

# World Journal of *Gastroenterology*

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## Cathepsin L, transmembrane peptidase/serine subfamily member 2/4, and other host proteases in COVID-19 pathogenesis – with impact on gastrointestinal tract

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### Abstract

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) seems to employ two routes of entrance to the host cell; *via* membrane fusion (with the cells expressing both angiotensin converting enzyme 2 (ACE2) and transmembrane peptidase/serine subfamily member 2/4 (TMPRSS2/4)) or *via* receptor-mediated endocytosis (to the target cells expressing only ACE2). The second mode is associated with cysteine cathepsins (probably cathepsin L) involvement in the virus spike protein (S protein) proteolytic activation. Also furin might activate the virus S protein enabling it to enter cells. Gastrointestinal tract (GIT) involvement in SARS-CoV-2 infection is evident in a subset of coronavirus disease 2019 (COVID-19) patients exhibiting GIT symptoms, such as diarrhea, and presenting viral-shedding in feces. Considering the abundance and co-localization of ACE2 and TMPRSS2 in the lower GIT (especially brush-border enterocytes), these two receptors seem to be mainly involved in SARS-CoV-2 invasion of the digestive tract. Additionally, *in vitro* studies have demonstrated the virions capability of infection and replication in the human epithelial cells lining GIT. However, also furin and cysteine cathepsins (cathepsin L) might participate in the activation of SARS-CoV-2 spike protein contributing to the virus invasiveness within GIT. Moreover, cathepsin L (due to its involvement in extracellular matrix components degradation and remodeling, the processes enhanced during SARS-CoV-2-induced inflammation) might be responsible for the dysregulation of absorption/digestion functions of GIT, thus adding to the observed in some COVID-19 patients symptoms such as diarrhea.

**Key Words:** COVID-19; SARS-CoV-2; Angiotensin converting enzyme 2; Transmembrane peptidase/serine subfamily member 2/4; Cathepsin L; Gastrointestinal tract

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**Core Tip:** Gastrointestinal tract (GIT) is believed to participate in severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) dissemination. The current research shows the abundance and co-localization of angiotensin converting enzyme 2 (ACE2) and transmembrane peptidase/serine subfamily member 2 receptors in the lower GIT. Furthermore, about half of coronavirus disease 2019 patients present with GIT symptoms, such as diarrhea, and exhibit viral-shedding in feces. Additionally, *in vitro* studies have demonstrated the virions capability of infection and replication in the human epithelial cells lining GIT. This paper reviews the possible routes of the virus infection with respect to the host-enzymatic systems responsible for the proteolytic priming of SARS-CoV-2.

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## INTRODUCTION

Coronaviruses may employ several host proteases for their invasion into target cells. The enzymes participating in the viruses activation include: proprotein convertases (PCs) (mainly furin), transmembrane serine proteases, especially transmembrane peptidase/serine subfamily member 2 (TMPRSS2), the lysosomal cathepsins (mainly cathepsin L), elastase, and coagulation factor Xa[1].

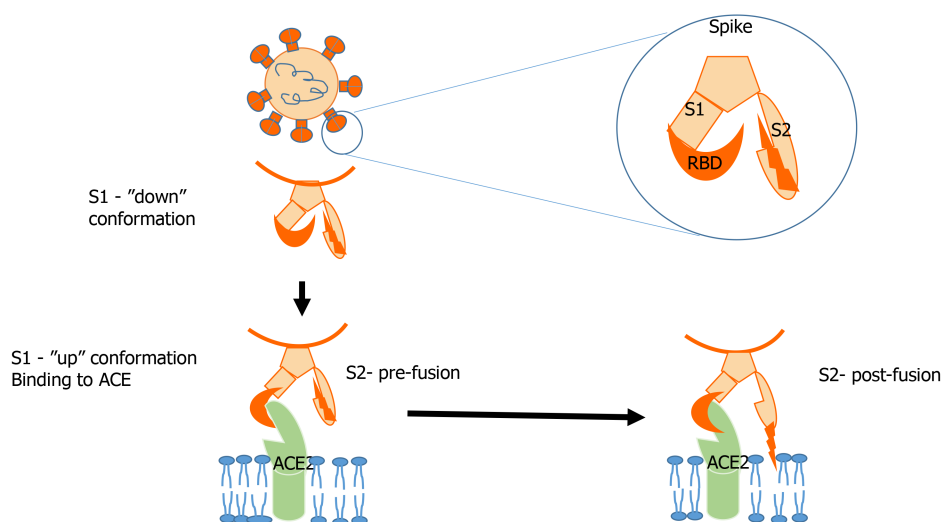
Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) virus responsible for coronavirus disease 2019 (COVID-19) pandemic exhibits many similarities when compared to SARS-CoV. It employs a similar mechanism of host cells invasion; recognizes and binds the same type of angiotensin converting enzyme 2 (ACE2) receptors to enter host cells (but with higher affinity[2]), and comparable processing of spike protein (S protein) seems to be necessary for the virion fusion with the host cell membrane[3-7] (Figure 1).

Two proteolytic events need to be conducted for SARS-CoV-2 activation; initially spike protein is cut in the specific cleavage site between S1 and S2 domain, then the second cleavage within S2 domain (S2' site) allows for the exposition of the fusion peptide, which enables membrane fusion. The first proteolytic step can happen in the producer cell, in the extracellular space, or within the host cell's endosome. This cleavage site in SARS-CoV-2 spike protein is recognized by various proteases, including furin (unlike in SARS-CoV lacking furin-cleavage site between S1 and S2)[6, 8] and TMPRSS2[3]. The second cleavage can be either performed by TMPRSS2 on the surface of the host cell, or in the endolysosomes by lysosomal proteases, most probably cathepsin L[9].

Therefore, as proposed by Pislari *et al*[10], two routes of SARS-CoV-2 entry to the host cell are likely; *via* membrane fusion with the host cells which expose both ACE2 and TMPRSS2 (and/or other transmembrane serine proteases such as TMPRSS4[11]) proteins, or *via* receptor-mediated endocytosis (RME) to the target cells expressing only ACE2 receptors. In the first case, both processing steps performed by TMPRSS2 before the virus entry enable membrane fusion, whereas in the second mechanism, the virion binding with ACE2 receptors induces endocytosis followed by spike protein activation by cathepsin L (and/or other cysteine cathepsins)[4,10]. As a result of either of these pathways, viral RNA is released in the host cell and undergoes the processes of replication (Figure 2).

## TYPES OF HOST PROTEASES IN SARS-CoV-2 INVASION

The findings of several studies support the notion that, apart from ACE2 receptors, the main host peptidases involved in SARS-CoV-2 spike protein processing include: TMPRSS2 (and/or TMPRSS4), lysosomal cysteine cathepsins (mainly cathepsin L), as well as furin-like PCs. They may participate in SARS-CoV-2 activation independently,



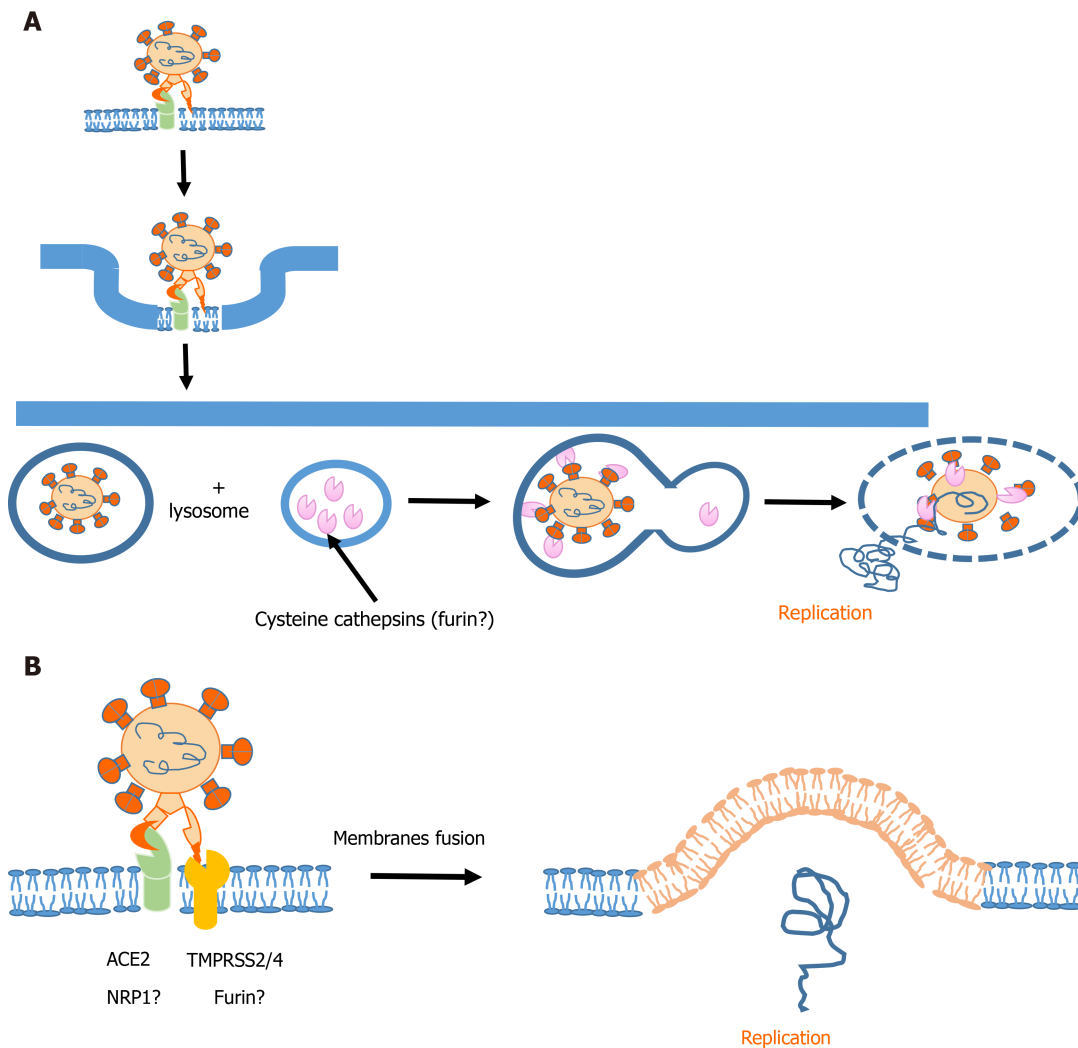
**Figure 1 Alterations in spike proteins conformation upon binding to ACE 2.** Spike S1 subunit contains receptor binding domain, that has to change from "down-conformation" state to "up-conformation" state to be accessible for ACE2. Changes in S1 subunit trigger conformational changes in S2 subunit, causing exposition of hydrophobic domain, changing it from "pre-fusion" to "post-fusion" state. This enables fusion of the virus with host membrane (after Zhu *et al*[7]).

or their actions may overlap or complete one another, depending on the pattern of the virion-recognized proteins exposed on the host cells (*e.g.* expressing or not TMPRSS2).

### Cysteine cathepsins (CCs) in pathology

Cysteine cathepsins belonging to the papain-like family of cysteine proteases (containing cysteine in their catalytic center) comprise 11 cathepsins (B, C, F, H, K, L, O, S, V, X and W) in the human organism[12,13]. They belong to lysosomal proteases involved mainly in intracellular protein breakdown, antigen processing, MHC-II mediated immune response, and apoptosis. However, their functions go far beyond this; they participate in various physio-pathological processes not only intracellularly, but also in the extracellular matrix (ECM), because, except for their endolysosomal sequestration, they have been observed in the nucleus, cytosol, mitochondria, at the plasma membranes and in the extracellular milieu[13,14]. Their secretion is observed in physiological conditions (*e.g.* in bone remodeling – conducted by osteoclasts-secreted cathepsin K, in wound healing performed by keratinocytes-secreted cathepsin B, in prohormone processing – thyroid hormones released from thyroglobulin by cathepsins B, L and K secreted from the thyroid epithelial cells). However, an excessive secretion of cysteine cathepsins is mostly observed in pathological states associated with inflammatory processes, such as cancer diseases (cathepsins B, C, K, L, S, H, X), cardiovascular diseases (cathepsins C, K, L, S, V), joint and bone diseases (cathepsins K, B, L, S, H), inflammatory bowel disease (cathepsin L), and many other disorders (summarized in[12-15]). In these pathological states CCs typically act extracellularly, where they participate in collagen, elastin, and other ECM components degradation directly or indirectly (activating other proteases) after being secreted from recruited immune cells (mostly), as well as from inflamed tissue cells (to the lesser extent). Macrophages and other immune cells infiltrating tissues seem to be the main extracellular source of CCs whose secretion is stimulated by inflammatory factors like cytokines. However, also other cell types secrete excessive amounts of CCs, including osteoclasts or chondrocytes (oversecreting cathepsin K and/or S in arthritis and osteoporosis), or cancer cells and tumor-associated fibroblasts (oversecreting cathepsin S, L and/or B in cancer invasion)[14]. Apart from degradation of main components of ECM, also more refined processing of ECM (undergoing both intra-, and extracellularly) is ascribed to CCs. This comprises modifying and shedding cell adhesion molecules and cell membrane receptors, which affects signal transduction pathways, as well as processing cytokines and chemokines, which upregulates immune response [14]. The resulting augmentation of inflammatory processes further induces the secretion of CCs, enhancing the destruction of ECM, which eventually leads to the acceleration of the processes observed in the aforementioned disorders.

Due to their roles in multiple inflammatory-based disorders, CCs have been considered for a long time as a target for medicinal drugs design. However, because of ubiquitous expression of most CCs, their constitutive, overlapping functions, broad substrate specificities, most of the studied so far medicines have exhibited unfavorable



**Figure 2 Two modes of virus entry.** A: Receptor-mediated endocytosis of severe acute respiratory syndrome coronavirus-2. After binding to ACE2 and formation of endosome, lysosomal cathepsins activate spike protein, which leads to the release of the viral RNA into host cell. B: Membrane fusion mechanism - priming of the spike proteins is mediated by transmembrane peptidase/serine subfamily member 2/4, which leads to fusion of viral and host membranes and release of the viral RNA into host cell.

side effects, thus not surviving clinical trials. However, the attempts to construct CCs-aimed drugs, taking advantage of the newest technology, are underway. The most clinical trials have been conducted on cathepsins K and S inhibitors, also cathepsin C seems to be a promising target, whereas the remaining cathepsins inhibitors have either got stuck in the initial stages of clinical trials or their trials have been discontinued (reviewed in[14]).

### Cathepsin L in inflammatory processes

Similarly to other cysteine cathepsins, cathepsin L exhibits pleiotropic activities in the human organism. One of the most evident actions of this enzyme is (beside cathepsins S, K and V) its participation in inflammatory processes associated with various pathological conditions[14-16]. For example Menzel *et al*[17] have demonstrated the up to 10-fold induction of cathepsin L expression (mRNA) in intestinal macrophages derived from inflammatory bowel disease (IBD) patients, and the clear improvement of the disorder symptoms in DSS (dextran-sulphate-sodium)-induced colitis mice model, when a simultaneous application of cathepsins L and B inhibitors was investigated. Xu *et al*[18] have exhibited the stimulatory function of cathepsin L in microglia-mediated neuroinflammation, which accompanies many neurological disorders including Parkinson's disease. Cao *et al*[19] have shown the correlation between serum cathepsin L activity and the markers of inflammation (such as neutrophil counts and hs-CRP) in the patients with chronic kidney disease.



### Cysteine cathepsins and TMPRSS2 in SARS-CoV-2 invasion

Cell line experimental systems creating the environment aimed at the inhibition of CCs have substantiated the function of these enzymes in the processes of SARS-CoV-2 activation. Raising pH in the endolysosomal compartments (with ammonium chloride and/or bafilomycin A), which inactivates lysosomal proteases working in acidic environment, or application of cysteine proteases inhibitors (such as E-64d inhibitor - inactivating cysteine cathepsins L, B, H, as well as cytosolic calpain) have significantly limited entry of SARS-CoV-2 into chosen cell lines[4]. Ou *et al*[20] (applying lentiviral pseudotype system) have demonstrated that the treatment of HEK-293/hACE2 cells with E-64d inhibitor reduced entry of SARS-CoV-2 S pseudovirions by over 90%. Further, the Authors compared the effect of two specific inhibitors of cathepsin L (SID 26681509), and cathepsin B (CA-074). Whereas the first inhibitor limited the pseudovirions entry by over 76%, the second one did not exhibit any significant effect, which suggests a prevalent function of cathepsin L in the receptor-mediated endocytosis mechanism of the virus invasion. RME mechanism in HEK-293/hACE2 cells has been confirmed by the Authors in the experiments showing the inhibition of the SARS-CoV-2 S protein entry by blocking the factors inevitable in the process of endolysosomal trafficking (PI(3)P 5-kinase (PIKfyve) and two-pore channel subtype 2 (TPC2)[20]. The involvement of CCs has been also observed by Hoffmann *et al*[3] who demonstrated that the increase in pH (with ammonium chloride) almost completely inhibited SARS-2-S-driven entry into 293T cells expressing ACE2 but devoid of TMPRSS2. This observation is in agreement with Ou *et al*[20] findings confirming that the virus entry into these cells undergoes *via* RME with the involvement of cathepsin L in spike protein activation. In their experiments on the human colon cell line - Caco-2 cells overexpressing TMPRSS2, Hoffmann *et al*[3] exhibited the participation of both TMPRSS2 and CCs in the mechanism of the virus entry into these cells. Alkalization of the environment caused around 90% reduction in SARS-2-S-driven entry, whereas incubation with E-64d inhibited the virus entry by around 40%. On the other hand, the application of camostat mesylate (inhibitor of TMPRSS2 and other serine proteases) reduced the virus entry by about 90%, and when the Authors used both inhibitors simultaneously, they achieved nearly complete virus-entry inhibition to Caco-2 TMPRSS2 (+) cells. They also found that E-64 inhibitor significantly reduced the virus entry into Vero and 293T cells not expressing TMPRSS2, which indicates the endolysosomal pathway. However, the transduction of these cell lines with TMPRSS2 markedly reversed the effect of CCs inhibition, emphasizing the function of TMPRSS2 in the S protein priming, agreeably with Ou *et al*[20] findings that the expression of TMPRSS 2, 4, 11 A, 11D, and 11E on 293/hACE2 cells enhanced SARS-CoV-2 S protein-mediated cell-cell fusion. Moreover, the Authors noted that, however the addition of trypsin (like TMPRSS belonging to serine proteases) stimulated the formation of syncytia in 293/hACE2 cells (indicative of activated by trypsin SARS-2-S protein-stimulated cell-cell fusion), this process was also noticed in the experiments without trypsin. These findings indicate that binding with ACE2 receptors may be a sufficient event inducing cell-cell fusion (without the proteolytic priming with the extracellular protease). Nevertheless, it is possible that other host extracellular peptidases are involved in spike protein activation. The findings derived from Hoffmann *et al*[3] and Ou *et al*[20] experiments are depicted in Table 1.

### Furin in SARS-CoV-2 invasion

As determined by Shang *et al*[8], another enzyme involved in SARS-CoV-2 spike protein priming is furin belonging to proprotein convertases (PCs). PCs are eukaryotic serine proteases, and ubiquitously expressed furin belongs to the PCs subfamily present in the organelles of the constitutive protein secretion pathway. These enzymes participate in the proteolytic post-translational modification of a variety of functionally important peptides and proteins, such as growth factors and hormones, both intra- and extracellularly (in the trans-Golgi network, endosomes, and pericellular environment). The amino acid sequence specifically recognized and cleaved by PCs including furin, is found in many viral surface proteins, so different viruses (like MERS-CoV) are activated by these enzymes[1]. Unlike SARS-CoV (exhibiting PC cleavage site motif only in the S2' site) [1], SARS-CoV-2 spike protein includes PCs specific motif at the S1/S2 boundary[8]. Shang *et al*[8] have performed experiments to examine whether this sequence is cut by furin. They demonstrated that PCs inhibitors reduced SARS-CoV-2 pseudovirus entry into three cell lines expressing hACE2 receptors; HeLa cells (human cervical cells), Calu-3 cells (human lung epithelial cells), and MRC-5 cells (human lung fibroblast cells). Moreover, the mutation of the PCs specific motif significantly reduced the pseudovirions entry to the studied cells. The

**Table 1 Receptors/proteases involved in Severe acute respiratory syndrome coronavirus-2 invasion of human cells**

Receptor/protease	Experimental model	Observation	Ref.
ACE2 and TMPRSS2/4	Human small intestinal enteroids; HEK-293T cell line transfected with ACE2, TMPRSS2, or TMPRSS4	Productive infection of SARS-CoV-2 in ACE2 (+) mature enterocytes; Correlation of ACE2, TMPRSS2 and 4 with SARS-CoV-2 invasiveness	Zang <i>et al</i> [11]
ACE2 and TMPRSS2	ACE2 and TMPRSS2 expressing C2BBel, Caco-2, and Calu-3 cell lines	Persistent invasion and replication of SARS-CoV-2 in the cells; Correlation of TMPRSS2 (but not ACE2) with SARS-CoV-2 RNA	Lee <i>et al</i> [33]
ACE2, TMPRSS2, and CCs	HEK-293T cell line transfected with ACE2; Caco-2 cells overexpressing TMPRSS2	Inhibition of SARS-CoV-2 pseudovirus entry into HEK-293T ACE2(+)/TMPRSS2(-) by pH increase and E-64 inhibitor; Inhibition of SARS-CoV-2 pseudovirus entry into Caco-2 TMPRSS2(+) by pH increase, CCs inhibitor (E-64d), and TMPRSS2 inhibitor (camostat mesylate)	Hoffmann <i>et al</i> [3]
ACE2, TMPRSS 2, 4, 11A, 11D, 11E, and CCs (cathepsin L)	HEK-293T cell line transfected with ACE2	Inhibition of SARS-CoV-2 pseudovirus entry into the cells with CCs inhibitor (E-64d) and cathepsin L inhibitor (but not cathepsin B inhibitor); Intensification of SARS-CoV-2 S protein-mediated cell-cell fusion, caused by expression of TMPRSS 2, 4, 11A, 11D, and 11E on 293/hACE2 cells	Ou <i>et al</i> [20]
ACE2 and TMPRSS2	ACE2 and TMPRSS2 expressing Caco-2 and T84 cell lines	Persistent invasion and replication of SARS-CoV-2 in the cells	Stanifer <i>et al</i> [35]
ACE2, furin, TMPRSS2, and CCs	ACE2-expressing HeLa, Calu-3, and MRC-5 cell lines	Reduction of SARS-CoV-2 pseudovirus entry into cells by inhibitors of PCs, CCs and TMPRSS2	Shang <i>et al</i> [8]
NRP1, ACE2, and TMPRSS2	HEK-293T cell line transfected with ACE2, TMPRSS2, or NRP1	Augmentation of SARS-CoV-2 infectivity when NRP1 was coexpressed with ACE2 and TMPRSS2	Cantuti-Castelvetri <i>et al</i> [21]

ACE2: Angiotensin converting enzyme 2; NRP1: Neuropilin 1 (receptor which binds furin-cleaved substrates); TMPRSS2/4: Transmembrane peptidase/serine subfamily member 2/4; CCs: Cysteine cathepsins; HEK-293T: Human embryonic kidney 293T cells; Caco-2: Human colorectal adenocarcinoma cell line; C2BBel: A subclone of Caco-2; T84: Human colon carcinoma cell line; HeLa: Human cervical cell line; Calu-3: Human lung epithelial cell line; MRC-5: Human lung fibroblast cell line; PCs: Proprotein convertases.

Authors detected no cleavage within the spike protein, when they packaged the pseudoviruses to HEK293T cells pretreated with furin-targeting siRNA. Additionally, they excluded the participation of matrix metalloproteinases (MMPs) in the experiment with the application of MMP inhibitor. Therefore, they confirmed the involvement of furin in SARS-CoV-2 entry into chosen cells. Additionally, furin priming of SARS-CoV-2 spike protein has been associated with the virions recognition and binding by neuropilin 1 (NRP1 - receptor which binds furin-cleaved substrates). Cantuti-Castelvetri *et al*[21] have shown that, except for ACE2, also NRP1 receptors are involved in SARS-CoV-2 invasion. Although the exact mechanism of NRP1 participation in this process is not elucidated, the Authors found the receptors to markedly enhance the virus infectivity. Apart from furin involvement, Shang *et al*[8] also observed the association of other aforementioned peptidases with SARS-CoV-2 invasion. They noticed the reduction in the pseudovirus entry to the three studied cell lines, caused by the application of both serine proteases (camostat) and cysteine cathepsins (E64) inhibitors. Furthermore, the pseudovirus entry to HeLa cells was more markedly reduced when either camostat or E64d was applied in the cells pretreated with proprotein convertases inhibitor[8]. Therefore, it might be concluded that depending on the type of target cells, TMPRSS2, lysosomal cathepsins, and furin might be involved in the activation of SARS-CoV-2 entry exhibiting cumulative/overlapping final effect (Figure 2). The observations of Shang *et al*[8] and Cantuti-Castelvetri *et al*[21] are collected in Table 1.

### **Cysteine cathepsins as a target in search for COVID-19 therapy**

Focusing on cathepsin L expression as a target in search for an anti-Covid-19 drug, Smieszek *et al*[22] have tested an array of medications applied in clinical practice. They found amantadine (a drug used previously to treat influenza A, and now applied in neurological diseases including Parkinson's disease) to be a promising compound. Being able to accumulate in lysosomes and alkalize them, amantadine belongs to lysosomotropic agents. Such compounds inactivate lysosomal enzymes including CCs whose optimal pH lies below 5. The Authors demonstrated a significant reduction in cathepsin L gene expression using amantadine, however also other cysteine cathepsin

genes expression was inhibited, including cathepsins B and K, with the most pronounced effect observed for cathepsin H. Therefore, it might be hypothesized that this is also cathepsin H which plays an important role in the processing of the SARS-2-S spike protein. The activity of cathepsin H would have been inhibited similarly to other lysosomal cathepsins in the aforementioned experiments (raising pH and/or using E64 inhibitor), which demonstrated a significant reduction of SARS-2-S-mediated entry to the studied cells. However, in comparison with thoroughly studied cathepsins B, L, S, and K, there is much less scientific data referring to cathepsin H.

Amantadine efficiency in COVID-19 treatment has been suggested by Rejdak *et al* [23] who documented no clinical manifestations of COVID-19 infection in 22 patients in spite of the confirmation of SARS-CoV-2 presence with rRT-PCR testing in all of these individuals. The patients had been treated with either amantadine or memantine, for at least 3 months prior to the infection exposure, due to their conditions (multiple sclerosis, Parkinson's disease or cognitive impairment). Therefore, amantadine seems to be a promising treatment for COVID-19 patients. The effect of amantadine may be associated with the down-regulation of cysteine cathepsins (L and/or H) in the endolysosomal compartment and/or the disturbance of viroporin protein channel probably involved in the viral RNA release, as suggested for SARS-CoV[24].

As discussed before, the generation of anti-CCs medicinal drugs is a problematic issue (associated with the observation of unfavorable side effects). Hence, the attitude aimed at screening the already existing therapeutics, which would lower the activity of proteases involved in SARS-CoV-2 activation, seems a rational approach.

## EFFECT OF SARS-CoV-2 ON GASTROINTESTINAL TRACT

### ***Gastrointestinal symptoms and fecal virus shedding in COVID-19 patients***

A significant amount of scientific evidence accumulated so far points to gastrointestinal tract (GIT), especially its lower part, as a target organ affected by SARS-CoV-2, beside the respiratory system[25]. Apart from the typical pulmonary symptoms (cough, fever, shortness of breath), some of the patients (around 4%-50% individuals) present with digestive symptoms like diarrhea, nausea, vomiting and abdominal pain[26]. Moreover, the virus mRNA presence in stool samples has been observed in some patients, often persisting long after its disappearance from the respiratory tract. Wu *et al*[27] have documented SARS-CoV-2 mRNA presence in the feces of more than half of the studied patients, with duration for up to 5 wk after its vanishing from the respiratory tract specimens. It might suggest active proliferation of the virus in the gastrointestinal tract of some patients. Similarly, Xiao *et al*[28] have observed fecal virus shedding in more than 50% of the studied patients, and in over 20% of them the duration of positive results in stool exceeded the virus presence in the respiratory samples. Additionally, the Authors detected the protein parts of the virus (as well as the presence of ACE2 receptors) in gastrointestinal epithelial cells. The virus replication in rectal tissue derived from a COVID-19 patient has been noted by Qian *et al*[29] who detected SARS-CoV-2 components in the intestinal epithelial cells (but mainly in intestinal lymphocytes and macrophages). The Authors hypothesize that, like in the case of influenza virus, it is possible for SARS-CoV-2 virions to be transported from the respiratory tract to the GIT *via* the immune cells. In the meta-analysis of 60 studies comprising over 4000 COVID-19 patients, performed by Cheung *et al*[30], 17.6% of the patients exhibited gastrointestinal symptoms, and in almost 50% (30%-70%) the virus mRNA was detected in the feces. Most of these positive stool samples (above 70%) were collected after the loss of the virus from the respiratory specimens.

However, it is still not clear, whether the virus is transmittable *via* fecal-oral route. Whereas Wang *et al*[31] detected live virus in the fecal samples, Zang *et al*[11] demonstrated the inactivation of the virions released into the intestinal lumen in the environment simulating human colonic fluid. Moreover, the Authors did not manage to recover infectious virus from the stool specimens.

### ***Ability of SARS-CoV-2 to productively infect human GIT cells via ACE2 and TMPRSS***

Several studies have demonstrated the invasion and replication of SARS-CoV-2 in the human GIT cells. Chu *et al*[32] in their ex-vivo experiments on human intestinal tissues have evidenced the ability of SARS-CoV-2 to infect, proliferate and release infectious virus particles from intestinal cells. In comparison with SARS-CoV, SARS-CoV-2 replicated less efficiently and brought about less damages in the human intestinal

epithelium, but evoked greater response of innate immune system (inducing the expression of proinflammatory mediators such as interferons and interleukins). Hence, the Authors suggested that the gastrointestinal tract might serve as an alternative route of virus dissemination. The vulnerability of the human GIT epithelial cells to SARS-CoV-2 invasion *via* ACE2 and TMPRSS-2 receptors has been confirmed in other studies with the application of intestinal cell lines models, as well as human small intestinal organoids – hSIOs[11,33-35]. Lee *et al*[33] investigated the growth of SARS-CoV-2 in a human GIT cell line model; C2BBE1 (a subclone of human epithelial colorectal adenocarcinoma cells: Caco-2). C2BBE1 (genetically and structurally resembling the brush border epithelial cells in the human GIT[36,37], and expressing moderate level of ACE2 and high level of TMPRSS2[33]) exhibited the greatest susceptibility to the virus. SARS-CoV-2 virions invaded and replicated in these cells, as well as in Caco-2[33,35] and T84 (human colon carcinoma) cells[35]. Furthermore, Stanifer *et al*[35] demonstrated SARS-CoV-2 infection of human colon organoids followed by active virions replication. These observations are in agreement with other studies on hSIOs[11,34]. Zang *et al*[11] reported productive infection of SARS-CoV-2 in ACE2<sup>(+)</sup> mature enterocytes, dependent on TMPRSS2 and TMPRSS4 receptors in human small intestinal enteroids. The Authors noted the role of an additional serine protease: TMPRSS4 which heightened the effect of TMPRSS2. Also, the two serine proteases enhanced SARS-CoV-2 spike protein-induced cell-cell fusion observed by the Authors.

ACE2 receptors (except for the respiratory system) are present in a variety of other organs including the gastrointestinal tract, where a great number of these proteins has been detected in the lower part of GIT[38]. Unlike in the upper segment (oral cavity, esophagus, stomach), high expression of ACE2 (both mRNA and protein) is observed in the small intestine (the greatest level), colon and rectum, as well as in the gall bladder[38,39]. Actually, ACE2 expression in the small intestine is much higher in comparison with all other organs in the human organism including the respiratory tract[11]. Specifically, ACE2 is present in the enterocyte cytoplasm and in the apical brush border, as well as in the glandular cells (in the lining epithelium of the lower GIT)[37]. The expression of ACE2 receptors has been exhibited to increase upon enterocytes differentiation. Lee *et al*[33] observed that (unlike constitutively expressed TMPRSS2), the expression of ACE2 receptors was significantly stimulated in the experiment inducing C2BBE1 enterocytes differentiation, associated with the generation of more pronounced features typical for brush border cells. Similarly, Zang *et al*[11] detected the greatest expression of ACE2 in mature brush border enterocytes, and Lamers *et al*[34] noticed around 1000-fold increase in ACE2 mRNA expression upon enterocytes differentiation. Zang *et al*[11] observed all studied receptors (ACE2, as well as TMPRSS2 and TMPRSS4) to be correlated with the virus invasiveness, similarly as Lee *et al*[33] noted a strong correlation between TMPRSS2 (although not ACE2) and viral RNA levels in the studied human epithelial cell lines (including Caco-2 and C2BBE1). Lee *et al*[33] found that the ectopic coexpression of ACE2 and TMPRSS2 in RPMI 2650 cells enhanced viral dissemination by 56.7 times (over 10-fold more in comparison with the sole ACE2 effect – 4.9 times, whereas TMPRSS2 transfection alone did not enhance the level of infectivity). It might be supposed that an effective level of ACE2 receptors is a prerequisite for the virions invasion of epithelial cells, but abundant expression of TMPRSS2 (and possibly TMPRSS4) greatly facilitates ACE2-mediated SARS-CoV-2 dissemination in the human GIT.

The involvement of both ACE2 and TMPRSS2 in SARS-CoV-2 invasion of GIT epithelial cells is in accordance with the reported co-localization of the two receptors in the lower GIT[38-40]. The most abundant expression of both proteins has been detected in the small intestine epithelial cells[38]; especially in the brush border cells [33]. Lee *et al*[39] evaluated single-cell RNA-sequencing datasets from the GIT in search for these two genes co-expression, and found the small intestine enterocytes as well as colonocytes to display the highest proportions of cells co-expressing ACE2 and TMPRSS2. Additionally, the Authors checked for the co-expression of ACE2, TMPRSS2 and TMPRSS4, and demonstrated the highest proportions of the three genes co-expression in the progenitor and stem-like epithelial cells in the small intestine. TMPRSS4 is an extra serine protease involved in SARS-CoV-2 activation and invasion, enhancing TMPRSS2 priming effect[11]. Therefore, both ACE2 and TMPRSS2 seem to be the main receptors responsible for SARS-CoV-2 invasion of GIT. The experimental data coming from Lee *et al*[33], Zang *et al*[11], and Stanifer *et al*[35] investigations are displayed in Table 1.



### **Putative association between SARS-CoV-2-mediated ACE2 disturbance and gastrointestinal symptoms development in COVID-19 patients**

The known function of angiotensin converting enzyme 2 (ACE2) is the regulation of systemic arterial blood pressure (renin-angiotensin system). ACE2 catalyzes the conversion of Ang I to angiotensin (1-9) and angiotensin II (Ang II) to angiotensin (1-7), what counteracts the effects of Ang II, leading to decrease in blood pressure and inflammatory processes attenuation (reviewed in[41]). However, the role of ACE2 in GIT (where it is abundantly expressed) seems to be rather associated with the processes occurring in this organ. The analysis of the digestive system specific functional enrichment map for ACE2 gene suggests the involvement of ACE2 in digestion (with reference to its proteolytic activity) and transport of metabolites (the regulation of amino acid transport)[38]. In fact, ACE-2 proteins have been demonstrated to be coupled with sodium-dependent amino acids and glucose transporters. ACE2 is a chaperone for the sodium-dependent amino acid transporter B0AT1 which is involved in transport of neutral amino acids[42]. Moreover, ACE2 participates in the regulation of gut microbiota homeostasis[43,44].

Therefore, as proposed by Kumar *et al*[38], it might be hypothesized that SARS-CoV-2-associated dysregulation of ACE2 receptors in the human GIT may be involved in the mechanism of GIT symptoms development in COVID-19 patients.

## **CONCLUSION**

In light of the presented studies, the impact of SARS-CoV-2 virus on GIT is evident, with the most substantiated involvement of ACE2 and TMPRSS2. However, except for these two receptors (and probably TMPRSS4), also other proteases might be implicated in SARS-CoV-2 invasion of GIT, as well as the development of the observed symptoms. Ubiquitously expressed furin and cathepsin L may be involved in spike protein processing, contributing to the virus invasiveness. Cathepsin L (and other CCs) might participate in the endolysosomal processing of the spike protein following ACE2-mediated endocytosis of the virion particles. Additionally, SARS-CoV-2 - induced inflammatory cytokines could stimulate the secretion of cathepsin L. Therefore, extracellular cathepsin L may contribute both to the spike protein processing, as well as degradation/remodeling of the ECM components and membrane-bound receptors including TMPRSS2/4 and ACE2. The resulting events might accelerate the inflammatory processes disturbing the digestion/absorption of nutrients yielding the observed symptoms such as diarrhea. However, more research is required, since the participation of furin and lysosomal cathepsins in SARS-CoV-2 GIT-invasion is more speculative.

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