Coronaviruses fusion with the membrane and entry to the host cell

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Abstract

Introduction. Coronaviruses (CoVs) are positive-strand RNA viruses with the largest genome among all RNA viruses. They are able to infect many host, such as mammals or birds. Whereas CoVs were identified 1930s, they became known again in 2003 as the agents of the Severe Acute Respiratory Syndrome (SARS). The spike protein is thought to be essential in the process of CoVs entry, because it is associated with the binding to the receptor on the host cell. It is also involved in cell tropism and pathogenesis. Receptor recognition is the crucial step in the infection. CoVs are able to bind a variety of receptors, although the selection of receptor remains unclear. Coronaviruses were initially believed to enter cells by fusion with the plasma membrane. Further studies demonstrated that many of them involve endocytosis through clathrin-dependent, caveolae-dependent, clathrin-independent, as well as caveolae-independent mechanisms.

Objectives. The aim of this review is to summarise current knowledge about coronaviruses, focussing especially on CoVs entry into the host cell. Advances in understanding coronaviruses replication strategy and the functioning of the replicative structures are also highlighted. The development of host-directed antiviral therapy seems to be a promising way to treat infections with SARS-CoV or other pathogenic coronaviruses. There is still much to be discovered in the inventory of pro- and anti-viral host factors relevant for CoVs replication. The latest pandemic danger, originating from China, has given our previously prepared work even more of topicality.

Key words
coronavirus, spike protein, membrane fusion, viral entry, nonstructural proteins, replication complex

INTRODUCTION

Coronaviruses (CoVs) are positive-strand RNA viruses from the Coronavirinae subfamily which, together with Torovirinae, belong to the large Coronaviridae family in the Nidovirales order. The Coronavirinae subfamily is divided into different genera of alphacoronavirus (α-genus), betacoronavirus (β-genus), gammacoronavirus (γ-genus) and deltacoronavirus (δ-genus) [1].

Coronaviruses are able to infect a variety of mammals and other species. CoVs infections cause especially enteric and respiratory diseases in humans, including the common cold, as well as diseases in animals. Infrequently they are the reason for hepatitis and multiple organ failure [2]. The first member of the coronaviruses was identified in the 1930s [3], and in the 2003 they have become known again and classified as agents of Severe Acute Respiratory Syndrome (SARS) [4]. SARS-CoV is a human enveloped coronavirus with single positive-strand RNA of 25–31kb with a 5'-cap and 3'-poly(A) tail [5]. In 2002–2003, SARS-CoV caused almost 8,000 infections with a mortality rate of 10% [6, 7].

Middle East Respiratory Syndrome Coronavirus (MERS-CoV), first identified in Saudi Arabia in 2012, is also highly pathogenic. As of 4 December 2015, 1,621 laboratory-confirmed cases of infection with MERS-CoV were notified with approximately 36% mortality (584 deaths related to MERS-CoV) [8]. Currently, the rapidly-spreading COVID-19 pandemic, caused by SARS-CoV2 is being observed. The number of patients increases rapidly and as of 2 April 2020, there are over a million confirmed cases, and more than 50 thousand deaths [9].

OBJECTIVES

The aim of this study is to summarise current knowledge about coronaviruses and review the results of studies on CoVs entry into the host cell. Advances in understanding coronaviruses replication strategy and the functioning of the replicative structures are also highlighted.

Coronavirus genome. The size of the Coronaviruses varies from 80–120nm. Their 5'-capped single-positive strand RNA genome encodes 4–5 structural, 15–16 nonstructural and 1–8 accessory proteins [10]. The 5'- and 3'-ends of the CoVs genome consists of Untranslated Regions (UTRs) with cis-acting elements essential for viral replication and transcription.
CoVs genome size ranges from 26.2–31.7 kb and contains 6–10 Open Reading Frames (ORFs) [11]. The first ORF (ORF1a and ORF1b) encodes the replicate proteins and extends over 2/3 of the CoV genome. The replicate is a mandatory factor for the transcription of both: genomic (full-length) minus-strand template and subgenomic (discontinuous transcription) minus strand synthesis. As a result of polyproteins processing by proteinases, 16 non-structural proteins are generated. No-structural protein 3 (nsp3), non-structural protein 4 (nsp4) and non-structural protein 6 (nsp6) are considered to be responsible for the stability of the CoVs Replication/Transcription Complex (RTC). The remaining 1/3 of the ORF1 encodes 4 structural proteins: S (spike protein), E (envelope protein), M (membrane protein) and N (nucleocapsid protein). The M and E proteins are responsible for the assembly of the virions, whereas the S protein allows viral entry into the host cell, while the N protein is required for encapsidation of viral RNA. However, some of the CoVs also contain the additional HE (hemagglutinin esterase) viral protein, which is known to be involved in the host cell membrane interactions [10, 11, 12]. These structural proteins are created by discontinuous transcription during the subgenome minus-strand RNA synthesis [13, 14]. The genomic positive-sense RNA is rewritten into a negative-sense RNA template by RNA-dependent RNA polymerase (RdRp). Then, RdRp has the possibility to move to the 5’-end to finish the negative subgenomic (sg) mRNA, which might be a template for the positive-sense sgRNA synthesis. It has been shown that only the first unique gene from 5’-end of all sgRNA is translated. It last approximately 75–90 minutes, until both minus and plus-strand RNA can be detected after the infection [15, 16, 17, 18].

**Spike protein as the main agent of coronavirus cell entry.**

The spike protein plays a major role in the entry to the host cell. The infection is caused by a viral particle which interacts with the host receptors on the cell surface. This viral and cellular fusion triggers conformation of the S protein [19] which regulates cell tropi and host range, and also is involved as a main target of neutralizing antibodies during infection [20].

The coronavirus spike is the class I of transmembrane proteins, with a typical size ranging from 1,160–1,400 amino aci, and contains 21–35 N-glycosylation sites. Many studies have demonstrated that fusogenic conformational changes cause the generation of trimers composed of harpins on the virion surface [21, 22, 23, 24]. As a member of the class I viral fusion proteins [25, 26, 27, 28], the spike protein consists of 3 segments: an ectodomain, a single-pass transmembrane anchor, and a short intracellular tail [29, 30]. The ectodomain of the S protein consists of 2 domains: S1 (N-terminal), which is responsible for binding to the receptor and subsequently attaching the virion to the cell membrane, and the S2 (C-terminal), the most conserved region of the spike protein. The S2 subunit includes domains involved in membrane fusion: Fusion peptide (FP), heptad repeat domains-1 and -2 (HR1, HR2) and the transmembrane domain (TM) [31]. Commonly, domains of the spike protein remain connected in alpha- and majority of beta-corona viruses, but in gamma- and also same cases of beta-coronaviruses the spike protein is divided between those domains [19]. The S1 domain has 2 independent subdomains: N-terminal (S1-NTD) and C-terminal (S1-CTD). Both exhibit ability to bind molecules, sugars or proteins, as receptor binding domains (RBDs) [19]. RBDs enclose primary neutralization epitopes, activate host response, and for this reason might be a part of vaccines directed against infections caused by coronaviruses [20, 32, 33, 34, 35, 36].

Trimers of harpins consist 2 heptad-repeat regions (HR) in the S2 domain. They are assembled as prolonged triple helical coiled-coil motif (HR1) surrounded by 3 HR2 motifs, which are much more shorter [37, 38, 39]. Some studies have already been conducted on the crystal structure of CoVs RBDs, together with their cognate receptors [40, 41]. Casais at al. also demonstrated the S protein importance in tropism. They decided to use a recombinant Infectious Bronchitis Virus (IBV), a member of the Coronaviridae that replicates primarily in the respiratory tract as well as in epithelial kidney cells [42]. IBV strains are able to infect only chicken embryo cells. Thus, the authors decided to produce the rIBV in which the Beaudette S glycoprotein gene in Beau-R was replaced with the corresponding sequence derived from IBV M41-CK [43, 44]. The IBV Beaudette strain is able to spread in CK, CEF, BHK-21, and also in Vero cells. As opposed to IBV Beaudette, IBV M41-CK can only infects CK cells [45]. These differences provide examination of the cell tropism mechanism. They investigated BeauR-M41(S), a recombinant infectious bronchitis virus (rIBV), in which the ectodomain region of the S gene from IBV M41-CK was replaced by the corresponding region of the IBV Beaudette genome. As a result, BeauR-M41(S) obtained the same cell tropism as IBV M41-CK in different cell types, and indicated that spike protein is a determinant of cell tropism [42].

The chimeric virus is another example that perfectly shows the importance of the S protein regarding cell tropism. Mouse hepatitis virus (MHV) strains cause hepatic, neurological, respiratory and enteric diseases. One of the most studied is the weakly neurovirulent A59 strain, which causes moderate hepatitis and acute encephalitis. It undergoes clearance from the central nervous system (CNS) and the liver by strong CD8 T-cell response. Unfortunately, the viral RNA usually remains in the spinal cord and causes acute infection [46, 47, 48]. The JHM strain (also called JHM, SD or MHV-4), is highly neurovirulent and causes fatal encephalitis and only minimal hepatitis, while the MHV-2 strain is highly hepatotropic. By introduction of the JHM genes to the MHV-A59 strain, Navas at al. have shown enhanced virulence of the recombinant virus, which occurs by MHV receptor CEACAM1a-dependent and independent mechanisms as well as hepatotropism, and thereby the major role of the S protein in determination of organ tropism and neurovirulence [49, 50]. Replacement of the spike protein in A59 by the S protein from JHM causes a robust neurovirulent phenotype of the recombinant A59 virus (rA59). This chimera caused increased inflammation and the rate of viral antigen spread, compared to the wild type of A59 [51, 52, 53].

Finally, Wallis et al. contributed to the understanding of CoVs entry. They produced a mutated MHV S ectodomain trimer with enhanced stability, and therefore high affinity to the CEACAM1 receptor, and determined its structure at 4.0 Å resolution by single particle cryo-electron microscopy. They proved that the S trimer has 3 central helices that are packed through a central part. The S1 subunit has a ‘V’ shape. The N-terminal subdomain is mostly composed of domain A, while C-terminal are β-rich domains. S2 domains contain long α-helices and are associated with membrane. Researches highlight that this domain is especially similar to
the paramyxovirus F proteins with the core, central helix and upstream helix. They also indicated that in the conformation of pre-fusion, the SI subunits interlock and therefore form a crown around the S2 trimer stabilizing it [54].

There is a very large similarity between the SINTD structures and human galectin. SINTD adopts β-sandwich fold where 2 anti-parallel β-sheet layers form a core structure. Depending on the genera, an α-helix (α and δ coronaviruses) or a ceiling-like structure (β - coronaviruses) is attached to the upper part of the core. Three-stranded β-sheet and another α-helix are attached to the bottom of the core structure, regardless of the type of virus. Research on the structure and functions S1-NTDs from different genera coronaviruses suggest that all of them have the same evolutionary origin, and acquired the galectin gene from the host and included it in their spike gene which began to encode S1-NTD. Next the galectin fold was preserved during evolution [55, 56].

Coronaviruses α, β and δ show the same structural topology but different structural folds in terms of their SI-CTDs. coronavirus α SI-CTD has a β-sandwich core structure and is similar to the δ coronaviruses SI-CTDs which containing 2 β-sheet layers: one is a 3-stranded anti-parallel β-sheet, the second is a 3-stranded mixed β-sheet. β-coronavirus SI-CTDs has a β-sheet core. Coronavirus α and δ SI-CTDs core contains receptor binding motifs (RBMs) on the top in the form of 3 loops, but the closed conformation of the SI-CTD prevents binding of the receptor. To bind its receptor, the SI-CTD would need change conformation to an open structure. The RBM in β-coronavirus SI-CTD is a long continuous subdomain.

The structures of SD1 and SD2 are similar to those of their counterparts in α- and β-coronavirus spikes. Both of them adopt a small β-sandwich fold, except that SD1 contains 2 antiparallel β-sheets (2-stranded and 5-stranded), and SD2 contains 2 three-stranded β-sheets. Two SI subdomains connect S1 and S2. There are both structural and mechanistic similarities between coronavirus S2 and influenza HA2. This suggests that the 2 viral membrane fusion proteins are evolutionarily related. Parts of S2 form 6-helix bundle structures corresponding to HR-N and HR-C. The similarity between the influenza virus and coronaviruses allows some assumptions to be made about the construction and principles of S2. Regardless of this assumption, should be confirmed by the atomic structure of po-fusion CoVs S2 [55, 56].

The N protein as a virulent principle. The nucleocapsid protein (N protein) is an RNA binding protein that interacts with the M protein during virion assembly. It is also involved in the formation of the viral capsid [57] and plays an important role in the replication. First of all, it associates with both the genomic and subgenomic mRNA [58], as well as with the microtubules [59]. Moreover, N protein blocks the activity of L RNase by being the antagonist of type I Interferon (IFN) [60], and also induces the fibrinogen-like protein 2, subsequently causing the increase of liver damage after infection [61, 62, 63].

The nucleocapsid protein consists of 2 domains, an N-terminal (N-NTD) and a C-terminal (N-CTD). It also has 3 conserved regions (I, II, III) that are detached by A and B regions. Region II is responsible for RNA binding [64, 65] while region III plays an important role in the binding of the M protein [57]. NTD domain begins from the conserved region I and ends with conserved region II. CTD domain is located inside the conserved region II and ends just before region B [66, 67, 68, 69].

Cowely at al. investigated the role of the N protein in MHV-induced disease using the A59 and JHS strains described above. Their research was based on the fact that almost 95% of the amino acid level in the N proteins of those strains is identical. They used 2 chimeric viruses with the exchange of N proteins between the A59 and JHS strains, compared to the wild-strains. Surprisingly, no morphological changes were observed, but the observation focused on the virulence obviously satisfied their expectations, and statistically significant differences were noted in the replication in CNS. Moreover, the antigen expression in the brain was enhanced when the A59 chimera was used with the N protein from JHS [70].

The same chimera (rA59/NJHM) conferred enhanced virulence by an approximately 1,000-fold lower LD50 (Lethal Dose 50). In comparison, the chimera (rJHS/NA59) showed similar, but rather less results [70] This confirmed that the MHV antigen expression in the CNS consistently correlates with the virulence [51, 71].

### Table 1. Coronaviruses fusion with the membrane and entry to the host cell

| Genera | CoV | Tropism | Receptor – Domain | Reference |
|--------|-----|---------|-------------------|-----------|
| α-genus | NL63 | alveolar cells type I and II, endothelial cells, ciliated bronchial cells | ACE2 – S1CTD | [40] |
| 229E | | alveolar epithelial cells type I, alveolar macrophages | APN – S1CTD | [165,166] |
| TGEV | non-ciliated bronchial cells | | APN – S1CTD, sugar – S1NTD | [74] |
| PRCoV | non-ciliated bronchial cells | | APN – S1CTD | [74] |
| β-genus | OC43 | epithelial and neuronal cells | 9-O-acetylated sialic acid – S1NTD domain A | [166,167] |
| HKU1 | ciliated airway epithelial cells, type II alveolar epithelial cells | | 9-O-acetylated sialic acid – S1NTD domain A | [166,168] |
| MHV | leukocytes, epithelia, and endothelia cells | CEACAM1 – S1NTD, distal loops domain A | [41] |
| BCoV | non-enteric epithelial cells | 9-O-acetylated sialic acid – S1NTD domain A | [75] |
| MERS | endothelial cells, endothelial tissues | DPP4 – S1CTD, β-motif, domain B | [39] |
| SARS-CoV-1 | | alveolar cells type I and II, endothelial cells, ciliated bronchial cells | ACE2 – S1CTD, β-motif, domain B | [37] |
| SARS- CoV-2 | | alveolar cells type I and II, endothelial cells, ciliated bronchial cells | ACE2 – S1CTD, β-motif, domain B | [37] |
| γ-genus | IBV | epithelial cells | sugar – S1NTD | [75] |
All the human CoVs receptors belong to the same family – membrane ectopeptidases. However, it has been shown that viral entry is not dependent on the catalytic activity of these enzymes. The crucial step is co-expression of other host peptidases [76, 77], e.g. human transmembrane serine proteases HAT and TMRPSSII are involved in the cleaving and activating of the MERS-CoVs and SARS-CoVs Spike proteins [78, 79].

Angiotensin Converting Enzyme 2 (ACE-2), the functional receptor for the spike glycoprotein of the human SARS-CoV, is a zinc-dependent mono-carboxypeptidase that catalyzes the cleavage of angiotensin [80]. It regulates cardiovascular function [81] and protects against severe acute lung failure [82]. ACE-2 is expressed on alveolar cells types I and II, endothelial cells, and also on ciliated bronchial cells [83].

Aminopeptidase N (APN), also known as CD13, is a zinc-dependent ectopeptidase that cleaves peptides proteins from N-terminal amino acid. APN is a type II transmembrane protein associated with various functions, such as pain sensation, blood pressure regulation, cancer angiogenesis and metastasis, immune cell chemotaxis as well as cell-cell adhesion [84]. Two common CoVs are able to recognize APN: TGEV that infects cells of the small intestine and respiratory track, and PRCov (Porcine Respiratory Coronavirus) that infects only pulmonary cells. The one and only difference between spikes of those strains is that the S protein from TGEV has a haemagglutinating activity that enables TGEV to replicate in the gut [74]. APN is mostly expressed on non-ciliated bronchus cells [85].

Carcinoembryonic Antigen-Related Cell Adhesion Molecule 1 (CEACAM1) is a cell adhesion molecule that occurs on leukocytes, epithelia, and endothelia. It is responsible for apoptosis, angiogenesis, tumour suppression, and the induction of immune responses [86, 87, 88]. However, it has been noted that the neurovirulence of the JHM strain correlates with the enhanced spread of the virus in the brain and not fully dependent on CEACAM1. Miura at al. demonstrated that ceacam1-/- mice could also be infect by the JHM strain, but with a 100-fold higher lethal dose [89].

Dipeptidyl Peptidase-4 (DPP4), a serine exopeptidase, plays a multifunctional role in physiological processes by removing N-termini from many peptides, including hormones, neuropeptides, chemokines and mitogenic growth factors [86, 90]. DPP4 is commonly expressed in endothelial cells as well as multiple epithelial tissues [91].

Finally, some coronaviruses, e.g. avian infectious bronchitis virus (IBV), bind sugars that play an essential role in a variety of biological processes, such as cell interactions or immunity [92, 93].

Unfortunately, those examples do not explain how coronaviruses are able to recognize their specific receptors. There are 6 representative structures composed of S1 domains and its receptor: SARS-CoVs S1-CTD and ACE2 [37], MERS-CoV S1-CTD and DPP4 [39,94] HCoV-NL63 S1-CTD and ACE2 [40], PRCov S1-CTD and APN [95], MHV S1-NTD and CEACAM1 [41], OC43, HKU1, BCoV 9-O-acetylated sialic acid and S1-NTD [75] (Tab. 1).

There is no rule for the receptor recognition. CoVs from the same genera recognize different receptors, e.g., MERS-CoV and SARS-CoV belong to the β-genus, but their S1CTDs bind DPP4 and ACE-2, respectively [96, 97]. Moreover, some CoVs from different genera are able to recognize the same receptor, e.g. although NL63-CoV belongs to α-genus and SARS-CoV belongs to β-genus, both S1-CTDs bind ACE-2 [98, 99, 100, 101, 102, 103, 104]. Finally, coronaviruses could use one or both SI domains as RBDs, e.g., S1-CTD of TGEV-CoV binds APN, while its SI-NTD recognizes sugar [105].

It is well known that SARS-CoV mostly replicates in alveolar tissues, and is therefore commonly observed with pneumonia. On the other hand MERS-CoV has wide cellular tropism. It is able to replicate in both bronchial and alveolar tissue, and could therefore be the reason for such high mortality [106].

**Coronavirus entry and entry into the host cell.** After binding with specific receptors, enveloped viruses have the necessity to fuse with a host membrane, and subsequently deliver the viral genome to achieve successful infection. There are 2 main pathways. The first is pH-independent, based on genome delivery to the cytosol, and subsequently the fusion of their envelopes with the plasma membrane. However, many viruses use receptor-dependent endocytosis as a way of viral entry. In this process, virions are endocytosed and surrendered to the crucial step which takes place in the endosomes. Environmental cues, such as pH decrease, changing of the redox status and proteolytic activity, are necessary for inducing conformational changes in viral proteins, and subsequently lead to fusion of viral envelope and endosomal host membranes. Finally, the viral positive-strand RNA genome is released into the cytoplasm [107, 108, 109, 110].

**Cellular endocitosis of viral entry** could induce clathrin-dependent, caveolae-dependent, clathrin-independent as well as caveolae-independent mechanisms [111, 112, 113]. SARS-CoV was at first thought to enter by direct fusion at the plasma membrane [114, 115, 116]. Further studies identified that low pH could have asignificant influence on this process [117], and an acid protease, cathepsin L could be involved [118, 116], which suggests that SARS-CoV could enter via endocytosis.

Many experiments have been carried out that have produced conflicting results. One of the most common pathways is the clathrin-dependent pasthway,based on viral entry and translocation into endosomes where they are degraded or recycled [119, 120]. This pathway starts from binding the adaptor protein 2 (AP2) to the cytoplasmic tail of the receptors using clathrins [121, 122]. The receptors are then invaginated to clathrin coated pits. Viruses connected with receptors are subsequently endocytosed and transported to early endosomes in a pH-dependent way. Low pH is necessary to mature vesicles to late endosomes, and subsequently set up an infection [111, 112]. Inoue at al. demonstrated that SARS-CoV binds ACE2 and penetrates endosomes in the clathrin-dependent mechanism. Spike protein is first cleaved into the S1 and S2 subdomains by cathepsin L. This process provides the fusion of the viral envelope and the membrane of the endosome. Clattern-coated pits are shaped because of the contact between the AP2 with the clathrin complex and ACE2 with the virus, which is finally translocated to endosomes. Uncaging of the virus is made by acid protease, e.g. cathepsin L [119].

Further studies demonstrated that there is also a caveolae-dependent pathway. Caveolae are a flask-shaped type of lipid
rafts. These small invaginations of the plasma membrane are composed of cholesterol, glycosphingolipids and caveolin [123]. Caveolin is able to oligomerize which leads to the formation of caveolin-rich microdomains in the plasma membrane, and subsequently to the endocytic vesicle. The released caveolar vesicle has gained the ability to fuse with the early endosomal compartment – caveosome or even endosome [124]. Choi at al. demonstrated on the MHV strain that lipid rafts must have been involved in virus entry to the host cell and also in cell–cell fusion [123], but to-date there is no data about the host lipid modulation by CoVs [125].

The current study concerned the inventory of pro- and antiviral host factors relevant for SARS-CoV replication. Using siRNA (small interfering RNAs), de Wilde et al. demonstrated that over 40 proteins, including molecules involved in lipid metabolism, promote SARS-CoV replication. They found that depletion of double-stranded RNA-activated protein kinase (PKR) promotes increased SARS-CoV protein expression. Moreover, cyclin-dependent kinase 6 (CDK6) was identified as a novel anti-viral host factor in SARS-CoV replication [126].

Wang et al. confirmed that CoVs could also infect cells in a clathrin- and caveolae-independent mechanism. This concerned especially CoVs that harbor a non-cleaved spike on their surface. Wang at al. discovered that SARS-CoV is able to enter cells by a pH-sensitive pathway in the absence of clathrin-mediated endocytosis [127]. Indeed, because of the low pH, SARS-CoV infection can be inhibited by lysosomotropic agents [128]. Thus, SARS-CoV fusion could probably be triggered by proteolytic processing of the spike. It has been proved in vitro that SARS-CoV infection is enhanced by a diversity of proteins, such as elastase, thermolysin or trypsin [129], which trigger fusion by dual cleavage: between S1 and S2 together, as well as at the N-terminal of the fusion peptide [130, 131, 132].

Another research investigated the endo- and lysosomal pathway in a proteolysis-dependent manner. Using siRNA, the investigators demonstrated that strain A59 of MHV-CoV requires proteins for the maturation of the endosome, and its subsequent fusion with lysosome during cell infection. They confirmed that MHV entry is dependent upon clathrin-mediated endocytosis by using replication-independent fusion assay and the MHV particles fuse in lysosomes. They hypothesized that the fusion site is determined by the proteolytic cleavage site, upstream of the FP in the spike protein [107].

Conducted studies demonstrated that viruses could use more than one pathway to enter into the host cell [133, 134]. Over the years, coronaviruses have altered their S proteins, which leads to the variety of the fusion mechanisms depending on the strain. It remains unclear how CoVs choose the way of entry. After binding with a specific receptor, fusion might appear at the cell surface or after endocytosis. Preferred entry could also be dependent on the cell type and pH acidification.

**Membrane rearrangements**. During the infection in mammalian cells, coronaviruses rearrange cellular membranes into organelle-like replicative structures including double-membrane vesicles (DMVs) and convoluted membranes (CMs). DMVs range vary from 150–300 nm [135]. Nsp3, nsp4 and nsp6 are believed to structure the DMV formation and subsequently attach the RTC to intracellular membranes [17, 136, 137, 138]. RTC is constructed through the interplay of the 16 CoVs non-structural proteins and as well as the N protein [137].

Coronaviruses also induce a wide range of membrane structures, some of which are responsible for viral RNA synthesis, such as DMVs and CMs. DMVs are present in a very high proportion in the perinuclear region of cytoplasm. CMs are present between the clusters of DMVs [139, 140, 141]. Many studies have demonstrated that DMVs do not remain isolated vesicles, but an interconnected membranous network continuous with Endoplasmic Reticulum (ER) [139]. Electron tomography studies confirmed that these replicative structures are localized in MHV-CoV and SARS-CoVs nps. [136, 142, 139, 140, 143, 144]. However, CMs are more highly enriched in SARS-CoV nsp5 than DMVs [139]. Therefore, it is thought that active replicate complex is localized to the CM [145].

Immunoelectron microscopy has shown that newly synthesized viral RNA was present near the replicative structures described above [146, 147]. Later, CoVs generate Large Virion-Containing Vesicles (LVCVs), Tubular Bodies (TBs) and also Cubic Membrane Structures (CMSs) [114, 139, 140, 148]. Subsequently, at later stages of SARS-CoV infection, DMVs are believed to fuse into packets of single-membrane vesicles surrounded by a common outer membrane, and the clustered single DMVs slowly disappeared. Those Vesicle Packets (VPs) are quite large (1–5μm) and tend to include over 25 inner vesicles [139, 148]. However, electron tomography has not revealed any connections between the interior of DMVs and the cytosol. This finding leads to the hypothesis that the replication carries out in DMVs until the network with the cytosol is maintained [145].

It remains unclear where nascent viral RNA synthesis takes place. Although nps include DMVs and CMs, the connection with the cytoplasm has not yet been detected. The presence of viral enzymes involved in RNA synthesis is not equal to active RTCs. Sites of RNA synthesis certainly include RdRp, dsRNA, as well as nascent viral RNA. The latter has been visualized close to antibodies that recognize nsp5 and the C-terminal end of pp1a [147]. Further studies demonstrated that newly-synthesized coronavirus RNA were observed in MHV-infected cells in the vicinity of DMVs [149]. Furthermore, nascent viral RNA was also observed in close proximity to nsp12 that contains RdRp [135].

However, during later stages of CoVs infection, not all DMVs remain active and are not involved in RNA synthesis. Moreover, those which persist actively, frequently enclose DMVs. DMVs remain isolated vesicles, but an interconnected membranous network continuous with Endoplasmic Reticulum (ER) [139]. Many studies have demonstrated that DMVs do not remain isolated vesicles, but an interconnected membranous network continuous with Endoplasmic Reticulum (ER) [139].

Reticulovesicular Network (RVN) is a part of a membrane associated with the virus replication. Many studies have suggested that ER could be involved in the biogenesis of this compartment [150, 151]. It has been shown that the Endoplasmic-Reticulum Associated Protein Degradation (ERAD) is connected with CoVs replicative structures [152]. On the other hand, there are many membrane proteins, such as Sec13, Arf1, GBF1 or synaxin 5 that have not been detected in RVN, despite functioning downstream of the ER in the secretory pathway.
Treatment of CoVs infections. Despite many scientific studies on human CoVs, there is currently no efficient therapy available. Commonly, patients are treated ineffectively with interferon or/and ribavirin [153, 154, 155, 156, 157]. The treatment with IFN alpha or lambda is only reasonable for the preventive treatment of exposed people. The rise of newer and more acute CoVs has emphasized the demand to develop effective therapeutic tools against CoVs. Therefore it highlights the need to discover an effective therapeutic tool against CoVs.

Over the years, a wide range of molecules have been determined against coronaviruses, i.e. protease inhibitors [158, 159, 160]. Lundin at al. screened a collection of 1,6671 varied compounds against human CoVs and identified an inhibitor, designated K22, which specifically targets membrane-bound RNA synthesis. It proved to be efficient in primary human epithelia cultures against a wide spectrum of human coronaviruses, including MERS. K22 expend the most effective antiviral activity at an early step of the viral life cycle [161].

The ideal target of vaccinology seems to be the accessibility of the conservation sequence of the fusion peptide at the periphery of the trimer [54]. Studies may be based on raising neutralizing antibodies of CoVs S proteins, which overlap with the fusion peptide [162]. Antigenic determinants that binds to this conserved site could block the insertion of the fusion peptide into target membrane, and also prevent conformational changes [54].

Some trials conducted on Spike proteins have achieved in vivo a therapeutic threshold, i.e. against SARS [163] or MERS [164], which suggests that it might be possible to discover a vaccine against human CoVs.

CONCLUSIONS
Coronaviruses has the largest RNA plus strand genome which encodes a variety of RNA-modifying enzymes, often absent in most virus strains. Many studies have demonstrated that the spike protein is the crucial molecule responsible for CoVs tropism and cell entry. Recent research has shown that modifications of the S protein could affect the viral pathogenicity. The development of host-directed antiviral therapy seems to be promising way to treat infections with SARS-CoV or other pathogenic coronaviruses. There is still much to be discovered in the inventory of pro- and antiviral host factors relevant for CoVs replication. Therefore, studies on coronaviruses have a significant impact on health and the economy. Further research on the interactions between coronaviruses proteins in viral replication may also be of appreciable importance.

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