Evolution of SARS-CoV-2 entry mechanism into host cells during infection

WRITING ASSIGNMENT

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Abstract

SARS-CoV-2 was responsible for the highly destructive pandemic that started in early 2020, with the Omicron lineage being dominant for half of the pandemic due to its higher transmission rate. However, Omicron variants appear to be less infectious and thought to enter the target cell less efficiently. Omicron's lack of infectivity has been associated with an increased usage of the endosomal pathway for viral entry and lower dependance on TMPRSS2. In this review, we aim to provide an outlook on the available data regarding the Omicron entry mechanisms and the current lack of consensus in the topic. It remains unclear which mechanism is preferred by SARS-CoV-2 Omicron variants to infect host cells as well as Omicron's relationship with TMPRSS2 during viral entry. We propose to conduct further mutations and variants of concern-based studies on human cell organoids and *in vivo* to understand SARS-CoV-2 Omicron entry pathway mechanism and access TMPRSS2 and the dependency.

Summary

The COVID-19 pandemic, caused by the SARS-CoV-2 virus, has haunted the world since late 2019. Since then, the virus has been specially exposed to evolution events that cause the accumulation of mutations and consequential alteration in its characteristics – including pathogenesis, and transmissibility – creating different lineages and variants. A SARS-CoV-2 lineage includes all the viral variants that share a common ancestor.

In this review we focus on the Omicron lineage, which has been the dominant circulating lineage for half of the pandemic duration. Omicron is characterized by lower pathogenicity and higher transmissibility between individuals. The source of these changes may rely on mutations on the spike protein, which is responsible for mediating the virus to enter the cells of its host as well as escaping the immune system upon infection. Traditionally, the Spike is highly dependent on a protein named TMPRSS2 and is consequentially able to enter the cells to infect them through (manly) fusing the virus outside to the cell's. For Omicron variants, alternatively, the endosomal entry pathway is proposed to dominate, combined with a decrease in TMPRSS2 usage.

We have reviewed the most recent data regarding Omicron and its mechanisms to enter the cells of the host. It was concluded that it is currently not possible to make a full judgement on the mechanisms behind Omicron cell entry and that they require additional research. Further, we discuss possible approaches to further understand Omicron and its variants behavior while considering previous attained results in the field.

Introduction

Coronaviruses belong to the viral family *Coronaviridae*, from the *Nidovirales* order, and consists of four different genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronaviruses* and *Deltacronaviruses*. These viruses are known to infect animals, including humans, with the potential of being transmissible zoonotically, which have previously been the cause of epidemical and pandemical outbreaks of disease in humans[1,2].

History, Origin, And Impact Of Human Coronaviruses

The first Coronavirus to be identified originated from livestock animals in the 1930. Human coronaviruses (HCoVs) causing a cold were later identified in the 1960s and found to have similar characteristics to the avian IBV previously discovered. Interestingly, up until 2002, coronaviruses were downplayed and only associated with the common cold symptoms. However, winter of the same year, an often-lethal form of pneumonia titled acute respiratory syndrome (SARS) arose in Guangdong, China. These developments and the epidemic potential of the SARS-CoV virus led to the increase interest in the viral family. Later in 2012, the Middle East respiratory syndrome coronavirus (MERS-CoV) epidemic was also recorded[1–3]. Lastly, the COVID-19 pandemic, caused by the SARS-CoV-2 virus whose outbreak started in Wuhan, China, in late 2019. The pandemic currently registers more than 771 million cases and almost 7 million deaths reported worldwide[1,4].

All the HCoVs mentioned have a zoonotic origin, meaning that the viruses were initially transmitted from animals to humans. Importantly, all HCoVs are considered to have an origin in bats as their natural hosts. However, before being transmitted to humans, HCoVs most likely infect intermediary hosts which are responsible for the zoonotic jump. The intermediary hosts can be determined by analyzing both animal and human viruses level of genetic similarity. On this note, pangolins (*Manis javanica*) are suggested to be SARS-CoV-2 may have originated intermediary hosts, hinted due to the found genomic similarities between the pangolin CoV genome and SARS-CoV-2[3,5,6].

Morphology And General Characteristics Of Coronaviruses

The Coronavirus distinguished morphology and characteristics are essential to describe the impact of these viruses. Coronaviruses comprise the largest single-strand positive-sense RNA viral genomes known and require the production of nested mRNA transcripts through a complex process within the host cell to progress their life cycle. Moreover, the name of the *Coronaviridae* family stems from their crown-like ("corona") fringe, the name attributed to the bulbous distal ends of embedded envelope glycoproteins that comprise the outside of each spherical virion[2,7].

Coronaviridae virions can measure between 100-150nm in diameter and are round, somewhat pleomorphic and include a viral envelop. On the outside, virions are covered by characteristically crown-like structure of glycoproteins, known as the spike protein (S). The transmembrane glycoprotein (M) and the internal phosphorylated nucleocapsid protein (N) are the other crucial proteins integrating these virions[2,7]. The virion structure is detailed in ***Figure 1***.

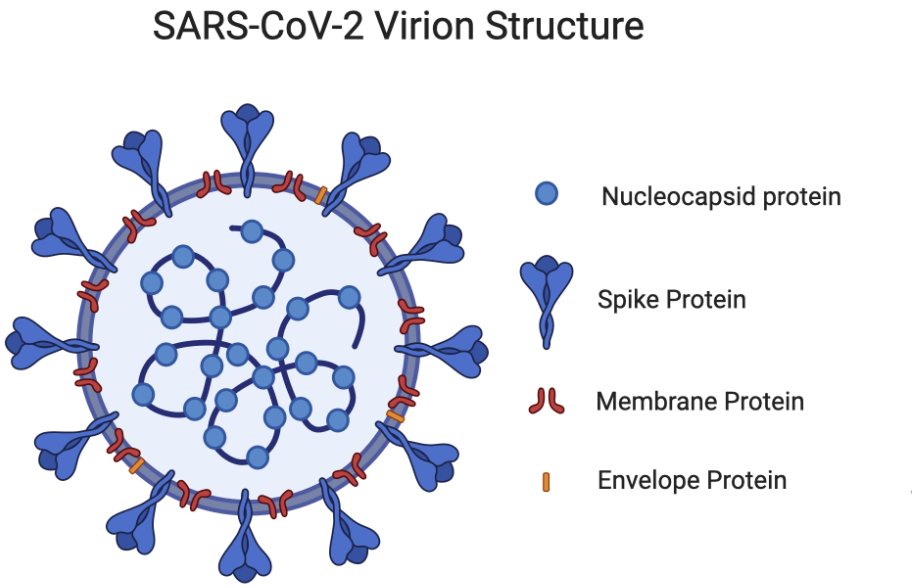

Figure 1 - SARS-CoV-2 shares its basic structure with other members of the Coronaviridae family, including the presence of the spike protein. *Simplified representation of SARS-CoV-2, including all the main structural proteins. Inside the virion, the viral genome is organized by the nucleocapsid (N) proteins. Protecting the virion is the membrane which harbors the other structural proteins. The envelope glycoproteins (E) are represented in orange, the membrane proteins (M) are represented in red, and the spike (S) proteins are in blue. Created with BioRender.*

The large genome of the coronaviruses can reach 25.4-31.8kb in size. It is comprised by a single-strand of RNA in the positive sense while being capped and adenylated. The majority of the coronaviruses follow a standard genomic organization: 5'– replicase – spike – envelope – membrane protein – nucleocapsid – 3'. Many species include additional genes, such as the hemagglutinin esterase gene and other accessory proteins[7]. Moreover, the genome of coronaviruses includes 7-14 open reading frames (ORFs), the first one starting at gene one and overlapping two ORFs, 1a and 1b, which code for the replicase[2].

Depending on the coronavirus, different cellular receptors can be selected to ensure the entry of the virions into the host cell[7]. For example, the MERS-CoV requires the dipeptidyl peptidase-4 (DPP4/CD26) protein, which can be found in many cellular tissues, such as the respiratory endothelium[3]. These cellular receptors are responsible for the virus cellular tropism and help trigger conformational changes in the spike protein that help further guide viral entry. More specifically, coronaviruses can enter a cell via membrane fusion - mediated by the spike protein and cellular receptors – as well as via endosomal pathway – which requires the fusion between the viral capsid and the endosomes post endocytosis. The viral entry in the host cells culminates in the release of the viral material in the cytoplasm of the cell, leading to the continuation of the viral life cycle: replication and further release of new infectious virions to the extracellular space[2].

To sum up, coronaviruses are responsible for a multitude of disease in animals, including humans, with the ability to cause pandemical events as seen with SARS-CoV-2. Bats are thought to be the overall natural host of the virus, where it tends to evolve and recombine. On the other hand, livestock seems to be the most probable intermediary host, which than is responsible for the human infections and spread. Nonetheless, coronaviruses are also responsible for the common cold and can usually target respiratory and gastrointestinal tissues to replicate. Consequentially, all these characteristics make coronaviruses an important research target to avoid later catastrophic pandemics.

SARS-CoV-2 And COVID-19

The acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was the virus responsible for the destructive pandemic which started in the end of 2019 and spread throughout the world in 2020 by causing the coronavirus disease 19 (COVID-19). The virus is transmitted between humans through respiratory droplets and aerosols, having a 4–5-day incubation period before the host displays symptomology. Infected individuals can develop an asymptomatic infection, a mild to moderate respiratory disease or severe illness. In its mild to moderate state, individuals experience cough, fever, headache, myalgia, and diarrhoea. For severe cases, the symptoms tend to include hypoxaemia, culminating in dyspnea. The latest symptoms can thus lead to progressive respiratory failure and to the development if ARDS, a form of lung injury which includes extensive inflammation and pulmonary vascular leakage[1,8,9].

Worldwide efforts have been allocated in the past years to develop treatment and prevention options against COVID-19. For example, vaccines were developed only 18 months after the start of the pandemic. These vaccines include the first ever approved nucleic acid-based vaccine and contribute to an overall immunization by stimulating acquired immunity and preventing further dissemination[10]. However, by late 2020, new variants of concern (VOCs) emerged, leading to an increased immune escape and lessening of the vaccination efficiency[11]. In addition, severe COVID-19 cases require therapeutic strategies to prevent multi-organ failure, ARDS and death[12]. Therapeutical strategies might include anti-viral drugs that target the viral life cycle or the adverse effects of COVID-19[11,13]. Nevertheless, the development of treatment and prophylactic measures to combat COVID-19 require the comprehensive understand of its entry mechanisms and biology. More specifically, the targeting of the virus towards the host infection and cellular entry, which greatly relies on the spike viral protein.

The Spike Protein

The Spike (S) protein is the viral mediator for SARS-CoV-2 attachment and entry to the host cell. Its crown-like appearance integrates the glycoprotein arrangement in the outside of the virions titled “corona” that gives coronaviruses their name[2].

Due to its role in infectivity, spike proteins are targeted by the immune system and anti-viral drugs. Therefore, the S protein requires strategies to evade the immune system. One of its techniques is by being coated with polysaccharides, which work as camouflage. The other common viral strategy is viral evolution. Specifically, the appearance of new SARS-CoV-2 variants usually relies on S

protein mutations that tailor its specificity and reduce the impact of neutralizing antibodies capable of its targeting[14]. Therefore, understanding the different patterns and mutations of the S protein is essential to decode infectivity, pathophysiology, fusogenicity and other aspects of SARS-CoV-2, essential to combat the infection.

The S protein in SARS-CoV-2 includes two different subunits – S1 and S2 – as well as a signal peptide on the N-terminal domain (NTD). The protein ranges from 180-200KDa in size and comprises 1273 amino acids. The S1 subunit is responsible for binding with the host cellular receptors and encompasses a NTD and the receptor biding domain (RBD). On the other hand, the S2 subunit is responsible for membrane fusion and includes the fusion peptide (FP), heptapeptide repeat sequence 1 and 2 (HR1 and HR2), the transmembrane domain (TM) and the cytoplasm domain. Comparable to other coronaviruses, the S protein in SARS-CoV-2 is cleaved into the S1 and S2 subunits upon infection using cellular proteases, proven to be the same as in SARS-CoV. The S protein can be visualized as trimers surrounding the viral particle in its characteristic "corona" structure[14]. The S protein constituents are graphically displayed in ***Figure 2***.

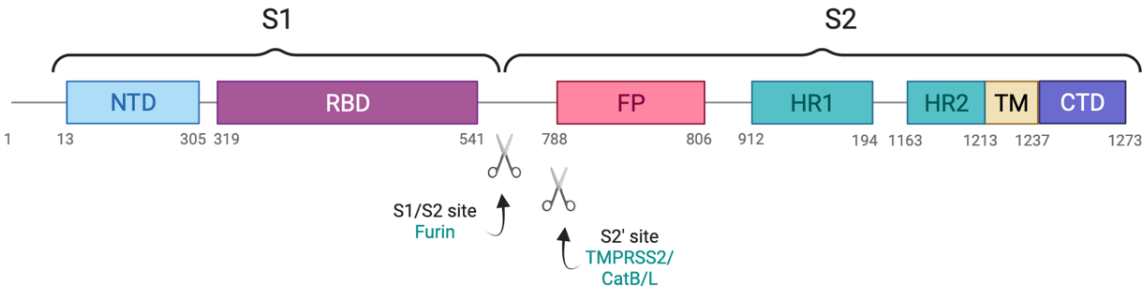

Figure 2 - Schematic illustration of the SARS-CoV-2 Spike protein organization. *The spike protein is constituted by two different domains, S1 and S2, and presents as trimers in the virions surface. The S1 includes the NTD and the RBD. The S2 includes the FP, HR1, HR2, TM and CTD. The amino acid residues are represented under the subunits in grey. To be activated, the spike protein must be cleaved in both S1/S2 site by furin and on the S2' by TMPRSS2 or CatL. Abbreviations: NTD: N-terminal domain, RBD: receptor binding domain, FP: fusion peptide, HR1/2: heptapeptide repeat sequence 1/2, TM: transmembrane domain, CTD: C-terminal domain. Created with BioRender.*

The S1 subunit is responsible for the first part of the viral infection, as it includes the RBD, accountable for recognizing the cellular receptors and binding the virions to the host cell. In SARS-CoV-2, the RBD binds to ACE2, allowing for S protein activation. Nevertheless, the S1 subunit includes both a NTC as well as a C-terminal domain (CTD), the latest one being the most mutated in new VOCs and the segment that interacts directly with the ACE2. Thus, acquired mutations seem to impact the binding efficiency of SARS-CoV-S to the ACE2 cellular receptor, leading to different overall viral infectivity outcomes. Therefore, the RBD region is usually the target for neutralizing antibodies (nAbs), with this region being up to 76% similar in sequence between SARS-CoV and SARS-CoV-2. The majority of the similarities reside with the residues directly responsible for binding ACE2

even though recent studies suggest that the differences acquired might be enough to prevent nAbs to bind to the SARS-CoV-2 S RBD[11,14].

The viral fusion and entry relies on the S2 subunit of the SARS-CoV-2 spike protein. The FP is composed of 15-20 conserved residues within the viral family and is responsible for the target anchoring with the target's membrane upon S protein conformational change. This segment is especially important to disrupt/reconnect lipids in the host membranes, aiding in membrane fusion. Moreover, the HR1 and HR2 domains of the S2 subunit likewise aid in the viral entry and fusion and are designated as the "fusion core region". This region is conserved amongst coronaviruses, making it an enticing drug target[14].

Cellular Entry Mechanisms of SARS-CoV-2

The SARS-CoV-2 virions are required to enter the host cells in order to continue their life cycle and replicate. The virus goal is thus to entry the host cell and release its genome to proceed with the production of new infectious virions that can endure the infection. Importantly, the spike glycoprotein (S) is considered the most relevant viral protein to mediate targeted cellular entry[11].

The entry of the SARS-CoV-2 virions into the cells require two bonds to be broken within the spike protein. The first bond is the S1-S2 bond that connects both subunits of the spike protein and the second bond in the S2' cleavage site. The S1-S2 bond is cleaved by furin whereas the S2' cleavage is cleaved by the host cell proteases either in the plasma membrane (PM) or inside endosomes. There are two entry mechanisms used by the virus to infect the host cells: plasma membrane fusion (PMF) and endosomal pathway (EP)[15]. The PMF entry pathway requires TMPRSS2-mediated cleavage in the plasma membrane. On the other hand, EP relies on cathepsin L to activate the S protein within the endolysosome and does not necessarily require previous furin-mediated cleavage of the S1-S2[15].

Furin-Mediated S1-S2 Site Cleavage Is Unique To SARS-CoV-2

The maturation of the Spike protein requires its cleavage by furin in the infected cell where the virions are being produced. The cleavage site is localized in the junction of the spike protein subunits S1 and S2. This is a key difference between SARS-CoV-2 and SARS-CoV, because the later exclusively uses TMPRSS2 for both cleavage sites (S1-S2 and S2'). Importantly, cleaving the S1-S2 site within the spike protein appears to be a crucial step in maturing the S protein into both trimereric subunits for the PMF pathway[16,17].

Notably, furin cleaves the spike in the Golgi apparatus during the last steps of assembling the new virions. Despite this cleavage, both subunits remain attached to each other, forming the characteristic crown-like appearance of the coronaviruses. Both subunits embark different functions upon infection of a new target cell. S1 is responsible for binding to ACE2 whereas S2 will ensure that the virions is anchored to the PM as well as mediating fusion[15]. Nevertheless, it is important to highlight that Furin-mediated binding is only required during the plasma membrane fusion pathway, because it allows TMPRSS2 to access the second cleavage site. In contrast, the endosomal pathway does not rely on furin as the conformational change is induced by the pH change inside the endosome instead of furin.

ACE2 Is Common In Both Entry Pathways

In general, coronaviruses require different sets of host cell receptors and are thus dependent on their presence to infect cell and proceed with their life cycle. This factor has a multitude of consequences, especially in viral pathogenesis and infectivity. Therefore, the first step for the virions to infect a new target cell is to bind to a specific receptor present in the target cell's membrane. For SARS-CoV-2, the spike protein binds to the cellular receptor human angiotensin converting enzyme 2 (ACE2)[2].

As highlighted in this review, the *Coronaviridae* family harbors a multitude of viruses capable of interspecies transmissibility due to the versatility of the spike protein. In fact, the spike is responsible for determining which cells the virions are capable of entering, depending on the S protein specificity. Likewise, the majority of these cellular receptors are present across different mammals, potentiating the zoonotic transfer between species. More specifically, the ACE2-Spike interface is the cause of specie spillover and viral infection outbreaks. In humans, due to the ACE2 expression pattern, virions tend to replicate in epithelial cells of the respiratory and enteric tracs[5,6]. The S protein is also responsible for determining

key factors regarding the overall viral behavior, such as its basic biology (e.g. entry mechanisms), epidemiology and phylogeny[18].

Upon viral binding to the ACE2 receptor, the S1 spike subunit undergoes conformational changes. This alteration exposes the S2' cleaving site in the S2 subunit which can then be cleaved by a specific cellular protease, depending on the chosen entry route[15]. Among coronaviruses, the RBD is the peptidic interface responsible for establishing contact with the target cell receptor that will later cleave the CoV spike in the S2' site[18]. Interestingly, both SARS-CoV and SARS-CoV-2 translate into a similar pandemic potential due to their resemblances, including their usage of ACE2 as their cellular receptor. Moreover, SARS-CoV-2 potentiates the entry in similar tissues as the previous SARS-CoV[19].

The ACE2 receptor integrates the angiotensin metabolism by transforming angiotensin II into angiotensin$_{1\text{-}7}$ as well as angiotensin I in angiotensin$_{1\text{-}9}$. SARS-CoV-2 (and other coronaviruses) utilize this widely available cellular receptor – present in the heart, vessels, gut, lungs, kidney, testis, and brain – to enter cells and infect them to proceed with its viral life cycle. During the infection stage, the NTD of the S1 subunit binds to the ACE2 pocket, allowing for the viral entry process[20]. Similarly to SARS-CoV, SARS-CoV-2 also incites a down-regulation of the ACE2 receptor in infected cells, which led to questioning whether SARS-CoV-2 was more efficient in binding the human receptor due to its higher transmissibility and consequential enhanced pandemic potential. This proved to be true as SARS-CoV-2 is indeed more efficient in binding ACE2 compared with the previous SARS-CoV[21]. Moreover, upon its down-regulation in the body due to the infection, ACE2 depletion is associated with intensification of severe COVID-19 symptoms caused by the angiotensin II accumulation[20].

ACE2/TMPRSS2 S Protein Binding Mediates SARS-CoV-2 Target Cell Entry In The Membrane Fusion Pathway

Similarly to SARS-CoV, Hoffmann *et al.* has proved that the new SARS-CoV-2 is depended not only on the ACE2 as well as the cellular serine protease TMPRSS2 during infection. More specifically, the TMPRSS2 is responsible for priming the viral S protein upon plasma membrane binding to enable entry of the virus into the host cell. This was proven using a camostat treatment on both SARS-CoV and SARS-CoV-2, which blocked the TMPRSS2-mediated entry of both viruses.

Vero cells do not naturally include the TMPRSS2 cellular protease, thus making it an exceptional vessel to understand the impact of this protease in the entry mechanism. Additionally, both E-64d (Cathepsin B/L blocker) and Camostat (TMPRSS2 blocker) can be used to study the impact of viral entry. Interestingly, upon the usage of Vero, inhibition using E-64d and camostat simultaneously appears to be more dramatic for SARS-CoV rather than for SARS-CoV-2. Using only camostat revealed that SARS-CoV-2 pseudotype entry was a bit higher than SARS-CoV. However, SARS-CoV-2 maintained the same pattern overall as SARS-CoV regarding its usage of TMPRSS2 and CatL/B[19].

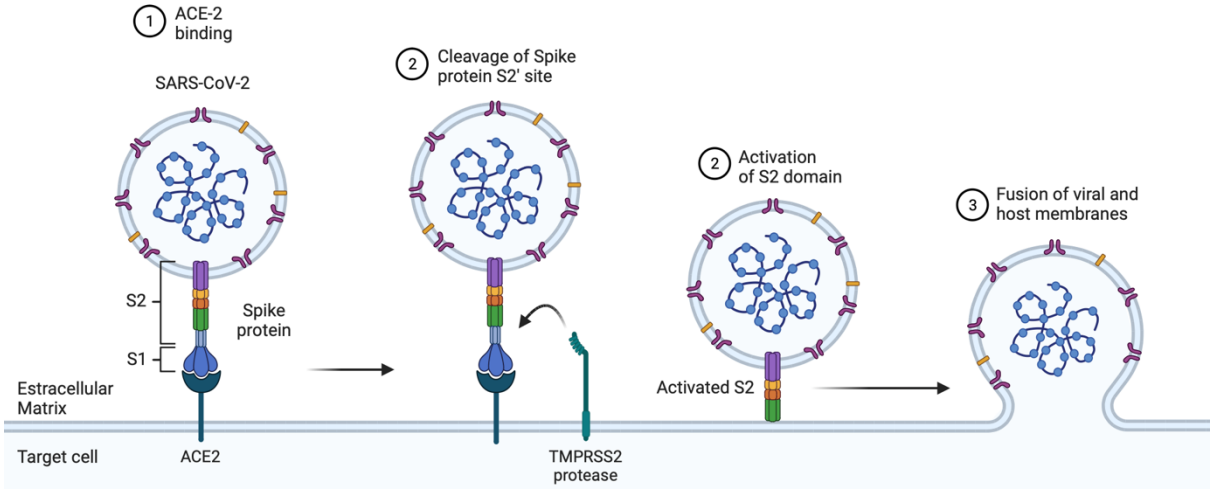

Figure 3 - SARS-CoV-2 plasma membrane fusion (PMF) entry pathway. *The PMF pathway requires the spike to bind to ACE2, enabling the cleavage of the S2' site by TMPRSS2 protease. The spike then enters a successive sequence of conformational changes that allow for the viral membrane to fuse with the host plasma membrane, releasing the viral genome to the host cell, allowing for the continuation of the viral life cycle. Created with BioRender.*

The membrane fusion pathway starts when the S protein binds to the ACE2 cellular receptor. When this interface is established, TMPRSS2 mediates the S2' site cleavage if the spike was previously cleaved by furin. The S2' site processing activates the spike protein and allows a series of conformational changes to occur. During the fusion process, the activated S protein brings both the viral envelope and the plasma membrane from the host cell to close. The proximity between phospholipidic bilayers, aided by other cellular/viral proteins, trigger the membranes to fuse and consequential freeing of the viral genetic material into the cell cytoplasm[22]. The membrane fusion entry pathaway is schematically described in ***Figure 3***.

Lastly, it is important to note that, when TMPRSS2 was proven to be essential to the SARS-CoV-2 entry mechanism to induce PMF, SARS-CoV-2 had been in circulation for a short period of time. Therefore, early SARS-CoV-2 circulating strains prefer to entry cells using the PMF entry pathway, which translates molecularly to the use of TMPRSS2 to cleave the S2' site[15].

Cathepsin L Mediates SARS-CoV-2 Target Cell Entry In The Endosomal Pathway

Cathepsins are a family of proteolytic enzymes which can cleave aspartyl, serine, or cysteine residues. Mostly Cathepsin L (CatL) and B (CatB) have been reported to be involved in viral entry processes, including for SARS-CoV[23]. For example, Simmons *et al.* highlighted how inhibiting CatL proteases could prevent SARS-CoV from entry the cells[24]. Contrastingly, according to Hoffmann *et al.*, cathepsin B and L was determined as non-essential[19]. However, recent studies have suggested that CatL levels are elevated in patients with COVID-19 and appears to enhance the cleavage of spike protein and consequential viral entry efficiency[25].

Therefore, similarly to SARS-CoV, SARS-CoV-2 can also utilize CatL to enter cells via the endosomal pathway. From a physiological point of view, CatL is a lysosomal enzyme that integrates a multitude of natural processes, such as apoptosis, extracellular matrix (ECM) remodeling and antigen processing. Interestingly, this protease appears to also have an array roles in pathological events, including tumor metastasis, renal disease, diabetes, inflammation as well as viral infection[26].

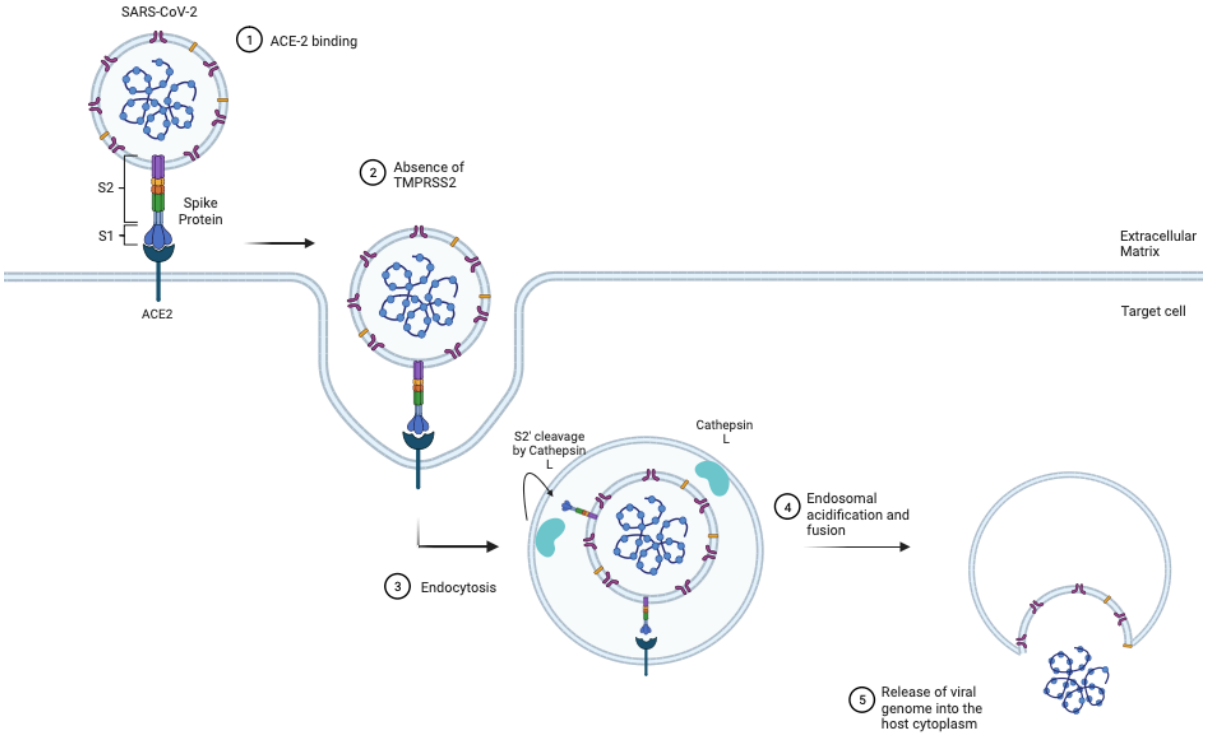

Figure 4 - SARS-CoV-2 endosomal entry pathway (EP). *The EP requires the spike to bind to ACE2. If the spike cannot find TMPRSS2 to cleave it, the virions are endocytosed via clathrin-mediated endocytosis. The lower pH of the endolysosome allows for a conformational change in the spike, allowing the available Cathepsin L to cleave the S2' site. The following activation of the spike protein allows to bring the viral membrane and the endosomal membrane together, leading to their fusion and consequential release of the viral genome into the host cell. Created with BioRender.*

The endosomal pathway is not the main entry pathway chosen by SARS-CoV-2 to infect host cells. The PMF pathway where the ACE2-TMPRSS2 pair is used to bind S1 and consequentially hydrolyze the S2' site is the preferred route for viral entry[15]. However, SARS-CoV-2 is capable of infecting cells with a low ACE2 and/or TMPRSS2 surface content due to its ability to utilize the endosomal entry pathway as an alternative route of entry[26]. A comprehensive outlook on the endosomal pathway is available in ***Figure 4***.

For the endosomal pathway to be chosen as the entry route for the virion, the viral particles must encounter and bind to the ACE2 cellular receptors on the surface. Then, if the amount of TMPRSS2 is insufficient in the target cell membrane, then the particles binding ACE2 will be endocytosed. The same can also happen if the S protein had not previously been processed on the S1-S2 site by furin[15]. After undergoing clathrin-mediated endocytosis, CatL cleaves the S2' site, inducing the same conformational changes in the S protein as TMPRSS2, leading to the fusion of the viral capsid to the endosomal membrane and consequential freeing of the genetic viral material into the cell's cytoplasm[15,26]. Additionally, SARS-CoV-2 makes use of CatL/B's capacity of degrading the extracellular matrix (ECM), carving a path to facilitate viral infection of the host cells, as well as its upregulation during chronic inflammation[26,27].

Interestingly, there seems to be contradictory results regarding CatL importance on viral entry. Whereas Hoffmann *et al.* argues that CatL/B is not essential for SARS-CoV-2 viral entry, even upon inhibition using E-64d, Gomes *et al.* hints that more than 76% of viral entry decreases upon CatL inhibition[19,26]. These results might suggest that different Cathepsin inhibitors and cell lines might produce differential results in viral entry assessment. Therefore, it is suggested that multiple Cat inhibitors should be accessed and compared in order to evaluate the differences between drugs – for example obatoclax and E-64d[19,27]. Moreover, these disagreements can also be due to the usage of different viral variants for testing, especially considering the high variability of the virus due to its large global distribution and fast evolution[28].

SARS-CoV-2 Variants

Due to the pandemic scale of SARS-COV-2, multiple resources were implemented to combat the virus. Scientific efforts included not only by developing vaccines and anti-viral drugs effective against the virus, but also regarding databases with updated information on this virus. In late January 2020, the China National Center for Bioinformation (CNCB) was responsible for creating the 2019 Novel Coronavirus Resource (2019nCoVR), which includes a complete overview on all the aspects of the virus[29].

With the development of the pandemic, Song *et al.* highlighted the importance of integrating the variants and haplotypes of the virus in the data base. This is especially important considering high circulating virus have increased chances of accumulating mutations that can alter the track of the transmissibility course of the virus[30]. Morais *et al.* hypothesizes that the viral variants who appeared later are connected with base mutations that happened in the beginning of the pandemic onset. Moreover, these basic subtypes proposed concord with the geographical distribution of the different SARS-CoV-2 populations[31].

Despite being noted as a low rate mutation virus, its world-wide onset and pandemical potency lead to new mutations emerging and impacting viral behavior at a fast pace[28]. Moreover, it is most likely that mutations in the spike protein are kept in virions to enhance their fitness. This is due to spike's involvement in the viral life cycle, as the immune escape and viral entry responsible protein[32].

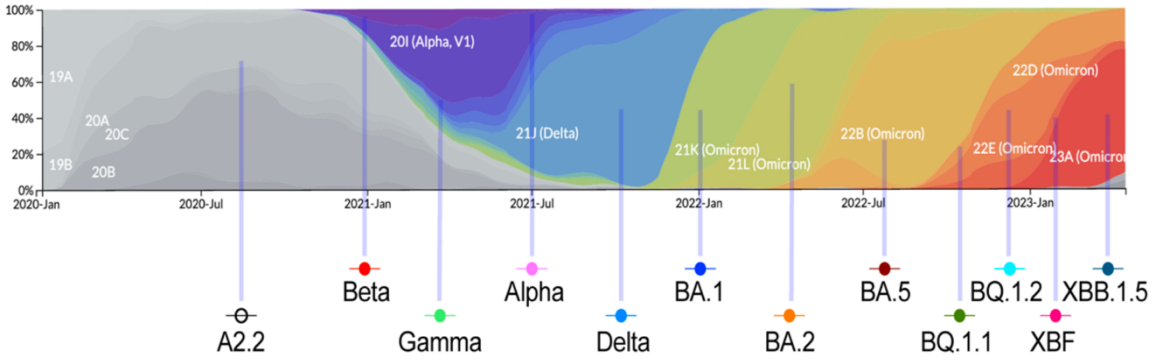

Figure 5 – Major SARS-CoV-2 lineages and VOCs. *It is represented the prevalence of each main variant circulating since early 2020. The Omicron lineage has dominated the circulating variants since late 2021. At the time of writing, the SARS-CoV-2 Omicron XBB.1.5 variant is the dominant circulating variant of concern. Figure attained from Aggarwal et al.[48].*

SARS-CoV-2 has currently seven lineages: Alpha, Beta, Delta, Gamma, Lambda, Mu, and Omicron. To the different lineages and types of SARS-CoV-2 that emerged and that pose an important role in the epidemiology its titled variants of interest (VOCs). VOCs can integrate different lineages[32,33]. For example, the Alpha variant was detected for the first time in the UK and included a significant amino acid change affecting the ACE2 binding site. This lineage was the dominant circulating by early 2021. The Delta lineage substituted the Alpha

and was the dominant circulating by mid-2021. The Delta variants include other two mutations on the spike that rewarded this VOC with 40-80% higher transmissibility, leading to higher RNA copies to be detected[34]. The SARS-CoV-2 lineages and dominating VOCs over time are represented in ***Figure 5***.

The Omicron Lineage and SARS-CoV-2 Evolution

The Omicron variant emerged in late 2021 and became the predominantly circulating variant shortly after. The Omicron lineage (and sub-lineages) has since diversified and spread globally/locally, causing infection waves that concur with its branching. Important VOCs include the BA.1, BA.2 and BA5, along with the respective sublineages. Despite having enhanced transmissibility and immune evasion compared with previous VOCs, Omicron variants were contrastingly recorded as less pathogenic[35,36]. It is suggested that the emerging of the highly different SARS-CoV.2 lineages is related with chronical infections that allow the accumulation of mutations and subsequent transmission of a new variant to a new host (recombination events).

As of the time of writing, the XBB Omicron sublineages are the dominant strains since early 2023 and have substituted the previously circulating BA.2.75. BA.2.75 was the most outspread VOC from the second-generation BA.2 variant lineage. The VOC BA.2.86 is currently growing and represents 40 mutations when compared with BA.2, which is comparable to the original Omicron lineage BA.1 when compared to its ancestor (B.1.1). Interestingly, the current dominating XBB lineage of Omicron is the most globally dispersed recombinant interlineage until now. The Omicron XBB originates from a recombination event between two BA.2 lineages. Therefore, this VOC exhibits a variety of differential RBD mutations, leading to its neutralizing antibodies-resistant spike protein[36].

Part of the Omicron lineage, more specifically the BA.5 sublineage, has displayed an antigenic drift behavior opposing to the previous "saltatory" behavior observed in other VOCs. In the BA.5 sublineages, the recombination-type events (simple and complex) – originated from the lack of intermediary sequences – gave place to more sequential and gradual mutations that can easily be traced back to the original sequence. This evolutionary pattern is more consistent with other respiratory viruses, such as the Influenza A virus. Contrastingly, events of convergent evolution within the SARS-CoV-2 lineages is also common, especially regarding factors that affect the infectivity and immune escape. For example, mutations that enhance ACE2-biding in the NTD of the Spike protein will always be favored[36].

However, none of the previous variants essentially changed the viral approach and its intrinsic behavior[35]. However, the Omicron lineage and subsequential variants comprise a new set of viral characteristics. The biggest gap between the ancient SARS-CoV-2 and the new Omicron lineage is especially underlined when looking into infection probability in groups of naïve, natural immunity and three-dose regimen individuals. Compared to the Delta, Omicron recorded an increase in all of the three groups, including 2,805% increase in infectivity of three-dose vaccinated individuals. These results also revealed that Omicron has an enhanced immune escape rather than being more highly transmissible[37]. Moreover, Hirose *et al.* compared all the major variants with the original Wuhan strain regarding their environmental stability, concluding that the Omicron variants BA.1 and BA.2 have similar stability in the environment as well as the highest survival time compared to all the other tested variables[38].

Despite the high variety of sublineages and being the dominant lineage for half of the pandemic duration, the Omicron lineage has maintained its common novelty traits, distinct from previous lineages. These traits include a similar viral, clinical, and epidemiological characteristics with mild variance between VOCs. Compared with previous lineages, Omicron has been proposed to exhibit a changed tropism, lower fusogenicity and an altered preference for its entry mechanism into the host cell[36]. This, however, lacks consensus within the scientific community, especially regarding the active reason behind Omicron's changed characteristics.

Omicron Sublineages Rely More On The Endosomal Pathway And Less On TMPRSS2 Due To Spike Protein Mutations

Since its appearance, the Omicron variants have been suggested to prefer the endosomal pathway entry over the plasma membrane fusion pathway common for ancestor lineages. The entry pathway preferred by the virus is correlated with Omicron having a lower efficiency in recruiting TMPRSS2, presumably leading to its higher dependence on CatL/B to cleave the S2' compared with previous lineages[35,36].

Before Omicron lineage establishment as the dominant circulating variant in early 2022, SARS-CoV-2 utilized TMPRSS2 as its entry catalyst into the host cell. Due to its importance, TMPRSS2 potent and well-established blocker, camostat mesylate – oral drug that inhibits serine proteases – could be used as an anti-viral drug to combat COVID-19. Indeed, camostat was proven to decrease viral entry *in vitro*, thus inhibiting viral replication and halting the viral life cycle[19,39]. However, recent studies highlight that this drug appears to be the most

successful only in patients with COVID-19 pneumonia[40]. Contrastingly, Breining *et al.* highlighted that camostat usage is most appealing only as a prophylactic measure to prevent severe COVID-19 onsetting by blocking viral replication. This correlates directly with the fact that, the beginning of the infection is the most associated with increased viral load and replication compared to lower viral levels in more severe cases[39]. The recent data suggesting that Omicron variants have lower dependency on TMPRSS2 would compromise the usage of camostat as an effective COVID-19 drug, thus posing a great concern from the clinical standpoint.

Two of the first Omicron VOCs are BA.1 and BA.2. Hu *et al.* studied the impact of each individual mutation recorded on the viral behavior and attributed the main triggers for the observed alterations in the Omicron variants. It was proposed that the S1-S2 deletion of 10 amino acids in the flanking region (S1-S2 10Del) increased endosomal entry pathway perveance over the traditionally preferred PMF entry coupled with TMPRSS2 dependency compared with the Wuhan variant in pseudoviruses. Therefore, it was concluded that the BA.1 Omicron variant is less efficient in performing PMF entry and more efficient in performing EP. However, Calu3 cells (used as the PMF pathway model), in the absence of an endosomal inhibitor, still record the lowest entry for Omicron BA.1 and S1-S2 10Del[35]. This result points out that, despite being more efficient in entering the target cell using the endosomal pathway, this variant still prefers to utilize the PMF pathway when available. On this note, one can hypothesize that, upon S1-ACE2 binding, the spike protein searches for the TMPRSS2 protease by default to cleave its S1-S2 site and trigger the PM fusion entry pathway. Thus, this could be the reason why the Omicron BA.1 variant is less pathogenic than previous VOCs.

Another interesting remark made by Hu *et al.* included the study of two other mutations. These were found to increase the ACE2 affinity as well as decrease TMPRSS2 dependence[35]. However, the authors discard mutations that apparently induce the same physiological alteration in the virus but that are not present in both BA.1 and BA.1 simultaneously. Not considering variant-specific mutations could impair the comprehensive understanding of Omicron differential behavior. Spike cleavage and reduced cell-cell fusion were also accessed in this study and were attributed to the S375F substitution and S1-S2 10Del, respectively[35].

Importantly, the Omicron BA.1 and BA.2 variants mutations S1-S2 10Del, S1-S2- AAAA, and H655Y appear to fund the increase usage of Cathepsin L (and B). These results are consistent with the fact that the Omicron lineage is more efficient in the endosomal pathway rather than in the PMF entry route. Notably, camostat only reduced H655Y mutation carrying pseudovirus entry by 34.81% and E-64d reduced the entry by 69,99% under the same conditions in VeroE6-TMPRSS2 cells[35]. These results hint towards Omicron relying on a more balanced

entry mechanism preference, rather than a full shift where it depends mainly on the endosomal pathway as stated by the authors.

Multiple genetic studies have focused on the specific mutations responsible for the entry pathway shift. For example, Qu *et al.* hints that the Omicron subvariants BA.2, BA.2.12.1, BA.4/5 and BA.2.75 display lower fusogenicity and depend on the endosomal pathway to enter the target cells by the means of the H655Y mutation. This data was attained by addressing relative infection in HEK293T cells and Calu-3 cells using pseudovirus with the specific mutations. On this note, H655Y caused higher sensitivity to E64-d regarding the pseudoviruses entry in HEK293T-ACE2-TMPRSS2 cells compared to other mutations and maintain the infection levels for the same cell line in the presence of camostat. Reverting the H655Y mutation ultimately decreased entry in HEK293T and barely increased infectivity in Calu-3 cells[41]. Accordingly, substantial evidence has since emerged that points to this key mutation – as well as others like the N969K substitution – for being responsible for inducing the entry pathway shift in the new Omicron variant[42,43].

The hypothesis that the Omicron variants tends to depend more heavily on the endosomal pathway is currently not fully agreed upon. Despite the suggestive data regarding Omicron variants entry preferentially using CatL/B and the endosomal entry mechanism to infect target cells, other studies have refuted this hypothesis. One of the concerns proposed regarding the premise included the use of VeroE6/VeroE6-TMPRSS2 cells to test for viral entry. These cells isolated from the kidney of an African green monkey lack representation for the *in vivo* human perspective[44]. Therefore, recent studies have resorted to broadening the cell line spectrum as well as representative airway human organoids and mice to test Omicron variants dependence on the endosomal pathway, their dependence on CatL/B and lower TMPRSS2 usage efficiency detected[44,45].

For instance, Mykytyn *et al.* focused on utilizing different cell lines as well as organoid models to address Omicron's variable behavior regarding entry into the target cells. Firstly, the authors prove that Omicron spike is poorly cleaved, despite the cell line in which it is produced. However, the poor cleavage of the spike does not correlate with its inefficiency in using TMPRSS2-mediated entry directly. Moreover, the authors demonstrate that, despite Omicron BA.1 being less efficient in utilizing the PMF entry pathway – thus relying partially on the CatL/B-mediated entry – all the other tested variants (614G, Alpha, and Delta) are equally capable of using the endosomal pathway to enter cells in the absence of TMPRSS2[44]. However, when the same assays were performed on human airway organoids, camostat lead to similar inhibition results in entry for Delta and BA.1 variants as camostat with E64-d, whereas E64-d alone did not pose significant entry inhibition results. Moreover, the Omicron XBB1.5 (current dominant variant at the time of writing) entry was fully inhibited by camostat, revealing its dependence on the PMF entry pathway – and proving that the Omicron variants

still heavily depend on the TMPRSS2 cellular protease[44]. Finally, knocking-out TMPRSS2 in the organoid models had a similar effect on infectivity as deleting the ACE2 receptor, whereas CatL/B deletion had no significant decrease on replication[44].

Omicron dependence on the serine protease TMPRSS2 was further corroborated by Gartner *et al.* through the usage of different models representing the respiratory track in different locations. The Delta variant appears more capable of infecting all cells tested whereas Omicron is suggested to have a replication advantage for the large-airway epithelial cells. In addition, the dependence on TMPRSS2 of Omicron was highlighted for all systems. Nonetheless, camostat revealed to be weaker in decreasing viral titers of Omicron in human nasal epithelial cells even though the endosomal entry was not enhanced in this situation[45].

Tests on mice also revealed important *in vivo* data. Despite being necessary for disease development for the Beta variant, Omicron-infected mice did not have a significant clinical score decrease upon TMPRSS2 knock out. Moreover, TMPRSS2 is evidenced to be indispensable for Omicron spreading in the tissues, however not as significantly as for the Beta variant. Nevertheless, it is important to note that TMPRSS2 does not appear to lead to inflammatory lung damage in SARS-CoV-2 Omicron compared with the Beta variant. Despite disagreeing with Omicron being fully independent of TMPRSS2, the authors mention that viral entry for Omicron is less dependent on this serine protease compared with the Beta variant[46]. Iwata-Yoshikawa *et al.* have also highlighted through an *in vivo* mice study that Omicron does infect murine airways *in vivo* by utilizing TMPRSS2, even though it is not possible to understand if the protease is directly related to the viral entry. Moreover, nafamostat – protease inhibitor – did not significantly inhibit SARS-CoV-2 Omicron infection on mice, contrary to what was observed for the Beta and Gamma variants[47].

Recently, some light has been shed over the physiological role of TMPRSS2 in cells and how it correlates with the SARS-CoV-2 infection mechanisms. TMPRSS2 and other proteases like ADAM17 and HAT are able to naturally cleave the ACE2 receptor. More specifically, TMPRSS2-mediated cleavage was shown to remove a 13-kDa fragment from the C-terminal end of ACE2. Despite not being a necessary step, it was found that SARS-CoV entry was enhanced upon binding with TMPRSS2-cleaved ACE2 molecules[48]. SARS-CoV-2 was later suggested to also make use of this system[49]. Interestingly, the differential behavior of Omicron might be related directly with a loss of ability to bind to previously TMPRSS2-cleaved ACE2 receptors in the surface of the host cell. Results show that higher expression of ACE2 and TMPRSS2 in cell lines (Vero) decreased viral entry for the Omicron variants contrasting with the increase of entry for the pre-Omicron lineages tested. Moreover, by removing ACE2 cleavage mediated by TMPRSS2 it was possible to revert the Omicron entry defect[50]. Therefore, one can conclude that

upon high availability of both ACE2 and TMPRSS2 in cells, the PMF entry mechanism become constrained due to ACE2 TMPRSS2-mediated cleavage. Thus, the virus depends more heavily on the endosomal pathway to enter the cells, which is naturally less efficient. This hypothesis also aligns with the results attained *in vivo*, where the ACE2/TMPRSS2 ratio allows Omicron lineages to continuously prefer the PMF entry as the previous lineages, despite the contradictory results attained *in vitro*[42,47,50].

Zipeto *et al.* additionally mention that ACE2 surface expression is heavily decreased during both SARS-CoV infections and COVID-19. Importantly the lack of ACE2 may be related with disease severity and progression, specially by the lack of protection against inflammation[49]. Omicron VOCs present lower pathogenicity patterns which may be related with its lack of entry efficiency and consequential keeping of ACE2 expression levels in the surface.

On this note, the aforementioned studies have unveiled the significance of the Omicron contradictory information regarding actual changes in the viral behavior. Therefore, it is still challenging to determine whether or not the Omicron lineage and its VOCs have made a full shift from preferring the ACE2-TMPRSS2 PMF entry pathway to the ACE2-CatL endosomal entry pathway. In some cases, data suggests the virus to be more efficient in utilizing the EP to enter cells when compared to previous lineages, even though it still utilizes (and prefers) the PMF pathway.

It is possible to conclude that the SARS-CoV-2 Omicron lineage tends to utilize the endosomal pathway more often compared to previous lineages. We hypothesize this shift to be directly influenced by the spike protein lack of binding to TMPRSS2-processed ACE2. If TMPRSS2 is more abundant in the host cell, ACE2 is more likely to be cleaved by the protease, disabling spike binding. Thus, SARS-COV-2 Omicron VOCs might tend to prefer low TMPRSS2 expressing host cells, which ultimately culminates in upgraded endosomal pathway usage due to the absence of TMPRSS2 to cleave the S2' site. Because naturally SARS-CoV-2 performs the membrane fusion pathway more efficiently than the endosomal pathway to replicate, this could be the reason why SARS-CoV-2 Omicron variants present themselves as less pathogenic and more transmissible. Nevertheless, more data it is required to understand the causes behind SARS-CoV-2 Omicron differential behavior depending on the target cell as well as why the available data might be controversial at the time of writing.

Conclusions and Future Perspectives

SARS-CoV-2 was the perpetrator of the catastrophic COVID-19 pandemic that started in late 2019 in Wuhan, China. Since then, the virus has spread throughout the globe and evolved into different lineages[28,30,31]. The Omicron lineage has been the circulating dominant variant since early 2022, half the pandemic duration. The Omicron lineage has brought up a different set of viral characteristics from a pathophysiological point of view: Omicron variants are less likely to form syncytia, are more transmissible but less pathogenic, less efficient in performing viral entry and improved immune escape ability[36].

The Spike protein is responsible for immune evasion, fusogenicity, and viral entry[32]. Therefore, S protein mutations have been greatly associated with the observed changes in Omicron[43]. However, there is no consensus regarding the origin of Omicron's differential behavior. Data highlights the decrease dependence of Omicron on the previously essential TMPRSS2 for viral entry directly[35–37,43] while other sources condemn the serine protease as essential for Omicron infectivity in respiratory track tissues just equally as previous lineages and VOCs[44–47,50].

In this review we have displayed the current data available on SARS-CoV-2 Omicron variants and how they compare with previous lineages and VOCs. Results point out a few key conclusions: *in vitro* testing in both VeroE6 and Calu-6 cell lines indicate that Omicron is only capable of entering target cells if the endosomal pathway is available for both pseudoviruses and the circulating strains[35,47]. However, *in vivo* mice infection with Omicron variants as well as respiratory track organoids reveal that Omicron requires TMPRSS2 for efficiently infecting target cells[44,45,47,50]. Nevertheless, multiple studies suggest that Omicron is indeed less dependent on TMPRSS2 compared with previous lineages but that it is still a requirement, which is specially observed when using full virions instead of engineered pseudoviruses with a single spike mutation. In these situations, Omicron VOCs still appear to have a slight tendency to use more the endosomal pathway compared with previous lineages[45,47,50]. Lastly, it has been recently highlighted that the observed shifts might be related with ACE2 TMPRSS2-mediated cleavage, which potentiated viral entry for pre-Omicron lineages but prevent entry for Omicron, especially for the BA.1 sublineage[50].

It is thus possible to draw some general conclusions regarding TMPRSS2-dependency as well as entry pathway preference for Omicron variants. In one hand, it has been highly suggested that Omicron does still depend on TMPRSS2 to maintain infectious capacity, even though this requirement is lower compared with previous strains, especially *in vivo*. This event might be explained by the ACE2/TMPRSS2 ratio available in each tissue, leading to different behaviors *in vitro versus in vivo*. Therefore, SARS-CoV-2 Omicron's pathophysiological

behavior appears to differ depending on the target cell type as well as the infection environment. One can then hypothesize that SARS-CoV-2 spike depends on a balance between its two entry mechanisms and that SARS-CoV-2 Omicron variants might be equally efficient in performing both PMF and EP, depending on cleaved ACE2 availability.

The current available data does not allow to draw definitive conclusions over the entry mechanism used by Omicron variants and requires more in dept analysis. Moving forward would be important to analyze how pseudoviruses with single (and grouped) Spike mutations (for example H544Y, S1-S2 10Del, and S1-S2- AAAA[35]) impact viral entry *in vivo* as well as in organoid cultures. Moreover, addressing different cell lines with circulating variants as well as previous VOCs could possible give some inside over the result inflation single mutation spike pseudoviruses seem to cause *in vitro*. It would also be beneficial to perform all the previous analysis by modulating ACE2/TMPRSS2 ratios and availability in the host cells in order to understand the impact of ACE2 cleavage in all systems (*in vivo* and *in vitro*). Finally, it would be plausible to address other physiological patterns that might be involved in Omicron decrease entry efficiency and TMPRSS2-dependency. This includes inflammation, cell signaling and other sources of modulation to fully unveil how ACE2 presence might be responsible for post-Omicron VOCs decreased pathogenesis, including in the presence of camostat and E64-d.

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