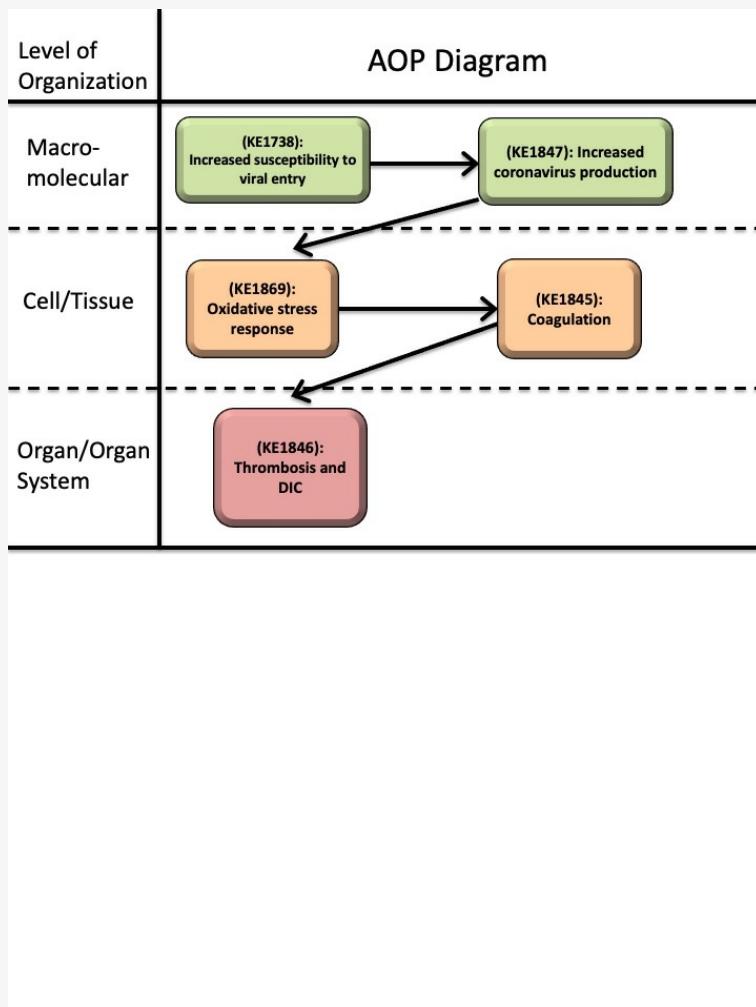


AOP ID and Title:

AOP 379: Binding to ACE2 leading to thrombosis and disseminated intravascular coagulation
Short Title: SARS-CoV2 to thrombosis and DIC

Graphical Representation**Authors**

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Status

Author status	OECD status	OECD project	SAAOP status
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Abstract

Coronavirus disease-19 (COVID-19) is circulating all over the world. To understand and find a way of the COVID-19 treatment, the signaling pathway and therapeutic mechanism of COVID-19 should be investigated. The pathogenesis of COVID-19 includes molecular networks such as the binding of the membrane proteins, signaling pathways, and RNA replication. The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is a new type of coronavirus causing COVID-19, infects the cells via the binding of the membrane proteins of human cells and is internalized by the cells. The viral genome is replicated by RNA-dependent RNA polymerase (RdRp), followed by the packaging and releasing of the viral particles. These steps can be the main targets for the therapeutics of COVID-19. The AOP379 "Increased susceptibility to viral entry and coronavirus production leading to thrombosis and disseminated intravascular coagulation" consists of the molecular initiating events (MIE) as "Increased susceptibility to viral entry" (KE1738) and "Increased coronavirus production" (KE1847), key events (KEs) as "Oxidative stress response" (KE1869) and "Coagulation" (KE1845), and adverse outcome (AO) as "Thrombosis and Disseminated Intravascular Coagulation" (KE1846).

Summary of the AOP**Events****Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)**

Sequence	Type	Event ID	Title	Short name
1	MIE	1739	Binding to ACE2	Binding to ACE2
2	MIE	1738	SARS-CoV-2 cell entry	SARS-CoV-2 cell entry
3	MIE	1847	Increased SARS-CoV-2 production	SARS-CoV-2 production

Sequence	Type	Event ID	Title	Short name
	KE	1901	Interferon-I antiviral response, antagonized by SARS-CoV-2	IFN-I response, antagonized
4	KE	1869	Diminished protective oxidative stress response	Diminished Protective Response to ROS
5	KE	1845	Coagulation	Coagulation
6	AO	1846	Thrombosis and Disseminated Intravascular Coagulation	Thrombosis and DIC

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Binding to ACE2	adjacent	SARS-CoV-2 cell entry	High	Moderate
SARS-CoV-2 cell entry	adjacent	Interferon-I antiviral response, antagonized by SARS-CoV-2	Moderate	Moderate
Interferon-I antiviral response, antagonized by SARS-CoV-2	adjacent	Increased SARS-CoV-2 production	Moderate	Moderate
Increased SARS-CoV-2 production	adjacent	Diminished protective oxidative stress response	Moderate	Not Specified
Diminished protective oxidative stress response	adjacent	Coagulation	Moderate	Not Specified
Coagulation	adjacent	Diminished protective oxidative stress response	Moderate	Not Specified
Coagulation	adjacent	Thrombosis and Disseminated Intravascular Coagulation	High	

Stressors

Name	Evidence
Stressor:624 SARS-CoV-2	High

Overall Assessment of the AOP

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	Moderate

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI

Sex Applicability

Sex	Evidence
Unspecific	High

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Appendix 1

List of MIEs in this AOP

[Event: 1739: Binding to ACE2](#)

Short Name: Binding to ACE2**Key Event Component**

Process	Object	Action
receptor binding	angiotensin-converting enzyme 2	occurrence

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:320 - Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality	MolecularInitiatingEvent
Aop:374 - Binding of Sars-CoV-2 spike protein to ACE 2 receptors expressed on brain cells (neuronal and non-neuronal) leads to neuroinflammation resulting in encephalitis	MolecularInitiatingEvent
Aop:381 - Binding of viral S-glycoprotein to ACE2 receptor leading to dysgeusia	MolecularInitiatingEvent
Aop:385 - ACE2 binding to viral S-protein leading to microvascular dysfunction via ACE2 dysregulation	MolecularInitiatingEvent
Aop:394 - SARS-CoV-2 infection of olfactory epithelium leading to impaired olfactory function (short-term anosmia)	MolecularInitiatingEvent
Aop:395 - Binding of Sars-CoV-2 spike protein to ACE 2 receptors expressed on pericytes leads to disseminated intravascular coagulation resulting in cerebrovascular disease (stroke)	MolecularInitiatingEvent
Aop:406 - SARS-CoV-2 infection leading to hyperinflammation	MolecularInitiatingEvent
Aop:407 - SARS-CoV-2 infection leading to pyroptosis	MolecularInitiatingEvent
Aop:426 - SARS-CoV-2 spike protein binding to ACE2 receptors expressed on pericytes leads to endothelial cell dysfunction, microvascular injury and myocardial infarction.	MolecularInitiatingEvent
Aop:427 - ACE2 downregulation following SARS-CoV-2 infection triggers dysregulation of RAAS and can lead to heart failure.	MolecularInitiatingEvent
Aop:422 - Binding of SARS-CoV-2 to ACE2 in enterocytes leads to intestinal barrier disruption	MolecularInitiatingEvent
Aop:428 - Binding of S-protein to ACE2 in enterocytes induces ACE2 dysregulation leading to gut dysbiosis	MolecularInitiatingEvent
Aop:430 - Binding of SARS-CoV-2 to ACE2 leads to viral infection proliferation	MolecularInitiatingEvent
Aop:379 - Binding to ACE2 leading to thrombosis and disseminated intravascular coagulation	MolecularInitiatingEvent
Aop:468 - Binding of SARS-CoV-2 to ACE2 leads to acute respiratory distress (via cell death)	MolecularInitiatingEvent

Stressors**Name**

Sars-CoV-2

Biological Context**Level of Biological Organization**

Molecular

Evidence for Perturbation by Stressor**Overview for Molecular Initiating Event**

Receptor recognition is an essential determinant of molecular level in this AOP. ACE2 was reported as an entry receptor for SARS-CoV-2. The viral entry process is mediated by the envelope-embedded surface-located spike (S) glycoprotein. Jun Lan and Walls, A.C et al (Nature 581, 215–220; Cell 180, 281–292) demonstrated a critical initial step of infection at the molecular level from the interaction of ACE2 and S protein. ACE2 has shown that receptor binding affinity to S protein is nM range. To elucidate the interaction between the SARS-CoV-2 RBD and ACE2 at a higher resolution, they also determined the structure of the SARS-CoV-2 RBD–ACE2 complex using X-ray crystallography. The expression and distribution of the ACE2 in human body may indicate the potential infection of SARS-CoV-2. Through the developed single-cell RNA sequencing (scRNA-Seq) technique and single-cell transcriptomes based on the public database, researchers analyzed the ACE2 RNA expression profile at single-cell resolution. High ACE2 expression was identified in type II alveolar cells (Zou, X. et al. Front. Med. 2020)

SARS-CoV-2 belongs to the Coronaviridae family, which includes evolutionary related enveloped (+) strand RNA viruses of vertebrates, such as seasonal common coronaviruses, SARS-CoV and CoV-NL63, SARS-CoV (Kim Young Jun et al)

Human viruses strains	Genus	Major cell receptor	First report	Animal reservoir	Intermediate host	Pathology	Diagnostic test	Evidence
HCoV-NL63	Alphacoronavirus	ACE2	2004	Bat	Unknown	Mild respiratory tract illness	RT-PCR, IF, ELISA, WB	Strong
SARS-CoV	Betacoronavirus	ACE2	2003	Bat	Pangolin	Severe acute respiratory syndrome	RT-PCR, IF, ELISA, WB	Strong
SARS-CoV-2	Betacoronavirus	ACE2	2020	Bat	Pangolin	Severe acute respiratory syndrome	RT-PCR, IF, ELISA, WB	Strong

Domain of Applicability**Taxonomic Applicability**

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
mouse	Mus musculus	High	NCBI
Mustela lutreola	Mustela lutreola	High	NCBI
Felis catus	Felis catus	Moderate	NCBI
Panthera tigris	Panthera tigris	Moderate	NCBI

Canis familiaris Canis lupus familiaris Low Evidence NCBI

Life Stage Applicability

Life Stage	Evidence
Adult, reproductively mature	High
During development and at adulthood	High

Sex Applicability

Sex	Evidence
Mixed	High

Key Event Description

Angiotensin-converting enzyme 2 (ACE2) is an enzyme that can be found either attached to the membrane of the cells (mACE2) in many tissues and in a soluble form (sACE2).

A table on ACE2 expression levels according to tissues (Kim *et al.*)

	Sample size	ACE2 mean expression	Standard deviation of expression
Intestine	51	9.50	1.183
Kidney	129	9.20	2.410
Stomach	35	8.25	3.715
Bile duct	9	7.23	1.163
Liver	50	6.86	1.351
Oral cavity	32	6.23	1.271
Lung	110	5.83	0.710
Thyroid	59	5.65	0.646
Esophagus	11	5.31	1.552
Bladder	19	5.10	1.809
Breast	113	4.61	0.961
Uterus	25	4.37	1.125
Protaste	52	4.35	1.905

ACE2 receptors in the brain (endothelial, neuronal and glial cells):

The highest ACE2 expression level in the brain was found in the pons and medulla oblongata in the human brainstem, containing the medullary respiratory centers (Lukiw *et al.*, 2020). High ACE2 receptor expression was also found in the amygdala, cerebral cortex and in the regions involved in cardiovascular function and central regulation of blood pressure including the sub-fornical organ, nucleus of the tractus solitarius, paraventricular nucleus, and rostral ventrolateral medulla (Govrisankar and Clark 2016; Xia and Lazartigues 2010). The neurons and glial cells, like astrocytes and microglia also express ACE-2.

In the brain, ACE2 is expressed in endothelium and vascular smooth muscle cells (Hamming *et al.*, 2004), as well as in neurons and glia (Gallagher *et al.*, 2006; Matsushita *et al.*, 2010; Govrisankar and Clark, 2016; Xu *et al.*, 2017; de Moraes *et al.*, 2018) (from Murta *et al.*, 2020). Astrocytes are the main source of angiotensinogen and express ATR1 and MasR; neurons express ATR1, ACE2, and MasR, and microglia respond to ATR1 activation (Shi *et al.*, 2014; de Moraes *et al.*, 2018).

ACE2 receptors in the intestines

The highest levels of ACE2 are found at the luminal surface of the enterocytes, the differentiated epithelial cells in the small intestine, lower levels in the crypt cells and in the colon (Liang *et al.*, 2020; Hashimoto *et al.*, 2012; Fairweather *et al.* 2012; Kowalcuk *et al.* 2008).

How it is Measured or Detected

In vitro methods supporting interaction between ACE2 and SARS-CoV-2 spike protein

Several reports using surface plasmon resonance (SPR) or biolayer interferometry binding (BLI) approaches to study the interaction between recombinant ACE2 and S proteins have determined a dissociation constant (Kd) for SARS-CoV S and SARS-CoV-2 S as follow,

Reference	ACE2 protein	SARS-CoV S	SARS-CoV2 S	Method	Measured Kd
doi:10.1126/science.abb2507	1–615 aa	306–577 aa	1–1208 aa	SPR	325.8 nM
		306–577 aa			14.7 nM

doi:10.1001/jama.2020.3786	19–615 aa	306–527 aa	319–541 aa	SPR	4UD. / 11M
					133.3 nM
Lan et al., 2020	19–615 aa	306–527 aa	319–541 aa	SPR	31.6 nM
					4.7 nM
doi:10.1016/j.cell.2020.02.058	1–614 aa	306–575 aa		BLI	1.2 nM
			328–533 aa		5 nM
doi:10.1126/science.abb2507	1–615 aa	306–577 aa		BLI	13.7 nM
			319–591 aa		34.6 nM

Pseudo typed vesicular stomatitis virus expressing SARS-CoV-2 S (VSV-SARS-S2) expression system can be used efficiently infects cell lines, with Calu-3 human lung adenocarcinoma epithelial cell line, CaCo-2 human colorectal adenocarcinoma colon epithelial cell line and Vero African grey monkey kidney epithelial cell line being the most permissive (Hoffmann et al., 2020; Ou et al., 2020). It can be measured using a wide variety of assays targeting different biological phases of infection and altered cell membrane permeability and cell organelle signaling pathway. Other assay measured alteration in the levels of permissive cell lines all express ACE2 or hACE2-expressing 293T cell (e.g. pNUO1-hACE2, pFUSE-hlgG1-Fc2), as previously demonstrated by indirect immunofluorescence (IF) or by immunoblotting are associated with ELISA(W Tai et al., nature 2020). To prioritize the identified potential KEs for selection and to select a KE to serve as a case study, further in-silico data that ACE2 binds to SARS-CoV-2 S is necessary for virus entry. The above analysis outlined can be used evidence-based assessment of molecular evidence as a MIE.

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Event: 1738: SARS-CoV-2 cell entry

Short Name: SARS-CoV-2 cell entry

Key Event Component

Process	Object	Action
membrane fusion	transmembrane protease serine 2	occurrence
endocytosis involved in viral entry into host cell	cathepsin L1 (human)	occurrence

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:320 - Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality	KeyEvent
Aop:379 - Binding to ACE2 leading to thrombosis and disseminated intravascular coagulation	MolecularInitiatingEvent
Aop:394 - SARS-CoV-2 infection of olfactory epithelium leading to impaired olfactory function (short-term anosmia)	KeyEvent
Aop:395 - Binding of Sars-CoV-2 spike protein to ACE 2 receptors expressed on pericytes leads to disseminated intravascular coagulation resulting in cerebrovascular disease (stroke)	KeyEvent
Aop:406 - SARS-CoV-2 infection leading to hyperinflammation	KeyEvent
Aop:407 - SARS-CoV-2 infection leading to pyroptosis	KeyEvent
Aop:426 - SARS-CoV-2 spike protein binding to ACE2 receptors expressed on pericytes leads to endothelial cell dysfunction, microvascular injury and myocardial infarction.	KeyEvent
Aop:422 - Binding of SARS-CoV-2 to ACE2 in enterocytes leads to intestinal barrier disruption	KeyEvent
Aop:430 - Binding of SARS-CoV-2 to ACE2 leads to viral infection proliferation	KeyEvent
Aop:468 - Binding of SARS-CoV-2 to ACE2 leads to acute respiratory distress (via cell death)	KeyEvent

Stressors

Name
Sars-CoV-2

Biological Context

Level of Biological Organization

Molecular

Cell term**Cell term**

cell

Organ term**Organ term**

organ

Domain of Applicability**Taxonomic Applicability**

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Manis javanica	Manis javanica	Low	NCBI
Canis familiaris	Canis lupus familiaris	Moderate	NCBI
Macaca fascicularis	Macaca fascicularis	Not Specified	NCBI
Mesocricetus auratus	Mesocricetus auratus	Not Specified	NCBI
Mustela putorius furo	Mustela putorius furo	Not Specified	NCBI
Felis catus	Felis catus	Moderate	NCBI
Mustela lutreola	Mustela lutreola	High	NCBI
Neovison vison	Neovison vison	High	NCBI
Panthera tigris	Panthera tigris	Moderate	NCBI

Life Stage Applicability**Life Stage Evidence**

All life stages High

Sex Applicability**Sex Evidence**

Unspecific High

TMPRSS2 vertebrates (Lam et al., 2020)

NRP1 in human & rodents (but also present in monkey and other vertebrates (Lu and Meng, 2015)

The ability for SARS-CoV-2 to use multiple host pathways for viral entry, means that it is critical to map which viral entry pathway is prevalent in specific cell types. This is key for understanding coronavirus biology, but also use informed decisions to select cells for cell-based genetic and small-molecule screens and to interpret data. In fact, a combination of protease inhibitors that block both TRMPSS2 and cathepsin L is the most efficient combination to block coronavirus infection (Yamamoto et al., 2020, Shang et al., 2020, Shirato et al., 2018). In accordance, SARS-CoV-2 entry processes are highly dependent on endocytosis and endocytic maturation in cells that do not express TMPRSS2, such as VeroE6 or 293T cells (Murgolo et al., 2021, Kang et al., 2020, Mirabelli et al., 2020, Riva et al., 2020). However, even in these cells, heterologous expression of TMPRSS2 abrogates the pharmacological blockade of cathepsin inhibitors (Kawase et al., 2012, Hoffmann et al., 2020a). Treatment of SARS-CoV-2 with trypsin enables viral cell surface entry, even when TMPRSS2 is absent. Moreover, TMPRSS2 is more efficient to promote viral entry than cathepsins (Lamers et al., 2020), as when both factors are present, cathepsin inhibitors are less effective than TMPRSS2 inhibitors (Hoffmann et al., 2020b). Therefore it is critical to map which cells contain the different types of proteases.

In summary, TMPRSS2 appears to be expressed in a wide range of healthy adult organs, but in restricted cell types, including:

- AT2 and Clara cells of lungs
- sinusoidal endothelium, and hepatocyte of the liver,
- endocrine cells of the prostate,
- goblet cells, and enterocytes of the small intestine,
- intercalated cells, and the proximal tubular of the kidney.
- Ciliated, secretory and suprabasal of nasal
- spermatogonial stem cells of testes
- cyto tropoblast and peri vascular cells of placenta
- The nasal epithelium expresses various combinations of factors that, in principle, could facilitate SARS-CoV-2 infection, but it also expresses robust basal levels of RFs, which may act as a strong protective barrier in this tissue.

There is a shift in TMPRSS2 regulation during nasal epithelium differentiation in young individuals that is not occurring in old individuals (Lin et al., 1999, Lucas et al., 2008, Singh et al., 2020).

Only a small minority of human respiratory and intestinal cells have genes that express both ACE2 and TMPRSS2. Amongst the ones that do, three main cell types were identified: A) lung cells called type II pneumocytes (which help maintain air sacs, known as alveoli); B) intestinal cells called enterocytes, which help the body absorb nutrients; and C) goblet cells in the nasal passage, which secrete mucus (Ziegler et al., 2020).

The clinical manifestations of COVID-19 include not only complications from acute myocardial injury, elevated liver enzymes, and acute kidney injury in patients presenting to hospitals, but also gastrointestinal symptoms in community patients experiencing milder forms of the disease (Madjid et al., 2020, Pan et al., 2020).

NRP-1:

All life stages

The expression of isoforms 1 (NRP1) and 2 (NRP2) does not seem to overlap. Isoform 1 is expressed by the blood vessels of different tissues. In the developing embryo it is found predominantly in the nervous system. In adult tissues, it is highly expressed in heart and placenta; moderately in lung, liver, skeletal muscle, kidney and pancreas; and low in adult brain. Isoform 2 is found in liver hepatocytes, kidney distal and proximal tubules. Expressed in colon and 234 other tissues with low tissue specificity (UniProtKB).

The expression of NRP1 protein in gastric cancer was not related to gender or age (Cao et al., 2020).

Sex Applicability:

TMPRSS2:

Androgen receptors (ARs) play a key role in the transcription of TMPRSS2 (Fig. 1). This may explain the predominance of males to COVID-19 infection, fatality, and severity because males tend to have a higher expression and activation of ARs than females, which is due to the presence of dihydrotestosterone (DHT).

Regulation of COVID-19 severity and fatality by sex hormones. Females have aromatase, the enzyme that converts androgen substrates into estrogen. On the other hand, males have steroid 5 α reductase, the enzyme that is responsible for the conversion of testosterone into dihydrotestosterone (DHT). In case of males, DHT activates androgen receptor (AR) that binds to the androgen response element (ARE) present in the promoter of TMPRSS2 gene, leading to its transcription. This ultimately results into enhanced processing of viral spike protein for greater entry and spread of SARS-CoV-2 into host cells. On the other hand, in females, estrogen activates estrogen receptor (ER), which binds to the estrogen response element (ERE) present in the promoter of eNOS gene to drive its transcription and catalyze the formation of nitric oxide (NO) from L-arginine. This NO is involved in vasodilation as well as inhibition of viral replication.

NRP-1:

For more information difference of NRP1 expression between male and female see <https://www.proteinatlas.org/ENSG00000099250-NRP1/tissue>.

The expression of NRP1 protein in gastric cancer was not related to gender, age. The expression of NRP1 protein in gastric cancer is closely correlated to clinical stage, tumor size, TNM stage, differentiation, and lymph node metastasis (Cao et al., 2020).

SARS-CoV-2 Spike protein co-opts VEGF-A/Neuropilin-1 receptor signalling to induce analgesia had same results on both male and female rodents (Moutal et al., 2020).

Key Event Description

Coronavirus is recognized by the binding of S protein on the viral surface and angiotensin-converting enzyme 2 (ACE2) receptor on the cellular membrane, followed by viral entry via processing of S protein by transmembrane serine protease 2 (TMPRSS2) (Hoffmann et al., 2020b). ACE2 is expressed on epithelial cells of the lung and intestine, and also can be found in the heart, kidney, adipose, and male and female reproductive tissues (Lukassen et al., 2020, Lamers et al., 2020, Chen et al., 2020, Jing et al., 2020, Subramanian et al., 2020).

SARS-CoV-2 is an enveloped virus characterized by displaying spike proteins at the viral surface (Juraszek et al., 2021). Spike is critical for viral entry (Hoffmann et al., 2020b) and is the primary target of vaccines and therapeutic strategies, as this protein is the immunodominant target for antibodies (Yuan et al., 2020, Ju et al., 2020, Robbiani et al., 2020, Premkumar et al., 2020, Liu et al., 2020). Spike is composed of S1 and S2 subdomains. S1 contains the N-terminal (NTD) and receptor-binding (RBD) domains, and the S2 contains the fusion peptide (FP), heptad repeat 1 (HR1) and HR2, the transmembrane (TM) and cytoplasmic domains (CD) (Lan et al., 2020). S1 leads to the recognition of the angiotensin-converting enzyme 2 (ACE2) receptor and S2 is involved in membrane fusion (Hoffmann et al., 2020b, Letko et al., 2020, Shang et al., 2020).

Upon binding to ACE2, the spike protein needs to be activated (or primed) through proteolytic cleavage (by a host protease) to allow membrane fusion. Fusion is a key step in viral entry as it is the way to release SARS-CoV-2 genetic material inside the cell. Cleavage happens between its spike's S1 and S2 domains, liberating S2 that inserts its N-terminal domain into a host cell membrane and mediates membrane fusion (Millet and Whittaker, 2018). Many proteases were identified to activate coronaviruses including furin, cathepsin L, trypsin-like serine proteases TMPRSS2, TMPRSS4, TMPRSS11, and human airway trypsin-like protease (HATs). These may operate at four different stages of the [virus infection cycle](#): (a) pro-protein convertases (e.g., furin) during virus packaging in virus-producing cells, (b) extracellular proteases (e.g., elastase) after virus release into extracellular space, (c) cell surface proteases [e.g., type II transmembrane serine protease (TMPRSS2)] after virus attachment to virus-targeting cells, and (d) lysosomal proteases (e.g., cathepsin L) after virus endocytosis in virus-targeting cells (Li, 2016). SARS-CoV-2 lipidic envelope may fuse with two distinct membrane types, depending on the host protease(s) responsible for cleaving the spike protein: (i) cell surface following activation by serine proteases such as TMPRSS2 and furin (Hoffmann et al., 2020b); or (ii) endocytic pathway within the endosomal-lysosomal compartments including processing by lysosomal cathepsin L (Yang and Shen, 2020). These flexibility for host cell factors mediating viral entry, highlights that the availability of factors existing in a cell type dictates the mechanism of viral entry (Kawase et al., 2012). When TMPRSS2 (or other serine proteases such as TMPRSS4 (Zang et al., 2020) or human airway trypsin-like protease [HAT] (Bestle et al., 2020a) is expressed, fusion of the virus with the cell surface membrane is preferred (Shirato et al., 2018), while in their absence, the virus can penetrate the cell by endocytosis (Kawase et al., 2012). A third factor has also been shown to facilitate SARS-CoV-2 entry in cells that have ACE2 and even promote, although to very low levels, SARS-CoV-2 entry in cells that lack ACE2 and TMPRSS2 which is the neuropilin-1 (NRP-1) (Cantuti-Castelvetro et al., 2020). This key event deals with SARS-CoV-2 entry in host cells and is divided in three categories: TMPRSS2, cathepsin L and NRP-1.

TMPRSS2 Spike cleavage:

TMPRSS2 (transmembrane serine protease 2, <https://www.ncbi.nlm.nih.gov/gene/7113>) is a cell-surface protease (Hartenian et al., 2020) that facilitates entry of viruses into host cells by proteolytically cleaving and activating viral envelope glycoproteins. Viruses found to use this protein for cell entry include Influenza virus and the human coronaviruses HCoV-229E, MERS-CoV, SARS-CoV and SARS-CoV-2 (COVID-19 virus).

TMPRSS2 is a membrane bound serine protease also known as epithelin. TMPRSS2 belongs to the S1A class of serine proteases alongside proteins such as factor Xa and trypsin. Whilst there is evidence that TMPRSS2 autoclaves to generate a secreted protease, its physiological function has not been clearly identified. However, it is known to play a crucial role in facilitating entry of coronavirus particles into cells by cleaving the spike protein (Huggins, 2020).

After ACE2 receptor binding, SARS-CoV-2 S proteins can be subsequently cleaved and activated by host cell-surface protease at the S1/S2 and S2' sites, generating the subunits S1 and S2 that remain non-covalently linked. Cleavage leads to activation of the S2 domain that drives fusion of the viral and host membranes (Hartenian et al., 2020, Walls et al., 2016). For other coronaviruses, processing of spike was proposed to be sequential with S1/S2 cleavage preceding that of S2. Cleavage at S1/S2 may be crucial for inducing conformational changes required for receptor binding or exposure of the S2 site to host proteases.

The S1/S2 site of SARS-CoV-2 S protein contains an insertion of four amino acids providing a minimal furin cleavage site (RRAR685↓) (that is absent in SARS-CoV). Interestingly, the furin cleavage site has been implicated in increased viral pathogenesis (Bestle et al., 2020b, Huggins, 2020). Processing of the spike protein by furin at the S1/S2 cleavage site is thought to occur following viral replication in the endoplasmic reticulum/Golgi intermediate compartment (ERGIC) (Hasan et al., 2020). The spike S2' cleavage site of SARS-CoV-2 possesses a paired dibasic motif with a single KR segment (KR815↓) (as SARS-CoV) that is recognized by trypsin-like serine proteases such as TMPRSS2. **The current data support a model for SARS-CoV-2 entry in which furin-mediated cleavage at the S1/S2 site pre-primes spike during biogenesis, facilitating the activation for membrane fusion by a second cleavage event at S2' by TMPRSS2 following ACE2 binding** (Bestle et al., 2020b, Johnson et al., 2020).

Virus	S1/S2 site	S2' site
SARS-CoV-2	TNSP RRAR SVA	PSKPSK R SFIEDL
SARS-CoV	S---LLR STS	PLKPT KR SFIEDL

Camostat mesylate, an inhibitor of TMPRSS2, blocks SARS-CoV-2 infection of lung cells like Calu-3 cells but not Huh7.5 and Vero E6 cells. Cell entry was assessed using a viral isolate and viral pseudotypes (artificial viruses) expressing the COVID-19 spike (S) protein. The ability of the viral pseudotypes (expressing S protein from SARS-CoV and SARS-CoV-2) to enter human and animal cell lines was demonstrated, showing that SARS-CoV-2 can enter similar cell lines as SARS-CoV. Amino acid analysis and cell culture experiments showed that, like SARS-CoV, SARS-CoV-2 spike protein binds to human and bat angiotensin-converting enzyme 2 (ACE2) and uses a cellular protease TMPRSS2 for priming. Priming activates the spike protein to facilitate viral fusion and entry into cells. Cell culture experiments were performed using immortalized cell lines and primary human lung cells (Hoffmann et al., 2020b, Rahman et al., 2020).

Spike binding to neuropilin-1:

Neuropilin-1 (NRP1) is a transmembrane glycoprotein that serves as a cell surface receptor for semaphorins and various ligands involved in angiogenesis in vertebrates. NRP1 is expressed in neurons, blood vessels (endothelial cells), immune cells and many other cell types in the mammalian body (maternal fetal interface) and binds a range of structurally and functionally diverse extracellular ligands to modulate organ development and function (Raimondi et al., 2016). NRP1 is well described as a co-receptor for members of the class 3 semaphorins (SEMA3) or vascular endothelial growth factors (VEGFs) (Gelfand et al., 2014). Structurally, NRP1 comprises seven sub-domains, of which the first five are extracellular; two CUB domains (a1 and a2), two coagulation factor V/VIII domains (FV/VIII; b1 and b2) and a meprin, A5 μ -phosphatase domain (MAM; c). NRP1 contains only a short cytosolic tail with a PDZ-binding domain lacking internal signaling activity. The different ligand families bind to different sites of NRP1; SEMA3A binding requires the first three sub-domains of NRP1 (a1, a2, and b1), whereas binding of VEGF-A requires the b1 and b2 domains (Muhl et al., 2017). Additional studies conducted by means of *in silico* computational technology to identify and validate inhibitors of the interaction between NRP1 and SARS-CoV-2 Spike protein are reported in (Perez-Miller et al., 2020). Represents a schematic picture of VEGF-A triggered phosphorylation of VEGF-R2. Screening of NRP-1/VEGF-A165 inhibitors by *in-cell* Western (Perez-Miller et al., 2020). NRP1 acts as a co-receptor for SARS-CoV-2.

NRP1 is a receptor for furin-cleaved SARS-CoV-2 spike peptide (Cantuti-Castelvetro et al., 2020, Daly et al., 2020, Johnson et al., 2020). Blockade of NRP1 reduces infectivity and entry, and alteration of the furin site leads to loss of NRP1 dependence, reduced replication in Calu3, augmented replication in Vero E6, and attenuated disease in a hamster pathogenesis disease model (Johnson et al., 2020). In fact, a small sequence of amino acids was found that appeared to mimic a protein sequence found in human proteins that interact with NRP1. The spike protein of SARS-CoV-2 binding with NRP1 aids viral infection of human cells. This was confirmed by applying a range of structural and biochemical approaches to establish that the spike protein of SARS-CoV-2 does indeed bind to NRP1. The host protease furin cleaves the full-length precursor S glycoprotein into two associated polypeptides: S1 and S2. Cleavage of S generates a polybasic RRAR C-terminal sequence on S1, which conforms to a C-end rule (CendR) motif that binds to cell surface neuropilin-1 (NRP1) and neuropilin-2 (NRP2) receptors. It was reported that

the S1 CendR motif directly bound NRP1 by X-ray crystallography and biochemical approaches. Blocking this interaction using RNAi or selective inhibitors reduced SARS-CoV-2 entry and infectivity in cell culture (Daly et al., 2020).

NRP1, known to bind furin-cleaved substrates, significantly potentiates SARS-CoV-2 infectivity, which was revealed by a monoclonal blocking antibody against NRP1. It was found that a SARS-CoV-2 mutant with an altered furin cleavage site did not depend on NRP1 for infectivity. Pathological analysis of olfactory epithelium obtained from human COVID-19 autopsies revealed that SARS-CoV-2 infected NRP1-positive cells faced the nasal cavity (Cantuti-Castelvetri et al., 2020). Furthermore, it has been found that NRP1 is a new potential SARS-CoV-2 infection mediator implicated in the neurologic features and central nervous system involvement of COVID-19. Preclinical studies have suggested that NRP1, a transmembrane receptor that lacks a cytosolic protein kinase domain and exhibits high expression in the respiratory and olfactory epithelium, may also be implicated in COVID-19 by enhancing the entry of SARS-CoV-2 into the brain through the olfactory epithelium. NRP1 is also expressed in the CNS, including olfactory-related regions such as the olfactory tubercles and paraolfactory gyri. Supporting the potential role of NRP1 as an additional SARS-CoV-2 infection mediator implicated in the neurologic manifestations of COVID-19. Accordingly, the neurotropism of SARS-CoV-2 via NRP1-expressing cells in the CNS merits further investigation (Davies et al., 2020).

Up-regulation of NRP1 protein in diabetic kidney cells hints at its importance in a population at risk of severe COVID-19. Involvement of NRP-1 in immune function is compelling, given the role of an exaggerated immune response in disease severity and deaths due to COVID-19. NRP-1 has been suggested to be an immune checkpoint of T cell memory. It is unknown whether involvement and up-regulation of NRP-1 in COVID-19 may translate into disease outcome and long-term consequences, including possible immune dysfunction (Mayi et al., 2021).

The main feature of NRP1 co-receptor is to form complexes with multiple other receptors. Hence, there is a competition between receptors to complex with NRP-1, which may determine their abilities both quantitatively and qualitatively to transduce signals. It is tempting to hypothesize that the occupancy of NRP-1 with one receptor may thus decrease its availability for virus entry. Recent proteomics work has shown that NRP-1 can form a complex with the $\alpha 7$ nicotinic receptor in mice. Both receptors are expressed in the human nasal and pulmonary epithelium (Mayi et al., 2021).

NRP1, is highly expressed in the respiratory and olfactory epithelium; it is also expressed in the CNS, including olfactory related regions such as the olfactory tubercles and paraolfactory gyri (Davies et al., 2020).

More information on tissue distribution and protein expression of NRP1 can be found in <https://www.proteinatlas.org/ENSG00000099250-NRP1>

Spike entry via lysosomal cathepsins and endocytosis:

Evidence shows the role of TMPRSS2 and other serine proteases in activating the coronavirus spike protein for plasma membrane fusion. However, studies using various cell culture systems showed that SARS-CoV2 could enter cells via an alternative endosomal-lysosomal pathway. Evidence came from studies demonstrating that lysosomotropic agents reduced SARS-CoV replication in cells lacking TMPRSS2 and other studies, using highly potent and specific small-molecule cathepsin inhibitors, to understand the role of cathepsins in processing and activating the spike for membrane fusion, mainly of cathepsin L (one of the 11 cathepsins) (Rossi et al., 2004, Simmons et al., 2005). SARS-CoV-2 and other coronaviruses can establish infection through endosomal entry in commonly used *in vitro* cell culture systems. Of relevance, inhibitors of the endosomal pathway, as the cathepsin inhibitor Z-FA-FMK and PIKfyve inhibitor apilimod, blocked viral entry in Huh7.5 and Vero E6 cells but not Calu-3 cells.

Viral entry leads to delivery of virion proteins and translation of viral proteins immediately:

Coronavirus is a class of viruses that have single-stranded positive-sense RNA genomes in their envelopes [Kim D, et al., 2020]. The virus contains a 29.7 kB positive-sense RNA genome flanked by 5' and 3' untranslated regions of 265 and 342 nucleotides, respectively that contain cis-acting secondary RNA structures essential for RNA synthesis [Huston N. C. et al., 2021]. The genome just prior to the 5' end contains the transcriptional regulatory sequence leader (TRS-L) [Budzilowicz C.J., et al., 1985]. The SARS-CoV genome is polycistronic and contains 14 open reading frames (ORFs) that are expressed by poorly understood mechanisms [Snijder E. J., et al., 2003]. Preceding each ORF there are other TRSs called the body TRS (TRS B). The 5' two-thirds of the genome contains two large, overlapping, nonstructural ORFs and the 3' third contains the remainder ORFs [Di H., et al., 2018]. Genome expression starts with the translation of two large ORFs of the 5' two-thirds: ORF1a of 4382 amino acids and ORF1ab of 7073 amino acid that occurs via a programmed (-1) ribosomal frameshifting [Snijder E. J., et al., 2003], yielding pp1a and pp1ab. These two polyproteins are cleaved into 16 subunits by two viral proteinases encoded by ORF1a, nsp3, and nsp5 that contain a papain-like protease domain and a 3C-like protease domain [Sacco M. D. et al., 2020]. The processing products are a group of replicative enzymes, named nsp1-nsp16, that assemble into a viral replication and transcription complex (RTC) associated with membranes of endoplasmic reticulum (ER) with the help of various membrane-associated viral proteins [Klein S., et al., 2021, Snijder E. J., et al., 2020, V'Kovski P., et al., 2021]. This association leads to replication factories or organelles, that are originate new membranous structures that are observed by electron microscopy. They are a feature of all coronaviridae and the site of viral replication and transcription hidden from innate immune molecules.

How it is Measured or Detected

SARS-CoV2 entry can be determined by many different ways:

1) quantitative RT-PCR specific to the subgenomic mRNA of the N transcript, in cells manipulated with host factors that express or not TMPRSS2, cathepsinL, neuropilin-1, hACE2 [Glowacka I, et al. (2011)], or exogenous addition of HAT or furin.

2) using spike-pseudotyped viral particles expressing GFP/luciferase/bgalactosidase and comparing with vesicular stomatitis virus G pseudotyped particles expressing the same reporter analysed in manipulated cultured with cell lines, followed by determining fluorescence, bioluminescence, luciferase activity in cell lysates [Hoffmann M, et al. (2020)].

TMPRSS2:

TMPRSS2 gene expression can be measured by RNAseq and microarray (Baughn et al., 2020).

Expression levels of TMPRSS2 can be measured by RNA *in situ* hybridization (RNA-ISH) (Qiao et al., 2020)

NRP-1:

Several methods have been identified in the literature for measuring and detecting NRP1 receptor binding. Briefly described:

1. X-ray crystallography and biochemical approaches help to show that the S1 CendR motif directly bound NRP1 (1). Binding of the S1 fragment to NRP1 was assessed and ability of SARS-CoV-2 to use NRP1 to infect cells was measured in angiotensin-converting enzyme-2 (ACE-2)-expressing cell lines by knocking out NRP1 expression, blocking NRP1 with 3 different anti-NRP1 monoclonal antibodies, or using NRP1 small molecule antagonists (Centers for Disease Control and Prevention, 2020, Daly et al., 2020).

Key findings (Centers for Disease Control and Prevention, 2020, Daly et al., 2020):

- The S1 fragment of the cleaved SARS-CoV-2 spike protein binds to the cell surface receptor neuropilin-1 (NRP1).
- SARS-CoV-2 utilizes NRP1 for cell entry as evidenced by decreased infectivity of cells in the presence of: NRP1 deletion ($p < 0.01$). Three different anti-NRP1 monoclonal antibodies ($p < 0.001$). Selective NRP1 antagonist, EG00229 ($p < 0.01$).
- 2. Cell lines were modified to express ACE2 and TMPRSS2, the two known SARS-CoV-2 host factors, and NRP1 to assess the contribution of NRP1 to infection. Autopsy specimens from multiple airway sites were stained with antibodies against SARS-CoV-2 proteins, ACE2, and NRP1, to visualize co-localization of proteins (6, 15).

Key findings (Cantuti-Castelvetri et al., 2020, Centers for Disease Control and Prevention, 2020):

- Infectivity of cells expressing angiotensin converting enzyme-2 (ACE2, receptor for SARS-CoV-2), transmembrane protease serine-2 (TSS2, primes the Spike [S] protein), and neuropilin-1 (NRP1) with pseudovirus expressing the SARS-CoV-2 S1 protein was approximately 3-fold higher than in cells expressing either ACE2 or TSS2 alone ($p < 0.05$).
- Analysis of autopsy tissue from COVID-19 patients showed co-localization of the SARS-CoV-2 spike (S) protein and NRP1 in olfactory and respiratory epithelium.

Virtual screen of nearly 0.5 million compounds against the NRP-1 CendR site, resulting in nearly 1,000 hits. A pharmacophore model was derived from the identified ligands, considering both steric and electronic requirements. Preparation of receptor protein and grid for virtual screening, docking of known NRP-1 targeting compounds, ELISA based NRP1-VEGF-A165 protein binding assay; more details on methodology in the referenced paper (Perez-Miller et al., 2020)

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Event: 1847: Increased SARS-CoV-2 production

Short Name: SARS-CoV-2 production

Key Event Component

Process	Object	Action
viral RNA genome replication	viral RNA-directed RNA polymerase complex	increased
positive stranded viral RNA replication	viral RNA-directed RNA polymerase complex	increased
viral RNA genome packaging	viral assembly compartment	increased
mRNA transcription	ssRNA viral genome	increased
viral translation	ssRNA viral genome	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:379 - Binding to ACE2 leading to thrombosis and disseminated intravascular coagulation	MolecularInitiatingEvent
Aop:320 - Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality	KeyEvent
Aop:406 - SARS-CoV-2 infection leading to hyperinflammation	KeyEvent
Aop:407 - SARS-CoV-2 infection leading to pyroptosis	KeyEvent
Aop:422 - Binding of SARS-CoV-2 to ACE2 in enterocytes leads to intestinal barrier disruption	KeyEvent
Aop:430 - Binding of SARS-CoV-2 to ACE2 leads to viral infection proliferation	KeyEvent
Aop:394 - SARS-CoV-2 infection of olfactory epithelium leading to impaired olfactory function (short-term anosmia)	KeyEvent
Aop:468 - Binding of SARS-CoV-2 to ACE2 leads to acute respiratory distress (via cell death)	KeyEvent

Stressors

Name
Sars-CoV-2

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
cell

Organ term

Organ term
organ

Domain of Applicability

Taxonomic Applicability				
Term	Scientific Term	Evidence	Links	
Homo sapiens	Homo sapiens	High	NCBI	
Mus musculus	Mus musculus	Moderate	NCBI	
Mustela putorius furo	Mustela putorius furo	Moderate	NCBI	

Life Stage Applicability

Life Stage **Evidence**

All life stages High

Sex Applicability

Sex **Evidence**

Unspecific Evidence

Broad mammalian host range has been demonstrated based on spike protein tropism for and binding to ACE2 [Conceicao *et al.* 2020; Wu *et al.* 2020] and cross-species ACE2 structural analysis [Damas *et al.* 2020]. No literature has been found on primary translation and molecular interactions of nsps in non-human host cells, but it is assumed to occur if the virus replicates in other species.

Very broad mammalian tropism: human, bat, cat, dog, civet, ferret, horse, pig, sheep, goat, water buffalo, cattle, rabbit, hamster, mouse

Key Event Description

This KE1847 "Increase coronavirus production" deals with how the genome of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is translated, replicated, and transcribed in detail, and how the genomic RNA (gRNA) is packaged, and the virions are assembled and released from the cell.

Coronavirus is a class of viruses that have single-stranded positive-sense RNA genomes in their envelopes [D. Kim *et al.*]. The virus contains a 29.7 kB positive-sense RNA genome flanked by 5' and 3' untranslated regions of 265 and 342 nucleotides, respectively [E. J. Snijder *et al.*] that contain cis-acting secondary RNA structures essential for RNA synthesis [N. C. Huston *et al.*]. The genome just prior to the 5' end contains the transcriptional regulatory sequence leader (TRS-L) [C.J. Budzilowicz *et al.*]. The SARS-CoV genome is polycistronic and contains 14 open reading frames (ORFs) that are expressed by poorly understood mechanisms [E. J. Snijder *et al.*]. Preceding each ORF there are other TRSs called the body TRS (TRS B). The 5' two-thirds of the genome contains two large, overlapping, nonstructural ORFs and the 3' third contains the remainder ORFs [H. Di *et al.*]. Genome expression starts with the translation of two large ORFs of the 5' two-thirds: ORF1a of 4382 amino acids and ORF1ab of 7073 amino acid that occurs via a programmed (- 1) ribosomal frameshift [E. J. Snijder *et al.*], yielding pp1a and pp1ab. These two polyproteins are cleaved into 16 subunits by two viral proteinases encoded by ORF1a, nsp3, and nsp5 that contain a papain-like protease domain and a 3C-like protease domain [M. D. Sacco *et al.*]. The processing products are a group of replicative enzymes, named nsp1-nsp16, that assemble into a viral replication and transcription complex (RTC) associated with membranes of endoplasmic reticulum (ER) with the help of various membrane-associated viral proteins [S. Klein *et al.*, E. J. Snijder *et al.*, P. V'Kovski, *et al.*]. Besides replication, which yields the positive-sense gRNA, the replicase also mediates transcription leading to the synthesis of a nested set of subgenomic (sg) mRNAs to express all ORFs downstream of ORF1b that encode structural and accessory viral proteins. These localize to the 3' one-third of the genome, as stated above, and result in a 3' coterminally nested set of 7-9 mRNAs that share ~70-90 nucleotide (nt) in the 5' leader and that is identical to the 5' end of the genome [P. Liu, and J. Leibowitz]. sgRNAs encode conserved structural proteins (spike protein [S], envelope protein [E], membrane protein [M], and nucleocapsid protein [N]), and several accessory proteins. SARS-CoV-2 is known to have at least six accessory proteins (3a, 6, 7a, 7b, 8, and 10). Overall the virus is predicted to express 29 proteins [D. Kim *et al.*]. The gRNA is packaged by the structural proteins to assemble progeny virions.

Viral translation:

SARS-CoV-2 is an enveloped virus with a single-stranded RNA genome of ~30 kb, sequence orientation in a 5' to 3' direction typical of positive sense and reflective of the resulting mRNA [D. Kim *et al.*]. The SARS-CoV-2 genome contains a 5'-untranslated region (UTR; 265 bp), ORF1ab (21,289 bp) holding two overlapping open reading frames (13,217 bp and 21,289 bp, respectively) that encode two polyproteins [D. Kim *et al.*]. Other elements of the genome include are shown below [V. B. O'Leary *et al.*]. **The first event upon cell entry is the primary translation of the ORF1a and ORF1b gRNA to produce non-structural proteins (nsps).**

This is completely dependent on the translation machinery of the host cell. Due to fewer rare "slow-codons", SARS-CoV-2 may have a higher protein translational rate, and therefore higher infectivity compared to other coronavirus groups [V. B. O'Leary *et al.*]. The ORF1a produces polypeptide 1a (pp1a, 440-500 kDa) that is cleaved into nsp-1 through nsp-11. A -1 ribosome frameshift occurs immediately upstream of the ORF1a stop codon, to allow translation through ORF1b, yielding 740-810 kDa polypeptide pp1ab, which is cleaved into 15 nsps [D. Kim *et al.*]. Two overlapping ORFs, ORF1a and ORF1b, generate continuous polypeptides, which are cleaved into a total of 16 so-called nsps [Y Finkel *et al.*]. Functionally, there are five proteins from pp1ab (nsp-12 through nsp-16) as nsp-1-11 are duplications of the proteins in pp1a due to the ORF overlap. The pp1a is approximately 1.4-2.2 times more expressed than pp1ab. After translation, the polyproteins are cleaved by viral proteinases nsp3 and nsp5. Nsp5 protease can be referred to as 3C-like protease (3CL^{pro}) or as main protease (M^{pro}), as it cleaves the majority of the polyprotein cleavage sites. [H.A. Hussein *et al.*] Nsp1 cleavage is quick and nsp1 associates with host cell ribosomes and results in host cellular shutdown, suppressing host gene expression [M. Thoms *et al.*]. Fifteen proteins, nsp2-16 constitute the viral RTC. They are targeted to defined subcellular locations and establish a network with host cell factors. Nsp2-11 remodel host membrane architecture, mediate host immune evasion and provide cofactors for replication, whilst nsp12-16 contain the core enzymatic functions involved in RNA synthesis, modification and proofreading [P. V'Kovski *et al.*]. nsp-7 and nsp-8 form a complex priming the RNA-dependent RNA polymerase (RdRp or RTC) - nsp-12. nsp14 provides a 3'-5' exonuclease activity providing RNA proofreading function. Nsp-10 composes the RNA capping machinery nsp-9. nsp13 provides the RNA 5'-triphosphatase activity. Nsp-14 is a N7-methyltransferase and nsp-16 the 2'-O-methyltransferase. Many of the nsps have multiple functions and many viral proteins are involved in innate immunity inhibition. Nsp-3 is involved in vesicle formation along with nsp-4 and nsp-6 where viral replication occurs. Interactions between SARS-CoV-2 proteins and human RNAs thwart the IFN response upon infection: nsp-16 binds to U1 and U2 splicing RNAs to suppress global mRNA splicing; nsp-1 binds to 40S ribosomal RNA in the mRNA entry channel of the ribosome to inhibit host mRNA translation; nsp-8 and nsp-9 bind to the 7SL RNA to block protein trafficking to the cell membrane [A. K. Banerjee *et al.*]. Xia *et al.* [H. Xia *et al.*] found that nsp-6 and nsp-13 antagonize IFN-I production via distinct mechanisms: nsp-6 binds TANK binding kinase 1 (TBK1) to suppress interferon regulatory factor 3 (IRF3) phosphorylation, and nsp-13 binds and blocks TBK1 phosphorylation.

Viral transcription and replication:

Viral transcription and replication occur at the viral replication organelle (RO) [E. J. Snijder *et al.*]. The RO is specifically formed during infection by reshaping ER and other membranes, giving rise to small spherular invaginations, and large vesiculotubular clusters, consisting of single- and/or double-membrane vesicles (DMV), convoluted membranes (CM) and double-membrane spherules invaginating from the ER [S. Klein *et al.*, E. J. Snijder *et al.*]. There is some evidence that DMV accommodate viral replication which is based on radiolabelling viral RNA with nucleoside precursor ([5,3]³H]uridine) and detection by EM autoradiography [E. J. Snijder *et al.*].

Viral replicative proteins and specific host factors are recruited to ROs [E. J. Snijder *et al.*]. RNA viral genome is transcribed into messenger RNA by the viral RTC [P. Ahlquist *et al.*]. Viral RTC act in combination with other viral and host factors involved in selecting template RNAs, elongating RNA synthesis, differentiating genomic RNA replication from mRNA transcription, modifying product RNAs with 5' caps or 3' polyadenylate [P. Ahlquist *et al.*]. Positive-sense (messenger-sense) RNA viruses replicate their genomes through negative-strand RNA intermediates [M. Schwartz *et al.*]. The intermediates comprise full-length negative-sense complementary copies of the genome, which functions as templates for the generation of new positive-sense gRNAs, and a nested set of sg mRNAs that lead to the expression of proteins encoded in all ORFs downstream of ORF1b. The transcription of coronaviruses is a discontinuous process that produces nested 3' and 5' co-terminal sgRNAs. Of note, the synthesis of sg mRNAs is not exclusive to the order *Nidovirales* but a discontinuous minus-strand synthesis strategy to produce a nested set of 3' co-terminal sg mRNAs with a common 5' leader in infected cells are unique features of the *coronaviruses* and *arteriviruses* [W. A. Miller and G. Koev.]. Of note, the produced genomic RNA represents a small fraction of the total vRNA [N. S. Ogando *et al.*].

The discontinuous minus-strand synthesis of a set of nested sg mRNAs happens during the synthesis of the negative-strand RNA, by an interruption mechanism of the RTC as it reads the TRS-B preceding each gene in the 3' one-third of the viral genome [I. Sola, F. Almazan *et al.*, I. Sola, J. L. Moreno, *et al.*]. The synthesis of the negative-strand RNA stops and is re-initiated at the TRS-L of the genome sequence close from the 5' end of the genome [H. Di *et al.*]. Therefore, the mechanism by which the leader sequence is added to the 5' end requires that the RTC switches template by a jumping mechanism. This interruption process involves the interaction between complementary TRSs of the nascent negative-strand RNA TRS-B and the positive-strand gRNA at the positive-sense TRS-L. The TRS-B site has a 7-8 bp conserved core sequence (CS) that facilitates RTC template switching as it hybridizes with a near complementary CS in the TRS-L [I. Sola, F. Almazan *et al.*, I. Sola, J. L. Moreno, *et al.*]. Upon re-initiation of RNA synthesis at the TRS-L region, a negative-strand copy of the leader sequence is added to the nascent RNA to complete the synthesis of negative-strand sgRNAs. This means that all sg mRNAs as well as the genomic RNA share a common 5' sequence, named leader sequence [X. Zhang *et al.*]. This programmed template switching leads to the generation of sg mRNAs with identical 5' and 3' sequences, but alternative central regions corresponding to the beginning of each structural ORF [I. Sola *et al.* 2015, S. G. Sawicki *et al.*, Y. Yang *et al.*]. Of note, the existence of TRSs also raises the possibility that these sites are at the highest risk of recombining through TRS-B mediated template switching [Y. Yang]. The set of sg mRNAs is then translated to yield 29 identified different proteins [F. Wu *et al.*], although many papers have identified additional ORFs [D. Kim *et al.*, Y. Finkel *et al.*, A. Vandelli *et al.*]. The translation of the linear single-stranded RNA conducts to the generation of the following proteome: 4 are structural proteins, S, N, M, and E; 16 proteins nsp: the first 11 are encoded in ORF1a whereas the last 5 are encoded in ORF1ab. In addition, 9 accessory proteins named ORF3a, ORF3b, ORF6, ORF7a, ORF7b, ORF8, ORF9b, ORF9c, and ORF10 have been identified [F. Wu *et al.*]. At the beginning of infection, there is the predominant expression of the nsp that result from ORF1a and ORF1ab, however, at 5 hpi, the proteins encoded by the 5' last third are found in higher amounts, and the nucleoprotein is the protein found in higher levels [Y. Finkel *et al.*].

Viral assembly:

The final step of viral production requires virion assembly and this process is not well explored for SARS-CoV-2. For example, the role of the structural proteins of SARS-CoV-2 in virus assembly and budding is not known. In general, all beta-coronavirus structural proteins assemble at the endoplasmic reticulum (ER)-to-Golgi compartment [J. R. Cohen *et al.*, A. Perrier *et al.*] and viral assembly requires two steps: Genome packaging which is a process in which the SARS-CoV-2 gRNA must be coated by the viral protein nucleoprotein (N) protein, forming viral ribonucleoprotein (vRNPs) complexes, before being selectively packaged into progeny virions [P. V'Kovski *et al.*], a step in which vRNPs bud into the lumen of the ER and the ER-Golgi intermediate compartment (ERGIC) [N. S. Ogando *et al.*]. This results in viral particles enveloped with host membranes containing viral M, E, and S transmembrane structural proteins that need to be released from the cell.

SARS-CoV-2 gRNA packaging involves the N protein. The N protein of human coronaviruses is highly expressed in infected cells. It is considered a multifunctional protein, promoting efficient sub-genomic viral RNA transcription, viral replication, virion assembly, and interacting with multiple host proteins [P. V'Kovski *et al.*, D. E. Gordon *et al.*, R. McBride, and M. van Zyl, B. C.].

relation to transcription and replication, the N protein could provide a cooperative mechanism to increase protein and RNA concentrations at specific localizations. S. Alberti, and S. Carra, S. F. Banani *et al.*, and this way organize viral transcription. Five studies have shown that N protein undergoes liquid-liquid phase separation (LLPS) *in vitro* [A. Savastano *et al.*, H. Chen *et al.*, C. Iserman *et al.*, T. M. Perdikari *et al.*, J. Cubuk *et al.*], dependent on its C-terminal domain (CTD) [H. Chen *et al.*]. It has been hypothesised that N could be involved in replication close to the ER and in packaging of gRNA into vRNPs near the ERGIC where genome assembly is thought to take place [A. Savastano *et al.*], but so far this is still speculative. Phosphorylation of N could adjust the physical properties of condensates differentially in ways that could accommodate the two different functions of N: transcription and progeny genome assembly [A. Savastano *et al.*, C. Iserman *et al.*, C. R. Carlson *et al.*].

The ERGIC constitutes the main assembly site of coronaviruses [S. Klein *et al.*, E. J. Snijder *et al.*, L. Mendonca *et al.*] and budding events have been seen by EM studies. For SARS-CoV-2, virus-budding was mainly clustered in regions with a high vesicle density and close to ER- and Golgi-like membrane arrangements [S. Klein *et al.*, E. J. Snijder *et al.*, L. Mendonca *et al.*]. The ectodomain of S trimers were found facing the ERGIC lumen and not induce membrane curvature on its own, therefore proposing that vRNPs and spike trimers [S. Klein *et al.*].

Finally, it has been shown that SARS-CoV-2 virions de novo formed traffic to lysosomes for unconventional egress by Arf8b-dependent lysosomal exocytosis [S. Ghosh *et al.*]. This process results in lysosome deacidification, inactivation of lysosomal degradation enzymes, and disruption of antigen presentation [S. Ghosh *et al.*].

How it is Measured or Detected

Viral translation:

SARS-CoV-2 Nsp1 binds the ribosomal mRNA channel to inhibit translation [Schubert *et al.* 2020]

- Sucrose pelleting binding assay to verify Nsp1–40S complex formation
- In vivo translation assay
- Transient expression of FLAG-Nsp1 in HeLa cells and puromycin incorporation assay

SARS-CoV-2 disrupts splicing, translation, and protein trafficking [Banerjee *et al.* 2020]

- SARS-CoV-2 viral protein binding to RNA
- Interferon stimulation experiments
- Splicing assessment experiments
- IRF7-GFP splicing reporter, 5EU RNA labeling, capture of biotinylated 5EU labeled RNA

Membrane SUSET assay for transport of plasma membrane proteins to the cell surface

Viral transcription:

The mRNA transcripts are detected by the real-time reverse transcription-PCR (RT-PCR) assay. Several methods targeting the mRNA transcripts have been developed, which includes the RT-PCR assays targeting RdRp/helicase (Hel), spike (S), and nucleocapsid (N) genes of SARS-CoV-2 [Chan *et al.*]. RT-PCR assays detecting SARS-CoV-2 RNA in saliva include quantitative RT-PCR (RT-qPCR), direct RT-qPCR, reverse transcription-loop-mediated isothermal amplification (RT-LAMP) [Nagura-Ikeda M, *et al.*]. The viral mRNAs are reverse-transcribed with RT, followed by the amplification by PCR.

Viral replication:

viral replication is measured by RT-qPCR in infected cells, formation of liquid organelles is assessed in vitro reconstitution systems and in infected cells. Labelling by radioactive nucleosides.

Viral production:

Plaque assays, infectivity assays, RT-qPCR to detect viral RNA in released virions, sequencing to detect mutations in the genome, electron microscopy.

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List of Key Events in the AOP

Event: 1901: Interferon-I antiviral response, antagonized by SARS-CoV-2

Short Name: IFN-I response, antagonized

Key Event Component

Process	Object	Action
type I interferon signaling pathway	interferon alpha	decreased
type I interferon signaling pathway	interferon beta	decreased
cellular response to exogenous dsRNA	RNA viral genome	occurrence

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:430 - Binding of SARS-CoV-2 to ACE2 leads to viral infection proliferation	KeyEvent
Aop:422 - Binding of SARS-CoV-2 to ACE2 in enterocytes leads to intestinal barrier disruption	KeyEvent
Aop:320 - Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality	KeyEvent
Aop:379 - Binding to ACE2 leading to thrombosis and disseminated intravascular coagulation	KeyEvent
Aop:468 - Binding of SARS-CoV-2 to ACE2 leads to acute respiratory distress (via cell death)	KeyEvent

Stressors

Name
Stressor:624 SARS-CoV-2

Biological Context

Level of Biological Organization
Cellular

Cell term**Cell term**

cell

Organ term**Organ term**

organ

Domain of Applicability**Taxonomic Applicability**

Term	Scientific Term	Evidence	Links
humans	<i>Homo sapiens</i>	High	NCBI
mink	<i>Mustela lutreola</i>	High	NCBI
cat	<i>Felis catus</i>	High	NCBI
rhesus macaque	<i>Macaca mulatta</i>	High	NCBI
dog	<i>Canis lupus familiaris</i>	Moderate	NCBI
mammals	mammals	High	NCBI

Life Stage Applicability**Life Stage Evidence**

All life stages High

Sex Applicability**Sex Evidence**

Unspecific High

Broad mammalian host range based on spike protein tropism for and binding to ACE2 (Conceicao et al. 2020; Wu et al. 2020) and cross-species ACE2 structural analysis (Damas et al. 2020). Some literature found on non-human hosts indicates that NSPs and accessory proteins can interact in a similar manner with bird (chicken) and other mammal proteins in the IFN-I pathway (Moustaqil et al. 2021; Rui et al. 2021).

Key Event Description

SARS-CoV-2 is an enveloped virus with a single-stranded RNA genome of ~30 kb, sequence orientation in a 5' to 3' direction typical of positive sense and reflective of the resulting mRNA (doi:<https://doi.org/10.1016/j.cell.2020.04.01>). The SARS-CoV-2 genome contains a 5'-untranslated region (UTR; 265 bp), [ORF1ab](#) (21,289 bp) holding two overlapping open reading frames (13,217 bp and 21,289 bp, respectively) that encode two polyproteins (Kim et al. 2020; O'Leary et al. 2020). Viral transcription and replication is explained in depth in [KE1847](#). Briefly, the first event upon cell entry is the primary translation of the ORF1a and ORF1b genomic RNA to produce non-structural proteins (NSPs). The ORF1a produces polypeptide 1a (pp1a, 440–500 kDa) that is cleaved into NSP-1 through NSP-11. A -1-ribosome frameshift occurs immediately upstream of the ORF1a stop codon, to allow translation through ORF1b, yielding 740–810 kDa polypeptide pp1ab, which is cleaved into 15 NSPs (duplications of NSP1-11 and five additional proteins, NSP12-16). Viral proteases NSP3 and NSP5 cleave the polypeptides through domains functioning as a papain-like protease and a 3C-like protease, respectively (doi:<https://doi.org/10.1016/j.cell.2020.04.01>). The NSPs, structural proteins, and accessory proteins are encoded by 10 ORFs in the SARS-CoV-2 RNA genome. They may have multiple functions during viral replication as well as in evasion of the host innate immune response, thus augmenting viral replication and spread (Amor et al. 2020). Extensive protein-protein interaction (Gordon et al. 2020) and viral protein-host RNA interaction networks have been demonstrated between the viral NSPs and accessory proteins and host molecules.

This key event is focused on the specific viral:host protein interactions within the infected cell that are involved in the [IFN-I antiviral response pathways](#). IFN-I is the main component of the innate immune system that is suppressed by the SARS-CoV-2 coronavirus early in infection. The primary form of host intracellular virus surveillance detects viral components to induce an immediate systemic type I interferon (IFN) response. Cellular RNA sensors called pattern recognition receptors (PRRs) such as RIG-I, MDA5 and LGP2 detect the presence of viral RNAs and promote nuclear translocation of the transcription factor IRF3, leading to transcription, translation, and secretion of IFN- α and IFN- β . This in turn leads to interaction with the IFN receptor (IFNAR), phosphorylation of STAT1 and 2, and transcription and translation of hundreds of antiviral genes (Quarleri and Delpino, 2021).

Interactions between SARS-CoV-2 proteins and human RNAs thwart the IFN response upon infection: NSP1 binds to 40S ribosomal RNA in the mRNA entry channel of the ribosome to inhibit host mRNA translation; NSP8 and NSP9 displace signal recognition particle proteins (SRP54, 27 and 19) to bind to the 7SL RNA and block protein trafficking to the cell membrane (Banerjee et al. 2020; Gordon et al. 2020). Xia et al. (2020) found that NSP6 and NSP13 antagonize IFN-I production via distinct mechanisms: NSP6 binds TANK binding kinase 1 (TBK1) to suppress interferon regulatory factor 3 (IRF3) phosphorylation, and NSP13 binds and blocks TBK1 phosphorylation. NSP14 induces lysosomal degradation of type I IFNAR to prevent STAT activation (Hayn et al. 2021). ORF6 hijacks KPNA2 to block IRF3, and Nup98/RAE1 to block STAT nuclear import, to silence IFN-I gene expression (Xia and Shi, 2020). ORF7a suppresses STAT2 phosphorylation and ORF7b suppresses STAT1 and STAT2 phosphorylation to block ISGF3 complex formation with IRF9 (Xia and Shi, 2020). ORF8 interacts and downregulates MHC-I (Zhang et al 2020), and has been reported to block INF β expression, but the mechanism has not been identified (Rashid et al. 2021; Li et al. 2020). ORF9b antagonizes Type I Interferons by targeting multiple components of RIG-I/MDA-5-MAVS, TOMM70, NEMO and cGAS-STING signalling (Han et al. 2020; Jiang et al. 2020; Wu et al. 2021; Gordon et al 2020).

Following is a table of the current state of knowledge of SARS-CoV-2 protein putative functions in relation to IFN-I antiviral response antagonism.

Gene	Protein	Function	Role in early innate immune evasion
ORF1a	NSP1	NSP1 antagonizes interferon induction to suppress host antiviral response.	DNA Polymerase Alpha Complex: Regulates the activation of IFN-I through cytosolic RNA-DNA synthesis (POLA1/2-PRIM1/2) and primes DNA replication in the nucleus (Gordon et al. 2020; Chaudhuri et al. 2020). Can also inhibit host gene expression by binding to ribosomes and modifying host mRNAs (Shi et al. 2020; Schubert et al. 2020; Thoms et al. 2020).
NSP2		While not essential for viral replication, deletion of NSP2 diminishes viral growth and RNA synthesis	Translation repression through binding GIGYF2 and EIF4E2 (4EHP) (Gupta et al. 2021)
NSP3		Papain-like protease	Suppresses IFN-I: Cleaves IRF3

		(Plpro); Cleaves the ORF1a and 1ab polypeptides	(Moustaqil et al. 2021); binds/cleaves ISG15 (Rui et al. 2021; Shin et al. 2020; Liu et al. 2021; Klemm et al. 2020)
NSP5	3C-like protease (3CLpro); Cleaves the ORF1a and 1ab polypeptides	Binds STING (Rui et al. 2021)	
NSP6	Limits autophagosome expansion	Suppresses IFN-I expression: Binds TBK-1 to suppress IRF3 phosphorylation (Xia et al. 2020; Quarleri and Delpino, 2021)	
NSP7	In complex with NSP8 forms primase as part of multimeric RNA-dependent RNA replicase (RdRp)		
NSP8	Replication complex with NSP7, NSP9 and NSP12	Binds SRP72/54/19 (Gordon et al. 2020) and 7SL RNA to block IFN membrane transport (Banerjee et al. 2020)	
NSP9	Replication complex with NSP7, NSP8 and NSP12	Binds SRP and 7SL RNA with NSP8 to block IFN membrane transport (Banerjee et al. 2020)	
ORF1b	NSP13	Helicase and triphosphatase that initiates the first step in viral mRNA capping.	Binds TBK1 (Xia et al. 2020)
	NSP14		Induces lysosomal degradation of IFNAR1 (Hayn et al. 2021)
ORF2	Spike (S)	ACE2 interaction, cell entry	
ORF3a	ORF3a	Interacts with M, S, E and 7a; form viroporins; immune evasion	Binds STING (Rui et al 2021)
ORF4	Envelope (E)	Viral assembly and budding	
ORF5	Membrane (M)	Viral assembly	Interacts with RIG-I and MAVS sensors of viral RNA (Fu et al 2020)
ORF6	ORF6	Viral pathogenesis and virulence; interacts with ORF8; promotes RNA polymerase activity	Hijacks the nuclear importin Karyopherin a 2 (KPNA2) to block IRF3 (Xia and Shi, 2020) and Nup98/RAE1 to block STAT nuclear import (Miorin et al. 2020; Kato et al. 2020), leading to the silence of downstream ISGs
ORF7a	ORF7a	Interacts with S, ORF3a; immune evasion	Suppresses STAT2 phosphorylation to block IFN-I response (Xia and Shi, 2020).
ORF7b	ORF7b	Structural component of virion	Suppresses STAT1 and STAT2 phosphorylation to block IFN-I response (Xia and Shi, 2020)
ORF8	ORF8	Immune evasion	Interacts and downregulates MHC-I (Zhang et al. 2020). May inhibit type I interferon (IFN- β) and interferon-stimulated response element (ISRE) (Rashid et al. 2020; Li et al. 2020)
ORF9	Nucleocapsid (N)	Stabilizes viral RNA	Attenuates stress granule formation: G3BP1/2 (Chen et al. 2020; Cascarina et al. 2020); G3BP1 also interacts with RIG-I (Kim et al. 2019) and STAT1/2 (Mu et al. 2020)
ORF9b	ORF9b	Immune evasion	Membrane protein antagonizes Type I Interferons by targeting multiple components of RIG-I/MDA-5-MAVS, TOMM70, NEMO, and cGAS-STING signaling pathways (Fu et al. 2020; Chen et al. 2020; Han et al. 2020; Jiang et al. 2020; Wu et al. 2021; Gordon et al 2020)

How it is Measured or Detected

Detection of IFN-I suppression involves measuring gene promoter/transcription activation (luciferase assays), gene up/down regulation (quantitative PCR), protein-protein interaction (immunoprecipitation, immunoblotting) or in-situ co-location of viral and host proteins (immunofluorescent or confocal microscopy) in cell culture. Examples of methods used include the following:

Interferon I decrease (Xia et al. 2020):

- IFN-I production and signaling luciferase reporter assays
- Co-immunoprecipitation and western blot
- Indirect immunofluorescence assays
- DNA assembly and RNA transcription of a luciferase replicon for SARS-CoV-2
- Replicon RNA electroporation and luciferase reporter assay

SARS-CoV-2 ORF9b inhibits RIG-I-MAVS antiviral signaling (Wu et al. 2021)

- Viral- and host-protein-specific antibodies
- Immunoprecipitation
- Immunofluorescent microscopy
- Dual-luciferase reporter assays

- Fluorescence quantification immunoblotting

SARS-CoV-2-Human Protein-Protein Interaction Map (Gordon et al. 2020)

- Cloning and expression of viral proteins via plasmid transfection into HEK293T cell line
- Protein affinity purification using MagStrep beads with detection by anti-strep western blot of cell lysate
- Global analysis of SARS-CoV-2 host interacting proteins using affinity purification-mass spectrometry

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Event: 1869: Diminished protective oxidative stress response

Short Name: Diminished Protective Response to ROS

Key Event Component

Process	Object	Action
cellular response to oxidative stress	reactive oxygen species	increased
response to reactive oxygen species	reactive oxygen species	increased
AOPs Including This Key Event		
AOP ID and Name		Event Type
Aop:379 - Binding to ACE2 leading to thrombosis and disseminated intravascular coagulation		KeyEvent
Aop:406 - SARS-CoV-2 infection leading to hyperinflammation		KeyEvent
Aop:407 - SARS-CoV-2 infection leading to pyroptosis		KeyEvent
Aop:377 - Dysregulated prolonged Toll Like Receptor 9 (TLR9) activation leading to Multi Organ Failure involving Acute Respiratory Distress Syndrome (ARDS)		KeyEvent
Stressors		
Name		
Stressor:624 SARS-CoV-2		
Biological Context		
Level of Biological Organization		
Cellular		
Cell term		
Cell term		
cell		
Organ term		
Organ term		
organ		
Domain of Applicability		
Taxonomic Applicability		
Term	Scientific Term	Evidence
Homo sapiens	Homo sapiens	High
NCBI		
Life Stage Applicability		
Life Stage	Evidence	
All life stages	High	
Sex Applicability		
Sex	Evidence	
Unspecific	High	
Response to ROS occurs in many cell types and tissues in all life stages and the broad range of mammals.		
Key Event Description		
Oxidative stress is caused by an imbalance between the production of reactive oxygen and the detoxification of reactive intermediates. Reactive intermediates such as peroxides and free radicals can be very damaging to many parts of cells such as proteins, lipids, and DNA. Severe oxidative stress can trigger apoptosis and necrosis. [Ref. IPA, NRF2-mediated Oxidative Stress Response, version60467501, release date: 2020-11-19]		
The cellular defence/defense response to oxidative stress includes induction of detoxifying enzymes and antioxidant enzymes. Nuclear factor-erythroid 2-related factor 2 (Nrf2) binds to the antioxidant response elements (ARE) within the promoter of these enzymes and activates their transcription. Inactive Nrf2 is retained in the cytoplasm by association with an actin-binding protein Keap1. Upon exposure of cells to oxidative stress, Nrf2 is phosphorylated in response to the protein kinase C, phosphatidylinositol 3-kinase and MAP kinase pathways. After phosphorylation, Nrf2 translocates to the nucleus, binds AREs, and transactivates detoxifying enzymes and antioxidant enzymes, such as glutathione S-transferase, cytochrome P450, NAD(P)H quinone oxidoreductase, heme oxygenase, and superoxide dismutase. [Ref. IPA, NRF2-mediated Oxidative Stress Response, version60467501, release date: 2020-11-19]		
Nrf2, a master regulator of oxidative stress through enhanced expression of anti-oxidant genes of glutathione and thioredoxin-antioxidant systems, has anti-inflammatory, anti-apoptotic, and antioxidant effects. Dimethyl fumarate (DMF), an activator of Nrf2, can decrease inflammation and reactive oxygen species (ROS) through the inhibition of NF- κ B by inducing anti-oxidant enzymes [Hassan et al., MED ARCH. 2020 APR; 74(2): 134-138] [Timpani et al., Pharmaceuticals 2021, 14, 15.]		
Inactivation of Nrf2 causes diminished protective responses to ROS.		
How it is Measured or Detected		
Oxidative stress can be measured as follows:		
1. Direct detection of reactive oxygen species (ROS)		
ROS can be detected by intracellular ROS assay, <i>in vitro</i> ROS/RNS assay. Nitric oxide can be detected in intracellular nitric oxide assay.		

AOP379

Hydroxyl, peroxyl, or other ROS can be measured using a fluorescence probe, 2', 7'-Dichlorodihydrofluorescein diacetate (DCFH-DA), at fluorescence detection at 480 nm/530 nm.

Hydrogen peroxide (H₂O₂) can be detected with a colorimetric probe, which reacts with H₂O₂ in a 1:1 stoichiometry to produce a bright pink colored product, followed by the detection with a standard colorimetric microplate reader with a filter in the 540-570 nm range.

2. Measurement of anti-oxidants

The level of catalase, glutathione, or superoxide dismutase can be measured as anti-oxidants. Catalase is an anti-oxidative enzyme that catalyzes the resolution of hydrogen peroxide (H₂O₂) into H₂O and O₂. The chemiluminescence or fluorescence of HRP catalytic reaction can be detected with residual H₂O₂ and probes (DHBS+AAP, or ADHP (10-Acetyl-3, 7-dihydroxyphenoxazine)).

Anti-oxidant capacity is also one of the oxidative stress markers. Oxygen radical antioxidant capacity (ORAC), hydroxyl radical antioxidant capacity (HORAC), total antioxidant capacity (TAC), the cell-based exogenous antioxidant assay can be used for measuring the antioxidant capacity.

3. Detection of damages in protein, lipid, DNA or RNA

Oxidation of protein can be measured by the detection of protein carbonyl content (PCC), 3-nitrotyrosine, advanced oxidation protein products, or BPDE protein adduct.

DNA oxidation can be detected with 8-oxo-dG / 8-hydroxy-2'-deoxyguanosine (8-OHdG) by ELISA.

Lipid peroxides decompose to form malondialdehyde (MDA) and 4, hydroxynonenal (4-HNE), natural bi-products of lipid peroxidation. Lipid peroxidation can be monitored by thiobarbituric acid (TBA) reactive substances in biological samples. MDA and TBA form MDA-TBA adduct in a 1:2 stoichiometry and are detected by colorimetric or fluorometric measurement.

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[Event: 1845: Coagulation](#)

Short Name: Coagulation

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:379 - Binding to ACE2 leading to thrombosis and disseminated intravascular coagulation	KeyEvent

Stressors

Name
Sars-CoV-2

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
blood cell

Organ term

Organ term
blood plasma

Domain of Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Moderate	NCBI

Life Stage Applicability**Life Stage Evidence**

All life stages Moderate

Sex Applicability**Sex Evidence**

Unspecific Moderate

Homo sapiens

Key Event Description

Coagulation is a process that responds to injury by the rapid formation of a clot. Activation of coagulation factor proteins are involved in coagulation. In the extrinsic pathway, platelets, upon the contact with collagen in the injured blood vessel wall, release thromboxane A2 (TXA2) and adenosine 2 phosphates (ADP), leading to the clot formation. Extravascular tissue factor (TF) binds to plasma factor VIIa (FVIIa) and promotes the activation of FXa. Activated FXa assembles with cofactors FVa and FVIIa on the surface of aggregated platelets, which lead to generation of thrombin (FIIa). Thrombin catalyzes the production of fibrin (FG) which creates a clot. The binding of prekallikrein and high-molecular weight kininogen activate FXIIa in the intrinsic pathway. Many regulators are involved in coagulation system. Plasmin is one of the modulators required for dissolution of the fibrin clot. Plasmin is activated by tissue plasminogen activator (tPA) and urokinase plasminogen activation (uPA). SERPINS inhibit thrombin, plasmin and tPA. For example, SERPINE1 or plasminogen activator inhibitor-1 (PAI-1) inhibits tPA/uPA and results in hypofibrinolysis [Bernard I, et al. *Viruses*. 2021; 13(1):29.]. In addition, SERPING1 inhibits FXII, and thus down-regulation of SERPING1 lifts suppression of FXII of the intrinsic coagulation cascade [Garvin et al. *eLife* 2020;9:e59177]. Protein C, protein S and thrombomodulin degrade FVa and FVIIa. [Ref. IPA, Coagulation System, version60467501, release date: 2020-11-19]

How it is Measured or Detected

Coagulation and inflammatory parameters are measured in COVID-19 patients [Di Nisio et al. 2021]. Coagulation parameters include platelet count, prothrombin time, activated partial thromboplastin time, D-dimer, fibrinogen, antithrombin III [Di Nisio et al. 2021]. These parameters are measured in the blood.

In vitro systems

Whole human blood model for testing the activation of coagulation and complement system, as well as clot formation [Ekstrand-Hammarström, B. et al. *Biomaterials* 2015, 51, 58-68, Ekdahl, K.N., et al. *Nanomedicine: Nanotechnology, Biology and Medicine* 2018, 14, 735-744, Ekdahl, K.N., et al. *Science and Technology of Advanced Materials*, 20:1, 688-698,]

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List of Adverse Outcomes in this AOP**Event: 1846: Thrombosis and Disseminated Intravascular Coagulation****Short Name: Thrombosis and DIC****Key Event Component****Process Object Action**

Venous thrombosis platelet increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:379 - Binding to ACE2 leading to thrombosis and disseminated intravascular coagulation	AdverseOutcome
Aop:395 - Binding of Sars-CoV-2 spike protein to ACE 2 receptors expressed on pericytes leads to disseminated intravascular coagulation resulting in cerebrovascular disease (stroke)	KeyEvent
Aop:377 - Dysregulated prolonged Toll Like Receptor 9 (TLR9) activation leading to Multi Organ Failure involving Acute Respiratory Distress Syndrome (ARDS)	KeyEvent

Stressors**Name**

Sars-CoV-2

Biological Context**Level of Biological Organization**

Organ

Organ term

Organ term

blood

Domain of Applicability**Taxonomic Applicability**

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	Not Specified

Sex Applicability

Sex	Evidence
Unspecific	Not Specified

Homo sapiens

Key Event Description

Thrombosis is defined as the formation or presence of a thrombus. Clotting within a blood vessel may cause infarction of tissues supplied by the vessel. Extreme aggravation of blood coagulation induces multiple thrombi in the microvasculature, which leads to consumption coagulopathy followed by disseminated intravascular coagulation (DIC).

DIC is a pathological syndrome resulting from the formation of thrombin, subsequent activation and consumption of coagulation proteins, and the production of fibrin thrombi. The initial pathologic events are thrombotic in nature resulting in thrombotic vascular occlusions. The initial clinical events are usually hemorrhagic resulting in oozing from mucosa and massive gastrointestinal blood loss. The occlusive events occur as a result of fibrin microthrombi or platelet microthrombi that obstruct the microcirculation of organs. This obstruction can result in organ hypoperfusion and ischemia, infarction, and necrosis. All organs are potentially vulnerable to the effects of thrombotic occlusions.

The renal effects of DIC are multifactorial and may be associated with hypovolemia or hypotension. If the hypotension is not corrected it may lead to renal failure due to acute tubular necrosis. Fibrin thrombi may also block glomerular capillaries causing ischemic, renal cortical necrosis (Colman, 1984).

The cerebral effects of DIC often result in nonspecific changes such as altered state of consciousness, convulsions, and coma. Major vascular occlusions, subarachnoid hemorrhage, multiple cortical and brain stem hemorrhages may occur following microvascular occlusions (Schwartzman RJ, 1982).

The pulmonary effects of DIC may be caused by interstitial hemorrhage resulting in a clinical effect resembling acute respiratory distress syndrome (Schwartzman RJ, 1973; Shah RL, 1984).

How it is Measured or Detected

Clinical laboratory tests are used to diagnose DIC.

Prothrombin time (PT) is a blood test that measures how long it takes blood to clot. PT measures the time required for fibrin clot formation after the addition of tissue thromboplastin and calcium. The average time range for blood to clot is about 10 to 13 seconds.

Activated partial prothrombin time (APTT). Platelet poor plasma [PPP] is incubated at 37°C then phospholipid (cephalin) and a contact activator (e.g. Kaolin, micronized silica, or ellagic acid) are added. This leads to the conversion of Factor XI [FXI] to FXIa. The remainder of the pathway is not activated as no calcium is present. The addition of calcium (pre-warmed to 37°C) initiates clotting. The APTT is the time taken from the addition of calcium to the formation of a fibrin clot. The clotting time for the APTT lies between 27-35 seconds.

Decreased fibrinogen concentrations

Diluted plasma is clotted with a high concentration of Thrombin. The tested plasma is diluted (usually 1:10 but this may vary if the Fibrinogen concentration is very low or very high) to minimize the effect of 'inhibitory substances' within the plasma e.g. heparin, elevated levels of FDPs. The use of a high concentration of Thrombin (typically 100 U/ml) ensures that the clotting times are independent of Thrombin concentration over a wide range of Fibrinogen levels.

The test requires a reference plasma with a known Fibrinogen concentration and that has been calibrated against a known international reference standard. A calibration curve is constructed using this reference plasma by preparing a series of dilutions (1:5 –1:40) in the buffer to give a range of Fibrinogen concentrations. The clotting time of each of these dilutions is established (using duplicate samples) and the results (clotting time(s)/Fibrinogen concentration (g/L) are plotted on Log-Log graph paper. The 1:10 concentration is considered to be 100% i.e. normal. There should be a linear correlation between clotting times in the region of 10-50 sec.

The test platelet-poor diluted plasma (diluted 1:10 in buffer) is incubated at 37°C. Thrombin [\sim 100 U/ml] added (all pre-warmed to 37°C). The time taken for the clot to form is compared to the calibration curve and the Fibrinogen concentration deduced. Test samples whose clotting times fall out with the linear part of the calibration curve should be re-tested using different dilutions.

Most laboratories use an automated method in which clot formation is deemed to have occurred when the optical density of the mixture has exceeded a certain threshold.

Platelet Measurements-

A platelet count is the number of platelets a person has per microliter. The ideal platelet range is 150,000 – 400,000 per microliter in most healthy people.

Fibrinolysis measurements-

d-dimer concentration ALERE TRIAGE® D-DIMER TEST

D-Dimer can be measured by a fluorescence immunoassay. To determine cross-linked fibrin degradation products containing D-dimer in EDTA anticoagulated whole blood and plasma specimens. The test is used as an aid in the assessment and evaluation of patients suspected of having disseminated intravascular coagulation or thromboembolic events including pulmonary embolism

Procedure:

Commercially available kits are available to measure d-dimer in whole blood or plasma. The kits contain all the reagents necessary for the quantification of cross-linked fibrin degradation products containing D-dimer in EDTA anticoagulated whole blood or plasma specimens.

Regulatory Significance of the AO

Thrombosis is one of the world's main concerns in terms of severe symptoms or adverse responses of the vaccine for COVID-19 which is caused by SARS-CoV-2. Excess thrombosis leads to DIC, which might be mortal. For safely developing the therapeutics and vaccines of COVID-19, it is regulatory significant to understand the cellular and molecular mechanisms in the pathogenesis of coronaviral infection, which may include thrombosis and DIC, AO1846.

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

[Relationship: 2056: Binding to ACE2 leads to SARS-CoV-2 cell entry](#)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality	adjacent	High	High
SARS-CoV-2 infection of olfactory epithelium leading to impaired olfactory function (short-term anosmia)	adjacent		
SARS-CoV-2 infection leading to hyperinflammation	adjacent		
Binding of SARS-CoV-2 to ACE2 in enterocytes leads to intestinal barrier disruption	adjacent	High	High
Binding of SARS-CoV-2 to ACE2 leads to viral infection proliferation	adjacent	High	Moderate
Binding to ACE2 leading to thrombosis and disseminated intravascular coagulation	adjacent	High	Moderate
Binding of SARS-CoV-2 to ACE2 leads to acute respiratory distress (via cell death)	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Key Event Relationship Description

This KER deals with the evidence supporting the individual weight that the surface protein of SARS-CoV-2 spike needs to bind:ACE2, and of being cleaved in two different sites, for viral entry to occur. Viral entry is essential for initiating a cascade of events leading to COVID19.

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[COVID19 References related to CNS:](#)

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[Relationship: 2496: SARS-CoV-2 cell entry leads to IFN-1 response, antagonized](#)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding of SARS-CoV-2 to ACE2 leads to viral infection proliferation	adjacent	High	Not Specified
Binding of SARS-CoV-2 to ACE2 in enterocytes leads to intestinal barrier disruption	adjacent		
Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality	adjacent	High	
Binding to ACE2 leading to thrombosis and disseminated intravascular coagulation	adjacent	Moderate	Moderate
Binding of SARS-CoV-2 to ACE2 leads to acute respiratory distress (via cell death)	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mammals	mammals	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Key Event Relationship Description

Upon entry of a virus into the host cell (KE1738), the virus is unpackaged from the structural nucleocapsid (N), envelope (E), and membrane (M) proteins. The viral RNA is detected by Pattern Recognition Receptor (PRR) proteins including RIG-I and MDA5 but the M proteins can interact with these PRRs directly, and block this initial host reaction (<https://doi.org/10.1038/s41423-020-00571-x>). The viral genomic RNA can then be translated directly at the host ribosomes. The viral proteins may be processed through cleavage by viral protease enzymes. This releases a repertoire of non-structural proteins (NSPs) and accessory open reading frame (ORF) proteins that has evolved, for example in the SARS-CoV-2 virus, to bind and block the proteins in the interferon I (IFN-I) antiviral cascade (KE1901). The timely production of type I IFN by host cells is critical for limiting viral replication and promoting antiviral immunity (10.1126/science.abm8108).

Evidence Supporting this KER

Biological Plausibility

Biological plausibility comes from several studies indicating that if IFN is administered just before or upon exposure, viral production is reduced or eliminated (DOI 10.1007/s10517-021-05330-0; <https://doi.org/10.1038/s41467-021-27318-0>; doi.org/10.1016/j.immuni.2021.01.017), which supports the linkage that viral entry causes IFN antagonism. Also, it has been found that some people have developed autoimmunity toward their own IFN proteins (<https://doi.org/10.1084/jem.20210554>; <https://doi.org/10.1084/jem.20211211>). They produce autoantibodies that block even exogenously administered IFN, and this has resulted in more severe disease in these patients.

Empirical Evidence

There are studies with empirical evidence in support of temporal concordance, showing that interferon expression is delayed by SARS-CoV-2 compared to other viruses like influenza, which is also described as an untuned or imbalanced response between interferons being initially low and inflammatory cytokines elevated in moderate to severe cases (10.1016/j.cell.2020.04.026; <https://doi.org/10.1038/s41590-020-00840-x>; <https://www.science.org/doi/10.1126/science.abc6027>; <https://doi.org/10.1038/s41467-021-27318-0>; <https://doi.org/10.1038/s41598-021-95197-y>).

Uncertainties and Inconsistencies

There are uncertainties based on differing disease outcomes, especially associated with timing of administering IFN. Effectiveness of IFN treatment is still uncertain due to some studies evaluating IFN along with other drugs (DOI: 10.1002/jmv.27317). In the small intestine of infected hamsters, a mild antiviral gene signature was observed coinciding with a low-level inflammatory response and low replication similar to some human cases (<https://doi.org/10.1016/j.immuni.2021.01.017>), in contrast to the robust replication seen in human small intestinal organoids (<https://doi.org/10.1126/science.abc1669>) and in severely ill patients. See also <https://doi.org/10.1186/s12943-021-01363-1>.

Quantitative Understanding of the Linkage

Response-response relationship

Viral exposure dose threshold to result in IFN antagonism.

Time-scale

The viral entry MIE and early KEs coincide with the time from exposure to symptoms, within which are the latent period, or time from exposure to infectiousness, and the serial interval, or the time interval between the onset of symptoms in the primary (infector) and secondary case (infectee). Viral entry leading to antagonism of the IFN response occurs during the latent period of the disease prior to symptom onset. Latent period calculation is based on serial interval and median pre-symptomatic infectious period: Serial interval 5.2 days (Rai et al. 2021) – 2.5 days pre-symptom infectious period (Byrne et al. 2020) ≈ 2.7 days. The latent period was longer in asymptomatic cases (4-9 days); pre-symptomatic transmission occurs from about 3 days after exposure to symptom onset at about day 5-7, viral load peaks from about day 5-7 to day 9-11, and the host can remain infectious to symptom clearance or death (Byrne et al. 2020). As noted, IFN administered prior to exposure or within the latent period window can stop replication. However, IFN administered too late, in the inflammatory stage (post-symptom onset), led to long-lasting harm and worsened disease outcome (DOI: 10.1002/jmv.27317). In a study using a primary nasal cell model (differentiated at air-liquid interface), the virus did not proliferate beyond the limit of assay detection if treated with IFN beta or lambda 16 hours prior to infection, and virus was significantly reduced in cultures treated 6h post-infection compared to untreated cultures. Treatments 24h post infection were not significantly different from untreated controls for either type of IFN (<https://doi.org/10.1038/s41467-021-27318-0>).

Known modulating factors

- Autoimmunity as noted above (patients with autoantibodies that attack IFN).
- Factors affecting entry (TMPRSS2 or neuropilin) - therapeutics?
- Androgen signaling leads to increased TMPRSS2 production (<https://doi.org/10.1016/j.mce.2009.12.022>) in males, and therefore potentially enhanced viral entry.
- Weakened IFN antiviral response in the elderly (doi: [10.3390/cells10030708](https://doi.org/10.3390/cells10030708)).
- Environmental exposures to aryl hydrocarbons and dioxins may block IFN production (doi: [10.1016/j.cotox.2018.01.004](https://doi.org/10.1016/j.cotox.2018.01.004)).

Known Feedforward/Feedback loops influencing this KER

Initial exposure to the virus is exogenous, but disease progression results from viral proliferation and release from the initial site of infection to spread to adjacent cells and

eventually distal tissues.

[Relationship: 2497: IFN- \$\beta\$ response, antagonized leads to SARS-CoV-2 production](#)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding of SARS-CoV-2 to ACE2 leads to viral infection proliferation	adjacent	High	Not Specified
Binding of SARS-CoV-2 to ACE2 in enterocytes leads to intestinal barrier disruption	adjacent		
Binding of SARS-CoV-2 to ACE2 receptor leading to acute respiratory distress associated mortality	adjacent	High	
Binding to ACE2 leading to thrombosis and disseminated intravascular coagulation	adjacent	Moderate	Moderate
Binding of SARS-CoV-2 to ACE2 leads to acute respiratory distress (via cell death)	adjacent	High	Moderate

[Relationship: 2358: SARS-CoV-2 production leads to Diminished Protective Response to ROS](#)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding to ACE2 leading to thrombosis and disseminated intravascular coagulation	adjacent	Moderate	Not Specified
SARS-CoV-2 infection leading to hyperinflammation	adjacent		

[Relationship: 2359: Diminished Protective Response to ROS leads to Coagulation](#)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding to ACE2 leading to thrombosis and disseminated intravascular coagulation	adjacent	Moderate	Not Specified

[Relationship: 2360: Coagulation leads to Diminished Protective Response to ROS](#)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding to ACE2 leading to thrombosis and disseminated intravascular coagulation	adjacent	Moderate	Not Specified

[Relationship: 2290: Coagulation leads to Thrombosis and DIC](#)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Binding to ACE2 leading to thrombosis and disseminated intravascular coagulation	adjacent	High	

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Key Event Relationship Description

Many regulators are involved in coagulation system. Plasmin is one of the modulators required for dissolution of the fibrin clot. Plasmin is activated by tissue plasminogen activator (tPA) and urokinase plasminogen activation (uPA). SERPINS inhibit thrombin, plasmin and tPA. For example, SERPINE1 or plasminogen activator inhibitor-1 (PAI-1) inhibits tPA/uPA and results in hypofibrinolysis [Bernard I, et al. *Viruses*. 2021; 13(1):29.]. In addition, SERPING1 inhibits FXII, and thus down-regulation of SERPING1 lifts suppression of FXII of the intrinsic coagulation cascade [Garvin et al. *eLife* 2020;9:e59177]. Protein C, protein S and thrombomodulin degrade FVa and FVIIa. [Ref. IPA, Coagulation System, version60467501, release date: 2020-11-19]

Quantitative Understanding of the Linkage

Known Feedforward/Feedback loops influencing this KER

Decreased fibrinolysis is involved in coagulation system. Coagulopathy may also be involved in this KER. [Mast AE et al, Garvin MR et al.]

References

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2. Garvin et al. A mechanistic model and therapeutic interventions for COVID-19 involving a RAS-mediated bradykinin storm. *eLife* 2020;9:e59177. DOI: <https://doi.org/10.7554/eLife.59177>
3. Mast AE, Wolberg AS, Gailani D, Garvin MR, Alvarez C, Miller JI, Aronow B, Jacobson D (2021) SARS-CoV-2 suppresses anticoagulant and fibrinolytic gene expression in the lung. *eLife* 10:e64330. doi:10.7554/eLife.64330
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