dissociated cells, primary isolated cells from different organs of the human body. "In silico" is an expression meaning "performed on computer or via computer simulation" in reference to biological experiments.
Both "*in vitro*" testing methods and "*in silico*" methods have been used to characterize for instance specific adsorption, distribution, metabolism, and excretion processes of drugs or general chemicals (kinetic processes, "What the body does with the drug or chemical?") or adverse effects of drugs and chemicals on specific target organ systems of the human body (dynamic processes, "What the drug or chemical does with the human body?") inside a living organism. [16]

Similarly, these types of kinetic and dynamic processes can be studied, keeping in mind that the SARS-CoV-2 virus is a foreign entity entering the human body (Figure 1).

When SARS-CoV-2 research work is performed in vitro, it happens outside of a living organism and its normal biological context. In vitro cell and tissue culture work provide a controlled environment for generating SARS-CoV-2 related experimental mechanistic data. Since the in vitro work occurs outside of a living organism, results must be considered carefully and need to be interpreted knowing the system biological differences in comparison with the intact organism, or the target organ, tissue, or cells systems of that organism one tries to mimic.

In vitro and in silico method-based experimental studies can permit species-specific comparison and extrapolation, provide simple, standardized, harmonized, higher throughput, and specific mechanistic investigations. Such studies can facilitate understanding the mode-of-action of compounds foreign to the human body (xenobiotics) and invaders (bacteria, virus, etc.) that are more difficult or impossible to investigate in the whole organism. Just as studies in whole animals have been carried out pre-clinically to inform human clinical trials, in vitro and in silico studies

are used to replace, reduce and/or refine (3Rs, [17]) experimental animal investigations, or even better to directly inform human biological processes which cannot be studied in animals due to species differences. Indeed, SARS-CoV-2 has been shown to provoke highly heterogeneous responses in individuals, and as such the new generation of well-chosen in vitro and in silico methods can give the necessary data to diagnose the pathological features of COVID-19 disease (kinetics and dynamics) and also be used to understand the clinical symptoms, its risk factors and potential novel alternative treatments.

The primary disadvantage of in vitro and in silico experimental studies is the difficulty to extrapolate the results of the in vitro and in silico work back to the biology of the intact organism. With all the new life science, scientists have moved to integrate the knowledge obtained with these new approach methodologies (NAMs) [18] [19]), so contributing to accelerating knowledge on organismal and systems biology which is of critical importance in COVID-19 research.

An animal method-driven approach is not always the ideal methodology to represent human diversity, genetic make-up, polymorphism, risk factors and vulnerabilities, nor can it provide a complete insight on this very challenging new viral disease which causes a wide-variety of adverse effects in the human body, both pulmonary and extra-pulmonary, with subsequent multi-organ system effects. SARS-CoV-2 animal studies have employed mammalian species like bats, cats, ferrets, hamsters, mice (including GMO mice e.g., hACE2 mice due to ACE2 species difference), mink and non-human primates. The animal studies looked at aspects such as:

- Virus replication in the upper and lower respiratory tract and other organs,
- Clinical signs like fever, nasal discharge, labored breathing,
- Pneumonia and details on bilateral lung involvement, ground-glass opacities, focal oedema, inflammation, or acute respiratory distress syndrome (ARDS),
- SARS-CoV-2 transmission,
- Immunology, like aspects of seroconversion, neutralizing antibody titres, T cell immunity or pro-inflammatory cytokines,
- Demographics, like differential clinical effects between by females and males or younger and older patients [20] [21] [22]

The contribution and the impact of using the available human-relevant novel methodological approaches like in vitro and in silico methods or the novel generation of clinical methods using directly patient-derived cell and tissue material - within this new global research area - is yet to be properly quantified. This quantification is important since biomedical research and drug discovery and development includes two steps of

knowledge transfer, and it is critical to evaluate the effectiveness of both in order to properly benefit from the added value of COVID-19 research, compliant with the 3R principles (Replacement, Reduction and Refinement) as described in the European Directive 2010/63/EU.

XX.2.1 Evaluating COVID-19 biomedical literature research papers describing human-relevant novel approaches like in vitro and in silico methods

For this paper, the SCOPUS database from Elsevier was used to identify citations of original research papers on in vitro and in silico by using the following search string: *("COVID-19" OR covid OR "COVID-19" or "SARSCoV-2" OR "SARS-CoV-2") AND ("in vitro" OR "in silico" OR PBPK OR "physiologically based pharmacokinetic modelling" OR "alternative methods" OR "new approach method" OR "cell based" OR "cell-based" OR "tissue based" OR "tissue-based" OR "organ on a chip" OR "organ-on-a-chip" OR "in vitro simulator" OR "in silico simulator" OR "replacement methods")*. The search was defined to include the synonyms for COVID-19 and those for *in-silico* that correspond to the second set of keywords.

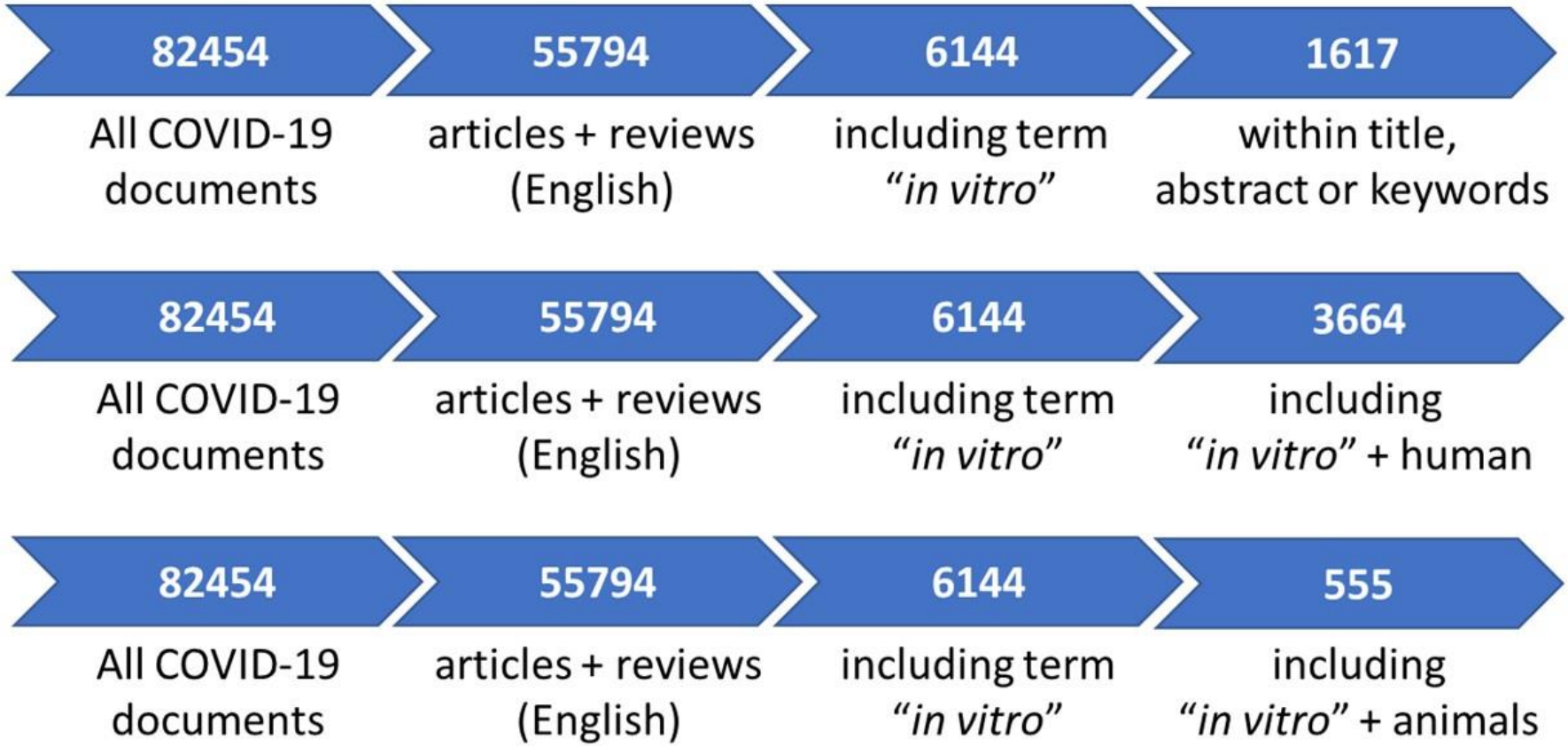

82454
All COVID-19 documents
55794
articles + reviews (English)
6144
including term "in vitro"
1617
within title, abstract or keywords
82454
All COVID-19 documents
55794
articles + reviews (English)
6144
including term "in vitro"
3664
including "in vitro" + human
82454
All COVID-19 documents
55794
articles + reviews (English)
6144
including term "in vitro"
555
including "in vitro" + animals

Figure 5. Scheme showing the literature on COVID-19 in the database SCOPUS (at 17/12/2020). The different steps show different filters applied to the search a) all articles, b) just articles and reviews (67.7 %), c) those including "*in vitro*" (7.5 %), d) those including "*in vitro*" in the abstract, title or author keywords (2 %, on top) or "*in vitro*" and human (4.5 %, second row) or "*in vitro*" and animals (0,7 %, third row).

As shown in Figure 5, the number of documents containing keywords related to "in vitro" or alternative methods to animal research represents just around 7.5 % of all research publications available for COVID-19. This number decreases even further to approximately 2 % when the search focus just on the "title", "abstract" or "author keyword" sections.

The value goes down to 2-3 % if we focus on the tissues and organs associated to the scope of this paper as shown in the Figure 6. For this reason, the documents resulting from the searches were enriched with others based on expert advice.

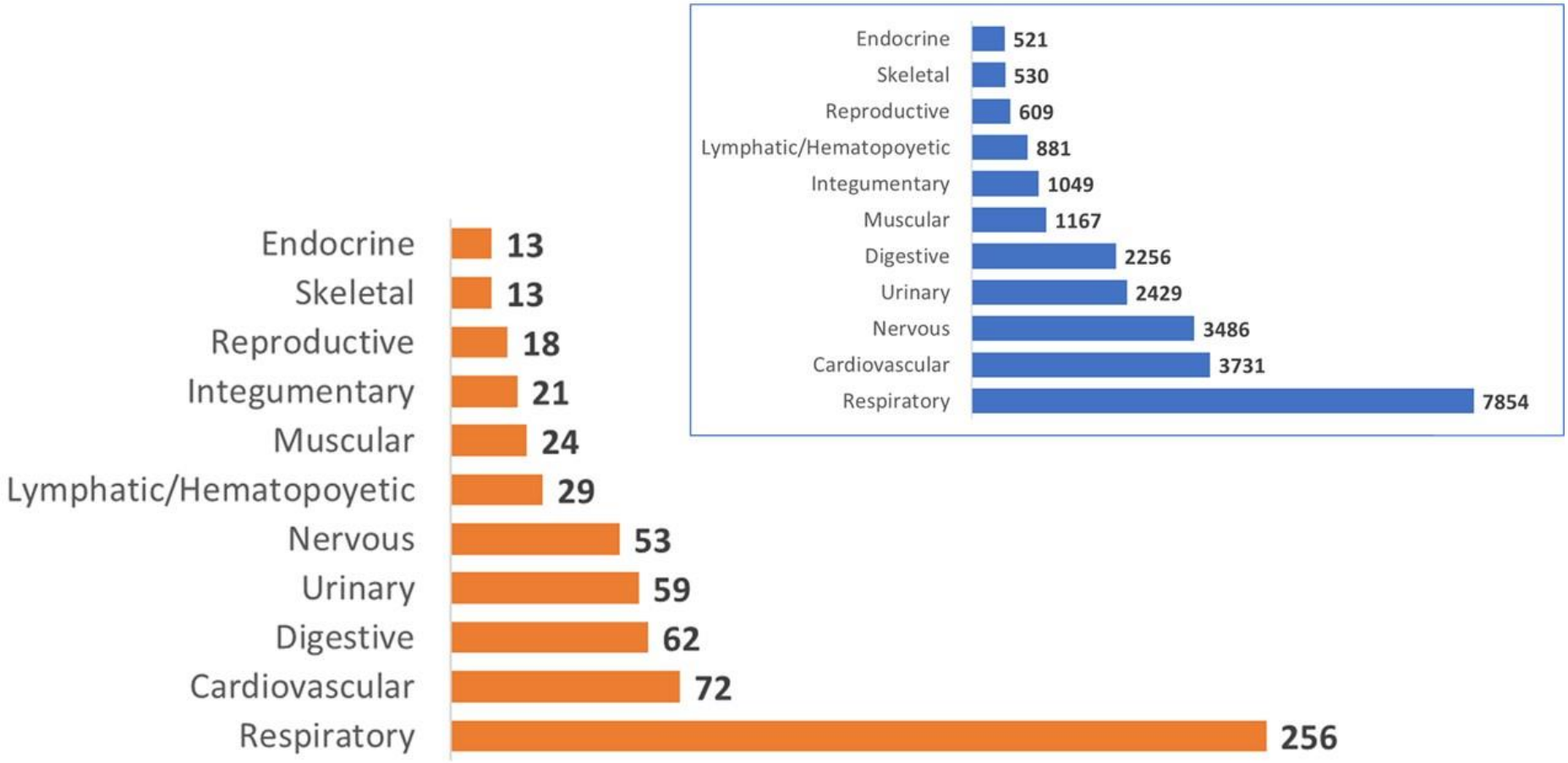


Figure 6 Number of documents found in the SCOPUS database (as 17/12/2020) when searching for COVID-19 (including its synonyms) is shown in the orange bar-diagram referring to each specific organ systems (Figure 11 and Table 2). Similarly, in the blue bar-diagram the SCOPUS literature search is shown omitting “in vitro” or its synonyms in the search string.

In general, biomedical research utilizing in vitro and in silico data is not the norm. In contrast to the animal research community publications, the human medical community tends to refer more frequently to available mechanistic knowledge from in vitro and in silico methods as knowledge support for understanding disease and its dynamics and kinetics. Although, in the case of COVID-19, circumstances are diverse. Due to the pandemic and its impact on population and economy, it seems that the SCOPUS literature findings follow a similar pattern. One can argue that if the animal research community is not citing in vitro and in silico papers on COVID-19, this research might be of limited use. However, that is inconsistent with their substantial use by the human medical community, which cites more these research findings than research based on animal studies. Additionally, this lack of transferability of knowledge between the animal and the human medical research communities is further evidenced by the fact that, in general, most citations received by animal research papers are referring to other animal-based studies, rather than to human medical papers.

Cross-community research on SARS-CoV-2 is essential to understand its detailed pathophysiology and mechanisms of infection, the host-virus interaction, replication kinetics and targets for therapeutic screening. As such "*in vitro*" cell and tissue-based models that can faithfully reproduce the viral life cycle and reproduce the pathology of COVID-19 are required. Two-dimensional (2D) cell cultures, the standard model in *in vitro* studies, but also more complex three-dimensional (3D) cultures, are the most widely used models in in vitro studies over the last decades. This is due to the efforts to standardize and harmonize in vitro methods for global use in research and routine testing by applying Good In Vitro Method Practices (GIVIMP). [23] [24] However, more and more, more complex technologically innovative 2D and three-dimensional (3D) cultures are used to comprehend disease features and other life science questions including the biology of the SARS-CoV-2 virus.

XX.2.2 SARS-CoV-2 in vitro target organ system 2- and 3-dimensional cell and tissue models

As such, with advances of genetic engineering, tissue engineering and novel technologies, in vitro target organ system models have emerged and are used as more realistic in vitro models, allowing for instance the construction of complex cytoarchitecture, with better representation of cell heterogeneity, extracellular matrix (ECM) composition, and functionality of native tissues. [25] 3D in vitro models consist of scaffold-free (spheroids and organoids) or scaffold-based (3D scaffolding and 3D bioprinting) systems used to study infectivity, replication kinetics, and host–viral interactions of SARS-CoV-2, showing increased physiological relevance as compared to 2D models.

Different types of in vitro cellular 2D and 3D derived-test systems are reported in the COVID-19 research papers (Figures 5, 6 and 7) e.g.

- Tissue or organ fragment
- Organ explant

- Dissociated cells
- Primary cells culture
- Continuous or finite cell lines
- Immortalized cell lines
- Human stem cells and human-derived induced pluripotent 2D and 3D stem (hiPSC) cell cultures (see Figure 7)
- Complex 3-dimensional culture system like spheroids, organoids, organs-on-a-chip, 3D-scaffold or 3D-bioprinting cell and tissue-based systems (see Figure 7)
- Re-differentiated cells
- Sub-cellular fractions like cytosol and microsomes or subcellular organelles
- Proteins
- Cellular derived genetic material

It was demonstrated that SARS-CoV-2 is able to infect a number of the above human-2D or 3D- derived cell systems. Some examples are listed below [26], [27], [21], [28], [29], [20], [30], [31], [32] and further detailed in this chapter for various application in COVID-19 related to the dynamics, kinetics, symptoms, risk factors and treatments.

Some generic examples of reported 2D in vitro models (Figure 7) for SARS-CoV-2 research are:

- Primary cell cultures in 2D set-ups from various target organ systems
- Human peripheral blood mononuclear cells studying the immune response of SARS-CoV-2
- U251 derived from a malignant glioblastoma tumor by explant technique
- BEAS-2B derived from human bronchial tracheal cells
- A549 derived from explanted cultures of human lung adenocarcinoma
- Calu-3 derived from human bronchial submucosal glands
- Huh-7 derived from human liver carcinoma
- Caco-2 derived from human colorectal adenocarcinoma
- HUVEC derived from human endothelium of veins from the umbilical cord
- HT-29 derived from human primary colon adenocarcinoma
- HEK293T derived human embryonic kidney cells that expresses a mutant version of the SV40 large T antigen

- HuH7 derived from human liver epithelial-like tumorigenic cells

Some generic examples of reported 3D in vitro models (Figure 7) for SARS-CoV-2 research are:

- Human intestine-on-chip-device
- Human induced pluripotent stem cell (iPSC)- derived brainsphere neurons
- Primary human airway epithelial cells derived from primary nasal (hAECN) or bronchial (hAECB) origin that can be cultured at an airway-liquid interface
- Human embryonic, adult stem cell or human iPSC-derived gut, heart, kidney, pancreatic, liver, etc. organoids
- Human blood vessel organoids
- Etc.

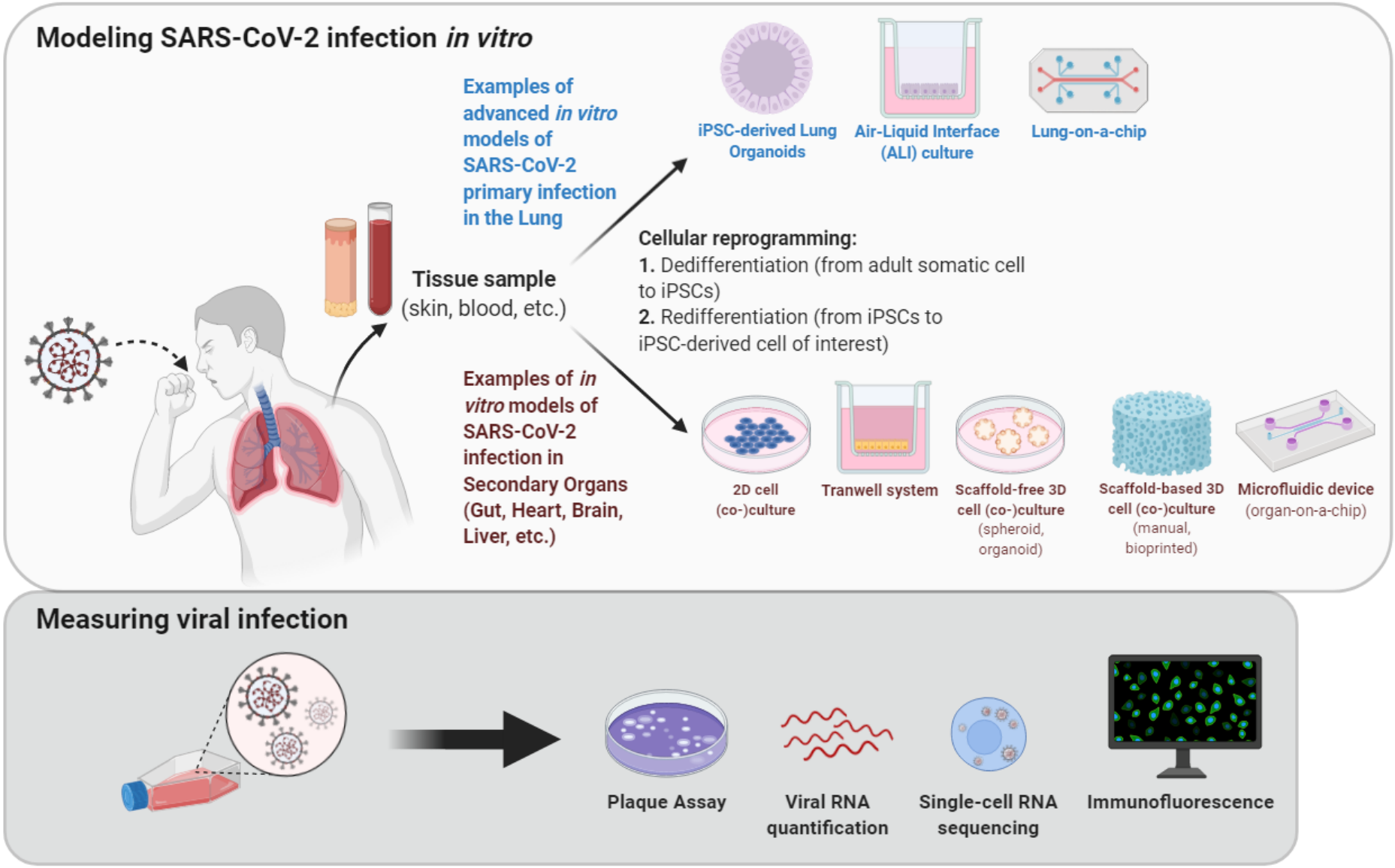

Modeling SARS-CoV-2 infection *in vitro*
Examples of advanced *in vitro* models of SARS-CoV-2 primary infection in the Lung
iPSC-derived Lung Organoids
Air-Liquid Interface (ALI) culture
Lung-on-a-chip
Tissue sample
(skin, blood, etc.)
Cellular reprogramming:
1. Dedifferentiation (from adult somatic cell to iPSCs)
2. Redifferentiation (from iPSCs to iPSC-derived cell of interest)
Examples of *in vitro* models of SARS-CoV-2 infection in Secondary Organs (Gut, Heart, Brain, Liver, etc.)
2D cell (co-)culture
Tranwell system
Scaffold-free 3D cell (co-)culture (spheroid, organoid)
Scaffold-based 3D cell (co-)culture (manual, bioprinted)
Microfluidic device (organ-on-a-chip)
Measuring viral infection
Plaque Assay
Viral RNA quantification
Single-cell RNA sequencing
Immunofluorescence

Figure 7: (created in **BioRender.com**) More complex 3-dimensional culture system like spheroids, organoids, organs-on-a-chip, 3D-scaffold or 3D-bioprinting cell and tissue-based systems. [28], [32], [31]

The application of in silico mathematical modelling and simulation in COVID-19 research may be used to understand the dynamics, kinetics, transmission, symptoms, risk factors, prevention strategies and treatments of the COVID-19 disease.

In silico methods can also be used to actively design better vaccine prototypes, support decision making, decrease experimental costs and time, and eventually improve success rates of the therapeutic and vaccine trials. In silico clinical trials (ISTs), for design and testing of therapeutic interventions, can accelerate and speed-up the therapy and vaccine discovery pipeline, predicting any therapeutic failure and very importantly minimizing undesired effects, a key focus for the toxicological research community. Also, in silico machine-learning models are demonstrating efficacy in predicting critical COVID-19 adverse effects defined as mechanical ventilation, multi-organ failure, admission to the ICU, and/or death. Hence, artificial intelligence may be applied for accurate risk prediction of patients with COVID-19, to optimize patients triage and in-hospital allocation, better prioritization of medical resources and improved overall management of the COVID-19 pandemic [33] which is an avenue to further explore with the data scientist community.

Mathematical approaches are also of critical importance for understanding the dynamics and kinetics of SARS-CoV-2 infection. Some generic examples of how in silico mathematical models are used for SARS-CoV-2 mechanistic research are: [34], [35]

- SARS-CoV-2 host tropism and analysis of the main cellular factors
- In silico prediction of molecular targets by systems network pharmacology and bioinformatic gene expression analysis
- Protein-protein interaction network construction and key SARS-CoV-2 pathways
- In silico molecular docking of key SARS-CoV-2 targets
- Studying host selectivity of SARS-CoV-2 components, spike protein, putative alternative receptors and putative co-receptors of ACE2

XX.3 Human body barriers and cell & mathematics-based methods as tools for COVID-19 infection kinetics understanding

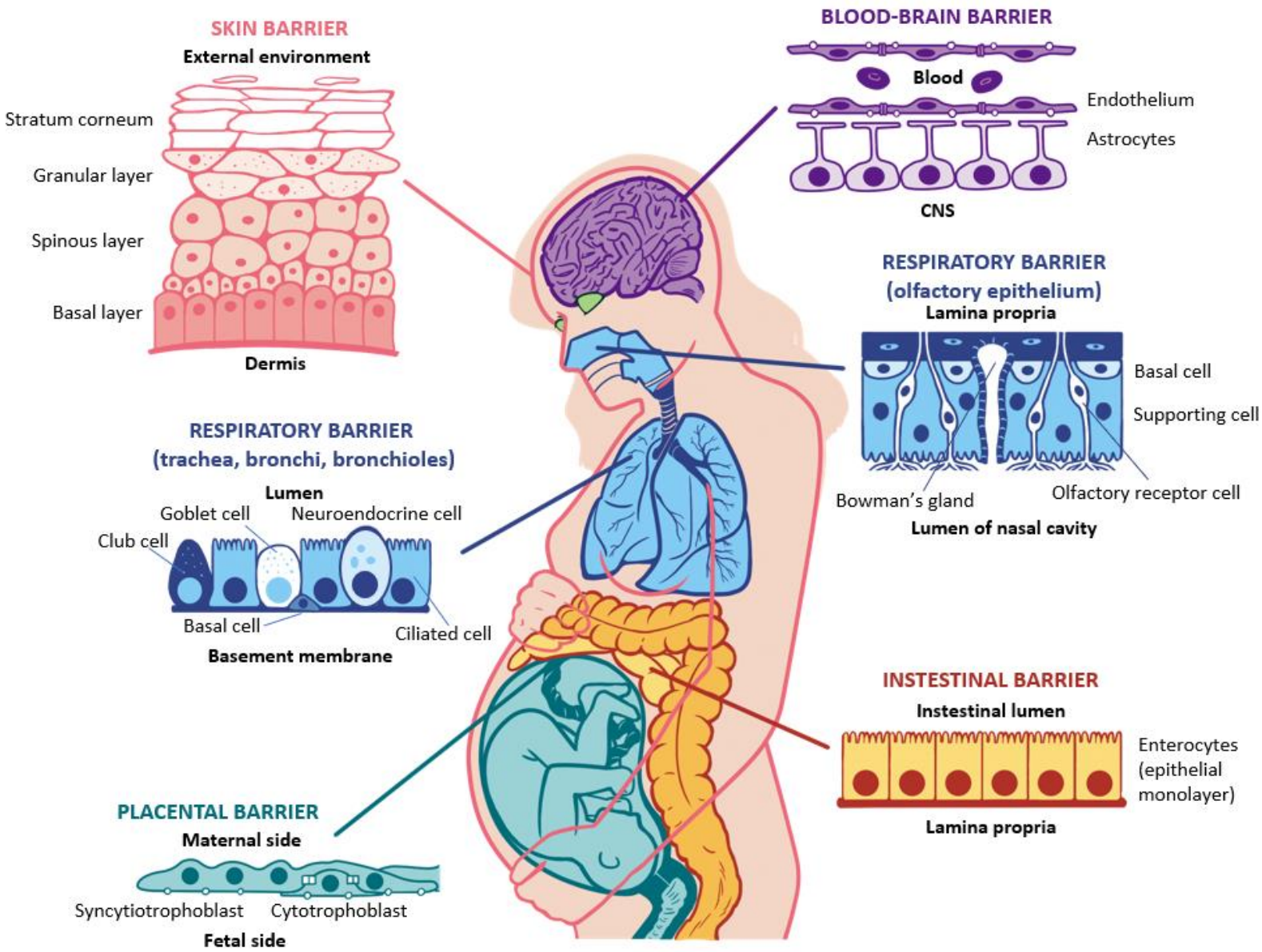

SKIN BARRIER
External environment
Stratum corneum
Granular layer
Spinous layer
Basal layer
Dermis
BLOOD-BRAIN BARRIER
Blood
Endothelium
Astrocytes
CNS
RESPIRATORY BARRIER
(olfactory epithelium)
Lamina propria
Basal cell
Supporting cell
Bowman's gland
Olfactory receptor cell
Lumen of nasal cavity
RESPIRATORY BARRIER
(trachea, bronchi, bronchioles)
Lumen
Goblet cell
Neuroendocrine cell
Club cell
Basal cell
Ciliated cell
Basement membrane
INSTESTINAL BARRIER
Instestinal lumen
Enterocytes
(epithelial
monolayer)
Lamina propria
PLACENTAL BARRIER
Maternal side
Syncytiotrophoblast
Cytotrophoblast
Fetal side

Figure 8: Human external and internal body barriers

The human body is enclosed by the external skin and mucosa barriers, which are the cellular barriers that separate the internal human body milieu and the external non-sterile environment. Additional cellular internal barriers, such as the blood–brain and placental barriers, define protected niches within the human body. In addition to their physiological roles, these host barriers provide both physical and immune defense against SARS-CoV-2 infection. The high contagion rate of SARS-CoV-2 occurs through direct transmission (coughing, sneezing, and inhalation of viral particles) or through contact (oral, nasal or ocular mucous membranes) that occur between humans, thus favoring their rapid spread around the world by efficiently crossing the respiratory, intestinal and blood retinal human body barriers. Like many pathogens, SARS-CoV-2 has a cellular mechanism to penetrate these barriers via cellular entry, resulting in a SARS-CoV-2 invasion of host barrier cells, disruption of barrier integrity, and systemic dissemination and invasion of deeper organs and tissues. The skin and linings of the mouth, pharynx, esophagus, urethra and vagina are protective epithelia. The protective epithelia prevent exchanges between the external and internal environments and protect the regions subjected to chemical and mechanical stress. These epithelia are layered tissues, composed of numerous overlapping cell layers. Their resistance is pronounced thanks to the presence of keratin, a protein that is also found in hair and nails. Since protective epithelia, such as the skin, are exposed to chemical irritants and pathogens, their cells die and are replaced by new ones quite frequently. It has therefore been of interest to study whether a certain human barrier could be involved or not in SARS-CoV-2 infection, and to what extent, several cellular types play a role in this process. One of the first evidences on the role of barriers in COVID-19 comes from a study using a single-cell RNA-sequencing (scRNA-seq) approach across various barrier tissues and model organisms to identify the potential initial cellular targets of SARS-CoV-2 infection, showing that ACE2 and TMPRSS2, the main host protein involved in the initial viral entry, are present in different cell types at different levels. [36] Indeed, ACE2 has been found in type II pneumocytes in the lung, absorptive enterocytes within the gut, and goblet secretory cells of the nasal mucosa, while ACE2 and TMPRSS2 co-expression in respiratory tissues has been found only among a rare subset of epithelial cells.
A review of some representative examples of SARS-CoV-2 interactions with human body barriers, including the external respiratory (lung and nose), intestinal, eye and cutaneous barriers and the internal, placental, testis and blood–brain barriers, is presented below, including a discussion, in the dynamics section [37], on how SARS-CoV-2 adhere to, invade, breach, or compromise these barriers and its subsequent dynamic effects on the 11 target organ systems.

XX.3.1 Respiratory barrier

The human respiratory tract starts in the nasal cavity and ends in the respiratory zone (the alveoli). In addition to its role as physical barrier, preventing the access to pathogens and foreign particulate matter, the epithelium within the mucosae bears potent innate immune functions, that allows the identification of pathogens through pattern recognition receptors (PRRs) and the release of antiviral mediators such as interferons (IFNs) and pro-inflammatory cytokines. Type I (α and β) interferons (IFNs) play a central role in defense against viral pathogens and type III (λs).

The most robust *in vitro* model of the respiratory epithelium is based on primary airway epithelial cells and is used to study molecular and functional aspects of SARS-CoV-2 virus infection *in vitro*. Primary nasal epithelial cells (NECs) can non-invasively be isolated from the inferior surface of the middle turbinate of nostrils with cytology brushes. Progress in modelling the upper respiratory tract led to the establishment of well-differentiated cell cultures. In an air-liquid interface (ALI) culture system, airway epithelial cells grow on a porous membrane, and their basal surface is in contact with culture medium in the basolateral chamber; after reaching confluency, the removal of apical medium and the exposure of the apical side of the cells to air mimic the conditions found in the airways. Airway epithelial cells grown at the ALI are now widely used to study airway disease mechanisms or for drug discovery, as they are robust surrogates of the structure and barrier functions of native respiratory epithelium. [38]

In the lower respiratory tract, the respiratory epithelium provides a highly effective barrier acting as the first line of defense against SARS-CoV-2 invasion. The pseudostratified epithelium of the conducting airways is primarily comprised of multi-ciliated cells, mucus-secreting goblet cells, neuroendocrine cells and basal cells, which secrete surfactant. It constitutes the first site of interaction with SARS-CoV-2 and is designed to facilitate effective mucociliary clearance of the virus. Multi-ciliated cells have cilia on the apical surface that beat coordinately to shuttle inhaled particulates and mucus out of the airways whilst goblet and secretory cells trap inhaled particulates and microorganisms. Basal cells are progenitor cells that act as resident stem cells for the trachea and proximal airways. These cells are capable of self-renewal as well as repopulating the pseudostratified epithelium during homeostasis and after injury.

The tracheal region and large airways harbor stromal cells such as interstitial fibroblasts that aid in the regulation of the regenerative response of the airway epithelium after injury. In contrast, the alveolar surfaces in the peripheral lung are lined by flat alveolar epithelial type 1 cells (AEC1) that form a continuous cell layer and are specialized in gas exchange, while cuboidal alveolar epithelial type 2 cells (AEC2) act as progenitor cells and secrete pulmonary surfactant, which reduces surface tension in order to prevent alveolar collapse during respiration. A common feature shared by these cell types is the presence of intracellular tight junctions that are localized at the apical surface and are pivotal for epithelial adhesion and barrier function. These tight junctions ensure the cells adhere together to form a regulated impermeable barrier. This intracellular adhesion complex consists of interconnections of proteins and receptors including, zonular occluden (ZO)-1, -2 and -3, occludins, claudins, and transmembrane

junctional adhesion molecules. Tight junctions control paracellular permeability, and immediately below them are the adherent's junctions, composed of β-catenin and E-cadherin, which mechanically connect the adjacent cells and initiate proliferation and differentiation. The presence and function of these complexes can be influenced by cellular differentiation, exposure to SARS-CoV-2 and may be altered in disease conditions, contributing severity in COVID-19 disease. [39]

XX.3.2 Intestinal barrier

The intestinal epithelium behaves like a physical and functional barrier, for pathogens and noxious stimuli, between the external and internal environment of the human organism. It is highly linked to the vascular and nervous system to perform gastrointestinal functions.
The digestive system, like the respiratory system, is particularly vulnerable to microbial infections as it is characterized by extensive areas of thin epithelium in direct contact with the external environment. In addition, the intestinal barrier interacts with commensal microbes and the immune system. Consequently, abnormalities in its function lead to several diseases such as viral, bacterial as well as parasitic infections. SARS-CoV-2 has been shown to interact with the gut [40], and for this reason, it becomes crucial to investigate the molecular mechanisms underlying the viral infection. This approach can be useful in understanding the effect that novel gut barrier associated treatments could have on COVID-19 patients. In fact, patients are often affected by gastrointestinal symptoms such as diarrhea, vomiting and abdominal pain. The virus enters the cell through its binding to the angiotensin I converting enzyme 2 (ACE2) receptor and transmembrane serine protease 2 (TMPRSS2). Both are highly expressed in enterocytes. In particular, enterocytes from the small intestine and colonocytes showed the highest proportions of cells co-expressing ACE2 and TMPRSS2. Therefore, the lower GI tract represents the most likely site of SARS-CoV-2 entry leading to GI infection. [41] However, the exact mechanism of COVID-19–induced gastrointestinal symptoms largely remains elusive. In light of this, ACE2-based strategies against COVID-19 such as ACE2 fusion proteins and TMPRSS2 inhibitors as well as gene silencing experiments should be tested to investigate the molecular pathways underlying the viral infection of the intestinal barrier by using suitable experimental models. Several *in vitro* models that simulate the barrier function of the intestinal epithelial layer have been developed and applied to assess gut infection.
To evaluate absorption and permeability across the intestinal membrane, 2D monolayer culture systems have been developed. The main representative example is the Transwell system, which is composed of a well plate and a well insert. The bottom of the insert is a semipermeable membrane on which intestinal epithelial cells can be cultured to form cell layers. The compounds to be tested are added to the apical side of the cell layer, then the absorption and permeability can be assessed by analyzing the amount of compound in the basolateral side. In most cases, Caco-2 cells, an intestinal epithelial cell line derived from colon carcinoma, are cultured on the membrane to form cell layers. Interestingly, SARS-CoV-2 showed high replication capacity in Caco-2 cells. [42] However, the Transwell system is a very simplified approximation of the real *in vivo*

situation because it lacks the gut epithelium distinctive 3D structures, consisting of villi and crypts, that not only increase the surface area, but also affect the physiology of gut epithelial cells, such as enzymatic activity and the expression of specific membrane proteins in the cells. For this reason, the development of three-dimensional *in vitro* structures assumes considerable importance in the study of human physiopathology.
It has been shown that SARS-CoV-2 can infect human intestinal organoids, or "mini-gut", resulting in the production of large amounts of infective virus particles in the intestine. [43].ç This evidence demonstrates that intestinal organoids can serve as a model to understand SARS-CoV-2 biology and infectivity in the gut, suggesting that SARS-CoV-2 can take advantage of this and infect intestinal enterocytes.
To overcome conventional static cell culture limitations, physiological parameters, such as mechanical movement and flow of medium, have been integrated into culture systems using microtechnology known as Organ-on-a-chip (OOC) technology. For example, microfluidics-based flow environments have been applied to cell culture, using top and bottom microchannels separated by a porous membrane. It has been shown that, by culturing Caco-2 cells on this membrane, is possible to measure the absorption permeability and the functionality of the intestinal wall. [44] Also, it has been developed a microfluidic cell culture device in which Caco-2 cells grew into a 3D structure on the porous membrane forming villus-like complexes as well as tight junctions with expression of occluding under fluidic conditions. [45] As previously reported, Organ-on-chips (OOCs) are also versatile tools to study organ interactions, by combining 3D cultures of different organs and allowing them to share biological signaling. Given the casual link between SARS-CoV-2 infection, gut alterations and the disruption of the gut-lung axis [40], could represent a good option to study in a more physiologically relevant way the effect of supportive treatment strategies against the viral infection would be the use of multi-organ-on-a-chip (MOC) platforms.

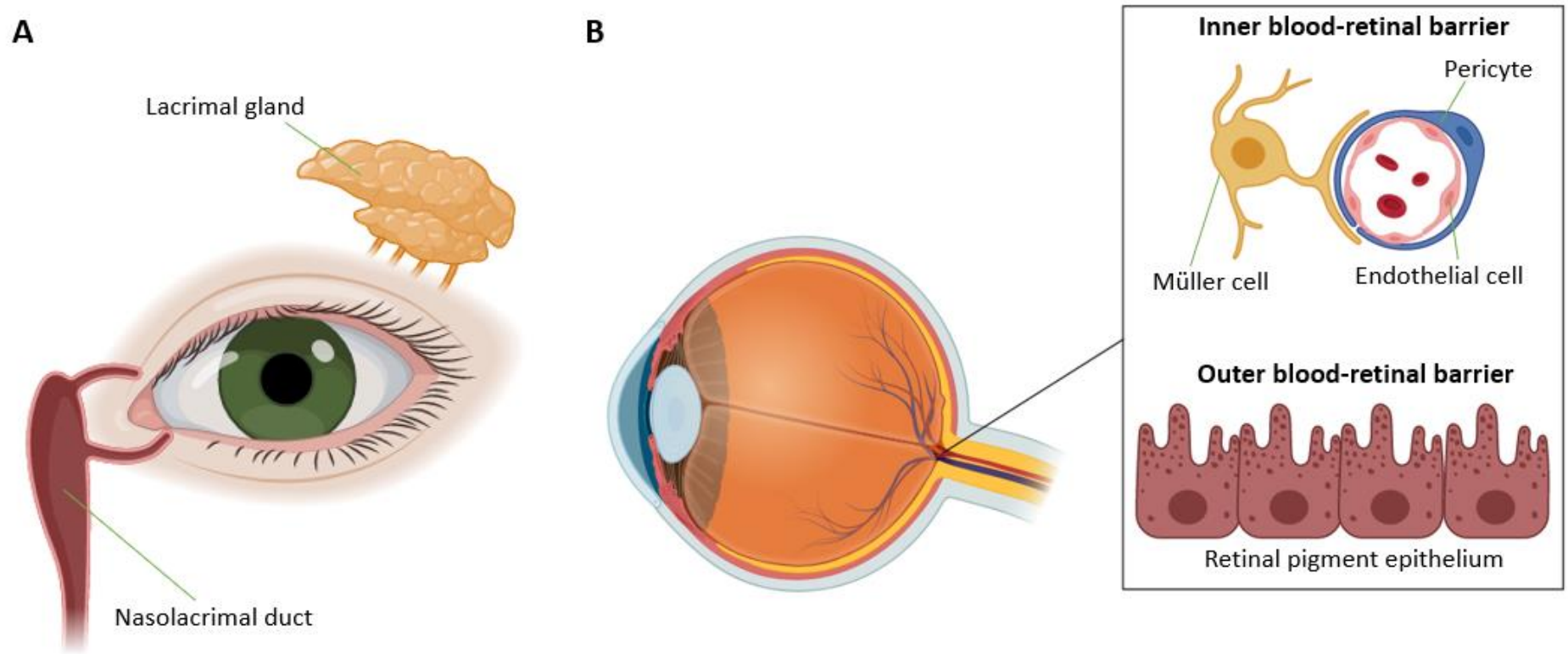


Figure 9: Eye A) frontal B) sagittal (created in **BioRender.com**)

XX.3.3 Anterior segment barrier

The eyes are an important entrance port and replication site of SARS-CoV-2. [14]
If the virus first contacts the conjunctiva of the patients' eyes, or the hand touches the virus and rubs the eye, the virus will invade the patient's eye conjunctiva, infect and reproduce, causing eye swelling which can even lead to conjunctivitis. The replicated virus may pass through the tear fluid

to patient's nasolacrimal duct and enter the respiratory infection pathway when coughing or enter the digestive tract infection pathway when swallowing food.

XX.3.4 Blood-Retinal barrier

The cells that are lining the blood-retinal barrier (BRB), the retinal endothelium, and retinal pigment epithelium (RPE) are highly permissive and susceptible to SARS-CoV-2-induced to microvascular alterations and cell death. Retinal veins diameter seems directly correlated with the disease severity. [46]
Another potential role of the eye in viral transmission of SARS-CoV-2 could be as a transport conduit to respiratory tract tissues. When ocular secretions and tears are drained through the nasolacrimal duct to the inferior meatus of the nose, viral particles may be transported from the ocular surface to the airway tissue causing respiratory infection and diseases.

XX.3.5 Skin barrier

It has also been suggested that injured skin barrier can act as a reservoir for SARS-CoV-2. ACE2 receptors have been detected in the basal cell layer of the epidermis and hair follicles, eccrine glands, blood vessels and capillaries, sebaceous glands, and its surrounding smooth muscle cells. Patients with injured skin can be exposed to the virus via respiratory droplets, saliva, or contaminated surfaces. This can provide an opportunity for the virus to survive for a while after binding to ACE2 receptors expressed in skin cells. This can further facilitate cellular viral entry and replication and protects SARS-CoV-2 from washing and antiseptics. However, further studies requiring isolation of the virus from active skin lesions would be necessary to prove this hypothesis.
In a study by Hirose, R., et al., researchers developed an ex vivo human skin model using 1-day old abdominal skin autopsy samples. [47] The model was designed such that the skin samples did not deteriorate because of drying even after long-term incubation. Using this model, the authors found that SARS-CoV-2 could survive on human skin for more than 9 hours; however, more studies would be required to study if the virus could cross the skin barrier.

XX.3.6 Blood-brain barrier

The human blood-brain barrier (BBB) represents a significant level of protection for the central nervous system (CNS). It is a highly selective functional barrier between the interstitial fluid and the blood. Its role is to isolate the body's main control center from potentially harmful substances such as toxins, fluctuations in the concentration of hormones, ions and neuroactive substances and bacteria present in the blood. The selective permeability of the blood brain barrier depends on its transport properties. The endothelium of cerebral capillaries has specific channels and membrane carriers through which nutrients and useful substances move from the blood to the interstitial fluid. Other transporters serve instead to pass waste substances from the interstitial liquid to the plasma. The BBB is formed by brain capillary endothelial cells and is characterized by tight junctions and the absence of fenestrations and pinocytotic vesicles. Moreover, astrocytes and the supporting pericytes are crucial for the expression of the BBB phenotype. Thus, the functional unit of BBB includes specialized endothelial cells, astrocytes, pericytes, and neurons along with extracellular matrix.

Thanks to the BBB, the human brain is well-shielded against many viruses, bacteria, and chemical agents. However, early reports from Wuhan, China, the origin of the COVID-19 pandemic, have suggested that 36% of patients with the disease, especially those in severe condition, show neurological symptoms. [48] In addition, it has been demonstrated that certain human neurons, as well as brain endothelial cells, express ACE2 receptor, suggesting that SARS-CoV-2 can also have access to the brain. [49] Given the species-specific nature of viral infections, like the one triggered by SARS-CoV-2, it becomes urgent to find good human-relevant *in vitro* models to better understand the molecular mechanisms underlying the COVID-19 pathogenesis in humans. In this regard, to answer the question of whether human brain cells can be infected with SARS-CoV-2 some experimental *in vitro* models have been challenged. For instance, it has been found that a 3D human iPSC-derived organotypic brain model, known as BrainSpheres, can be infected by SARS-CoV-2. [27]

Considering these findings there is huge interest in modelling *in vitro* the BBB; thus several *in vitro* models have been developed. [50] In fact, as suggested above, the BBB permeability is a key determinant for CNS exposure, also to viral particles, and it must be considered to evaluate the kinetics of brain penetration. According to a report of the European Centre for the Validation of Alternative Methods (ECVAM), there are a number of minimal requirements for any BBB model to be useful, such as the presence of restrictive paracellular permeability, the possession of a physiologically realistic cell architecture, the expression of the functional transporter mechanisms present *in vivo* and the ease of culture. [51] A negative effect, due to a pro-inflammatory response of the endothelial cells, of SARS-CoV-2 spike subunit S1 on BBB integrity has been demonstrated, in experiments performed using primary human brain microvascular endothelial cells in both confluent cell monolayers and 3D microfluidic models of the BBB. [52]

XX.3.7 Placental barrier

The placenta regulates the exchange of endogenous and exogenous materials between the mother and the fetus by diffusion or transported through specific membrane proteins. It is well known that adverse events in the placenta including hypoxia, reoxygenation and infection may damage the development of the fetus. [53] In particular, several pathogens, including viruses, can infect cellular components of the placenta, such as trophoblasts, syncytiotrophoblasts and other hematopoietic cells affecting the health status of the fetus. [54] For instance, it has been shown that ACE2, the primary receptor of SARS-CoV-2, is highly expressed in maternal-fetal interface cells, such as syncytiotrophoblasts, cytotrophoblasts and endothelial cells. In addition, pathological examinations have demonstrated that syncytiotrophoblasts are often infected by the virus.
Although the incidence of vertical transmission of SARS-CoV-2 from mother to fetus seems to be rare, some cases have been reported. [55] To confirm this evidence and better understand the molecular mechanisms of SARS-CoV-2 intrauterine infection, further investigations are needed. Therefore it could be useful to resort to preclinical *in vitro* models of the placental barrier consisting of multi-nucleated layer of syncytiotrophoblast and vascular endothelial cells. In this context, an *in vitro* model of the human placental barrier has been developed by co-culturing syncytialized trophoblastic cells and vascular endothelial cells on contralateral sides of ECM-coated Transwell inserts under physiologically relevant oxygen levels. [56] In fact, physiological oxygen levels in the human placenta are about 3-8%, substantially lower than the 21% of oxygen typical of the atmospheric air. Moreover, it has been reported a 1-day protocol for the production of a 3D *in vitro* placental barrier model using primary human trophoblasts and containing blood capillary networks which can be used to examine the mechanism of foeto-maternal dialogue during pregnancy. [57] It is also possible to replicate the microarchitecture of the placental barrier by using micro-engineered devices that mimic the structural and functional complexity of this specialized tissue *in vitro*. For example, a multi-layered microfluidic system has been created that enables co-culture of human trophoblast cells and human fetal endothelial cells in a physiologically relevant spatial arrangement to replicate the characteristic architecture of the human placental barrier observed *in vivo*. [58]

XX.3.8 Blood-testis barrier

The male reproductive system consists of the testes, epididymis and other accessory structures. The testes are the male gonads concerned with the production of sperm cells, and the process of spermatogenesis occurs in the seminiferous tubules. The epididymis is another essential structure concerned with sperm maturation. The seminiferous tubules are formed by two types of cells: spermatogonia in different stages of development and Sertoli cells. The tubule is surrounded on the outside by a basal lamina which acts as a barrier that prevents some large molecules present in the interstitial fluid from entering the tubule, while ensuring the passage of testosterone. The Sertoli cells are adjacent to each other and connected

by tight junctions formed by some structural proteins which includes occludin, tricellulin and scaffolding proteins, as well as desmosome and gap junctions that lead to the formation of an additional physiological, anatomical, and immunological barrier known as blood-testis barrier (BTB). [59] This barrier segregates the seminiferous epithelium into two compartments - basal and abluminal compartments - and it is considered as one of the closely fitted blood tissue barriers in the mammalian body. [60] Therefore, the BTB is an essential physical and functional barrier in the male reproductive tract located between the blood vessel and seminiferous tubules in the testes.

Several studies have shown that a wide range of viruses, including SARS-CoV-2, can penetrate the BTB and affect the urogenital tract, leading to testicular dysfunctions. In fact, as it is known SARS-CoV-2 infection is not restricted to the respiratory system alone but may also strike other vital tissues and organs in the body, including the male reproductive tract. It has been observed that the expression of ACE2 is high in testes if compared to other tissues, suggesting testicular pathogenesis in COVID-19 patients during the infection. [61] In addition, statistics have shown the increased vulnerability of men to COVID-19 infection compared to women due to the upregulation of ACE2 expression in the testis compared to ovarian tissue as well as the activity of androgen receptor to promote TMPRSS2 gene transcription. [62] In the light of this evidence, it is crucial to investigate the viral pathogenesis occurring in the human male reproductive tract by using advanced *in vitro* methodologies that could mimic the BTB in a physiologically and human-relevant way.

In recent years, there has been increasing interest in using Sertoli cells as a model to study BTB dynamics. [63] Sertoli cells cultured at high density on extracellular matrix-impregnated permeable supports have the ability to form a polarized monolayer that closely mimics the BTB *in vivo* both structurally and functionally. By maintaining the monolayers in appropriate culture chambers, the cells can develop a functional tight junction - permeability barrier and are able to achieve the separation of apical from basal compartments. [64] Although 2D culture methods have provided us with much information on testicular biology, they often fail to mimic organ specific physiology leading to inappropriate and biologically irrelevant cell–cell interactions. [65] Organ culture methods were applied to address the lack of 3D cell–cell interactions of 2D culture. In organ culture, small testicular tissue fragments rather than single cells are placed in cultures. [66] Although organ culture models have been used successfully to study testicular biology and toxicity in rodents, the system does not allow the study of testicular morphogenesis. [67] The rising interest in human induced pluripotent stem cell (hiPSC)-derived organoid culture has led to the development of tissue-specific organ-like structures to investigate testicular physiology *in vitro*. [68] Organoids can be used as a physiologically more relevant model system to study cell–cell interactions, development and tissue morphogenesis. [69] These testicular organoids might be useful in answering scientific questions about the link between the SARS-CoV-2 infection and the altered regulation and function of the spermatogonial stem-cell (SSC) niche as well as germ cell proliferation and differentiation in the presence of the viral particles.

XX.4 Tissue-specific distribution patterns

A detailed characterization of receptor(s) and protease(s) involved in SARS-CoV-2 entry is of major importance to understand SARS-CoV-2 cellular entrance, distribution patterns and the pulmonary and extra-pulmonary COVID-19 disease features. Indeed, while in the early phase of the pandemic, SARS-CoV-2 was believed to behave like other respiratory viruses leading to Acute Respiratory Distress Syndrome (ARDS), it is now very clear that it is a very unusual pathogen that provokes manifestations outside the respiratory apparatus leading to fatal outcomes in vulnerable people. Although research on receptors and proteases has highly benefited from accumulated knowledge on other human coronaviruses, notably the closely related SARS-CoV, much remains to be learned in the context of SARS-CoV-2 infection. Numerous data published support a main role of ACE2 as for SARS-CoV. However, there is still some doubt on the hypothesis as ACE2 as the unique SARS-CoV-2 cellular entrance port by the observation that ACE2 is at most poorly expressed in the respiratory epithelium. Whether other receptors than ACE2 or facilitators as TMPRSS2, such as the extracellular matrix metalloproteinase inducer CD147 (BSG-Basigin), furin, CD209L/L-SIGN and a related protein, CD209/DSIGN, neuropilins (NRP1), heparan sulfate or sialic acids exposed at the surface of target cells could be play a role should be further confirmed. [70], [71], [72], [73]

With COVID-19 emerging as a disease with vascular consequences and effects on many body target organ systems rather than just a respiratory syndrome, the role and qualitative and quantitative expression of the SARS-CoV-2 of entry receptor(s) is key to evaluate target organ system and tissue distribution and the related local pathogenic effects. Infection of endothelial cells is consistent with the role and expression of ACE2. [71]

However, more solid data, notably relying on post-mortem analyses or from patient-derived in vitro cell and tissue cultures, are urgently needed to validate this observation and determine whether it is anecdotic or more widespread among COVID-19 patients. Such data will help to elucidate the role of the modulation of the entry receptors in patients with comorbidities associated with severe symptoms and fatal outcome of COVID-19.

ACE2 in SARS-CoV-2 infection and its impact on the Renin Angiotensin System (RAS), Kallikrein-Kinin System (KKS) and coagulation systems is another area that can be further mechanistically investigated with in vitro cell and tissue culture tools to elucidate the inflammatory state of severe COVID-19. KKS is an intricate endogenous pathway involved in several physiological and pathological cascades in the brain. Due to the pathological effects of kinins in blood vessels and tissues, their formation and degradation are tightly controlled. Their components have been related to several central nervous system diseases such as stroke, Alzheimer's disease, Parkinson's disease, multiple sclerosis, epilepsy and others provoking some symptoms similar as those observed in COVID-19 disease. RAS is an important blood pressure regulator and controls both sodium and water intake. AngII is a potent vasoconstrictor molecule and angiotensin converting enzyme is the major enzyme responsible for its release. AngII acts mainly on the AT1 receptor, with involvement in several systemic and neurological disorders. Brain RAS has been associated with physiological pathways, but is also associated with brain disorders. [74], [75]

The tissue-wise distribution patterns of SARS-CoV-2 receptors in the human body can help in predicting the complications and early warning signs for the affected organs. [76]

ACE2 is expressed in the nasal and pulmonary respiratory airway epithelium at low levels compared to the intestine, kidney, heart, and pancreas. However, in nasal and bronchial tissues, ACE2 is mainly expressed by ciliated, club, and goblet cells. ACE2 is highly expressed in the sustentacular cells of the olfactory epithelium and the pericytes of the olfactory bulb. Low levels are also observed in the liver. It is also found in type-2 pneumocytes of alveoli and in endothelial cells of pulmonary capillaries.

Based on these findings studies looked at the tissue distribution of SARS-CoV-2 receptors and facilitators, including ACE2 andTMPRSS2 but also others such as the extracellular matrix metalloproteinase inducer CD147 (Basigin), furin CD209L/L-SIGN encoded by CLEC4M and a related protein, CD209/DSIGN, neuropilins (NRP1), heparan sulfate or sialic acids and BSG [77], [78] as mentioned above.

Tools like Gene ORGANizer (a phenotypic tool which directly links human genes to the organ systems they affect), Human Protein Atlas (HPA) and PubMed Gene database and literature survey are used to identify potentially high-risk organs vulnerable to COVID-19 infection such as lungs, intestine, kidney, testis, heart, liver, placenta, hematopoietic tissue and nerve tissue.

COVID-19 potential receptors		**Gene ORGANizer**	**Human Protein Atlas**	
			RNA expression *(consensus dataset)*	**protein expression**
ACE2		Blood vessels, heart, kidney	Small intestine, Colon, Duodenum, Kidney, Testis, Gallbladder, heart, Thyroid gland, Adipose tissue, Epididymis, etc.	Duodenum, Small intestine, gallbladder, Kidney, Testis, placenta, Nasopharynx, Bronchus, Colon, etc.
GRP78	(HSPA5) *(ENSG00000044574)*	Blood, Bone marrow, oesophagus, white blood cells	Thyroid gland, Liver, Bone marrow, Pancreas, Salivary gland, Epididymis, Heart, Placenta, Kidney, etc.	Cerebral cortex, Cerebellum, Thyroid gland, Placenta, Parathyroid gland, Adrenal gland, Nasopharynx, Bronchus, etc.

	(TMEM132A)		Cerebral cortex, Amygdala, Cerebellum, Hypothalamus, Salivary gland, etc.	Cerebellum, Nasopharynx, Bronchus, Fallopian tube, Cerebral cortex, Salivary gland, Muscle, etc.
TMPRSS2 (PRSS10)		Prostate	Small intestine, Prostate, Pancreas, Salivary gland, Colon, Stomach, Seminal vesicles, Lung, Thyroid gland, kidney, etc.	Kidney, parathyroid gland, Stomach, Pancreas, Epididymis, Prostate, Salivary gland, Duodenum, Small intestine, etc.
NPRs	NPR1	Blood vessels, heart, kidney	Breast, Adipose tissue, Lung, Kidney, Vagina, Heart, placenta, etc.	
	NPR2	Integumentary, respiratory, skeleton	Cerebellum, Endometrium, muscle, cerebral cortex, epididymis, Liver, retina, etc.	
	NPR3	prostate	Kidney, adipose tissue, Lund, heart, tongue, thyroid gland, muscle, prostate, liver, etc.	Adrenal gland, salivary gland, small intestine, gallbladder, kidney, muscle, endometrium, bone marrow, tonsil
CD209		Blood vessels, brain, head, intestine, kidney large intestine, spinal column, spinal cord, ureter, urinary bladder	Lymph node, Adipose tissue, Placenta, Small intestine, Heart, Rectum, Epididymis, Duodenum, etc.	Placenta, Lung, Testis, Bone marrow
CLEC4M			Liver, Lymph node, Placenta, Testis, Ovary, Lung, Oesophagus, etc.	Testis, Lung, Cerebral cortex, Colon
BSG (CD147)		Blood vessels, heart	Heart, Retina, Cerebral cortex, Skeletal muscle, placenta,	Stomach, Colon, Rectum, Kidney, Testis, Epididymis, Placenta,

		Colon, Bone marrow, Kidneys, testis, etc.	Cerebral cortex, oral mucosa, Oesophagus, etc

Table 1: ACE2 or facilitators as TMPRSS2, such as the extracellular matrix metalloproteinase inducer CD147 (BSG-Basigin), furin, CD209L/L-SIGN and a related protein, CD209/DSIGN, neuropilins (NRP1), heparan sulfate or sialic acids

Another study looked at the ACE2 expression differences between patterns in younger and older; male and female population; and within a single individual. [79] This was done using the RNA-Seq gene expression profiling datasets (RSEM normalized) downloaded for 31 human normal tissues from the UCSC Xena project and GenomicData Commons Data Portal. Among the human tissues, the small intestine, testis, kidneys, heart, thyroid, and adipose tissue had the highest ACE2 expression levels; blood, spleen, bone marrow, brain, blood vessels, and muscle had the lowest ACE2 expression levels; lungs, colon, liver, bladder, and adrenal gland showed medium ACE2 expression levels.

While ACE2 was not differentially expressed between males and females or between younger and older persons in many tissues, the study also looked at correlation between ACE2 levels and immune signatures. In the skin, digestive system, brain, and blood vessels, ACE2 expression levels were positively associated with immune signatures in both males and females. The immune signatures were determined by measuring the mean expression level of marker genes in various tissue. In the thyroid and lungs, ACE2 expression levels were positively associated with immune signatures in males and negatively associated in females. In the lungs, they had a positive correlation in the older groups, and a negative correlation in the younger groups. This suggests that differential host immune responses may explain the differences in disease severity between males and females, young and old.

XX.5 Human pulmonary and extra pulmonary target organ systems and cell & mathematics-based methods as tools for COVID-19 infection dynamics understanding

To describe the infection dynamics of SARS-CoV-2, the understanding of the role of the key processes, pathways and subcellular components following SARS-CoV-2 coronavirus cellular entrance via the ACE2 enzyme receptor is critical.

The ACE2 enzyme itself is a pivotal component of the renin-angiotensin system (RAS) exerting its physiological functions by modulating the levels of angiotensin II (Ang II) and Ang-(1-7) on human body organ systems. RAS plays an important role in inflammation and fibrosis. Figure 10 shows the Renin-angiotensin system modulatory role of cellular functions and molecular signaling pathways and their key role in COVID-19 pulmonary and extra-pulmonary system multi-organ system effects.

The classical axis of the RAS is formed by angiotensin converting enzyme (ACE), angiotensin II (Ang II) and angiotensin receptor type 1 (AT1) activating several cellular functions and molecular signaling pathways related to tissue injury, inflammation, and fibrosis.

In sharp contrast, the RAS axis composed by angiotensin converting enzyme 2 (ACE2), angiotensin-(1-7) and Mas receptor exerts opposite effects in relation to inflammatory response and tissue fibrosis. The Mas receptor has been identified as the protein transducing the vasodilator and antiproliferative actions of Ang-(1-7). The Mas protein is a G-protein couple receptor originally linked to modulation of growth regulating pathways involved in oncogenic effects and high expression in human eye, testis, hypothalamus and amygdala. [80]

For many years, modulators of RAS have been described to be essential for the control of essential hypertension. However, RAS modulators (including SARS-CoV-2 and chronic inflammation) have pleiotropic properties independent of their hypotensive effects, such as cognitive impairment of multiple etiologies. The spike glycoprotein S on the virion surface docking onto the ACE2 dimer (S1 and S2) (Figure 3) is an essential step in the process of SARS-CoV-2 infection in any cell of the human body organ system. It involves downregulation of ACE2 expression with systemic RAS imbalance and promotion of multi-organ damage.

In general, the RAS induces vasoconstriction, hypertension, inflammation, fibrosis, and proliferation via the ACE/Ang II/Ang II type 1 receptor (AT1R) axis and induces the opposite effects via the ACE2/Ang (1-7)/Mas axis. RAS may be activated by chronic inflammation in hypertension, diabetes, obesity, and cancer and create vulnerabilities to defend against SARS-CoV-2 coronavirus invasion (see chapter sections “Vulnerabilities and Risk factors”). The critical step in the viral infection dynamics is that SARS-CoV-2 induces the ACE2 internalization and shedding, leading to the inactivation of the ACE2/Ang (1-7)/Mas axis and the human body cells expressing ACE2 as entrance port for SARS-CoV-2 lose their natural protection against cell and tissue injury, inflammation, and fibrosis. As such two modulations to RAS may be critical in balancing the COVID-19 disease towards either defense or progression. The first modulation originates from chronic inflammation activating the ACE/Ang II/AT1R axis, and the second originates from the COVID-19 infection inactivating the ACE2/Ang (1-7)/Mas axis. Moreover, the two modulations to RAS may be a reason for increased mortality in patients with COVID-19 who have comorbidities and specific risk factors and may serve as a clinical and therapeutic target for COVID-19 intervention and treatment. [81]

Within the brain, different components of RAS have been extensively studied in the context of neuroprotection and cognition. Interestingly, a crosstalk between the RAS and other systems such as cholinergic, dopaminergic, and adrenergic systems have been demonstrated. The expression and function of the different receptor subtypes within the RAS, such as:

- Angiotensin II type I receptor (AT1R),
- Angiotensin II type II receptor (AT2R),
- Angiotensin IV receptor (AT4R), Mas receptor (MasR),

- and Mas-related-G protein-coupled receptor (MrgD), (see Figure 10)

on different cell types within the brain can be modulated by RAS modulators. As such the importance of RAS on cognition and different disease conditions caused by SARS-CoV-2 infection is an avenue central to understanding the overall COVID-19 disease etiology. [82]

The RAS signal transduction system is activated via the ACE/Ang II/AT1R axis; this promotes vasoconstriction, hypertension, inflammation, fibrosis, and proliferation. Studies on the biological functions of AT2R, a receptor of Ang II, are scant. Although AT2R activation induces the effects opposite to those of AT1R activation, AT2R expression is lower than AT1R expression in most adult tissues. Most functions of Ang II are performed via AT1R. The ACE2/Ang (1-7)/Mas axis induces the opposite effects to suppress the harmful effects of the ACE/Ang II/AT1R axis induction.

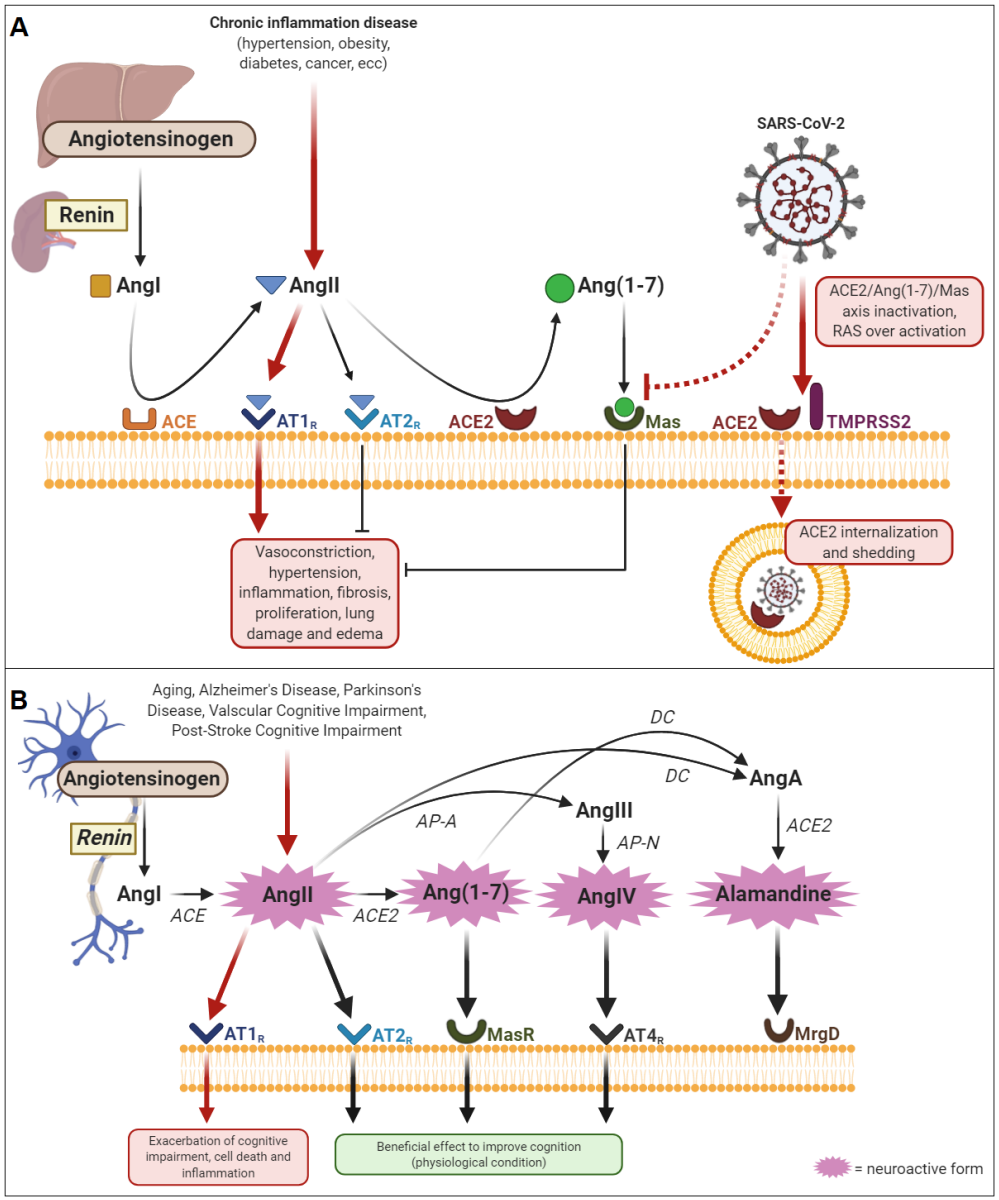

A
Chronic inflammation disease
(hypertension, obesity,
diabetes, cancer, ecc)
Angiotensinogen
Renin
AngI
AngII
Ang(1-7)
SARS-CoV-2
ACE2/Ang(1-7)/Mas
axis inactivation,
RAS over activation
ACE
AT1R
AT2R
ACE2
Mas
ACE2
TMPRSS2
Vasoconstriction,
hypertension,
inflammation, fibrosis,
proliferation, lung
damage and edema
ACE2 internalization
and shedding
B
Aging, Alzheimer's Disease, Parkinson's
Disease, Valscular Cognitive Impairment,
Post-Stroke Cognitive Impairment
Angiotensinogen
Renin
AngI
ACE
AngII
ACE2
Ang(1-7)
AngIII
AngIV
AngA
Alamandine
DC
DC
AP-A
AP-N
ACE2
AT1R
AT2R
MasR
AT4R
MrgD
Exacerbation of cognitive
impairment, cell death and
inflammation
Beneficial effect to improve cognition
(physiological condition)
= neuroactive form

Figure 10: (created in BioRender.com) Hits to the Renin-angiotensin system (RAS) modulatory role of cellular functions and molecular signaling pathways and their key role in COVID-19 pulmonary and extra-pulmonary multi-organ system effects depicting the critical step in the viral infection dynamics: SARS-CoV-2 induces the ACE2 internalization and shedding, leading to the inactivation of the ACE2/Ang (1-7)/Mas axis and the human body cells expressing ACE2 as entrance port for SARS-CoV-2 lose their natural protection against cell and tissue injury, inflammation and fibrosis. RAS modulation is critical in balancing the COVID-19 disease towards either defense or progression. [81]

In summary, both chronic inflammation and COVID-19 infection have an impact on the RAS modulation. The ACE/Ang II/AT1R axis of the RAS is activated through chronic inflammation, including hypertension, diabetes, obesity, and cancer. Furthermore, SARS-CoV-2 induces ACE2 internalization and shedding, which lead to inactivation of the ACE2/Ang (1-7)/Mas axis. Therefore, two modulating effects to RAS may be the primary reason for the mortality rate being high among patients with COVID-19 who have comorbidities. In brief, first modulation originates from chronic inflammation activating the ACE/Ang II/AT1R axis, and another modulation originates from the COVID-19 infection inactivating the ACE2/Ang (1-7)/Mas axis.

XX.5.1 Potential SARS-CoV-2 target organ systems

To better understand how downregulation of ACE2 expression with systemic RAS imbalance and other related mechanisms lowering cellular defense can lead to promotion of pulmonary and extra-pulmonary multi-organ damage in the human body, the human body has been divided in eleven potential SARS-CoV-2 target organ systems (Table 2 and Figure 11): [11]

1. *Respiratory organ system*
2. *Cardiovascular organ system*
3. *Digestive organ system*
4. *Urinary organ system*
5. *Nervous organ system*
6. *Lymphatic/Hematopoietic organ system*
7. *Muscular organ system*
8. *Integumentary organ system*

9. *Reproductive organ system*
10. *Skeletal organ system*
11. *Endocrine organ system*

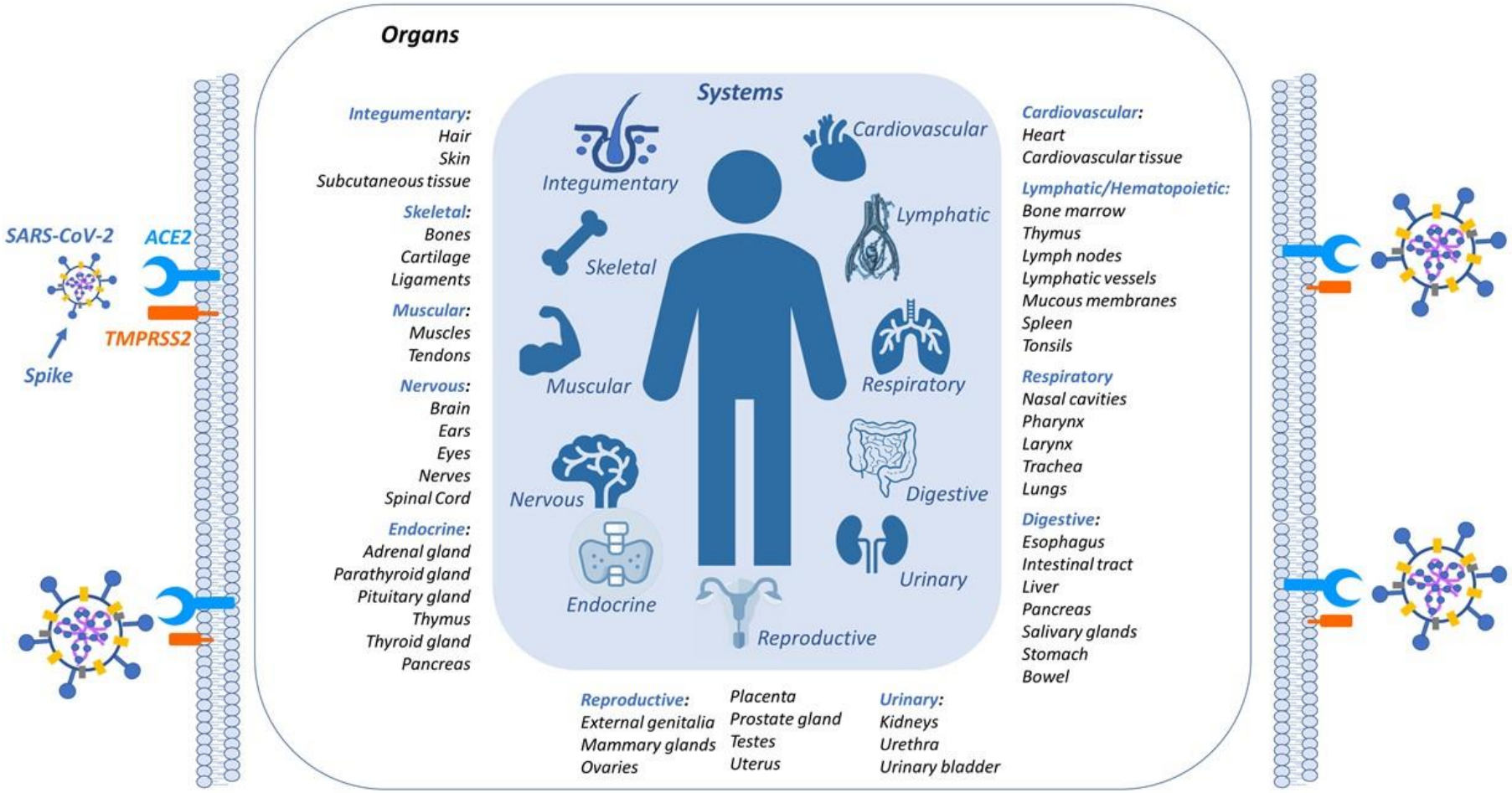


Figure 11 Human body target organ system containing several potential specific target organs where SARS-CoV-2 effects can be seen at the molecular, cellular, tissue or organ level.

Each target organ system contains several potential specific target organs where effects can be seen at the molecular, cellular, tissue or organ level. For the purpose of this chapter an organ is defined as a unique anatomic structure consisting of groups of tissues that work in concert to perform specific functions. Furthermore, potential mechanisms of cellular and molecular interaction and signaling pathways, elicited by functional receptors, in major targeted tissues/organs (e.g., such as from the respiratory, digestive, lymphatic, cardiovascular, urinary, and nervous systems) are listed. Moreover, the potential involvement of these pathways and processes in evoking the onset and progression of COVID-19 symptoms in these organ systems following SARS-CoV-2 coronavirus cellular target organ entrance are discussed.

XX.5.2 SARS-CoV-2 target organ systems relevant cell types, molecular and cellular events, methods and symptoms

Table 2 includes the structures and functions of these eleven organ systems describing their function, organ composition as well as the COVID-19 relevant cell types, molecular and cellular events, methods used to identify the effects and the symptoms associated. Patients who develop severe/critical illness are reported to have more frequently acute and sub-chronic effects observed at target organ systems listed in Table 2.

System (Function)	**Organs / Tissues**	**COVID-19 relevant**			
		Cell types	**Molecular and cellular effects**	**Examples of cell-based methods**	**Symptoms (%)** **Laboratory findings (%)**
1. Respiratory (Oxygen/carbon dioxide exchange)	Nasal cavities Pharynx Larynx Trachea	epithelial cells (lining the nasal turbinate, ethmoid sinus, trachea, and bronchus)	ACE2 is robustly expressed in the motile cilia of the respiratory tract [83]	immunohistochemistry using a panel of ACE2 antibodies on human tissue microarrays Human nasal tissue specimen collection Tissue microarrays Chromogenic immunohistochemistry IMCD3 cell culture, transfection, and staining [83]	Dry cough (31-80%) Nasal obstruction (0-4%) Sore throat (0-5%)
	Lung	Lung: Alveolar epithelial cell	ACE2 proteins are most significantly expressed on the surface of alveolar epithelial cells [84]	[87], [88], [89] Serum levels of proinflammatory cytokines	Dyspnea (30-66%) Acute respiratory distress syndrome = ARDS (29-67%)

		type II (AEC II)	Inflammatory cytokine storm [85], [86]	CT scan Hystopatology from deceased In vitro cell-derived human airway organoids and *ex vivo* bronchus cultures [90] Primary human airway epithelial cells derived from primary nasal (hAECN) or bronchial (hAECB) origin that can be cultured at an airway-liquid interface BEAS-2B derived from human bronchial tracheal cells A549 derived from explanted cultures of human lung adenocarcinoma Calu-3 derived from human bronchial submucosal glands	Pneumothorax (2%) Haemoptysis (2%) D-dimer levels were markedly higher in severe cases
2. Cardiovascular (Oxygen and nutrients transport Waste products elimination)	Heart Cardiovascular tissue	cardiac myocytes, fibroblasts, endothelial cells, smooth-muscle cells: ACE2 has high expression	a. direct viral injury: isolation of the virus from myocardial tissue has been reported in a few autopsy studies [91], [92], [93] b. inflammatory infiltrates without myocardial evidence of SARS-CoV-2 [94], [95] c. direct viral infection of the endothelium and accompanying inflammation [96] d. systemic inflammatory response syndrome (cytokine	Human engineered 3D heart tissue model to demonstrate that SARS-CoV-2 selectively infects cardiomyocytes provoking cell death and loss of contractility [98]	Chest tightness (11%) In severe cases: Cardiac injury (12-23%) (elevated creatine kinase, creatinine kinase MB)

			storm) is another putative mechanism of myocardial injury [97]		
3. Digestive (Food processing Nutrient absorption)	Oesophagus Intestinal tract Liver Pancreas Salivary glands Stomach Bowel	Differentiated entero cytes [99] intestinal glandular cells, gastric, duodenal, rectal epithelial cells, glandular enterocytes (presence of ACE2)	a.virus-mediated direct tissue damage [100], [101], [102], [99]	Hystopatology from deceased confocal and electron microscopy [99] Hystopatology from biopsy [101] Human primary gut epithelial cells used to establish small intestinal organoid in 3D culture [99], [31] Huh-7 derived from human liver carcinoma Caco-2 derived from human colorectal adenocarcinoma HT-29 derived from human primary colon adenocarcinoma HuH7 derived from human liver epithelial-like tumorigenic cells Human intestine-on-chip-device 3D liver and cholangiocytes for chemokine, IL-17, TNF and NFkB signaling [32]	Anorexia (14-30%) Diarrhea (9-13%) Nausea (3-6%) Vomiting (3-5%) abdominal pain (2-6%) In severe cases: Liver dysfunction (16-37%) with Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels significantly higher
4. Urinary (Waste elimination Blood volume and pH regulation)	Kidneys Urethra Urinary bladder	renal cells (presence of ACE2 receptors)	a. directly infection of renal cells [103], [91], [104] b. endothelial damage (glomerular capillary endothelial cells viral inclusion) [96]	Kidneys CT scan Histopathology (autopsy) [103] In silico analysis of publicly available data sets of single-cell RNA sequencing. [103], [91], [104] viral inclusion particles [96]	In severe case: Acute kidney injury (7-29%) Electrolyte abnormalities (hyperkalemia, hyponatremia, hypernatremia)

			c. cytokine storm [105], [97] d. immunocomplexes of viral antigen or virus-induced specific immunological effector mechanisms [106]	Biopsy [106] HEK293T derived human embryonic kidney cells that expresses a mutant version of the SV40 large T antigen Human embryonic stem cell derived and iPSC-derived kidney organoids where single-cell RNA sequencing showed ACE2 expression in tubular-like cells and SCL3A1, SCL27A2, SCL5A12 [30], [107]	Proteinuria hematuria Increased serum creatinine (10%) Metabolic acidosis
5. Nervous (Organ systems activity coordination Stimuli response)	Brain Ears Eyes Nerves Spinal cord	neurons, glial cells, endothelial cells, and arterial smooth muscles in the central nervous system	Hematogenous spread (2)- retrograde neuronal transport via the olfactory nerve, The cytokine cascade that follows potentiates the invasion of the virus into the brain. [108]	U251 derived from a malignant glioblastoma tumour by explant technique Human induced pluripotent stem cell (iPSC)-derived brainsphere neurons Pluripotent stem cells to produce a whole eye organoid model of retina, retinal pigment epithelium, ciliary margin, iris lens, and cornea [109]	Headache (6-13%) Dizziness (16%) Smell impairment (5-74%) Taste impairment (5-50%) In severe case: acute cerebrovascular disease (5-9%) Ischemic stroke (1%) Epilepsy (1%)
6. Lymphatic / Hematopoietic (Tissue fluid to blood return Defence against foreign organisms)	Bone marrow Thymus *Lymph nodes* *Lymphatic vessels* *Mucous membranes*			Human peripheral blood mononuclear cells studying the immune response of SARS-CoV-2 Human blood vessel organoids closely simulating human vascular capillary growth with a lumen, PDGFR+ pericyte coverage CD31+ endothelial lining, and formation of a basal membrane [30]	Fever (98-100%) Lymphopenia in severe cases (70%) procalcitonin tended to be higher in severe cases

	Spleen *Tonsils*				
7. Muscular (Locomotion Heat production)	Muscles Tendons	muscle stem cells (satellite cells), myonuclei (muscle fibers) vascular cells (i.e. endothelial cells, smooth muscle cells, pericytes) macrophage, adaptive immune cells (B, T, or natural killer cells)	a. vascular cells (endothelial cells, smooth muscle cells, pericytes), muscle stem cells (satellite cells), macrophages, adaptive immune cells (B, T, or natural killer cells), and myonuclei (muscle fiber) express TMPRSS2 b. Neuronal demyelination [110]	Autopsy [110] 3D human skeletal muscle culture model (reference [111]	Myalgia (11-40%)
8. Integumentary (Physical barrier Temperature control)	Hair Skin Subcutaneous tissue	keratinocytes	diffuse and dense lymphoid infiltrates, along with signs of endothelial inflammation immune hypersensitivity endothelial inflammation [112], [113], [114] Human skin keratinocytes and basal cells express more ACE2 than lung epithelial cells [115].	Human histopathology [112] [113] transcriptomic data [115] In vitro organoid model of dermal papilla of human hair follicle [116]	Cutaneous manifestations (0-20%) Erythematous rash (15%) Urticaria (3%) Chickenpox-like vesicles (1%) Maculopapular eruptions (47%) Vesicular eruptions (9%) Erythema with vesicles or pustules (pseudo-chilblain) (19%)

					Livedo or necrosis (6%)
9. Reproductive (Germ cell production (eggs and sperm) Environment for growth of foetus (female))	External genitalia Mammary glands Ovaries Placenta Prostate gland Testes Uterus	Female: Oocytes epithelial cells uterus and vagina Placental villi [11]	a. ACE2 is widely expressed in the ovary, uterus, vagina and placenta. b. modulating the levels of angiotensin II (Ang II) and Ang [11], [72]	GeneCards database [117] Bgee [118] [11] HUVEC derived from human endothelium of veins from the umbilical cord	Infertility?
		Male: spermatogonia (Spg), Leiden cells, Sertoli cells [119]	a. ACE2 is widely expressed in the testis b. Modulating the levels of angiotensin [119]	microarray profiling assay Autopsy [120] Potential to use in vitro testicular organogenesis from human fetal gonads to produce fertilization-competent spermatids to study SARS-CoV-2 effects [121]	Infertility Orchitis penetrate the barriers and induce testicular dysfunctions
10. Skeletal (Body support Internal organ protection Mineral storage)	Bones Cartilage Ligaments	Osteoclast Osteoblast chondrocytes	increased bone resorption and bone loss: including Angiotensin-converting enzyme 2 (ACE2)-dependent bone resorption, inflammatory cytokines storm, immunosuppression and hypoxaemia [122] Chondrocytes express ACE2	RNA sequencing identifying skeletal muscle, synovium and cortical bone as potential sites of direct SARS-CoV-2 infection [110] In vitro model of mesenchymal condensation during chondrogenic development [90]	Arthralgia (0-1%)
11. Endocrine	Adrenal gland		[123], [124], [125]	Potential for studying dynamics of calcium-regulated PTH	"an acute adrenal insufficiency may also be due to a thrombotic event at the adrenal level in

(Body function regulation (hormones))	Parathyroid gland Pituitary gland Thymus			secretion in vitro to investigate the relationship between the parathyroid cell cycle and the calcium-regulated PTH secretion [126]	COVID-19 patients. This could cause an acute adrenal insufficiency with impaired hormone production with consequent shock and worsening of the possibility of reacting to severe respiratory distress. A timely screening for pituitary–adrenal axis function and identification of this condition could allow adequate replacement therapy avoiding severe shock" [127]
	Thyroid gland		a. ACE2 and TMPRSS2 expression levels are high in the thyroid gland and more than in the lungs [128] b. thyroid damage may be secondary to a hypothalamic-pituitary system virus insult leading to thyroid disconnection [129],[128] Physiological concentrations of L-thyroxine (T4) and 3,3',5-triiodo-L-thyronine (T3) stimulate the production and release of cytokines. Thyroid hormones are capable to potentiate the antiviral action of IFN-γ. It is also of interest that some pathways (i.e., the cytokine and hyperactivation of Th1	Anatomopathological studies SARS genomic sequence positive lymphocytes and monocytes in the vessel of the thyroid gland from a SARS autopsy, [130], [131] Immunohistochemistry Evaluation of adenohypophysis from autopsies [129] In vitro models for assessing thyroid effects at TSAR "Test Methods Tracking" [132]	Thyrotoxicosis: fatigue, palpitations, inappetence, sweating, insomnia, anxiety, tremor, weight loss. Hypothyroidism: fatigue, stypsis Subacute thyroiditis

			helper cells responses) of immune responses to virus infection are observed in thyroid disorders [i.e. classical autoimmune thyroid diseases (AITD), interferon-alpha-related thyroid disease, immune checkpoint inhibitor mediated thyroiditis, alemtuzumab-induced thyroid dysfunctions [128]		
	Pancreas	Pancreatic cell	Via hematogena: ACE 2 receptor expression is present in the pancreas ACE 2 expression is noted both in the exocrine pancreatic glands as well as in the islets [133] In pancreas, binding to ACE2 with subsequent damages of islets and reduced insulin release [134]	Quantitative expression map for ACE RNA was extracted from 72 human tissues [135], [136] In vitro human pancreatic islets [137]	Hyperglycemia Ketoacidosis, including that in patients with previously undiagnosed diabetes or no diabetes Euglycemic ketosis Severe illness in patients with pre-existing diabetes and/or obesity [138]

Table 2. SARS-CoV-2 Target organ systems, functions, target organs, tissues, cell types of the human body and a listing of some of the molecular and cellular effects observed and the examples of the cell-based observation methods and some of the COVID-19 symptoms and laboratory findings reported [139], [140], [141], [142], [143], [144] (cutaneous), [48] (neuro), [145] (systematic review), [146] (smell and taste systematic review), [147], [148] (CNS systematic review) [149]

Respiratory organ system symptoms and dynamics

The second most common symptom, after fever, is dry cough (67.8%), as described in one of the first cohorts of patients (data regarding 1099 patients with laboratory-confirmed COVID-19 from 552 hospitals in 30 provinces, autonomous regions, and municipalities in mainland China through January 29, 2020). The median incubation period was 4 days. Fever was present in 43.8% of the patients on admission but developed in 88.7% during hospitalization. [150]

A systematic literature search (publications from 1 January 2019 to 3 April 2020) reported that the main symptoms of COVID-19 are fever, cough, fatigue, slight dyspnea, sore throat. [151]

ACE2 protein is abundantly expressed in multiciliated airway epithelial cells, spanning from the nasal cavity down to the lower bronchus. ACE2 protein expression is not only present in epithelial cells lining the human respiratory tract, ACE2 is robustly expressed in the motile cilia of the respiratory tract. Cilia are microscopic, finger-like protrusions that project above the apical surfaces of epithelial cells into the nasal and bronchial airway lumen. Since ~80% of the human respiratory epithelium from the nasal cavity down to the lower bronchus is densely covered with cilia (50–200 cilia per epithelial cell), the presence of ACE2 in the respiratory cilia represents an exceptionally large surface area for SARS-CoV-2 binding and cell entry. This may partially explain the high transmissibility of SARS-CoV-2 and supports the use of face masks to decrease upper airway transmission. [83]
ACE2 is not expressed in goblet cells of the respiratory tract. Secretory goblet cells make up ~20% of the epithelial cells in the airway and play an important function in mucus production for motile cilia to sweep out unwanted substances during mucociliary clearance. [83]
To study the dynamics of SARS-CoV-2 infection and to gain understanding of the cell-type-specific innate immune mechanisms triggered in response to viral entry, target cells of the adult human lung have been infected employing 3D alveolosphere cultures of primary human alveolar epithelial type-2 cells (AT2s), the stem cells of the distal alveolar region. [152]

Based on the cells that are likely infected, COVID-19 can be divided into three phases that correspond to different clinical stages of the disease. [153]

<u>Stage 1: Asymptomatic state (initial 1–2 days of infection)</u>

At this stage the patient is asymptomatic. The virus can be detected by nasal swabs. Although the viral burden may be low, these individuals are infectious. The RT-PCR value for the viral RNA might be useful to predict the viral load and the subsequent infectivity and clinical course but care should be taken in interpreting the values related to the RT-PCR cycle numbers and reference standards need to be used to allow a harmonized

global comparison. Nasal swabs have been reported to be more sensitive than throat swabs which might be explained due to differences in measurement and sampling aspects of the procedure.

From the dynamic point of view, the inhaled virus SARS-CoV-2 likely binds to epithelial cells in the nasal cavity and starts replicating. ACE2 is the main receptor for both SARS-CoV-2 but alternative receptors and co-receptors (Figure 3) might play an additional role. In vitro data on with SARS-CoV-2 infection dynamics indicate that the ciliated cells are primary cells infected in the conducting airways. There is local propagation of the virus but a limited innate immune response.

Stage 2: Upper airway and conducting airway response (next few days)

Nasal swabs or sputum should yield the virus (SARS-CoV-2) as well as early markers of the innate immune response. At this stage, the disease COVID-19 is clinically manifest. For around 80% of the infected patients, the disease will be mild and mostly restricted to the upper and conducting airways.

From the dynamic point of view the virus propagates and migrates down the respiratory tract along the conducting airways, and a more robust innate immune response is triggered. The level of signaling chemokine CXCL10 (or other innate response cytokine) may be predictive of the subsequent clinical course. SARS-CoV-2 virus infected epithelial cells are a major source of beta and lambda interferons. CXCL10 is an interferon responsive gene that has an excellent signal to noise ratio in the alveolar type II cell response to both SARS-CoV and influenza and as such a useful disease marker for COVID-19. Determining the host's innate immune response is used as a monitoring tool to improve predictions on the subsequent course of the disease and need for more aggressive monitoring.

Stage 3: Hypoxia, ground glass infiltrates, and progression to ARDS

About 20% of the infected patients will progress to stage 3 disease and will develop pulmonary infiltrates and some will develop very severe COVID-19 disease. Initial estimates of the fatality rate are around 2%, but this varies markedly with age and additional risk factors.

Return of fever, along with worsening lung consolidation and respiratory failure, were observed during the second week in about 20% of the patients, which could potentially result in acute respiratory distress syndrome (ARDS). [154] .

The common Chest X-ray findings show unilateral, or bilateral peribronchial thickening or airspace infiltrates (32, 46). High-resolution computer tomography (HRCT) can detect early lung parenchymal changes. Some of these include interlobular septal and intralobular interstitial thickening, consolidation, and ground-glass opacification, predominantly involving peripheral lung fields and lower lobes. [154]

The most common laboratory findings include lymphopenia, elevated C-reactive Protein (CRP), elevated aspartate aminotransferase, hypoalbuminemia, elevated procalcitonin level, elevated D-dimer and erythrocyte sedimentation rate (ESR). Serum levels of pro-inflammatory cytokines (interleukins, MCP1, MIP1A, MIP1BTNFα, IFNγ, IP10, and MCP1) were found to be elevated in patients with COVID-19. [155]

From a dynamic point of view, SARS-CoV-2 reaches the gas exchange units of the lung and infects alveolar type II cells. Both SARS-CoV and influenza preferentially infect type II cells compared to type I cells. The infected alveolar units are mainly peripheral and subpleural. SARS-CoV propagates within type II cells and a large number of viral particles are released, leading the cells to undergo apoptosis and die. Subsequently, the released viral particles infect type II cells in adjacent units. Parts of the lungs loose most of their type II cells, and secondary pathway for epithelial regeneration are triggered. Normally, type II cells are the precursor cells for type I cells. The pathological result of COVID-19 is diffuse alveolar damage with fibrin rich hyaline membranes and a few multi-nucleated giant cells. The wound healing process leads to severe scarring and fibrosis. Recovery of fibrosis requires a vigorous innate and acquired immune response and epithelial regeneration.

Following the SARS-CoV-2 infection, macrophages are released as a response to inflammatory signals by type II cells. Cytokines are released by macrophages that in turn results in the release of more immune cells to the injury site. Cytokines also cause vasodilatation. Fluid accumulation in alveoli causes surfactant (a surface-active lipoprotein complex – phospholipoprotein - formed by type II alveolar cells) damage, and thus alveolar collapse which subsequently affects gas exchange and extensive release of cytokines occurs including interleukin-6 (IL-6), resulting in subsequent increase in the vascular permeability. This further leads to entry of a large number of blood cells and fluid into the lungs, and causes dyspnea and respiratory failure. [156]

An overactive immune defect (by localizing neutrophils and increasing cytokines) leads to an increase in free radicals, cell debris and proteases, and edema resulting from the increase in proteins in the interstitial space. Consequently vasoconstriction occurs through platelet activation that further alters alveolar gas exchange with severe hypoxemia and tissue hypoxia which will lead to multi organ failure. [157]

It has been described that, in severe COVID-19 infection, the dysregulated immune system responds by secreting cytokines in an uncontrolled manner leading to marked increases in cytokine release, or a “cytokine storm” syndrome (Moore and June 2020; Zhang et al. 2020b). The neutrophil-to-lymphocyte ratio (NLR) is described as the leading indicator of cytokine storms (hypercytokinaemia), with increased NLR in the

blood due to the elevated cytokine levels. SARS-CoV-2 can infect monocytes, macrophages, dendritic cells, and lymphocytes, which together play an indispensable role in the development of cytokine storm. [158]

Some authors suggest that the descriptor cytokine storm does not appropriately describe the milieu in COVID-19-induced organ dysfunction since autopsy reports consistently described widespread dissemination of SARS-CoV-2 throughout diverse target organ systems. Lymphopenia is common and prognostic. T lymphocytes are directly susceptible to SARS-CoV-2 infection and are depleted in clinical COVID-19. As such, the less pronounced cytokine elevations in COVID-19 patients could reflect a regulated, or even inadequate, inflammatory response to the overwhelming viral infection. A predominantly hypoimmune state with subsequent (direct) virus-mediated target organs system and tissue damage, and dysregulated inflammation is consistent with clinical and pathological abnormalities in COVID-19 and the high concentrations of circulating acute-phase reactants reported here. [159]

That SARS-CoV-2 cellular entrance and spread stimulates a proliferation of immune cells in the lung, that are part of the aberrant immune system activation, is being increasingly recognized as a feature of severe COVID-19. [160]

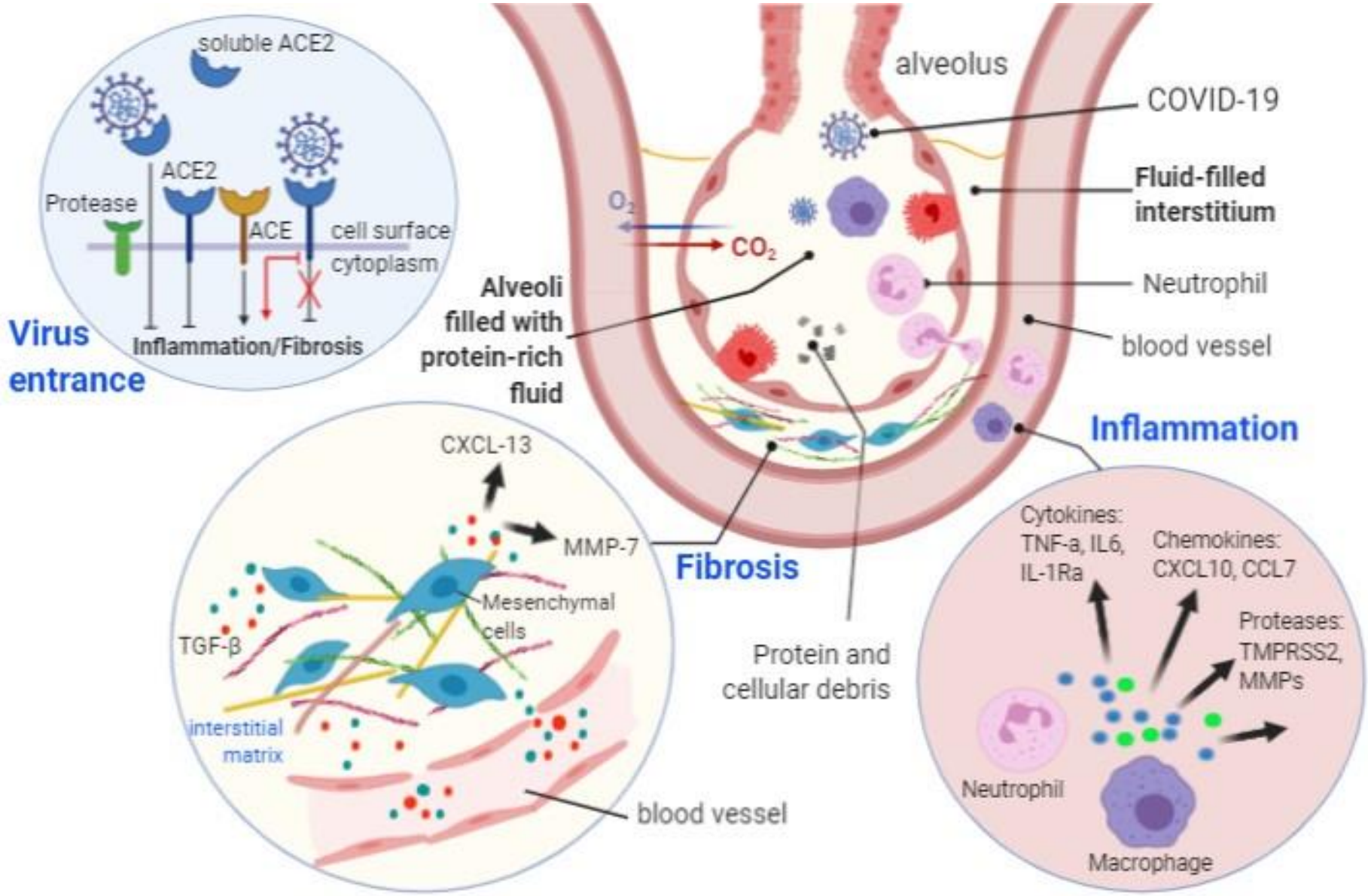

soluble ACE2
ACE2
Protease
ACE
cell surface
cytoplasm
Virus entrance
Inflammation/Fibrosis
alveolus
COVID-19
Fluid-filled interstitium
O_2
CO_2
Alveoli filled with protein-rich fluid
Neutrophil
blood vessel
Inflammation
CXCL-13
MMP-7
Fibrosis
Mesenchymal cells
TGF-β
interstitial matrix
blood vessel
Protein and cellular debris
Cytokines: TNF-a, IL6, IL-1Ra
Chemokines: CXCL10, CCL7
Proteases: TMPRSS2, MMPs
Neutrophil
Macrophage

Figure 12: Adapted from [86]. (created in **BioRender.com**)

Cardiovascular organ system symptoms and dynamics

By some estimates, 20% to 30% of hospitalized patients experience heart problems, and cardiovascular complications contribute to 40% of all deaths.
Some people exhibit a heart attack like syndrome, others a myocarditis like syndrome, and others heart failure (not appearing to be responsive to renin-angiotensin-aldosterone system inhibition) in response to an overwhelming pulmonary infection. Thus, there is a group of cardiac phenotypes related to SARS-CoV-2 infection.
In some cases, people who experienced heart problems with COVID-19 already had high blood pressure or heart disease, but in other instances, there were no known risk factors.
Most people recover from myocarditis on their own, but some people experience permanent damage to the heart muscle. There is also a known link between heart disease and respiratory infections, with a two to six-fold increase in the risk for heart attack and stroke, up to a month after having COVID-19, although the risk is highest during onset. Follow-up study of recovered SARS patients showed that 44% had various cardiovascular abnormalities 12 years after infection.

COVID-19 patients have been known to present with circulatory system symptoms like palpitations, chest tightness, and shortness of breath. Elevated creatinine kinase, creatinine kinase-MB, and high-sensitive cardiac troponin I (hs-cTnl) have also been reported in COVID-19 patients [161].

Cardiovascular dynamics are influenced by:

a. direct viral injury: isolation of the virus from myocardial tissue has been reported in a few autopsy studies [91], [92], [93]
b. inflammatory infiltrates without myocardial evidence of SARS-CoV-2 [94], [95]
c. direct viral infection of the endothelium and accompanying inflammation [96]
d. systemic inflammatory response syndrome (cytokine storm) is another putative mechanism of myocardial injury [97]

A human engineered heart tissue model demonstrated that SARS-CoV-2 selectively infects cardiomyocytes. Viral infection is dependent on expression of angiotensin-I converting enzyme 2 (ACE2) and endosomal cysteine proteases, suggesting an endosomal mechanism of cell entry. After infection with SARS-CoV-2, engineered tissues display typical features of myocarditis, including cardiomyocyte cell death, impaired cardiac contractility, and innate immune cell activation. Autopsy tissue obtained from individuals with COVID-19 myocarditis demonstrated these findings showing cardiomyocyte infection, cell death, and macrophage-predominate immune cell infiltrate. 3D in vitro models permits mechanistic studies of human cardiomyocyte SARS-CoV-2 tropism and provides an experimental platform for interrogating and mitigating cardiac complications of COVID-19. [98]

Digestive organ system symptoms and dynamics

The clinical features representing involvement of the digestive system are not very specific. Diarrhea, vomiting, and abdominal pain have been reported by affected patients. [161]

Overall, gastrointestinal symptoms were reported in 15% of patients with COVID-19 and liver injury in 19% of patients. As the severity of the disease increases, digestive symptoms and liver injury become more pronounced. [162]

Anorexia (26·1%, 95%) and diarrhea (13·5%) were the most common gastrointestinal symptoms, following by nausea (7·5%) and vomiting (6·0%). All gastrointestinal symptoms correlate with a more severe disease course and a larger proportion of intensive care unit (ICU) admission. The pooled prevalence of all gastrointestinal symptoms was higher in COVID-19 patients with severe disease than with non-severe disease (24·41% versus 16·31%, $P < 0·001$). [163]

In addition to digestive symptoms, patients with COVID-19 are also at risk of developing liver injury. Studies have shown that patients had varying degrees of liver function abnormalities—the incidence ranged from 1% to 53% — mainly indicated by abnormal ALT and AST concentrations, accompanied by slightly increased bilirubin concentrations. [162]

Severe acute liver injury has been reported with higher mortality. In fact, liver damage mainly arose with a pattern of elevated serum liver biochemistries in hospitalized patients with COVID-19 (primarily elevated AST and ALT, and slightly elevated bilirubin), ranging from 14% to 53%. Liver injury occurs more commonly in more severe COVID-19 cases than in mild cases. [164], [165]

Regarding the dynamics of the digestive system, SARS-CoV-2 virus-mediated direct digestive tissue damage has been reported. [99], [13], [102], [100] Intestinal glandular cells, gastric, duodenal, rectal epithelial cells, glandular enterocytes all have ACE2 receptor.

SARS-CoV-2 directly invades the digestive tract through binding with ACE2 receptors in glandular cells of gastric, duodenal and rectal epithelial cells, as well as in enterocytes of small intestinal. [101]

ACE2 receptors at the enterocyte level (glandular cells of gastric, duodenal and distal enterocytes) may explain gastrointestinal involvement, resulting usually in malabsorption, unbalanced intestinal secretion and activated enteric nervous system.

Relevant models are being developed, that can accurately reflect human response to SARS-CoV-2 viral infection, to study underlying infection mechanisms. [166] Biomimetic human intestine infection model on chip systems have been created containing a human intestinal epithelium (co-cultured human intestinal epithelial Caco-2 cells and mucin secreting HT-29 cells) lined upper channel and vascular endothelium (human umbilical vein endothelial cells, HUVECs) in a parallel lower channel under fluidic flow condition. These two channels sandwich a porous polydimethylsiloxane (PDMS) membrane coated with extracellular matrix (ECM).

Human primary gut epithelial cells have been used to establish small intestinal organoids in 3D culture. The model can be used for studying underlying SARS-CoV-2 mechanisms but also can be combined with other systems to the model interaction relevant immune constituents such as natural killer cells, macrophages, eosinophils. [31]

The SARS-CoV-2 cellular entrance port ACE2 expression is more abundantly expressed in ileum, gastrointestinal tract and liver [167]. Regarding liver, data supports cytopathic acute liver damage by SARS-CoV-2 through a direct cytopathic effect, due to infection of hepatocytes mediated by the highly expressed ACE2 receptors. Liver injury is caused by an uncontrolled immune response due to inflammatory activity, and/or as a consequence of therapies, manifesting as drug-induced liver injury. Other evidence based on autopsies in patients supports the theory that macrovascular and microvascular thrombosis may play a predominant role in the pathophysiological pathway, determining hypoxic-ischemic hepatic necrosis. [168]

Regarding liver involvement, the level of ACE2 expression in cholangiocytes is high (59.7%) and similar to type 2 alveolar cells, and higher than hepatocytes (2.9%).

When examining liver material from infected patients by electron microscopy, immunohistochemistry, terminal deoxynucleotidyl transferase dUTP nick end labeling staining (TUNEL assay) and pathological studies, conclusions were drawn that liver involvement is a crucial cause of hepatic impairment in COVID-19 patients. Indeed, SARS-CoV-2 causes conspicuous hepatic cytopathy, with massive apoptosis and presence of binuclear hepatocytes as predominant histological features. [169]

Post-mortem biopsy studies in patients with COVID-19 show moderate microvascular steatosis and mild lobular and portal activity, indicating that the injury could have been caused by either COVID-19 or drug-induced liver injury. [162]

SARS-CoV-2 infection and cholangiocyte damage have been studied in vitro with human liver ductal organoids as a new tool studying tropism and pathogenesis. [170]

Other 3D *in vitro* liver models consisting of scaffold-free (spheroids and organoids) or scaffold-based (3D scaffolding and 3D bioprinting) systems have been used to study infectivity, replication kinetics, and host–viral interactions of SARS-CoV-2, showing increased physiological relevance as compared to 2D models. [109], [32]

Urinary organ system symptoms and dynamics

A substantial proportion of patients with severe COVID-19 may show signs of kidney damage. Acute kidney injury (AKI) is a frequent complication of COVID-19 and is associated with mortality.

AKI has been reported to occur at high rates in critically ill patients ranging from 78% to 90%. Hematuria has been reported in around 50 % of COVID-19 patients, and proteinuria has been reported in up to 87% of critically ill patients. Hyperkalemia and acidosis are common electrolyte abnormalities that have been reported. [139]

Possible dynamic mechanisms for kidney injury in COVID-19 include direct infection of the kidney as well as cytokine storm related to sepsis. Renal cells have ACE2 receptors so a directly infection of renal cells is possible. [91], [103], [104] An endothelial damage is demonstrated by glomerular capillary endothelial cells viral inclusion. [96]

Cytokine storm has been reported in relation to the immunopathology of AKI. In fact, it has been speculated that this is an underlying mechanism of the clinical ‘viral sepsis’ and multiple-organ dysfunction. [97], [105]

Kidney organoids established from human embryonic stem cells or from induced pluripotent stem cells into 3D showing tubular structures, expressing different cell types (tubular-like cells and podicytes) and markers and the solute carrier SCL3A1 gene together with SCL27A2 and SCL5A12, have been used to study mechanistic effects such as the prediction about the effect of human recombinant soluble ACE2. [30], [107]

Microfluidic devices are also an attractive option for COVID-19 disease modeling of kidney injury, offering the opportunity to utilize human cells, control experimental and environmental conditions, and combine with other on-chip devices. Kidney "spheroids", spontaneously organized cells in 3D that mimic tubule polarization and organization, have been in wide use since the 1980s but are not amenable to shear stress conditions within the spheroid interior. To ensure shear flow conditions in the tubule "lumen" and maintain cells in a monolayer for simple visualization, the most common design for a kidney-mimicking microfluidic device is a sandwich of two chips of a non-porous material with a porous membrane between them. Instead of synthetic porous membranes, protein-derived hydrogels made of collagen or basement membrane extract allow for the study of extracellular matrix remodeling and 3D migration, both of which occur during fibrosis. [171]

For cell and tissue-based models, developed to model the urinary target organ system and the kidney, a challenge is to recapitulate blood clot or renin-angiotensin system, which is an important complex cascade of pathways that results in SARS-CoV-2 infection.

Nervous organ system symptoms and dynamics

There is mounting evidence that SARS-CoV-2 also impacts the brain. COVID-19 patients, experience neurological symptoms, including dizziness, headache, and cognitive impairment.
A number of non-specific mild neurological symptoms are notable in hospitalized patients with COVID-19, including headache (8–42%), dizziness (12%), myalgia and/or fatigue (11–44%), anorexia (40%), anosmia (5%), and ageusia (5%) ([172], [173], [174]) although the epidemiology may be different in milder outpatient presentations. [8] More-severe presentations of COVID-19 manifest with acute stroke (6% of those with severe illness), and confusion or impaired consciousness (8–9%).
Acute inflammatory demyelinating polyneuropathy (Guillain-Barré syndrome) has also been reported in some patients. In addition, meningoencephalitis, hemorrhagic posterior reversible encephalopathy syndrome and acute necrotizing encephalopathy, including the brainstem and basal ganglia, have been described in case reports. [139]
Most of the symptoms resolve over time, but some do not. Recovery depends, in large part on age, comorbidities [175], cognitive level before infection, and smoking habits.

Dynamics range from directly infecting neurons (e.g., olfactory neuroepithelium) to underlying inflammatory immune responses and oxygen deprivation.
New approach cell and tissue-based methods are contributing to understanding the root cause of the neurological problems and giving insights into the understanding of symptoms and their temporary or permanent features such as cognitive impairments (e.g., Intensive Care Unit delirium).

SARS-CoV-2 may access the central nervous system via the neuropethelium, lamina ribrosa, and olfactory bulb or via retrograde axonal transport. Sustentacular cells of the neuroepithelium display the highest expression of ACE2. This may account for the symptoms of altered or loss of sense of taste *(Hypogeusia: reduced ability to taste sweet, sour, bitter, or salty things; Ageusi: loss of sense of taste)* or smell *(Hyposmia: reduced ability to smell; Anosmia: loss of sense of smell*) frequently reported retrospectively in COVID-19 patients.
Other neurological manifestations support, at least, the neuroinvasion of COVID-19, perhaps by a hematogenous way reflecting the proinflammatory and prothrombotic cascade in the wake of cytokine storm as it affects brain vasculature and the blood–brain barrier. [139]

The infection of the central nervous system (CNS) and SARS-CoV-2 neuroinvasion, neuroinflammation, and blood-brain barrier (BBB) dysfunction may be implicated in the development of the observed disorders. Basic research is critical to understand the detailed mechanisms and pathway of infectivity underlying CNS pathogenesis. In order to understand why some patients develop such symptoms and others do not, mechanistic studies using advanced cell and tissue culture methods such as state-of-the-art 3D human induced pluripotent organotypic stem cell cultures, can give more insights into the infection dynamics and give more details on the onset of encephalitis and other neurological disorders. [27], [176]

Lymphatic/Hematopoietic organ system symptoms and dynamics

Hematological changes such as lymphopenia, and thrombocytopenia and leukopenia are not observed in COVID-19 patients. During the onset of the COVID-19 disease, patients exhibit a reduction in peripheral CD4+ and CD8+ T lymphocytes. It has been reported that patients having COVID-19 disease show lymphopenia (82.1%) and thrombocytopenia (36.2%) and leukopenia (33.7%) of patients on admission to the clinic. [177]

Regarding the dynamics, the inaccessibility of living bone marrow (BM) hampers the study of its pathophysiology under myelotoxic stress induced by SARS-CoV-2. Novel technologies like organ-on-a-chip systems offers the possibility to study such dynamic effects. Vascularized human BM-on-a-chip (BM chip) that support the differentiation and maturation of multiple blood cell lineages, facilitate CD34+ cell maintenance and recapitulate aspects of BM injury, are a novel elegant tool to use to study SARS-CoV-2 dynamics. The chips comprise a fluidic channel filled with a fibrin gel in which CD34+ cells and BM-derived stromal cells are co-cultured, a parallel channel lined by human vascular endothelium and perfused with culture medium, and a porous membrane separating the two channels. [178], [31]
Induced pluripotent stem cells, differentiated to endothelial lineage and cultured in matrigel–collagen gel in 96 well plates to form human capillary organoids, were successfully infected with SARS-CoV-2. Matrigel is a gelatinous protein mixture derived from mouse tumor cells and is commonly used as a basement membrane matrix for stem cells because it maintains the stem cells in an undifferentiated state. The in vitro 3D

model uses clinical-grade soluble human ACE2 and simulates human vascular capillary growth with a lumen, PDGFR+ pericyte coverage CD31+ endothelial lining, and formation of a basal membrane and models the early stages of infection. [30]
To avoid batch-to-batch variations of the Matrigel-collagen gel with attendant lack of reproducibility, biologically relevant and chemically defined matrigel alternatives are being made available by several commercial suppliers, tailored for different cell types. [24]

Muscular organ system symptoms and dynamics

Myalgias and generalized weakness have been reported, based on epidemiological data, to occur in one-quarter to one-half of symptomatic patients with COVID-19. [110], [179], [180]

Although some data have suggested that the occurrence of muscle pain does not increase with COVID-19 severity, in patients with abnormal computed tomographic (CT) or radiographic imaging of the lungs, myalgias were an important predictive factor in the severity of the overall disease. [110]

Hypoxia secondary to the decline of O_2/CO2 gas exchange in damaged alveoli (exudation and interstitial inflammation, peri lobular fibrosis, bronchial metaplasia) and prolonged ventilation induce muscular weakness. This is a potential risk factor of subsequent mortality in COVID-19 in view of the cardiopulmonary effort. [181] Moreover, decreased muscular function and muscle loss can lead to severe sarcopenia (from the Greek, sarx for: “flesh” and penia for “loss”), characterized by a catabolic state of the muscles. Sarcopenia is significantly associated with physical disability in both men and women, independent of ethnicity, age, morbidity, obesity, income, or health behaviors. Reduced muscle strength, usually with aging, and the loss of functional capacity is a major cause of disability, mortality, and other adverse health outcomes. [182] Primary sarcopenia is age related while secondary sarcopenia is related to chronic metabolic diseases, including pulmonary diseases and occurs during the recovery period following COVID-19 (Figure 13). The combination of cachexia and sarcopenia is a cause of mortality and disability. [183]

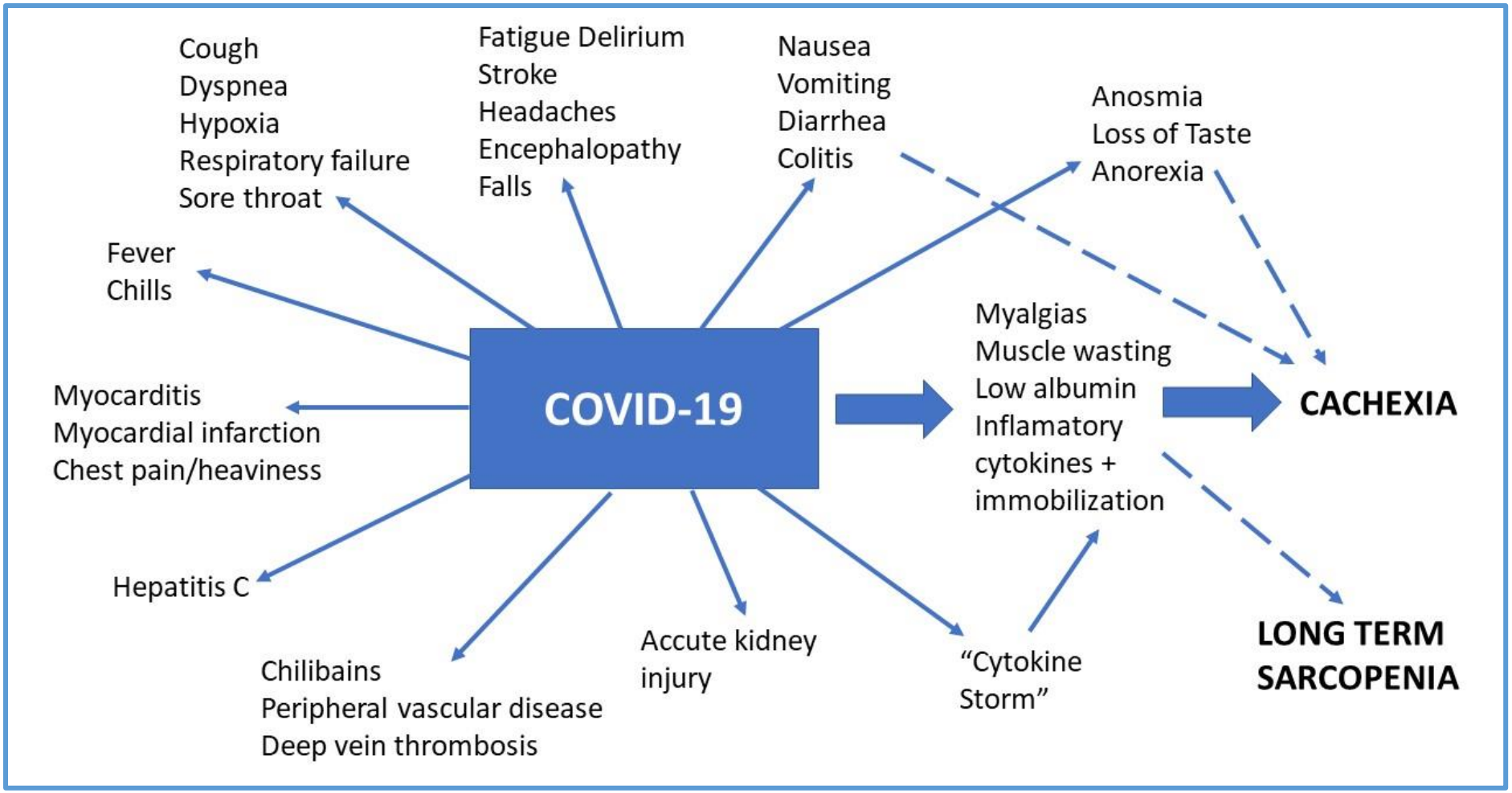


Figure 13. COVID-19 symptoms including cachexia and long-term sarcopenia. Adapted from [183]

In COVID-19 disease bone mass loss, muscle loss and weakness frequently coexist, and, for this reason, the term Osteo-sarcopenia should be applied. This syndrome is described by the combination of low bone density (osteopenia/osteoporosis) and decline of muscle mass, strength and/or

functional capacity (sarcopenia). [184] In addition to genetic traits such us glycine-N-acyltransferase (GLYAT) or myocyte enhancer factor-2 (MEF2C) that are associated with muscle atrophy and bone loss, mechanical gravitational loading or muscle contraction, metabolic activities (collagen synthesis related to protein turn-over), endocrine factors like testosterone and estrogens, parathyroid hormone facilitating calcium uptake, diet (D-Vitamin) are factors involved in muscle and bone kinetics. Moreover, prolonged use of corticosteroids catabolic inflammatory TNF-a and IL-6 cytokines can cause muscle and bone loss [185] as shown in figure 14.

Since bone and skeletal muscle are integrated organs, further and specific studies are needed to better understand these shared consequences of COVID-19.

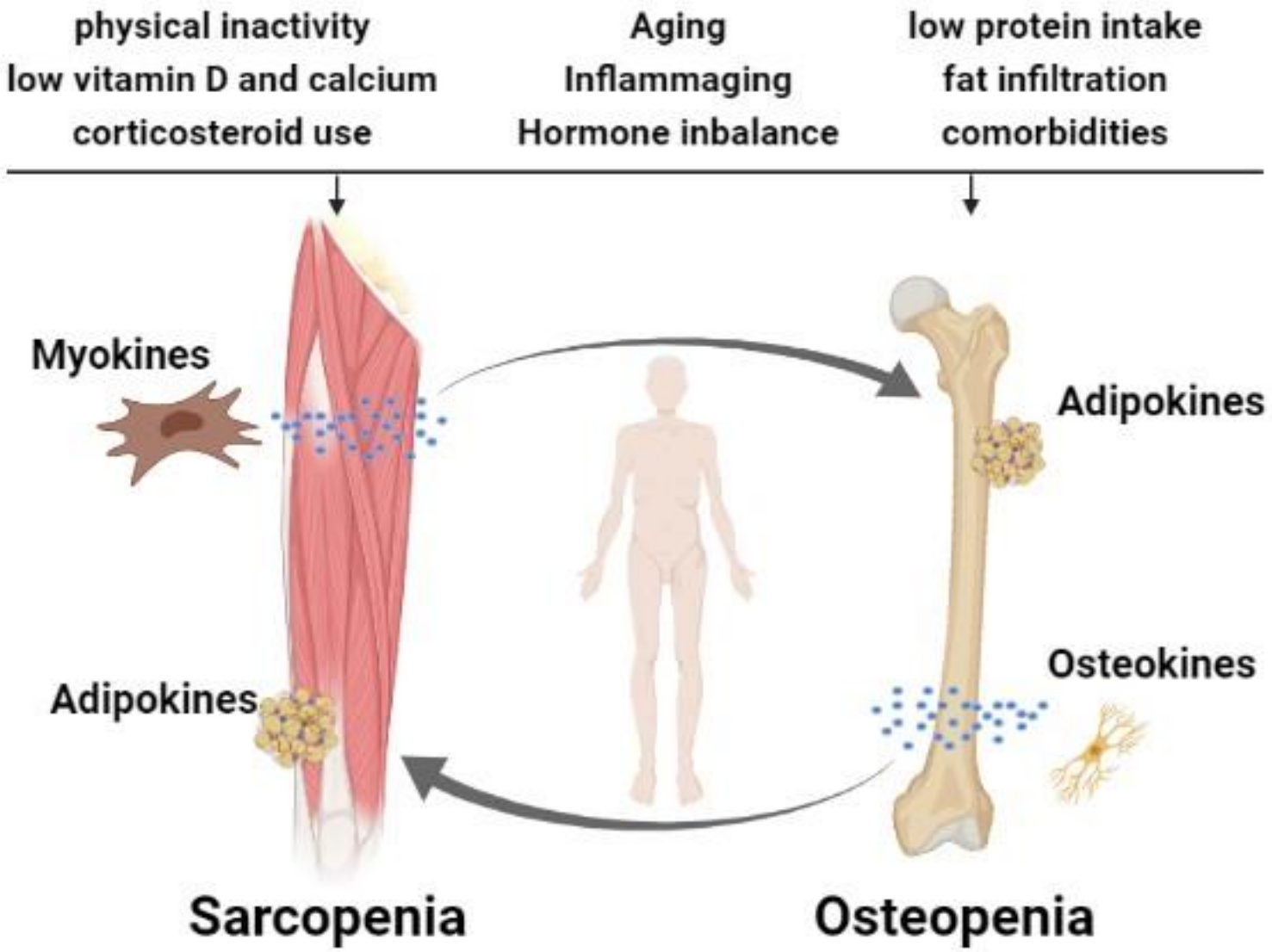


Figure 14. Muscular-skeletal manifestations of COVID-19. From [184]

For human skeletal muscle tissue, numerous cell types express TMPRSS2, including vascular cells such as endothelial cells, smooth muscle cells, pericytes, muscle stem cells (satellite cells), macrophages, adaptive immune cells (B, T, or natural killer cells), and myonuclei (muscle fibers). However, only smooth muscle cells and pericytes express ACE2 critical for the dynamics of muscular-skeletal manifestations. [110]

Immune-mediated muscle damage can be the cause of myositis and rhabdomyolysis by means of immune complex deposition in muscles and the release of myotoxic cytokines thanks to the homology between viral antigens and human muscle cells. [186]

In COVID-19 patients widespread muscle fiber atrophy was noted, with sporadic and focal muscle fiber necrosis and immune cell infiltration. Neuronal demyelination has also been reported, which may also contribute to muscle weakness and fatigue. In addition to potential direct viral infection, the cytokines and proinflammatory signaling molecules induced by the infection can lead to pathological changes in skeletal muscle tissue [110]. A subset of those proinflammatory cytokines is suggested to stimulate muscle atrophy and weakness during critical illness. Of these, three cytokines, tumor necrosis factor alpha (TNFα), interleukin 1 (IL-1), and interleukin 6 (IL-6) are the most investigated in critically ill patients. In vitro studies have shown that incubation of cultured myotubes with clinically relevant doses of TNFα caused progressive reduction in myotube diameter and total muscle protein content. [111]

Integumentary organ system symptoms and dynamics

Skin-related clinical manifestations in COVID-19 patients have been frequently reported. [187], [188]
A review observed different types of skin rashes: Erythematous maculopapular (n = 250/655), vascular (n= 146), vesicular (n = 99), urticarial (n = 98), erythema multiforme/generalized pustular figurate erythema/Stevens-Johnson syndrome (n = 22), ocular/periocular (n = 14), polymorphic pattern (n = 9), generalized pruritus (n = 8), Kawasaki disease (n = 5), atypical erythema nodosum (n = 3), and atypical Sweet syndrome (n = 1). Chilblain-like lesions were more frequent in the younger population and were linked to a milder disease course, while fixed livedo racemose and retiform purpura appeared in older patients and seemed to predict a more severe prognosis. [189]

Chilblain-like lesions in mildly symptomatic children, adolescents mainly affecting the feet and hands, especially toes and fingers and the plantar region and the heel have been reported. [190], [191]

Human skin keratinocytes and basal cells express more ACE2 than lung epithelial cells important for integumentary dynamic aspects. [115]

The pathophysiology of such manifestations remains largely unknown but lymphocytic perivascular and periadnexal infiltrates extending to the subcutis and occurrence of small thrombus in a capillary vessel in the upper part of the dermis has been observed. [190], [191]
In vitro vascularized human skin equivalents as a novel in vitro model of skin fibrosis can be an avenue to explore to study SARS-CoV-2 chilblain-like lesions. [192] There is growing evidence that adipose tissue plays a key role in the aggravation of COVID-19 where adipocytes act as a reservoir for SARS-CoV-2 and increase viral load in obese or overweight individuals. Furthermore, release of substances that boost the inflammatory reaction by adipocytes into the bloodstream has been described. This knowledge is important to consider when obesity is at play as the lipid profile is already disrupted, which may lead to increased susceptibility to infection which if occurs will further alter the lipid profile inducing hyper-inflammation. [193], [194]

Reproductive organ system symptoms and dynamics

Little is known about the effect of SARS-CoV-2 infection on reproductive functions.
SARS-CoV-2 may disturb the female reproductive functions, resulting in infertility, menstrual disorder and fetal distress. [11]
In males, studies have shown that a wide range of viruses can penetrate the barriers and induce testicular dysfunctions. [61], [119]

SARS-CoV-2 may infect the ovary, uterus, vagina and placenta through the ubiquitous expression of ACE2 important in reproductive dynamic processes. [11]

- ACE2 is most abundantly expressed in the ovary and also the expression level of *ACE2* in oocytes is relatively high. Therefore, the ovary and oocyte might be potential targets of SARS-CoV-2. Expression is also observed in uterus, vagina and placenta. [11]
- *ACE2* mRNA has been identified in the uterus of human after analyzing the data from the Human Protein Atlas [195], [196] and GeneCards. [11]
- the Human Protein Atlas and GeneCards database show the presence of *ACE2* in female breasts. [11]
- Ang II, ACE2 and Ang-(1-7) regulate follicle development and ovulation, modulate luteal angiogenesis and degeneration, and also influence the regular changes in endometrial tissue and embryo development. Taking these functions into account, SARS-CoV-2 may disturb female reproductive functions through its interaction with ACE2. [11]

Therefore, we believe that apart from droplets and contact transmission (Figure 4), the possibility of mother-to-child and sexual transmission also exists. Taking the facts mentioned above into account, SARS-CoV-2 may disturb female reproductive functions including follicle development and ovulation, modulate luteal angiogenesis and degeneration, and also influence the regular changes in endometrial tissue and embryo development.

In male studies showed mapping of ACE2 in human testicular tissue using single-cell transcriptome resolution with ACE2 protein expression localized in spermatogonia, Leydig, and Sertoli cells. The testis has been found to have the highest level of ACE2 expression compared to other tissue. [61]

Increased blood viral load (as seen in SARS-CoV infection) can increase the vulnerability and risk of male gonads to viral infection and predominantly affect testicular functions. ACE2 can be utilized as a potent receptor by the virus to penetrate the testes. The rapid replication of the virus after entry may promote pyroptosis of the immune cells and apoptosis of the endothelial cells in the testes with subsequent release of inflammatory biomolecules including cytokines, chemokines and adhesion molecules. Also, inflammatory cytokines (TNF-α, IL-1β) may promote oxidative stress in the Sertoli cells and compromise blood-testis-barrier integrity. A significant increase in cellular inflammation has been well-correlated with cell death and multiple organ damage. Local or systemic infection can cause orchitis (an inflammatory lesion of the testicles) due to the hematogenous propagation of the pathogens. Orchitis has been reported to be a risk factor for male infertility reported in 15% of infected or recovered patients undergoing fertility tests. [119]

Testicular autopsy of patients who died of SARS-CoV infection (a virus sharing similar pathogenicity with COVID-19 disease) revealed that there was inflammation of one or both testes resulting from the infection with substantial destruction and death of spermatogenic cells. Also, there is a significant reduction in sperm cells present in the germinal epithelium, macrophages infiltration into the tubular lumen and thickness of the basement membrane. Yang, M, Chen, S, Huang, B, Zhong, J-M, Su, H, Chen, Y-J, et al. have discussed pathological findings in the testes of COVID-19 patients and their clinical implications. [120]

A multitude of in vitro, ex vivo models to study reproduction have been made available and can be applied to the real needs of mechanistic understanding of SARS-CoV-2 on male and female reproduction. [197]

Skeletal organ system symptoms and dynamics

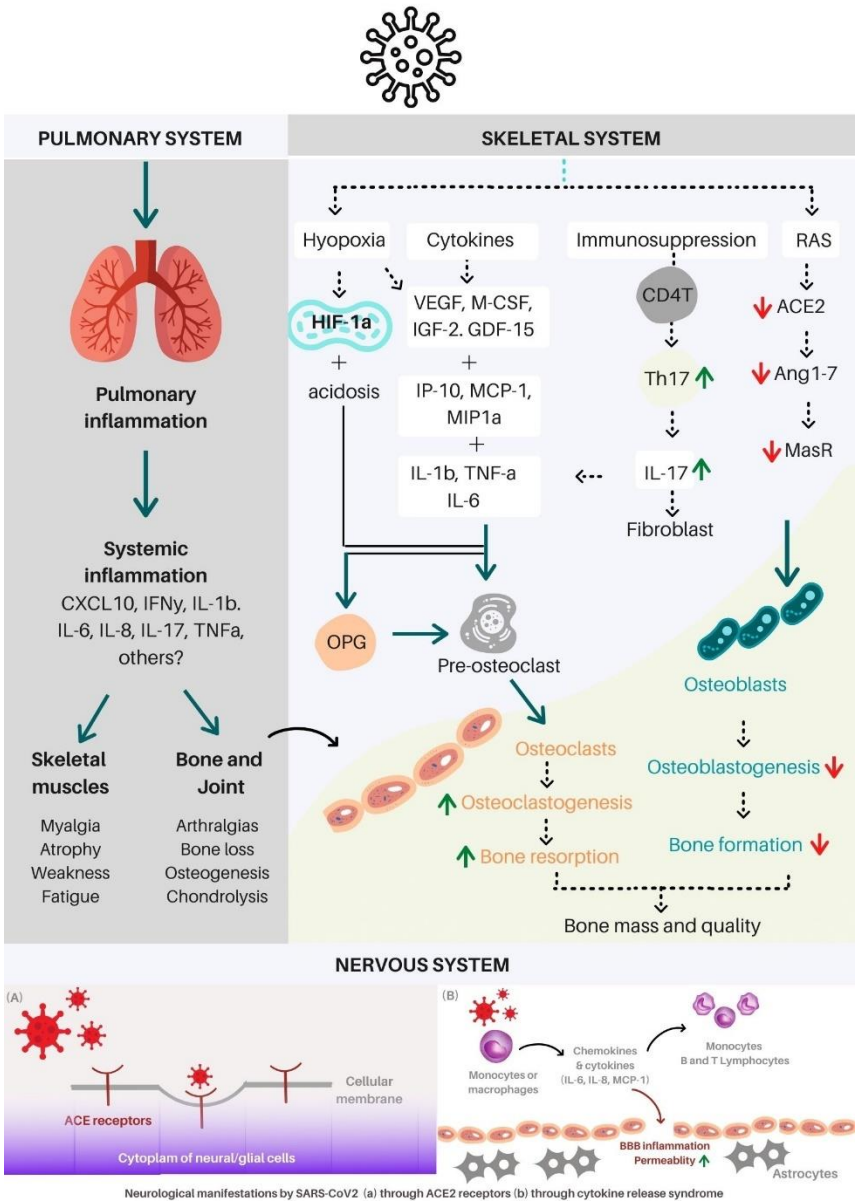
Patients infected with COVID-19 may face a risk of disruption of bone homeostasis balance increasing bone resorption and bone loss. [122] Among COVID-19 patients, 11% complain of arthralgia [179], while arthritis has been rarely reported. [180] Another 5-58 % of the patients have been described with osteonecrosis in the typical areas and is a possible consequence of SARS-CoV-2. [110]

Age, sex, the effects of prolonged immobilization, hypoxia and the use of corticosteroids contribute to bone resorption.

Osteonecrosis has been described in patients with severe SARS, (from 5 % to 58%) in the typical areas and is a possible consequence of SARS-CoV-2.

COVID-19 dynamic processes may affect the skeletal system through multiple mechanisms [122] (Figure 10):

1) ACE2 has been described as a critical player to induce bone resorption. ACE2 and MasR are expressed by osteoblasts and osteoclasts. The activation of ACE2/Ang-(1-7)/MasR axis can impact the bone resorptive milieu by inhibiting the expression of receptor activator of nuclear factor-kappaB ligand (RANKL)3. When COVID-19 targets ACE2, the ACE2 expression is downregulated, impacting the ACE2/Ang-(1-7)/MasR cascade resulting in unbalanced bone homeostasis and acceleration of both osteoclastogenesis and osteoblastogenesis.
2) High levels of inflammatory cytokines, such as IL-1β, IL-6, TNF-α, G-CSF, IP-10, MCP-1, MIP-1α may play a role in different aspects of the osteoclastogenic pathway from osteoclast formation to enhance bone resorptive capacity via RANK/RANKL/OPG signaling. Inflammatory cytokines may recruit osteoclasts, trigger bone loss and resist bone formation.
3) Immunosuppression also contributes to bone destruction. Reduction of lymphocytes in peripheral blood, mainly T cells and B cells is seen in COVID-19 infected patients. In such a situation, T cells are activated and differentiate into Th17 cells, which produce IL-17 and upregulate RANKL to facilitate osteoclast differentiation which degrades the bone matrix, leading to bone destruction.
4) Hypoxemia is a common symptom in COVID-19 infected patients needing high-level oxygen support with ICU care. Hypoxia stimulates the production of pro-osteoclastogenic cytokine and hypoxia inducible factor (HIF-1α) inhibits osteoprotegerin (OPG). The acidosis followed hypoxia upregulates RANKL and nuclear factor of activated T cells cytoplasmic 1 (NFATc1), acting to facilitate osteoclast formation and bone destruction.
5) Extra-traumatic osteonecrosis (sickle cell disease, hemoglobinopathies, autoimmune Systemic Lupus Erythematosus SLE, Gaucher disease, chronic renal failure, HIV infection, and intravascular coagulation) are common aspects of obstruction of vascular supply, autoimmune inflammatory and metabolic impairment that might be caused by SARS-CoV-2 infection and COVID-19 (Figure 15). [198]



Neurological manifestations by SARS-CoV2 (a) through ACE2 receptors (b) through cytokine release syndrome

Figure 15. COVID-19 infection affecting the skeletal system through multiple mechanisms.

Computational modelling and biochemical signaling studies have been used to predict musculoskeletal cellular targets and long-term consequences of the SARS-CoV-2 infection. [110]

Among the in vitro models of osteoclast differentiation, primary cell cultures and monocyte/macrophage-like cell linages with the capacity to form osteoclast-like cells, have been developed. Engineered bone tissue is available and is being used for SARS-CoV-2 mechanistic studies. [199], [31]

Several cells in the synovium express ACE2 and TMPRSS2, including fibroblasts, monocytes, B cells, and T cells (Figure 15-bottom). For articular cartilage, proliferative, hypertrophic, and effector chondrocytes (which are a subset of chondrocytes that appear to have a high level of metabolic activity) express ACE2, and only homeostatic chondrocytes (which appear to control circadian clock rhythm in cartilage) express TMPRSS2. In the meniscus, a small fraction of cartilage progenitors and regulatory fibrochondrocytes express ACE2, with no TMPRSS2 detected. [110]

Endocrine organ system symptoms and dynamics

Patients with diabetes mellitus and/or obesity are at risk of developing more-severe COVID-19 illness. Moreover, patients hospitalized with COVID-19 have exhibited a range of abnormalities of glucose metabolism including worsened hyperglycemia, euglycemic ketosis, and classic diabetic ketoacidosis. In a retrospective study from China, among a group of 658 patients hospitalized with COVID-19, 6.4% presented with ketosis in the absence of fever or diarrhea177. Of these, 64% did not have underlying diabetes (with an average hemoglobin A1c level of 5.6% in this group). [138], [139]

Pancreas related dynamics are affected by the fact that pancreatic cells highly express angiotensin-converting enzyme 2 (ACE2) so a direct cytopathic effect of SARS-CoV-2 may appear [200] and an indirect systemic inflammatory and immune-mediated cellular responses, results in organ damage.

COVID-19-related thyroid disorders could include thyrotoxicosis, hypothyroidism, nonthyroidal illness syndrome. COVID-19-related Subacute thyroiditis (SAT) is generally comparable to classical SAT and it can occur after or during COVID-19. Thyrotoxicosis in absence of neck pain is frequent in patients hospitalized for COVID-19. Low TSH and T3 and thyrotoxicosis appear to be predictors of poor outcome of patients hospitalized for COVID-19. [128]

ACE2 and TMPRSS2 expression levels are high in thyroid and more than in lungs affecting thyroid relegated infection dynamics. [79] Abnormal immune responses and cytokine storm associated with COVID-19 may induce thyroid gland inflammation. Two mechanisms (i.e., indirect and direct) might account for the changes in the thyroid gland and HPT axis. [173], [110], [201], [202], [128]

XX.5.3 SARS-CoV-2 cellular mechanisms

SARS-CoV-2 enter cells via the ACE2 receptor, critical actor itself in RAS renin-angiotensin system-driven cellular modulation using the serine protease TMPRSS2 (transmembrane protease, serine 2). Following receptor binding, proteolytic cleavage of the viral S protein by TMPRRS2 exposes a fusion peptide signal that permits mixing of viral and human membranes and release of viral RNA into the cellular cytoplasm. Once the viral RNA has access to the host cell cytoplasm (Figure 2), translation of viral proteins and replication of viral RNA can occur, ultimately leading to the assembly of virions. Once inside the host cell, the viral replicase protein complexes are translated and assembled, the viral RNA is synthetized, replicated and subsequently encapsulated resulting in the formation of the mature virus. Following assembly, virions are carried to the cell membrane into intracellular vesicles and released by exocytosis.

Several proteins encoded by SARS-CoV-2 viral RNA can also interact with various human cellular proteins to disrupt their function. Among the human proteins, processes, pathways and subcellular components predicted to be targeted by SARS-CoV-2 proteins are those involved with:

1. ***Intracellular vesicle trafficking***: SARS-CoV-2 exploits most of the cell machinery aimed at producing and releasing extra cellular vesicles, which in turn seem to be crucial components in the pathogenesis of virus infection. [203]
2. ***Ubiquitin ligases:*** The N-terminal of SARS-CoV-2 spike protein contains a PPxY L-domain motif that is known to hijack host cell WW-domain of Nedd4 E3 ubiquitin ligases and ultimately the Endosomal Sorting Complex Required for Transport (ESCRT) complex to enhance virus budding and spread. Importantly, this motif is absent in SARS-CoV and could explain why SARS-CoV-2 is more contagious than SARS-CoV, and can lead to severe and/or long-term systemic effects on pulmonary and extra-pulmonary organs. [204]
3. ***Nuclear transport:*** To successfully establish infection, SARS-CoV-2 overcomes the interferon (IFN)-mediated antiviral response using viral accessory protein Orf6, capable of inhibiting STAT1 nuclear translocation to block IFN signaling in the cells of body organ systems. [205]
4. ***Cytoskeletal stability:*** Cytoskeleton is an intricate network involved in controlling cell shape, cargo transport, signal transduction, and cell division. SARS-CoV-2's dynamic interactions between actin filaments, microtubules, and intermediate filaments of the host cell plays an

essential role for cytoskeleton in mediating the outcome of host–virus interactions. Disruption of host cytoskeleton homeostasis and modification state is tightly connected to a variety of organ systems cellular pathological processes, such as defective cytokinesis, demyelinating, cilia loss, and neuron necrosis. [205]

5. ***Mitochondrial respiration:*** The cellular innate immune system of a SARS-CoV-2 infected host target organ cell fighting the viral infection strongly relies on its membrane-bound cell organelles (mitochondrion, singular) that generate most of the chemical energy, adenosine triphosphate (ATP) needed to power the host cell's biochemical reactions. SARS-CoV-2 can evade host immune defense mechanisms by hijacking mitochondrial pathways, as recently suggested and the dysfunctional mitochondria could contribute to severe disease progression. [206]
6. ***Inflammatory signaling:*** The cytokines and signaling molecules, induced by SARS-CoV-2 infection, include C-X-C motif chemokine 10 (CXCL10), interferon gamma (IFN-γ), interleukin 1 beta (IL-1β), IL-6, IL-8, IL-17, and tumor necrosis factor alpha (TNF-α). [110]

SARS-CoV-2 infection of cells can lead to the production of more virus and can severely disrupt fundamental cellular functions and lead to eventual apoptosis. These apoptotic cells then contribute to tissue-level dysfunction and can also amplify local inflammation.

SARS-CoV-2 is thought to predominantly be a pulmonary disease targeting ACE2 and TMPESS217 expressing type-II pneumocytes that line the respiratory epithelium. The compromised alveolar epithelium in some patients with COVID-19 can lead to the development of viremias. Therefore, cells in other tissues may be susceptible to direct viral infection. **Viremia** is a medical condition where viruses enter the bloodstream and hence have access to the rest of the body. It is similar to bacteremia, a condition where bacteria enter the bloodstream. The name comes from combining the word "virus" with the Greek word for "blood" (haima). [110]

Since ACE2 is widely expressed in many of the human body organ systems COVID-19 symptoms are reported in many human body organ systems. Some of these effects are acute and easily detected via clinical measurement methods, other effects are more hidden and can be chronic long-term effects not always immediately identified by the onset of the disease. SARS-CoV-2 entry in the human body depends on the binding of spike (S) viral envelope protein to ACE2 expressing cells. Spike glycoprotein of SARS-CoV-2 not only binds the angiotensin converting enzyme 2 (ACE2) receptors, the same entry mechanism exploited by SARS-CoV-1, but in silico experiments indicated potential interaction also with dipeptidyl peptidase 4 DPP-4/CD26, a proposed pivotal event for hijacking and virulence, exactly how MERS-CoV works. [201]

DPP4/CD26 is variously expressed on epithelium and endothelial cells of the systemic vasculature, lung, kidney, small intestine and heart. In particular, DPP4 distribution in the human respiratory tract may facilitate the entrance of the virus into the airway tract itself and could contribute to the development of cytokine storm and immunopathology in causing fatal COVID-19 pneumonia. The cellular transmembrane serine protease

TMPRSS2, together with other proteinases is responsible for priming the S protein spike of the virus and allows viral cellular entrance via endocytosis. Alanyl aminopeptidase (ANPEP) and dipeptidyl peptidase-4 (DPP4), known receptors for other human CoVs may act as co-receptors or as auxiliary SARS-CoV-2 receptors facilitating SARS-CoV-2 cellular entrance. Furthermore, the identification of the transmembrane glycoprotein CD147 as well as the presence of furin-like cleavage sites in the spike (S) protein (absent for other SARS-CoVs) might be associated to viral–human cell molecular mechanisms of invasion and pathogenicity.

XX.5.4 Immunity dynamics following SARS-CoV-2 infection

Immunity to viral infection is caused by a variety of specific and nonspecific mechanisms. The activation of different immune functions and the duration and magnitude of the immune response depend on how SARS-CoV-2 interacts with host cells. SARS-CoV-2 can cause cytolytic, steady-state, latent, and/or integrated infection depending on the cell type and the related cellular viral load. Organ system damage is also linked to how the virus spreads (by local, primary hematogenous, secondary hematogenous, and/or nervous system spread). Therefore, viral antigens may be present in different parts of the body depending on the route of spread and phase of infection. Local infections at surfaces such as the mucosa can elicit humoral (IgA) and local cell-mediated immune responses, but not necessarily systemic immunity. The host has multiple immune defense functions that can eliminate virus and/or viral disease.

Humoral immunity occurs when the SARS-CoV-2 virus and/or virus-infected cells can stimulate B lymphocytes to produce antibody (specific for viral antigens). Antibody neutralization is most effective when the virus is present in large fluid spaces (e.g., serum) or on moist surfaces (e.g., the gastrointestinal and respiratory tracts). IgG, IgM, and IgA have all been shown to exert antiviral activity. Antibody can neutralize virus by 1) blocking virus-host cell interactions or 2) recognizing viral antigens on virus-infected cells which can lead to antibody-dependent cytotoxic cells (ADCC) or complement-mediated lysis. IgG antibodies are responsible for most antiviral activity in serum, while IgA is the most important antibody when viruses infect mucosal surfaces.

The term cell-mediated immunity refers to (1) the recognition and/or killing of virus and virus-infected cells by leukocytes and (2) the production of different soluble factors (cytokines) by these cells when stimulated by virus or virus-infected cells. Cytotoxic T lymphocytes, natural killer (NK) cells and antiviral macrophages can recognize and kill virus-infected cells. Helper T cells can recognize virus-infected cells and produce a number of important cytokines. Cytokines produced by monocytes (monokines), T cells, and NK cells (lymphokines) play important roles in regulating immune functions and developing antiviral immune functions. A number of cytokines, including IL-6, IL-1β, tumor necrosis factor α (TNF-α) and IFN-γ, have been frequently reported to be elevated in COVID-19. A putative systemic outcome due to this effect is known as

cytokine release syndrome (CRS), also called ‘cytokine storm’. CRS, characterized by an overactive immune response that results in an excessive systemic increase in pro-inflammatory cytokines in response to SARS-CoV-2 viral human cell body invasion, is believed to be a major cause of tissue damage in the pathophysiology of COVID-19. CRS is a two-step process where the primary response is characterized by the activation of innate immunity following viral infection in epithelial cells. Epithelial, innate immune and endothelial cells release several cytokines to block the viral replication, while effector cells are recruited to remove infected cells. A secondary cytokine cascade is induced downstream by the sustained release of primary cytokines or by immune cell signaling. IL-6, the most important CRS causative cytokine, is found to be increased in the serum of COVID-19 patients presenting with acute respiratory distress syndrome (ARDS).

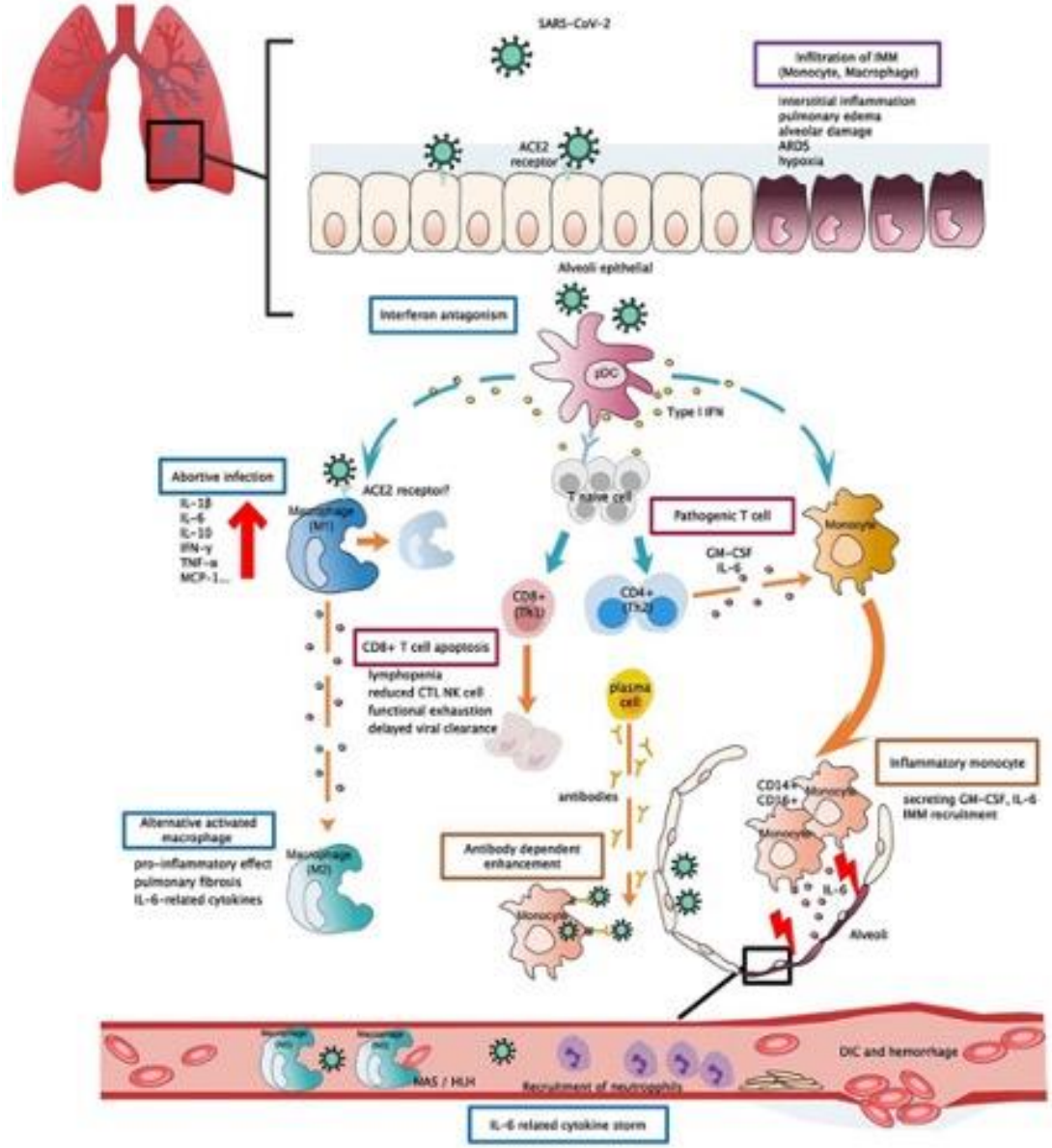

SARS-CoV-2
Infiltration of IMM
(Monocyte, Macrophage)
Interstitial inflammation
pulmonary edema
alveolar damage
ARDS
hypoxia
ACE2
receptor
Alveoli epithelial
Interferon antagonism
pDC
Type I IFN
Abortive infection
IL-1β
IL-6
IL-10
IFN-γ
TNF-α
MCP-1...
ACE2 receptor?
Macrophage
(M1)
T naive cell
Pathogenic T cell
Monocyte
GM-CSF
IL-6
CD8+
(Th1)
CD4+
(Th2)
CD8+ T cell apoptosis
lymphopenia
reducnd CTL NK cell
functional exhaustion
delayed viral clearance
plasma
cell
antibodies
Inflammatory monocyte
secreting GM-CSF, IL-6
IMM recruitment
CD14+
CD16+
Monocyte
Monocyte
IL-6
Alveoli
Alternative activated
macrophage
pro-inflammatory effect
pulmonary fibrosis
IL-6-related cytokines
Macrophage
(M2)
Antibody dependent
enhancement
Monocyte
DIC and hemorrhage
MAS / HLH
Recruitment of neutrophils
IL-6 related cytokine storm

Figure 16: Adapted from https://www.frontiersin.org/files/Articles/563286/fimmu-11-02033-HTML/image_m/fimmu-11-02033-g001.jpg

Naive T-helper cells (T_h0) can detect novel pathogens never encountered before, as is the current case of SARS-CoV-2. T_h0 then polarize the immune response into T_h1, the default response in immunocompetent subjects to intracellular or phagocytosable pathogens like SARS-CoV-2. This response is mediated by macrophages and T-cytotoxic (T_c) cells. Under some circumstances, the T_h0 polarize the immune response into T_h2, which is classically directed against extracellular non-phagocytosable pathogens, whose main effectors are eosinophils, basophils, mastocytes and B cells. Research on COVID-19, indicates that the immune system mounts a T_h2 response against SARS-CoV-2 in patients requiring intensive care, rather than a T_h1 response, which would keep the infection under control by means of macrophages and T_c cells.

It has been reported that a life-threatening escalation from T_h2 immune response to type 3 hypersensitivity (*immune complex disease*) in COVID-19 vasculitis takes place, and that the inflamed smooth muscle cells of blood vessels concur to the «cytokine storm» via interleukin-6. Faced with this scenario, it is possible that T_h1 and T_c lymphocytes are the immune cells most affected by the viral load, especially in the elderly, and that the immune system is forced to mount a T_h2 response, the only one still mountable, in the attempt to counteract SARS-CoV-2 by the action of T_h2 effectors. However, in this way, the symptomatic patient experiences all the negative effects of the T_h2 response, which can seriously aggravate the clinical picture. [202]

The two most frequently mentioned causes of multiorgan damage produced by SARS-CoV-2 are (i) the direct viral toxicity and (ii) cytokine-release syndrome or cytokine storm.
The direct viral toxicity is expected by the entrance of SARS-CoV-2 in the affected organs mediated by the protein ACE2 ([110], [201], [202], [112]), which is abundant in many human organs [112], and has been identified as the receptor mediating the endocytic cell entrance of SARS-CoV-2. [113]
The second attributed cause for extrapulmonary injury in COVID-19 patients is believed to be produced by the dysregulation of the immune system response. [1] It is a consequence of a hyperactive immune response of the innate immune system to the viral infection, releasing levels of interferons, interleukins, tumor-necrosis factors, chemokines and other mediators that result injurious to host cells. [114], [108], [91] This hypothesis is supported by the report of higher-than-normal levels of cytokines in patients' blood, particularly of IL-6. [92], [93], [94]
Other authors considered this impairment in the IL-6 levels typically produced by "cytokine storm" and the ones found in COVID-19 patients and observed that the median levels of IL-6 in patients with hyperinflammatory phenotype of ARDS are 10 to 200-fold higher [99], [1] than those observed in patients with severe COVID-19, so claiming that "cytokine storm" is not relevant to COVID-19.
Additionally, other key mechanisms of multiorgan damage mentioned in the literature include endothelial cell damage and thromboinflammation and the dysregulation of the renin-angiotensin-aldosterone system. [1]

According to the review of the literature by Gupta et al. [1], hematological, gastrointestinal, and endocrine manifestations are mainly produced by the direct toxicity of the virus mediated by ACE2. Dermatologic manifestations are however due to immune response to the virus, mainly produced by the cytokine-release syndrome. The rest of manifestations, cardiovascular, renal, hepatobiliary and neurologic, are considered to result from multifactorial causes with main components coming from direct viral toxicity and cytokine storm. Apart from a few cases reporting the isolation of the virus in myocardial tissue, as well as from gastrointestinal epithelial cells, the hypothesis of direct virus toxicity is supported by the abundance of ACE2 in the affected organs, i.e., this is the case in lymphocytes, kidney, liver, pancreas, and brain. It should be added here that ocular damage has also been reported in several COVID-19 patients. These ocular manifestations are believed to be caused by the viral interaction with ACE2 receptors which are abundant in the conjunctiva and cornea inferior parts.
Estrada proposed a new plausible mechanism by mean of which SARS-CoV-2 produces extrapulmonary damage in severe COVID-19 patients. The mechanism involves the existence of vulnerable proteins (VPs), which are (i) mainly expressed outside the lungs; (ii) their perturbation is known to produce human diseases; and (iii) can be perturbed directly or indirectly by SARS-CoV-2 proteins. These VPs are perturbed by other proteins, which are: (i) mainly expressed in the lungs; (ii) are targeted directly by SARS-CoV-2 proteins; (iii) can navigate outside the lungs as cargo of extracellular vesicles (EVs); and (iv) can activate VPs via subdiffusive processes inside the target organ. Using bioinformatic tools and mathematical modelling 26 VPs and their 38 perturbators were identified, which predict extracellular damage in the immunologic endocrine, cardiovascular, circulatory, lymphatic, musculoskeletal, neurologic, dermatologic, hepatic, gastrointestinal, and metabolic systems, as well as in the eyes. [207]

Understanding the multitude of signaling routes affected by SARS-CoV-2, in a cell/tissue-specific manner, can be crucial for the comprehension of the pathobiology as well as the therapeutics for COVID-19 [208]. Possible mechanisms of blood transmission after SARS-CoV-2 viral infection have been described. After SARS-CoV-2 enters into the lungs from the mouth and throat, and infects cells, the virus replicates in the cells and releases more new viruses. Massive accumulation of SARS-CoV-2 leads to a surge of immune cells and pro-inflammatory cytokines, which results in a rapid increase in CK levels in the blood or releasing more virus particles into the blood circulation. The virus and cytokines positively induce high expression of ACE2 in the intestinal epithelium and other organs, which accelerates overexpression of ACE2 and SARS-CoV-2 viral binding, causing target organ system effects [14].

XX.6 Risk factors

Numerous studies, including reviews of existing PubMed literature and retrospective cohort studies show that certain genetic, cellular, and organ-level phenotype and factors lead to an increased risk for both progression of the disease and increased mortality. Identification of these factors is important to predict the severity of disease and to make policy decisions regarding restrictions of movement and contact prohibition.

In this section, we review risk factors detected at various levels of biological organization, such as genetic, molecular, cellular, tissue and various in silico and cell-based methodologies that have been used to identify and validate those risk factors. The analysis of methodologies will also help in designing in vitro tests to identify the risks in various human populations.

XX.6.1 Genetic factors

Genome-wide association for severe COVID-19 have been conducted indicating potential molecular and genetic mechanisms involved in the disease. Associations between risk of severe COVID-19, a multigene locus at 3p21.31 and the ABO blood group locus at 9q34.2 has been reported. Patients with blood group A were reported to have an increased risk of severe COVID-19, and those with blood group O have been reported to have a decreased risk. The frequency of the rs11385942 insertion–deletion GA or G variant at locus 3p21.31 has been reported more in patients who received mechanical ventilation, indicating that this risk allele is associated with more severe forms of COVID-19. There are six different candidate genes at 3p21.31 locus, and LZFL1, and the proteins involved in protein trafficking to primary cilia, which have been highlighted as targets to consider. [209]

Regional distribution of frequent Human Leukocyte Antigen (HLA) haplotypes has been studied. [210] However, to understand if HLA haplotypes could be associated with disease susceptibility, global networks with patient information from varying geographic areas need to be built.

The entry of SARS-CoV-2 into the cells depends for the major part on the host factor ACE2 and the transmembrane serine protease TMPRSS2 for SARS-CoV-2 spike (S) protein priming. ACE2 is encoded on the X-chromosome and is involved in the catalysis of angiotensin II to angiotensin-(1–7) which is a vasodilator. It has been reported that unique DNA polymorphisms exists in ACE2 and TMPRSS2 in different populations which might lead to variations in genetic susceptibility towards SARS-CoV-2. For example, certain variants have been found to inhibit the interaction between ACE2 and spike protein. [211]

Toll-like receptors (TLR) are a family of immune sensor proteins and have a crucial role in the cytokine expression in the immune system. In addition, TLR genes show distinct population distribution pattern and are also subject to selection pressure and could thus play a key role in the

differential susceptibility to SARS-CoV-2 infection. [211] It has been shown that patients with severe form of COVID-19 were associated with a rare putative loss-of-function variants of X-chromosomal TLR7. [212]

While the analysis of gene polymorphisms is mainly carried out by whole exome sequencing, many of the annotation and association studies were carried out using Genome Wide Association studies (GWAS) and ANNOVAR, a bioinformatics tool for functional annotation of genetic variants.

XX.6.2 Cellular factors

Apart from polymorphisms, a genome-wide association study showed that the expression levels of ACE2 itself is significantly associated with a higher risk for severe COVID-19 infection. [213], [214]

Male sex, cardiovascular disease, and diabetes, all of which have been reported to be risk factors for severe COVID-19, were also associated with higher levels of ACE2. Similarly, the levels of TMPRSS2 have also been proposed as a biomarker for SARS-CoV-2. [215]

A coagulopathy has been reported in up to 50% of patients with severe COVID-19 manifestations. An increase in Ddimers is the most significant change in coagulation parameters in severe COVID-19 patients, and progressively increasing values can be used as a prognostic parameter indicating a worse outcome. [216]

In a study conducted to develop a model which could predict the risk to a fatal outcome in COVID-19 patients, a prospective study was conducted and the study found that high IL-6 level, C-reactive protein level, lactate dehydrogenase (LDH) level, ferritin level, D-dimer level, neutrophil count, and neutrophil-to-lymphocyte ratio were all predictive of mortality in COVID-19. [217]

Neutrophil extracellular traps (NETs) are extracellular webs of chromatin, microbicidal proteins, and oxidant enzymes released by neutrophils in response to infections. A study found correlation between high serum levels of NET and hospitalization of COVID patients. [218] However, further studies are required to estimate the predictive power and risk associated with circulating NETs.

Systemic inflammation is often associated with development of atherosclerosis, type 2 diabetes, and hypertension, which are well known comorbidities that can increase the risk for adverse outcomes associated with COVID-19. [219]

Studies show that SARS-CoV-2 infection pathogenesis is related to oxidative stress in patients in response to infection. [220] As mitochondria are the hub of cellular oxidative homeostasis, the increased inflammatory/oxidative state during COVID-19 infection may lead to mitochondrial dysfunction further causing platelet damage and apoptosis. The interaction of dysfunctional platelets with coagulation cascades can

also aggravate clotting and thrombus formation. With the critical role of mitochondria in inflammation and oxidative stress, low mitochondrial fitness has also been suggested as a risk factor for severe COVID-19. [221]

A study found that SARS-CoV-2 plasma viremia is commonly detected in hospitalized individuals and symptomatic non-hospitalized outpatients diagnosed with COVID-19. In addition, they found that SARS-CoV-2 viral loads, especially within plasma, are associated with systemic inflammation, disease progression, and increased risk of death.

The expression levels of cellular factors were determined using single cell RNA sequencing, and distribution patterns were visualized using cryo-electron microscopy and histopathology. In many cases, cellular events, such as death, injury, or presence of D-dimers were assessed by detecting biomarkers for specific events.

Genetic	Cellular	Tissue/Organ	Organism
HLA Haplotype [210] GA variant at 3p21.31 locus [209] ABO blood group [209] ACE2 polymorphism [211] TMRPSS2 polymorphism [211] TLR polymorphism [211] CCR5 polymorphism [222] IL6 polymorphism [223] effect of various cytokine SNPs on miRNAs (in silico work): [224] and 2) the heme oxygenase: [225]	ACE2 levels [214], [213] TMRPSS2 levels [215] Coagulopathy [216] Pro-NET-otic state [218] Presence of D-dimers [216] Inflammation [219] Apoptosis [226] IL-6, CRP levels [217] Low mitochondrial fitness [221] SARS-CoV-2 viral loads [227]	Pulmonary fibrosis [228] Myocardial infarction [229] Kidney injury [230] Arrythmia [231] Cytokine-mediated brain damage Sepsis [232] Necrosis of adrenal gland	Higher age Chronic lung disease Heart disease Diabetes Severe obesity (BMI>40) Kidney/liver diseases
Methodologies used			

Genome-wide association studies (GWAS) Whole exome sequencing ANNOVAR (ANNOtate VARiation)	Single cell RNA sequencing Biomarkers for cellular injury, cell death in serum D-dimer assay Cryo-Electron microscopy Histopathology Immunohistochemistry GO analysis (up/down regulation of genes)	Electrocardiogram (ECG) MRI CT scan Autopsy Radiography Metanalysis and correlation studies	

Table 3. Genetic, molecular, cellular, tissue and organism cell-based methodologies that have been used to identify and validate SARS-CoV-2 risk factors.

XX.6.3 Tissue/organ level

Pulmonary fibrosis is associated with permanent changes in lung tissue architecture and function, and it has been shown to complicate SARS-CoV-2 infection. Myocardial injury, visualized using biomarkers, is another risk factor which can exaggerate the clinical course and worsen the outcomes of COVID-19 infection.

Using multivariate analysis, a study showed association of acute kidney injury with not just disease severity, but also lymphopenia and D-dimer levels.

While bacterial infections are often the main cause of sepsis, viral sepsis, again monitored using biomarkers of sepsis, is often ignored and was shown to be a risk factor associated with COVID-19 mortality.

XX.6.4 Organism-level

Table 4 includes the risk factors that have been reported by the Centers for Disease Control and Prevention. The factors that are commonly mentioned include higher age, male gender, obesity, diabetes, and diseases associated with liver, heart, renal, and respiratory system. Higher age is one of the significant and consistent risk factors, and this could be due to declining immune function at older age, increased likelihood of comorbidities or the associated medications. [233]

While ethnicity has been reported as one of the risk factors, currently not enough evidence of the impact of ethnicity on the risk associated with acquiring the infection and progression to worse clinical outcomes has been demonstrated. [234]

Both organ and organism level risk information can be obtained using whole organ-level structure and function abnormalities can be captured using electrocardiogram (ECG), computed tomography (CT scans), magnetic resonance imaging (MRI), or histopathology of autopsy samples in some cases.

XX.7 Vulnerabilities

Epidemics and pandemics, such as COVID-19, often disproportionately effect vulnerable populations. Thus, there is a need to identify the populations that are either vulnerable to increased risk of infection or inferior health outcome as this information can help in informing where additional resources are most needed. In this section, we discuss the vulnerabilities towards severe prognosis of COVID-19 in various populations , such as malnourished, pregnant, and immunocompromised populations. We also discuss the cell-based strategies used to identify or visualize the cellular or molecular effects associated with COVID-19 vulnerability.

XX.7.1 Malnutrition

There is increasing evidence that suggests that COVID-19 may be associated with negative outcome in hypoalbuminergic patients and patients with high body-mass index (BMI). Interestingly, malnutrition is associated with immunosuppression, while overnutrition is associated with chronic inflammation, both of which are known risk factors of severe COVID-19.

Vulnerable population*	Cellular/tissue-level effects	Methodologies used to visualise cellular/molecular effects
Malnourished [235]	Immunosuppression Lymphopenia	
Population with high BMI [236]	Chronic inflammation Increased cytotoxic T cells Increased effector T cells	**For B and T cell numbers** Biomarkers for inflammation Lysispot assay [237] IFNγ Elispot [237] Flow cytometry Cytotoxic T lymphocyte (CTL) activity assay [238] (CTLp) frequency assessment [238] Tetramers [239] Immunoscope [239] OT T-cell system [238]
Old age [240]	Limited capacity of the thymus to generate "naive" T cells Reduced ability to regulate production of macrophages and neutrophils Immunosupression (reduced number of B and T cells)	**For Macrophage number and activity** Quantitative phase imaging and Raman spectroscopy [241]
AIDS Patients	Immune deficiency (Progressive loss of CD4 (+) T cells)	**For inflammation** Inflammatory biomarkers
Patients on immunosuppressants (such as corticosteroids)	Dose-dependent effects on: Reduced number of circulating B and T cells Suppression of lymphocyte activation and in the production of antibodies by B cells.	**Placental breach** Single cell RNA sequencing [174]
Pregnancy [242], [174]	Anti-inflammatory	

	Immunosuppression Placental breach	
Diabetes [243],[244]	Reduced forced vital capacity Reduced forced expiration volume Inflammation	
Hypertension	Increased troponin [245] Cardiac injury [246] Fibrosis [246]	**Tissue injury/fibrosis** Cardiovascular magnetic resonance [247] CT scan

**Known risk factors for severe COVID-19*

Table 4. Cell and tissue-based strategies used to identify or visualise the cellular or molecular effects associated with COVID-19 vulnerability.

XX.7.2 Immunocompromised conditions

Patients suffering from HIV infection, on immunosuppressant drugs or pregnant women, are in an immunodeficient state which could lead to inabilities in establishing immune response towards external infections. However, there is still insufficient evidence to suggest if reduced immune function is linked to more severe outcomes in COVID-19. The cellular responses in these conditions include suppression of circulating B and T cells and specifically CD4 (+) T cells in cases of HIV infection. Studies are looking at the proteomic and the transcriptomic profiles using RNA sequencing from lung samples of fatal COVID-19 cases and the authors suggest that patients' deaths may be due to uncontrolled host inflammatory processes. [248]

It has been reported that immunosuppressive therapies are not associated with increased risk for COVID-19. [249]

Using single cell RNA sequencing data, placental cells were found to express ACE2 and S protein priming protease TMPRSS2 during first and second trimester, indicating that placenta is permissive to SARS-CoV-2. Additionally, the placental cells also had the mRNA for proteins that physically interact with SARS-CoV-2 in host cells. [174]

XX.7.3 Presence of co-morbidities

Diabetes has been associated with reduced lung capacity, including forced vital capacity and forced expiratory volume in one second which could increase the risk for severe COVID-19. [243]

There is also increasing evidence that suggests that diabetes is an inflammatory disease where Type 1 diabetes is characterized by autoimmune-mediated destruction of pancreatic β cells. [250] Thus, existing inflammation in diabetes (detected using biomarkers for inflammation) may increase the risk of severe COVID-19.

Hypertension results in a number of pathophysiological changes in the cardiovascular system, including left ventricular hypertrophy and fibrosis. [246] These symptoms are also known risk factors for severe COVID-19, making the hypertensive heart particularly susceptible to SARS-CoV-2.

XX.8 Non-conventional or supportive treatments or approaches based on underlying cellular mechanisms

In this section, we list various molecular/cellular signaling pathways that are affected during COVID-19 infection, and treatment strategies that are based on effecting these pathways. We also discuss various *in silico*, cellular strategies and *in vitro* model systems that are being used to understand and assess the therapeutic potential of various therapeutic candidates.

The interruption of any stage of the viral life cycle can become a significant therapeutic approach for treating CoV-related diseases (Tables 5 and 6). A recent SARS-CoV-2-human protein-protein interaction analysis showed that SARS-CoV-2 contains many druggable proteins, each of which has several ligand binding sites. The most interesting coronavirus proteins are the S glycoprotein, proteases Mpro and PLpro, RdRP, and helicase. Broad-spectrum antivirals targeting the viruses and new alternative methods to identify damaged target organ systems are also used for identifying adverse target organ system features of the COVID-19 disease. [12], [16]

Based on the molecular, cellular, and tissue-level effects observed during COVID-19 infection, various drug targets are being investigated that affect one or more of these pathways.

Molecular/signalling effects	Treatment strategies

Binding of spike protein (S-RBD) and ACE2 host cell receptor	Decoy ACE2 receptors for virus attachment Blocking SARS-CoV-2 replication
Spike protein (S) priming by TMPRSS2	Blocking certain Serine proteases
Conversion of Ang I to Ang II by ACE2	ACE2 inhibitors
Activation of RAS signalling	RAS inhibitors
Activation of JAK/STAT pathway by IL-6	Downregulation of STAT3
Activation of MAPK pathway	MAPK inhibitors
Activation of NF-kB	Inhibition of NF-kB
Cellular/tissue effects	Treatment strategies
Pro-inflammatory cytokines (IL-6)	IL-6 receptor antagonists [251]
Venous thromboembolism Pulmonary embolism Hypercoagulability	Low molecular weight heparin (LMWH)
Pulmonary fibrosis	Antifibrotics (Nintedanib)
Organ injury	Systemic steroids
Hypoxemia	Bronchodilators, mechanical ventilation

Table 5. Molecular/cellular signalling pathways that are affected during COVID-19 infection, and treatment strategies that are based on effecting these pathways.

Methodologies					
In silico	**Cellular assays** **(to check drug mechanisms and efficacy)**	**Cell lines**	**Stem cells**	**Organoids**	**Organ-on-chip**
Molecular docking and simulation	*Cytokine biomarkers*	*Human airway epithelial cells*	*Mesenchymal stem cells*	*Lung organoid model*	*Human Airway Chip*

Network pharmacology *PK-PD models* *Simulating viral infection dynamics* *Tensor decomposition-based unsupervised feature extraction*	*Furin cleavage assay* *ELISpot cell-mediated immunity*	*In vitro* SARS-CoV-2 replication inhibition assay using VeroE6 and Caco-2 cells [252]		*Colon organoid model*	

Table 6: Mechanistic methods used to study effect of interfering with stages of SARS-CoV-2 viral life cycle at the molecular, cellular and tissue level as treatment strategies.

XX.8.1 In silico strategies

Molecular docking and simulation

Docking studies have been performed over the binding pocket of COVID-19 to find potential small molecules, including approved drugs and compounds undergoing clinical trials to combat SARS-CoV-2. [253]

Docking studies have also been performed to determine the binding energies of compounds/drugs in isolation or in combination with other drugs. For example, a study showed that Caulerpin and its derivatives could be used in combination with lopinavir, simeprevir, hydroxychloroquine, chloroquine, and amprenavir to disrupt the stability of SARS-CoV-2 receptor proteins and so increase the antiviral activity of these drugs. [254]

Network pharmacology

Network pharmacology and bioinformatics analysis, including KEGG molecular interaction/reaction network diagram pathway, Gene Ontology (GO) enrichment analysis and protein-protein interaction network have been evaluated. [255] The GO analysis results of SARS-CoV-2 differentially expressed genes (DEGs) revealed that the most significant terms were type I interferon signaling pathway, cellular response to type I interferon, response to type I interferon, defense response to the virus, and mRNA binding involved in posttranscriptional gene silencing. Influenza A, Measles, NOD-like receptor signaling pathway, Hepatitis C, Herpes simplex infection, Cytokine-cytokine receptor interaction were

the most enriched pathways in KEGG (database resource for understanding high-level functional and biological systems from large-scale molecular data generated by high-throughput experimental techniques) enrichment analysis. Identification of drug targets using network pharmacology approaches by identifying compounds that show overlapping differentially expressed genes with SARS-CoV-2 is an avenue that is routinely applied.

Pharmacokinetic-pharmacodynamic models (PK-PD)

A recent study by Garcia-Cremades et al. developed a mechanistic pharmacokinetic model to predict the SARS-CoV-2 rate of viral decline and QTc prolongation associated with various doses of Hydrochloroquinone (HCQ). The simulation showed that while HCQ doses > 400 mg predicted the rapid decline of viral loads, however, HCQ doses > 600 mg were also predicted to prolong QTc intervals. [256] Thus, the model helped to evaluate the benefit/risk analysis of high doses for a drug or a compound.

Simulating viral infection dynamics

A recent study by Wang et al. built a mathematical model to study various features of SARS-CoV-2 infection by examining the interaction between virus, cells and immune responses. [257] The simulation showed that in the first stage, the viral load increases rapidly and reaches the peak, followed by a plateau phase possibly due to lymphocytes as a secondary target of infection.

Then, the viral load decreases due to the emergence of adaptive immune responses. The model also showed that anti-inflammatory treatments or antiviral drugs combined with interferon reduced the duration of the viral plateau phase and the time to recovery. Thus, such models that help in understanding the infection dynamics might also help in designing appropriate treatment strategies.

Tensor decomposition-based unsupervised feature extraction for drug discovery

A study by Taguchi and Turki proposed a tensor decomposition (TD)-based unsupervised feature extraction (FE) method to predict drug candidate compounds without knowledge of known compounds. [258] TD-based unsupervised FE was applied to the gene expression profiles of multiple lung cancer cell lines infected with severe acute respiratory syndrome coronavirus 2 (SARSCoV-2). Around 163 genes were identified as differentially expressed genes (DEGs) in SARS-CoV-2 infection, and drugs were screened based on the coincidence of DEGs between drug treatments and SARS-CoV-2 infection.

XX.8.2 Fit for purpose cellular assays

Various bioassays have been devised to detect biomarkers, antibodies and therapeutic responses, and neutralization of the virus.

Cytokine biomarkers

The release of pro-inflammatory cytokines, post SARS-CoV-2 infection has been shown to be responsible for the respiratory complications associated with the disease. Thus, cytokine biomarkers can be monitored in patients enrolled in SARS-CoV-2 trials, to understand mechanism of action and effect of the treatment.

Furin cleavage assay

SARS-CoV-2 uses furin, a protease highly expressed in the lung, to cleave S-protein into S1 and S2 which then binds to ACE2 receptor. Intracellular furin also helps in packaging new viral particles. Certain therapies work by blocking the interaction of S protein and furin or by targeting furin. For this class of therapeutic, furin cleavage assay can be used as a proof of concept/mechanism of action.

ELISpot cell-mediated immunity

Many therapies are focused on affecting the T-cell cytokine as that is one of the primary drivers of progression to severe COVID-19. T-cell responses can be assessed using enzyme-linked immune absorbent spot (ELISpot) assays. This in vitro assay can screen responses in a cost-effective manner to an entire pathogen proteome and estimate memory response in patients.

XX.8.3 Cell lines

Human airway epithelial cells

Human airway epithelial cells highly express the angiotensin-converting enzyme 2 (ACE2) and transmembrane serine proteinase 2 (TMPRSS2), the receptor that the virus uses to prime the S protein (spike protein of SARS-CoV-2). These cells also show cytopathic effects 96h after infection. Drug validation studies were conducted on differentiated air-liquid interface (ALI) cultures of proximal airway epithelium. [259] ALI media containing drugs was added to the cultures 3 hours prior to infection, then, 100 μL of SARS-CoV-2 viral inoculum was added, and after 2 hours of viral adsorption, cells were washed and fresh media containing drugs was added. Remdesivir strongly suppressed viral infection/replication, indicating the potential relevance of this platform.

Vero-E6, Caco-2 cell lines

Vero-E6 cells, the most widely used cell lines to replicate and isolate SARS-CoV-2, are isolated from kidney epithelial cells of the African green monkey. Recently, the antiviral activity of 1,520 approved drugs from the Prestwick Chemical Library were tested on Vero-E6 and Caco-2 cells. [252] Of them, eleven compounds, including macrolides antibiotics, proton pump inhibitors, antiarrhythmic agents or CNS drugs showed antiviral potency with $2 < EC50 \leq 20$ μM.

XX.8.4 Mesenchymal stem cells

The role of mesenchymal stem cells (MSCs) in promoting regeneration and suppressing inflammation at sites of injury have been well-recognized. Many clinical trials have explored the role of MSCs to reduce inflammation in patients with osteoarthritis, multiple sclerosis, type 1 diabetes mellitus, and influenza virus-induced lung injury. Two reports from Chinese hospitals document the outcome for patients receiving MSCs for SARS-CoV-2 infections. Both studies showed significant clinical improvement in the test group compared to the control group. [260] MSCs have been shown to have broad immunoregulatory properties through the interaction of immune cells in both innate and adaptive immune systems. [261] This can lead to immunosuppression of many effector activities, and reduction of the cytokine storm, indicating the potential role of MSCs in COVID-19 treatment.

Extracellular vesicle (EV) includes both exosomes and microvesicles (MVs). The diameter of exosome is less than 200 nm, while the diameter of MV can reach up to 1000 nm. Exosomes secreted from MSCs have been studied in various model systems and have been shown to reduce edema in human injured lungs in an *ex vivo* lung perfusion model. [262] Thus, exosomes are also emerging as a therapeutic option for treatment of organ injury during COVID-19. [263]

XX.8.5 3D tissue/organoids

Lung organoid model

Han et al. developed a lung organoid model using human pluripotent stem cells (hPSCs). [264] Various cell types, including alveolar type II (AT2) cells, alveolar type I (AT1) cells, stromal cells, proliferating cells, a low number of pulmonary neuroendocrine cells and airway epithelial cells could be identified in the lung organoid. Post SARS-CoV-2 infection, there was a robust production of chemokines and cytokines with little type I/III interferon signaling, which is also present during human COVID-19 infection. A high throughput screen was performed using hPSC-derived lung organoids which identified FDA-approved drug candidates, including imatinib and mycophenolic acid, as inhibitors of SARS-CoV-2 entry.

Colon organoid model

Han et al. developed hPSC-derived colonic organoids (hPSC-COs) COs). [265] These organoids consisted of multiple colonic cell types, especially enterocytes, expressed ACE2 and were permissive to SARS-CoV-2 infection. The authors performed a high throughput screen of FDA-approved drugs, including imatinib, mycophenolic acid (MPA), and quinacrine dihydrochloride (QNHC). The treatment of these drugs at physiologically relevant levels inhibited the SARS-CoV-2 infection of colonic organoids, indicating these model systems as a valuable resource for drug screening to identify candidate COVID-19 therapeutics.

XX.8.6 Organ-on-chip

In a preprint, Si et al. developed human Airway Chip which is a microfluidic device where primary human lung airway basal stems cells are grown under an air-liquid interface (ALI) along with the primary human lung endothelium which is grown on the opposite side. [266] The lung epithelium is also exposed to continuous fluid flow. The chip supports differentiation of the lung airway basal stem cells into a mucociliary, pseudostratified epithelium; airway-specific cell types, such as ciliated cells, mucus-producing goblet cells, club cells, and basal cells. It also showed establishment of continuous ZO1-containing tight junctions and cilia; permeability barrier properties; and mucus production similar to a human airway *in vivo.* This system was used to test inhibitory activities of 7 clinically approved drugs, including chloroquine, arbidol, toremifene, clomiphene, amodiaquine, verapamil, and amiodarone. While all seven of these drugs inhibited infection by viral pseudoparticles expressing SARS-CoV-2 spike protein in cell lines (Huh-7 cells, Vero cells), only two of them inhibited infection by viral infection in airway chips, suggesting they can be used in conjunction with cell-based screening assays to expedite drug repurposing in SARS-CoV-2 infection.

XX.8.7 Diamagnetism as an alternative or integrating cellular therapy for COVID-19

Naturally occurring electric fields are not only important for cell-surface interactions but are also pivotal for the normal development of the organism and its physiological functions. Selective control of cell function by applying specifically configured, weak, time-varying magnetic fields has added a therapeutic dimension to biology and medicine, e.g., diamagnetic therapies. The word "diamagnetic" originated from diamagnetism, which refers to the magnetic property of some materials which, subjected to a sufficiently intense magnetic field, receive a repulsive and distancing force with respect to the magnetic source. One of the most common diamagnetic materials is body water, and most proteins and ions are diamagnetic. Using diamagnetic therapy, there is a powerful effect on cellular features including specific cell membrane mechanisms reducing edema and inflammatory processes and the damaged tissue is quickly repaired with an immediate analgesic effect. Field parameters for therapeutic, pulsed electromagnetic field (PEMFs) were designed to induce voltages similar to those normally produced during dynamic

mechanical deformation of e.g., in human body connective tissues. As a result, a wide variety of challenging musculoskeletal disorders have been treated successfully over the decennia. Patients with delayed union or non-union fractures have benefitted, from this surgically non-invasive method. Many of the athermal bioresponses, at the cellular and subcellular levels, have been identified and found appropriate to correct or modify the pathologic processes for which PEMFs have been used supported by double-blind trials. As understanding of underlying molecular and cellular mechanisms expand, specific requirements for field energetics are being defined and the range of therapeutic broadened. These include nerve regeneration, wound healing, graft behavior, diabetes, and myocardial and cerebral ischemia (heart attack and stroke), among other conditions. [267]

The biophysical stimulation induced by Low Frequency – High-Intensity Pulsed Electromagnetic Fields (LF- HI- PEMF) – Diamagnetotherapy, has recently been demonstrated to be effective in the treatment of lung fibrosis associated to autoimmune diseases as well as to fibrosis in post COVID-19 pneumonia.

In both conditions that share a dysregulation of the immune system, diamagnetotherapy, ameliorated the functionality of respiratory muscles in treated patients, improved dyspnoea, oxygen saturation, indicative of a better performance of the lung. In these cases, a double effect in lung parenchyma and in respiratory muscles has been evaluated, also considering that in post-intensive care syndrome, patients report muscle weakness, impaired mobility and balance, joint stiffness while the associated neuropathy and myopathy are a consequence of SARS-CoV-2 infection. These clinical conditions worsen the morbidity and the mortality in COVID-19 patients. Diamagnetoteraphy opens the possibility to treat symptoms in COVID-19 patients such as epilepsy, cognitive impairment, or motor imbalance, including the problems of the peripheral nervous system.

The rationale to employ diamagnetotherapy for medical problems is attributed to the intensity of the magnetic field that ensures the optimal and safe electric stimulation of the cell membranes. Moreover, the diamagnetic effect is also composed of a multi-variable spectrum of electromagnetic frequencies that selectively interact with the different electric state of the cell membrane in various situations. This phenomenon has been observed with electromyography fallowing the stimulation of the motor cortex in healthy individuals. [268] This opens the possibility to treat symptoms in COVID-19 patients such as epilepsy, cognitive impairment, or motor imbalance, including the problems of the peripheral nervous system.

Diamagnetotherapy takes its name from a mechanical repulsive effect on diamagnetic substances e.g., water. 60% of the human adult body is water. Already in 1945 the % of water content in human body organs was described for an adult (Table 7) with an average weight of 70.5 kg. [269]

Tissue	Water (%)
Lung	83.74
Striated Muscle	79.52
Kidney	79.47
Digestive tract	79,07
Spleen	78,69
Brain, spinal cord, nerve trunks	73.69
Hearth	73,69
Pancreas	73,08
Liver	71,46
Skin	64,86
Adipose tissue	50.09
Skeleton	31.81
Teeth	5
Liquid Tissues	93,33
Remaining Solid Tissues	70,40

Table 7. Water content in human body organs/tissues.

The diamagnetic phenomenon moves liquids and solutes of the extracellular matrix (ECM) and of the intracellular environment. This, positively stimulate the metabolic activities of the treated tissues, reduce fibrosis and ameliorate peripheric oedema in limbs and the possibility to treat lymphatic imbalance related to the prolonged COVID-19 related immobility is a possible scenario.

Although different in terms of variability and strength of the physical features, HI magnetic fields have therapeutic biological effects in comparison to the the more studied Low Intensity- PEMF with main regards to the anti-inflammatory, regenerative and trophic on the extracellular matrix (ECM). [270] The diversity consists in the interaction of HI-MF with a chain of diamagnetic nanoparticles of the cell membrane (ions, membrane receptor proteins, cholesterol, glycol, and phospholipids) and the intracellular cytosol. HI-MF modifies the hydrostatic pressure of the ECM and

the transmembrane flux of ions reflecting on the electric potential of the cells. The phenomenon is enforced by the variability of the frequencies of the Electromagnetic Field (Figure 17).

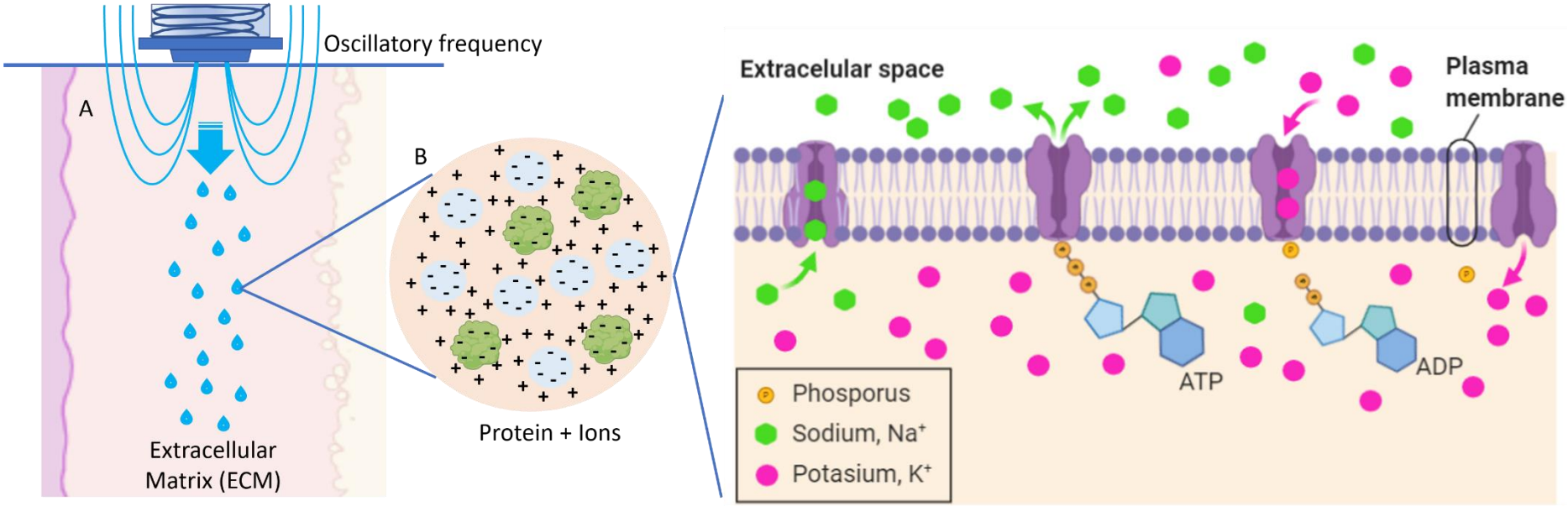


Figure 17. Diamagnetic Effect induced by the Low Frequency-High Intensity – Pulsed Magnetic Field (LF-HI-PEMF) – Diamagnetoteraphy (Simplified Model). The High Intensity of the Magnetic Flux mechanically moves liquids and solutes in the ECM (Extracellular Matrix) and inside the cell (Cytosol). B) Additionally, the electrochemical properties of Low Frequency – Variable Pulsed Magnetic Field induce in some ions (Ca^{2+}, Na^{+}, K^{+}, Li^{+}, Mg^{2+}) and in the diamagnetic proteins of the cell membrane oscillatory frequencies (C) able to enhance the transmembrane flows.

Variable intensities of the magnetic field inhibit IL-6 and TNF-α expression at the gene level as well as IL-6, IL-1β and TNFα expression at protein level in injured tissues. [271] This modulatory effect is confirmed in other studies that reveal the increase of the anti-inflammatory IL-10, observed in tendon cell cultures [272], and is also known for a strict relationship with the immune system in various, including autoimmune, pathologies.

[273] Reference to COVID-19 induced immune dysregulation is consequential. Finally, PEMF preserve ECM integrity of the cultured embryonic cartilage explants by modulating the metabolism of proteoglycans without affecting their gross structural nature.

These experiences show that diamagnetism-based therapeutic interventions should be further explored as an alternative or integrating cellular therapy for COVID-19 for e.g., brain, immune system muscles and parenchymal organs.

The chronobiology of the inflammatory process relates to the of IL-1, IL-6 and TNF. [274] This physiological self-control and cellular self-defense of inflammation is shown in the response time of IL-10, and TGF-Beta and increase in the genetic expression of the interleukins involved in the inflammatory process. [275]

However, we know that the rapid and exaggerated increase in IL-6 in patients with COVID-19 presents a response that can lead to a chronic increase in IL-6 leading to a chronic activation of IL-17. This leads to a response in the activity of specific antibodies to tissues, cells, cell membranes and/or intracellular organelles, which may have autoimmune or degenerative sequelae. However, the physiological response to the elevation of interleukin IL-6 and the subsequent elevation of IL-10 and TGF-beta [276] should be taken into account . As a consequence, we have an overexpression of these last two molecules, and the return to normality of TGF-beta is slow and usually occurs between 3-6 weeks after the acute pro-inflammatory event. However, the continuous activation of TGF-beta can cause changes in ECM [277], making it less soluble and evoking an increase in secondary fibrosis caused in the inflamated human body tissue areas. In the case of the post-COVID-19 patients, these tissues are identified by ACE receptors and through the stimulation given by chemotaxis and necrotaxis, these tissues change the inflammatory adaptation response of metalloproteinases. [278]

The ECM takes within its sol-gel regulation a management of remodeling and plasticity of the matrix. [279] This dynamic remodeling acts as a control in homeostasis, and protection of cell proliferation, migration and differentiation. As such, the ECM has a molecular filter control crucial for cell survival. [280] The ECM filter function is regulated by the electromagnetic and sterilization processes, which is maintained through the control of reactive oxygen species (ROS) and pH, the changes on the heparan sulfate chains thus stimulating the activation of metalloproteinases, capable of achieving changes in the sol-gel turn. [280], [281] The sol phase within the ECM has a protease activity, to initiate a reshaping of the matrix through the hydrolysis of proteoglycans. The maximum pH in this phase is 7.35, with sympathicotonic activation. The gel phase within the ECM has an anti-protease activity, performing a reconstruction and deposit of the matrix by means of the synthesis of matrix proteins. The pH peak in this phase is 7.45 so it is an alkaline phase with vagotonic activation. [282]

This regulation turn of the ECM is given by the activity of metalloproteinases (MMPs) that are 22 human proteolytic enzymes regulated by Th1 lymphocytes and therefore carry out degradation effects of the ECM. The regulation of these MMPs are made by the inhibitory tissue of metalloproteinases (TIMPs), which are 4 inhibitory proteins of MMP. These are regulated by Th2 lymphocytes and therefore carry out protein deposition in the ECM, this inhibition is carried out 1:1 from TIMPs to MMPs. [283], [284]

Therefore, inflammation as well as the increase in free radicals, increase the expression and secretion of the interleukins IL-1, IL-6 and TNF-alpha. This control in the degradation of matrix proteins is by means of the IL-4, IL-10 and TGF-beta1. This increases the deposit of glycoproteins and provides a control in physiological anti-inflammatory homeostasis. [285] Oxidative stress processes control cell aging. It is known that low levels of free radicals do not achieve the protection of cell survival that is needed and the high increase in oxidative stress causes a loss of oxidation reduction causing cell death and having low-grade chronic inflammation. [286]

ROS overexpression and pH reduction create pathological inflammation starting with an acute phase of inflammation like the one seen in COVID-19 in the acute and semi-acute phases, which enters into the sol phase as a degradative and inflammatory phase. At the same time, after the acute phase, a low-grade inflammation due to the increase in the expression and secretion of IL-6, causes as a regulatory control an increase in the secretion and expression of TGF-beta1, creating ECM rigidity due to an increase in the deposits of the proteoglycans giving the fibrotic phenotype. [287]
However, chronic alteration not only causes a rigidity of the ECM, it also creates an alteration of the intercellular junctions. In the case of inflammation caused by COVID-19, the mucosa represents alterations in the intercellular junctions of lung and receptors of the respiratory and oropharyngeal mucosa. In the processes of traditional inflammatory control, IL-10 activates the STAT3 receptors that decrease the expression of Claudin-2 and increase the expression of Claudin-4. TGF-beta activating the ERK and SMAD2 receptors, increasing the expression of Claudin-1, has been observed in autoimmune control processes in intestinal mucosa caused by Crohn's disease and treated with low interleukin dose and sequential kinetic activation. [288], [289], [290], [291]

An integrative treatment option with diamagnetic therapy is the implementation, penetration or implantation of therapeutic agents directly on the tissues under treatment. Due to the effect of diamagnetic repulsion movement, it is possible to introduce a product up to 8-10 cm deep (Figure 18).

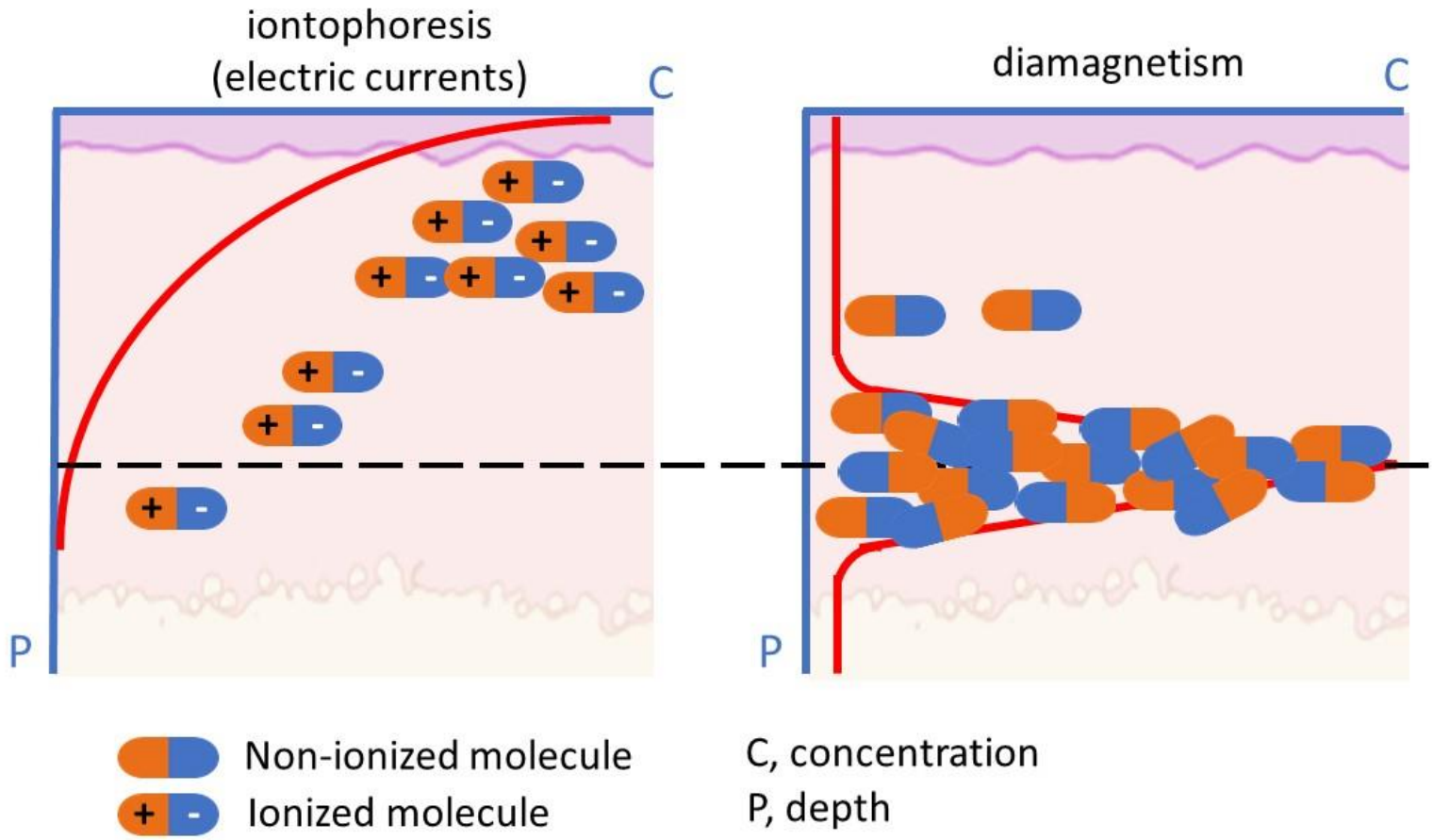

iontophoresis
(electric currents)
C
P
diamagnetism
C
P
Non-ionized molecule
Ionized molecule
C, concentration
P, depth

Figure 18. Distribution curve of pharmacological substances (physical model) expressed as concentration-depth ratio (PC) induced respectively by electric currents or High Intensity-PEMF Iontophoresis (electric currents). The delivery of the therapeutic agent decreases with the distance and depends on the ionization of the molecules. B) Homogeneous and deeper distribution of hydro-soluble (diamagnetic) therapeutic agent as a result of the impulse originating from the High Intensity of Magnetic Field. The ionization of the molecule is not necessary.

Therapeutic agents with the greatest possibility of transport are interleukins, hormones, neuropeptides or growth factors at low dose , which have already shown adequate control of the processes of low-grade chronic inflammation which causes COVID-19 inflammatory, autoimmune or degenerative sequelae, that is observed in patients between 2-3 weeks after the acute phase and up to 3-4 months. [292]

XX.8.8 Cellular effects of dietary supplement therapies and herbal medicines

Several dietary supplements, such as vitamin C and D, Zn etc. are being used in the treatment of COVID-19, despite the lack of undisputable evidence proving it beneficial effects. In this section, we discuss the possible mechanisms such as antioxidant effects (Figure 19), that could be behind the claims. However, it should not be read as an endorsement of the claims.

Zinc, Vitamin D, Vitamin C

Zinc is considered as a potential supportive treatment against COVID-19 infection due to its anti-inflammatory, antioxidant and antiviral effects. A study showed that Zn deficiency was associated with up-regulation of TNF-a, IFN-g, and FasR signaling and apoptosis in primary human upper airway and type I/II alveolar epithelial cultures obtained from human donors. [293] Another study showed that Zn-deficiency in primary hepatocytes and HepG2 cells up-regulates JAK-STAT3 and NF-κB pathways. [294]

Vitamin D plays a crucial role in immunomodulatory, antioxidant and antiviral responses.
GST pull-down assays show that Vitamin D receptor physically interacts with IκB kinase β (IKKβ) to block NF-κB activation. [295] Reports have also assessed the effects of Vitamin D on human CD4(+)CD25(-) T cells where stimulation of CD4(+)CD25(-) T cells in the presence of 1,25(OH)(2)D(3) [the active form of vitamin D] inhibited production of proinflammatory cytokines including IFN- gamma, IL-17, and IL-21. [296]

Vitamin C is also closely linked to the function of various immune cells. In a randomized controlled trial of hypertensive and/or diabetic patients, the experimental group was administered 500mg vitamin C twice a day. [297] After eight weeks of treatment, the levels of high-sensitivity C-reactive protein (hs-CRP) and interleukin 6 (IL-6) were measured in the control and treatment group. Vitamin D was found to have potential effects in alleviating inflammatory status by reducing hs-CRP, IL-6 levels in hypertensive and/or diabetic obese patients.

These signaling pathways regulated by these dietary supplements, such as inflammatory pathways; TNF-a, IFN, NF-kB pathways; JAK-STAT signaling are also involved in the COVID-19 response, indicating mechanisms via which they could have potential therapeutic effects.

Quercetin

Quercetin, a plant pigment (flavonoid) found in many plants and foods, such as red wine, green tea, etc., has been shown to have a variety of anti-inflammatory, antioxidant and anti-enzymatic effects. The major druggable targets of SARS-CoV-2 are 3-chymotrypsin-like protease (3CLpro), papain-like protease (PLpro), RNA-dependent RNA polymerase, and spike (S) protein. A recent study explored the anti-viral effects of Quercetin by conducting molecular docking studies. [298] The study showed that Quercetin can inhibit 3CLpro and PLpro with a docking binding energy corresponding to −6.25 and −4.62 kcal/mol, respectively. This suggests that Quercetin could show a relevant capability to interfere with SARS-CoV-2 replication.

Resveratrol

Resveratrol, a plant compound, has also been shown to have antioxidant and anti-viral properties. A recent preliminary study observed that Resveratrol can inhibit SARS-CoV-2 infection in the in vitro cell culture, suggesting the potential utility of RES as a novel therapy for COVID19 infection. [299]

Curcumin

Curcumin, a chemical produced by *Curcuma longa* plants, are known for pharmacological abilities, especially as an anti-inflammatory agent. Thus, their potential role in the therapeutic regimen for COVID-19 has been hypothesized. An *in silico* molecular docking and stimulation study demonstrated that both the viral S protein and ACE2 can bind to curcumin. [300] The binding of curcumin to receptor-binding domain (RBD) sites of viral S protein and to the viral attachment sites of ACE2 receptor, indicated the potential of curcumin against SARS-CoV-2 viral protein. Studies also show that curcumin can regulate the expression of angiotensin 2 type 1 receptor (AT1R) and angiotensin 2 type 2 receptor (AT2R) in myocardial cells. In animal models, treatment with curcumin attenuated the proinflammatory effects induced by Angiotensin II-AT1R axis leading to significant decrease in the level of proinflammatory cytokines TNF-α, IL-6 and reactive oxygen species. [301]

Melatonin

Melatonin is a well-known anti-inflammatory and anti-oxidative molecule and has known protective effects against acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) caused by viral and other pathogens. Melanin is thought to play a role in SARS-CoV-2 infection. The endogenous melatonin concentrations in bats range from 60 to 500 pg/mL during the night and 20–90 pg/mL during the day, dependent on the species. The melatonin production level in humans is significantly lower than in bats, especially in the older population. As elderly people are more affected by SARS-CoV-2 than younger people, it is hypothesized that higher levels of melatonin may exert protective properties in bats against the severity of SARS-CoV-2. In addition, melatonin supplementation has been shown to induce the production of cytokines, including IL-2, IL-6 and, IL-12 and reduces CD8+ cells generation in human peripheral blood mononuclear cell cultures. [302] All these studies indicate the protective effect of melatonin in COVID-19 infection.

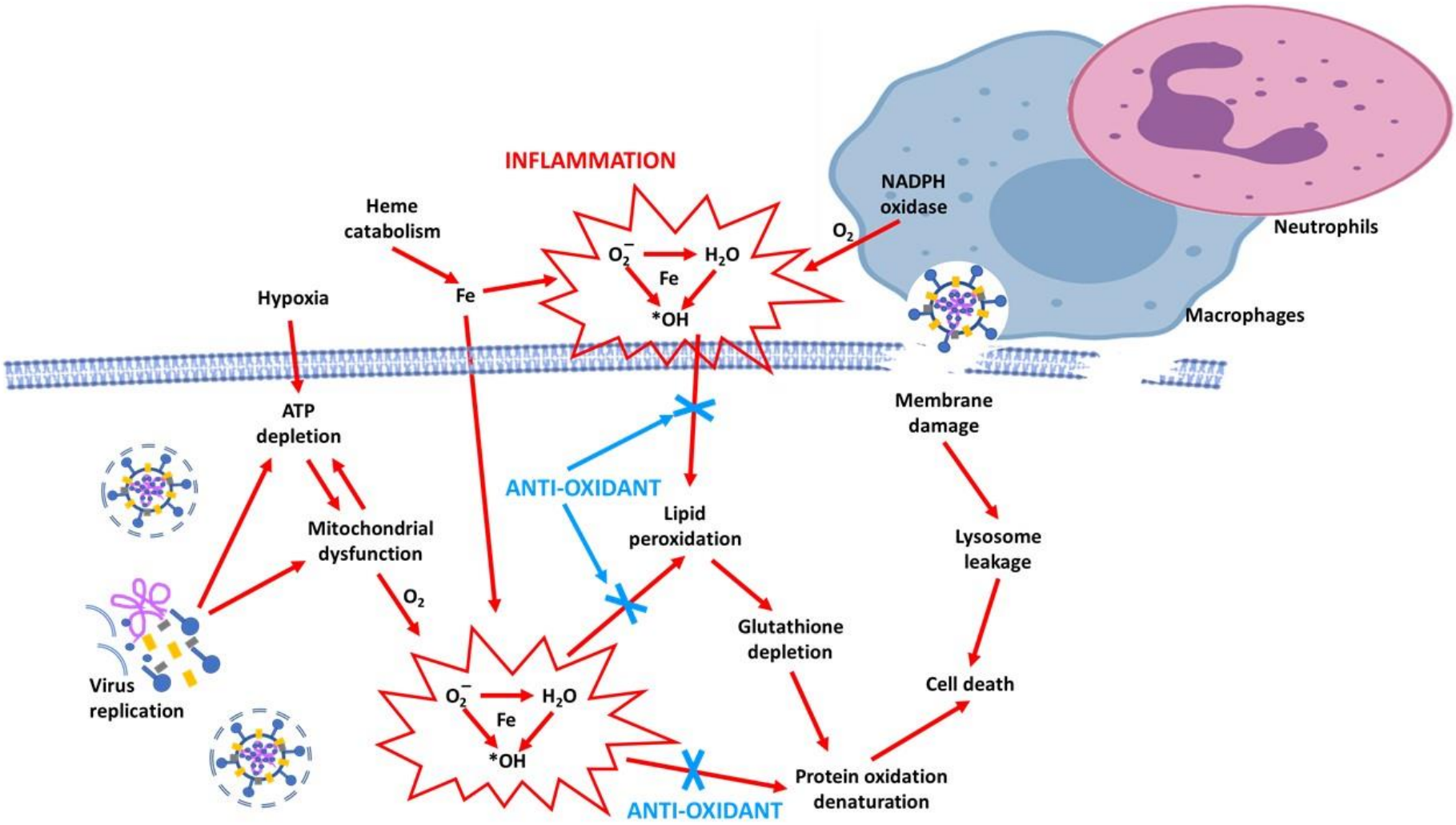


Figure 19. Schematic representation of the mechanisms of generation of oxygen free radicals in the course of COVID-19, and assumptions about the antioxidant action sites, indicated with "X" (Adapted from [303])

Chinese Herbal Medicines

The anti-viral effects of Chinese herbal medicines have been explored for a long time, previous studies have shown potential therapeutic effect of Chinese herbal medicines in SARS. [304] A recent study looked at five Chinese herbal medicines to assess their potential in treating COVID-19. [305] They predicted the binding affinities of these compounds and COVID-19 related targets, such as Spike protein, ACE2, 3CLpro, PLpro and RdRp using molecular docking. In addition, they also performed enzyme inhibition assays in vitro to determine the inhibitory activity against SARS-CoV-2 targets. Many of these herbs are known to display antiviral and immunomodulatory activities. [306] A study showed Ecalyptus extract is able to affect the phagocytic ability of human monocyte derived macrophages (MDMs) in vitro. [307]

XX.9 Concluding remarks

COVID-19 research suggests that there is a need for changing the paradigm that postulates that cell, tissue and mathematical based methods used for COVID-19 research inform animal studies that will subsequently inform human studies. In vitro models using 2D and 3D cell and tissue-based systems and in silico methods can be used as powerful tools to accelerate SARS-CoV-2 studies and discoveries. These models are proven to efficiently mimic a variety of subcellular, cellular, tissue and organ physiological aspects of relevance for the understanding of SARS-CoV-2 related risk factors, vulnerabilities, and potential therapies.

Due to the extraordinary crisis caused by COVID-19, medical researchers, cell biologists, life science experts, mathematical modelers and bioengineers across the world are actively collaborating to accelerate the rapid development of relevant cell, tissue and mathematical methods and approaches to gain detailed mechanistic knowledge of this new disease. This global collaborative knowledge sharing effort assists clinical approaches and treatments through the unravelling of the mechanistic understanding of SARS-CoV-2 to provide innovative solutions, such as the development of new diagnostic tools, and preventive and curative strategies.

The challenge is not only technological but also cultural. The perspective that sees only clinical studies at the center of the cognitive process of a disease needs change, but it must include the integration of in silico and in vitro methods with clinical studies. It is therefore necessary to disseminate knowledge on all new methodologies. Clinical doctors should strive to reconstruct the complexity of the organism from the molecular, cellular, tissue, organ and whole organism level and the researchers instead should strive to increase the level of complexity of the models and their integration in order to provide tools that are increasingly closer to the human organism. Biomedical research must be increasingly

multidisciplinary and integrate advanced technological aspects and large-scale clinical research to allow for faster knowledge gathering followed by concrete actions.

Information technology, in silico methods and artificial intelligence could support a more targeted and rational approach and to manage the complexity of the human body processes relatively quickly and at low costs. Furthermore, knowledge can be shared by optimizing resources and reducing waste and overlap. The positive effect of the worldwide sharing of scientific knowledge has permitted the rapid understanding of COVID-19 and trying together to combat the spread of the disease and its consequences.

In vitro methods dilucidated different aspects of the cellular function independently from the context, the organism, tissue and organ within the cells operate in physiological conditions. This is an advantage as it simplifies the model but may result in a rough approximation of reality. For example, the variations linked to body kinetics including the absorption, distribution, metabolism, and excretion are a challenge to overcome but progress is being made embracing new methodologies. For dealing with complex body dynamics and kinetics, advance technologies and increasingly complex dynamic systems such as 3D organoids and microphysiological systems have been developed.

Human biology is complex, and it is therefore important to remember that only integrated and complementary use of different experimental models and methods allows us to obtain useful information. The dissemination of this knowledge is fundamental and will allow an increasingly effective and multidisciplinary research to lower the disease burden of society.

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