AOP ID and Title:

AOP 385: Viral spike protein interaction with ACE2 leads to microvascular dysfunction, via ACE2 dysregulation
Short Title: Viral spike protein interaction with ACE2 leads to microvascular dysfunction

Authors

CIAO, the transdisciplinary collaborative effort to investigated the biological mechanisms underlying COVID-19 pathogenesis using the Adverse Outcome Pathway (AOP) framework.

Status

Author status	OECD status	OECD project	SAAOP status
Under Development: Contributions and Comments Welcome			

Background

This AOP was initiated within the context of the CIAO project (Project to establish a COVID-19 AOP | CIAO project (ciao-covid.net)) with the aim to test applicability of the AOP framework for exploring the available evidence for one hypothesized sequence of events leading to a particular pathophysiological outcome of COVID19, microvascular dysfunction.

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	KE	1854	Dysregulation, ACE2 expression and activity	ACE2 dysregulation
3	KE	1787	Downregulation, ACE2	Downregulation of ACE2
4	KE	1752	Increased Angiotensin II	Increased AngII
	KE	2096	Occurrence, (Micro)vascular dysfunction	(Micro)vascular dysfunction

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Binding to ACE2	adjacent	Dysregulation, ACE2 expression and activity		
Binding to ACE2	adjacent	Downregulation, ACE2		
Dysregulation, ACE2 expression and activity	adjacent	Occurrence, (Micro)vascular dysfunction		

Overall Assessment of the AOP

References

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 - Viral spike protein interaction with ACE2 leads 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 ID and Name	Event Type
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 hyperinflammation (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	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 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 (Gowrisankar 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; Gowrisankar and Clark, 2016; Xu et al., 2017; de Morais 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 Morais 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; Kowalczuk 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		SPR	325.8 nM
			1–1208 aa		14.7 nM
doi:10.1001/jama.2020.3786	19–615 aa	306–527 aa		SPR	408.7 nM
			319–541 aa		133.3 nM
Lan et al., 2020	19–615 aa	306–527 aa		SPR	31.6 nM
			319–541 aa		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-hIgG1-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.

References

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List of Key Events in the AOP

Event: 1854: Dysregulation, ACE2 expression and activity

Short Name: ACE2 dysregulation

Key Event Component

Process	Object	Action
mRNA transcription	angiotensin-converting enzyme 2	increased
carboxypeptidase activity	angiotensin-converting enzyme 2	increased
protein localization to cell surface	angiotensin-converting enzyme 2	decreased
	angiotensin-converting enzyme 2	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:385 - Viral spike protein interaction with ACE2 leads to microvascular dysfunction, via ACE2 dysregulation	KeyEvent
Aop:428 - Binding of S-protein to ACE2 in enterocytes induces ACE2 dysregulation leading to gut dysbiosis	KeyEvent
Aop:319 - Binding to ACE2 leading to lung fibrosis	KeyEvent

Stressors

Name
Sars-CoV-2
SARS-CoV
Influenza Virus

Biological Context

Level of Biological Organization
Molecular

Evidence for Perturbation by Stressor

Sars-CoV-2

Despite its significant role in the regulation/balancing of the RAS and KKS systems, the ACE2 enzyme has found its particular fame only since the outbreak of the SARS-COV2 pandemic in 2020 (ref…) because ACE2 turned out to act also as the receptor for the SARS-COV2, enabling virus entry into the cells, much like its cousin SARS-COV (Glowacka, 2010, ……..).

Interaction of the S proteins of SARS-COV1 and 2 with ACE2 on the cell membrane leads to:

- Down-regulation of its level and activity on the cellular membrane (ref)
- Up-regulation of the level of sACE2 and enzymatic activity in the supernatant of cultured cells (……..), enhanced enzymatic activity in vitro (Lu and Sun, 2020) and in vivo in COVID-19 patients ( )
- Up-regulation of ACE2 mRNA level in in BALF from infected patients (Garvin 2020, others?).

A number of reviews and hypothesis research papers have been able to link both up-regulation and down-regulation of ACE2 (at mRNA or protein/activity) to the observed perturbations in the RAS and KKS systems that ultimately drive organ damage in SARS and in COVID-19 (Garvin 2020, Nicplau, 2000, van de Veerdonk 2020, Jiqi Wang 2020) This is not surprising given the complexity and interconnectedness of these systems (Fig. 2).

It is possible that both mechanisms (up and down-regulation) may play a role in a tissue/organ specific and time dependent manner.

Gastrointestinal (GI) symptoms (anorexia, diarrhoea, vomiting, and abdominal pain) are commonly observed in patients with COVID-19 (Bourgonje et all, 2020 and references therein). Given the tissue distribution of ACE2 and its high expression in the GI tract luminal side, SARS-CoV-2 could directly invade the gastrointestinal epithelium via ACE2. Consistent with this, viral RNA in faeces could be detected in patients even after viral RNA in the respiratory tract became negative and infectious virus could be isolated from the stool [120, 121]. However, there are also conflicting studies that could not find evidence for the presence of infectious virus in RNA-positive stool samples [122].

Influenza Virus

The influenza virus is another biological stressor that appears to modulate ACE2 activity, but without binding to ACE2. Influenza infection also appears to up-regulate ACE2 mRNA in human nasal epithelia (Ziegler, 2020).

in epithelial kidney cells (Liu 2014, …..) and in mouse models of the disease where ACE2 modulation is associated with the severity of the virus induced disease (Yang 2014, Zou 2014,…….).

Domain of Applicability

ACE2 is expressed in a wide variety of tissues affecting their function.

ACE2 was initially identified in human lymphoma cDNA library (Tipnis, 2000), and from a human cDNA library of ventricular cells with heart failure (Donoghue 2000). Expression of ACE2 has later been also identified in the heart, kidney, and testis (Donoghue et al. (2000b). However, subsequent studies have shown a much broader distribution, including the upper airways, lungs, gut, and liver (reviewed recently by Saponaro, 2020).

Tissue and sub-cellular distribution of ACE2 (protein and mRNA)

Protein expression patterns

Immunostaining methods show that ACE2 is chiefly bound to cell membranes, predominantly in the smooth muscle cells and in the endothelium of the vasculature, while negligible levels can be detected in the circulation. In blood cells, it has been observed in platelets and macrophages, but not in B and T lymphocytes (Hamming et al., 2004; Fraga-Silva et al., 2011).

ACE2 shows differential sub-cellular distribution which can be significant for its basal/constitutive and modulated function. It is mainly detectable at the cell-surface with little intracellular localization, and the protein does not readily internalize (Warner et al., 2005). In polarized cells, ACE2 is exclusively targeted to the apical surface in vivo in kidney (Warner 2005) and in vitro in polarised cells derived from the colon, lung and kidney (Ren 2006). This is in contrast to its sequence homologue and functional "balancer" ACE, which distributes equally between apical and basolateral surfaces (Warner 2005).

In the small intestines, ACE2 is highly expressed on enterocytes and via its local RAS function participates in the regulation of the intestinal glucose transport. Intestinal ACE2 generates locally Ang 1-7 from luminal Ang II. Ang II was shown to inhibit SGLT1-dependent intestinal glucose uptake in a dose-dependent manner *in vitro* and *in vivo* in rats (Wong et al, 2007; 2009) and in human biopsies via AT1R activation (Casselbrant et al., 2015).

mRNA expression patterns

More recently, Qi. et al 2020 analysed 13 human tissues by scRA sequencing and report that ACE2 mRNA was mainly expressed in the ileum enterocytes, kidney proximal tubules and lung AT2 cells. ACE2 mRNA was also detected but to a lesser extent in the colon enterocytes, esophagus and keratinocytes and minimally in the cholangiocytes (biliary cells of the liver).

Key Event Description

The angiotensin-converting enzyme 2 (ACE2) is a membrane-anchored protein with wide tissue distribution https://www.proteinatlas.org/ENSG00000130234-ACE2. ACE2 has multiple functions, and is highly regulated at the transcriptional, post-transcriptional and post-translational levels. Modulation of the expression levels or functional activity of the ACE2 receptor is described in this KE.

ACE2 is bound to cell membranes, and in polarized cells it is exclusively targeted to the apical surface (Werner 2005; Ren 2006) [for more detailed description of the expression patterns of ACE2 see the applicability domain section].

ACE2 FUNCTIONS

Enzymatic function of ACE2 in interlinked bioactive peptide systems

ACE2 is mainly known and studied as a type I ectoenzyme i.e. a transmembrane protein with an extracellular amino-terminal domain harbouring a carboxypeptidase active site. ACE2 cleaves the carboxyterminal amino acid from a number of biologically active peptides (Figure 1 and 2), thus activating or deactivating them as agonists within the Renin Angiotensin System (RAS) (Santos et al., 2019) and within the Kinin–kallikrein system (KKS) (Kakoki & Oliver, 2009) in mammals.

The extracellular domain of ACE2 can itself be cleaved (shedding) in vitro and in vivo, releasing a soluble and catalytically active sACE2 (Guy et al., 2008 in human cardiac myofibroblasts; Peng Jia et al., 2009 in human lung epithelial cells and BALF; Werner et al., 2005 in canine epithelial polarised kidney MDCKII cells stabilly expressing ACE2). Constitutive shedding of ACE2 from the cellular membrane is mediated by another membrane-bound metalloprotease from the adamalysin family, ADAM17 (Iwata et al., 2009) also known as TACE, TNFalpha Converting Enzyme (Zunke 2017). Other proteins may also be involved in ACE2 protolithic modulation (Lambert et al, 2008).

The exact role of ACE2 shedding in modulating its function is not well understood, however, being catalytically active, the released sACE2 can, as the membrane-anchored full length ACE2, generate biologically active peptides which activate specific receptors in different cells/tissues/organs. Thus, the ACE2 function in the organism is mediated via its peptide products activating specific receptors on the same, (autocrine), nearby (paracrine) and potentially distant cells.

ACE2 is a homologue of angiotensin-converting enzyme (ACE), with whom it shares significant sequence similarity (Tipnis, 2000; Donoghue 2000), yet exhibits very distinct enzymatic activity. ACE2, as ACE, is a zink-metallopeptidase, however ACE2 is strictly a carboxypeptidase while ACE is an dipeptidase. Furthermore, the main substrate of ACE2 is the octopeptide Angiotensin II (Ang II), the enzymatic product of its homologue ACE. By cleaving a single amino acid from the C-terminus of Ang II, ACE2 generates a functionally different bioactive peptide Angiotensin 1-7 (Ang1-7) (Figure 1). ACE2 is key to the regulation of local and systemic Ang II levels.

Ultimately, the function of ACE2 at tissue level is mediated via the interaction of its main active product Ang1-7 with the Angiotensin II receptor 2 (AT2R), balanced by the activity of its homologue ACE and other peptidases in the RAS.

Figure1: Enzymatic activity of ACE2 compared to its homologue ACE and another protease relevant to the RAS (from Rice et al., 2004)

[to add]

Figure 2: Simplified representation of the biological function of the enzymatic products of ACE2 activity in the KKS (adopted from Kakoki & Oliver, 2009).

[to add]

Although most studies have focused on the role of ACE2 in angiotensin metabolism in the RAS, the enzyme has broad substrate specificity and it also hydrolyses a number of other biologically active peptides including des-Arg9-bradykinin (DABK), apelin-13, neurotensin(1–11), dynorphin A-1–13), β-casomorphin-(1–7), and ghrelin (Vickers 2002, Humming, 2007.

ACE2 cleavage of DABK to bradykinin 1-7 (Figure 2) has been demonstrated in chemico (Donoghue 2000, Vickers 2002), with human polarised primary lung cells in vitro and in mice broncho-alveolar lavage (BALF) (Sodhi 2018). Deactivation of DBAK, a preferencial bradikynin receptor 1 (B1K) agonist (Coulson et al., 2019), is an important regulatory function of ACE2 in the KKS (Figure 2).

1.2 ACE2 chaperone function for transporters of amino acid transfer (B0AT1)

Somewhat less known is the RAS independent function of ACE2 in the gut, where it regulates intestinal amino acid homeostasis, expression of antimicrobial peptides, and the gut microbiome (Camargo et al., 2020). ACE2 was identified as an important regulator of dietary amino acid homeostasis, innate immunity, gut microbial ecology, and transmissible susceptibility to colitis in mice (Hashimoto et al., 2012). The mechanism by which ACE2 regulates amino acid transport in the intestine involves interaction with the broad neutral (0) amino acid transporter 1 (B0AT1) (Slc6a19) which mediates the uptake of neutral dietary amino acids, such as tryptophan and glutamine, into intestinal cells in a sodium-dependent manner (Camargo et al, 2009). A crystal structure study revealed a complex dimer of ACE2/ B0AT1 heterodimers (Yan et al., 2020), previously suggested by immunoprecipitation of intestinal membrane proteins in mice (Fairweather et al., 2012). Immunofluorescence showed co-localization of B0AT1 with ACE2 at the luminal surface of human small intestine (Vuille-dit-Bille et al., 2015). ACE2 seems to be necessary not only for the amino acid transfer by B0AT1, but also for its membrane expression (Camargo et al., 2008; Hashimoto et al., 2012).

REGULATION OF ACE2 LEVELS and ACTIVITY

ACE2 is regulated at the transcriptional, post-transcriptional and post-translational level, the final potentially differing in the different organisational contexts: cell membrane versus tissue (plasma and/or interstitial). In addition, all of these regulatory processes may be differentially modulated in different tissues.

Age, sex and species specific differences in aspects of the regulation, and also tissue specific regulation, have been reported (reviewed in Saponaro 2020).

Loss of function of ACE2 in vivo in ACE2 knockout (KO) mice has been associated with elevated levels of Ang II in heart, kidney and plasma as well as histological and functional perturbations in the lungs and in the cardiovascular (Crackower et al, Nature 2002) and renal (Oudit et al, 2010) systems, mostly in the presence of a particular stress factor, in some cases potentiated by aging (reviewed in Humming 2007).

Furthermore, Ace2 KO mice exhibited reduced serum levels of tryptophan, together with downregulated expression of small intestinal antimicrobial peptides and altered gut microbiota, which was re-established by tryptophan supplementation (Hashimoto et a., 2012).

At transcriptional level

Overall, the transcriptional regulatory elements of the ace2 gene are not well characterised.

Ace2, human but not mouse, was identified as an Interferon Stimulated Gene (ISG) in airway epithelial cells (Ziegler, 2020), indicating species specific regulation and its importance for human viral infections mediated via ACE2 (e.g. SARS-COV2). Influenza virus infection also induced ACE2 mRNA synthesis in human lung tissue (Ziegler, 2020).

In vitro in normal kidney tubular epithelial cell line (HK-2) ACE2 mRNA is down-regulated following Ang II treatment (Koka 2008). The exact transcriptional regulatory mechanism is not clear, but the observed ACE2 mRNA up-regulation in this system appears to be mediated by the activation of the ERK1/2 and p38 MAP kinase pathway and dependent on the activation of AT1R receptor by AngII, as demonstrated by specific AT1R, MAP kinase and ERK1/2 MAP kinase inhibitors (Koka et al., 2008). Regulation of ACE2 expression mediated by AT1R activation is an important endogenous regulatory mechanism for ACE2 activity within the RAS system (ref…..).

Vitamin D Receptor (VDR) may also emerge as an ACE2 transcriptional regulator/repressor (Saponaro 2020 and Glinsky 2020, unreviewed pre-print). VDR has already been implicated in the transcriptional repression of Renin, at least in vitro (Yuan 2007).

17b-estradiol (E2) has also been indicated in the transcriptional regulation of ace2 in a tissue specific manner (recently reviewed by Saponaro 2020). E2 down-regulated ace2 transcription particularly in kidney and differentiated airway epithelial cells. However, in human atrial tissue, E2 appeared to up-regulate ACE2 mRNA and protein. This change was associated with decreased levels of ACE homologue protein. The exact mechanism for this regulation remains to be elucidated as it may represent a significant modulating factor in the differential sex susceptibility to ACE2 dysregulation under varied stress conditions (e.g SARS-COV infection).

Epigenetic transcriptional regulation of ace2 has also been indicated (recently reviewed by Saponaro 2020. Transcription of ace2 is repressed by histone methylation and stimulated by NAD+-dependent deacetylase SIRT1 during cellular energy stress. Interestingly, in children ACE2 is normally hypermethylated and poorly expressed in the lung and in other organs (Saponaro 2020, ref therein).

Gut microbiota have also been implicated in the transcriptional regulation of ACE2 expression in the gut (Yang et al., 2020) but also in the lung (Koester et al., 2021). Whether this is directly or indirectly occurring via microbial metabolites remains to be elucidated, but the study by Koester ar e al, 2020 observed variability in intestinal Ace2 expression in gnotobiotic mice colonized with different microbiota, partially attributable to differences in microbiome-encoded proteases and peptidases.

At post-transcriptional level

Generally, modulation of ACE2 mRNA and protein levels appear to follow consistent pattern. However, it has been demonstrated that under certain conditions and in some tissues, mRNA and protein levels appear to follow a different pattern, suggesting important role of post-transcriptional or post-translational (see next section) regulation of ACE2 expression and function.

For example, hypertension in humans has been associated with different modulation of mRNA and protein levels in the heart tissue (Koka 2008). Specifically, heart tissue from patients with hypertension showed decreased levels of ACE2 mRNA while protein levels were comparable to normal tissue. In contrast, ACE mRNA and protein levels appeared consistently up-regulated in hart tissue of hypertensive patients (Koka 2008). In the same study, in the kidney tissue from patients with hypertensive nephropathy, both, ACE2 mRNA and protein levels, appeared consistently down-regulated compared to normal kidney tissue (Koka 2008). Significant suppression of ACE2 mRNA and protein expression was also observed in vitro in normal kidney tubular epithelial cell line (HK-2) treated with AngII (linked to hypertension in vivo) in a dose and time dependent manner Koka 2008). AngII treatment in vitro with myocardia- derived cells was not examined in this study and the discrepancy of mRNA and protein level modulation in the hypertensive human heart tissue biopsies was attributed by the authors to limitations of the detecting methods (Koka 2008).

Clear discrepancy in the modulation of mRNA versus protein level has been observed in vivo in mice in myocardial tissue (Patel 2014). Namely, up-regulation of mRNA synthesis was associated with down-regulation of ACE2 protein levels following 1 or 2 week treatment by exogenous circulating AngII (Patel 2014). In this study, down-regulation of ACE2 protein levels was alleviated by AT1R blockage/inhibitors, while mRNA up-regulation was not dependent on AT1R signalling. This strongly suggests involvement of post-transcriptional mechanism step(s) mediated by AngII/AT1R fot the regulation of ACE2 protein/function, at least in myocardial tissue under certain stress conditions.

Modulation of ACE2 protein and activity levels by AngII is clinically relevant phenomenon and AngII activity blockers (ACE inhibitors and AT1R blockers) are used to move the balance of the RAS from the ACE/AngII/AT1R axis towards the protective ACE2/Ang1-7/MasR axis. This is particularly relevant in the lung where ACE/ACE2 activity ratio is high (Roca-Ho, 2017-mice, human and other ref……?..).

The up-regulation of ACE2 mRNA observed in mouse myocardial tissue by Patel et al., 2014 appears contradictory to the finding of ACE2 mRNA down-regulation observed in the heart of hypertensive patients observed in the study of Koka et al., 2008 (if the latter result is accepted despite potential method limitations). However, it should be noted that the base level of ACE2 and also the relative ACE2/ACE ratio in the case of chronic hypertensive patients, many of whom have been on AT1R inhibitor treatment (Koka 2008), and in healthy mice treated with AngII for relatively short time (Patel 2014), may be different leading to response to the stressor (hypertension and AngII) over time. Consistent with this, distinct ACE/ACE2 activity ratios have been demonstrated in different organs of normal, non-obese diabetic (NOD) and insulin treated NOD mice, which varied additionally over the time course after the onset of diabetes (Roca-Ho et al., 2014).

Finally, species specific regulatory differences may be involved that would warrant further examination. But, overall, the studies discussed above illustrate the complex regulatory mechanisms of ACE2 mRNA, protein and activity levels in different tissues and under different stress conditions for the RAS system.

Rapid and transient up-regulation of ACE2 mRNA followed by down-regulation of ACE2 protein levels has been reported in the lung as a result of LPS induced acute inflammation in mice (Sodhi 2018). In this case the increase of ACE2 mRNA appears to be a rapid and transient compensatory effect to ACE2 protein/activity down-regulation mediated by NFk- B signalling in response to acute inflammation. Inhibiting NF- B signaling by Bay11-7082 restored ACE2 activity, again demonstrating post transcriptional or translational regulation of ACE2 in the lungs. In addition, this study examined the effect of ACE2 dysregulation on the KKS and demonstrated that attenuation of ACE2 activity under conditions of LPS induced inflammation leads to impaired DABK inactivation and enhanced BKB1R signalling (Sodhi 2018).

The underlying mechanisms of post-transcriptional mRNA regulation remain to be elucidated further. There is evidence that small non-coding micro RNAs (miRNA or miRs) may be involved (Widiasta 2020; Lu 2020; Fang 2017, Lambert 2014).

At post-translational level – enzymatic activity including shedding

Complexity of analysing the regulation of ACE2 function is emphasized even further when enzymatic activity is considered, including its spatial distribution between cell/tissue versus interstitium/plasma, mediated by shedding.

The exact role of ACE2 shedding is not well understood, but proteolytic ectodomain shedding of membrane proteins is a fundamental post-translational regulatory mechanism of the activity/function of a wide variety of proteins, including growth factors, cytokines, receptors and cell adhesion proteins (Lichtenthaler et al., 2018).

sACE2 activity is increased in patients with heart failure (HF) and correlates with disease severity (Epelman 2008).

In mice in vivo, shedding of ACE2 by TACE was induced by sub-chronic (2 weeks) exogenous AngII treatment (mimicking HF), leading to decreased ACE2 protein level and increased ACE2 mRNA in myocardial tissue with concurrent elevated sACE2 activity (Patel et al., 2014).

How it is Measured or Detected

ACE2 activity

- using fluorescently labeled peptide substrates (Rice, 2004; Sodhi, 2008; Roca-Ho, 2017 1; Lu and Sun, 2020; Xiao 2017)
- measuring catalytic products (direct) or markers of activation of receptors for the products (indirect) of ACE2 activity (e.g Ferrario 2005)

ACE2 levels

- mRNA by RT-PCR (Sodhi 2018; Roca-Ho, 2017) or scRNA seq (e.g. Qi. et al 2020)
- protein in tissue extracts/preparations by immunoprecipitation or Western blotting (Koka 2008)
- protein in live tissues or cultured cells by immunostaining (Humming 2007; Fraga-Silva et al., 2011; Ren 2006; Warner 2005)

high throughput and quantitative measurement of protein by quantitative proteomic analysis (Park 2020; Stegbauer 2020)

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Event: 1787: Downregulation, ACE2

Short Name: Downregulation of ACE2

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:381 - Binding of viral S-glycoprotein to ACE2 receptor leading to dysgeusia	KeyEvent
Aop:385 - Viral spike protein interaction with ACE2 leads to microvascular dysfunction, via ACE2 dysregulation	KeyEvent
Aop:427 - ACE2 downregulation following SARS-CoV-2 infection triggers dysregulation of RAAS and can lead to heart failure.	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Event: 1752: Increased Angiotensin II

Short Name: Increased AngII

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:319 - Binding to ACE2 leading to lung fibrosis	KeyEvent
Aop:384 - Hyperactivation of ACE/Ang-II/AT1R axis leading to chronic kidney disease	KeyEvent
Aop:385 - Viral spike protein interaction with ACE2 leads to microvascular dysfunction, via ACE2 dysregulation	KeyEvent
Aop:381 - Binding of viral S-glycoprotein to ACE2 receptor leading to dysgeusia	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Event: 2096: Occurrence, (Micro)vascular dysfunction

Short Name: (Micro)vascular dysfunction

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:385 - Viral spike protein interaction with ACE2 leads to microvascular dysfunction, via ACE2 dysregulation	KeyEvent

Biological Context

Level of Biological Organization

Tissue

Key Event Description

The description of this KE aims to facilitate incorporation of evidence for distinct but relevant molecular level events influencing individual molecules and cells, but also important interactions driving higher (tissue/organ) organisational level events.

Microvasculature (MV) is the tissue system/organ of vessels (capillaries, arterioles and venules), which **enable delivery and exchange of gases (O2, CO2), nutrients, metabolites and circulating immune cells**, within all organs of the body. Thus proper function of the MV is essential for adequate response to changes in metabolic demand and blood flow to the organs.

Functional response of the microvasculature to normal physiologically changing or stressed tissue/organ environment is **mediated by its specific cellular and molecular structure** consisting of a variety of cell types intimately linked to the tissue environment.

Exsosome-mediated communication between different cell types within the vasculature is increasingly recognised as key aspect of (mycro)vascular function, while interference/perturbations of this intercellular communication emerges as an important factor driving dysfunction and potential target for therapy [10.1186/s12964-022-

00949-6; 10.1007/s12012-021-09700-y; 10.3389/fcvm.2022.912358; 10.3389/fcell.2019.00353; 10.1016/j.bbadis.2020.165833].

Depending on the tissue/organ type there may be subtle molecular differences but the general cellular outline of the MV can be illustrated as in Figure 1 include:

Endothelium: inner lining monolayer of closely juxtaposed squamous endothelial cells (ECs). The quiescent or non-proliferating endothelium has an active role in maintaining vascular homeostasis by receiving and generating diverse biochemical (autocrine, paracrine, and endocrine) and mechanical signals (Ricard et al., 2021-10.1038/s41569-021-00517-4).

One of the most commonly used surface markers for identification/sorting/enriching viable quiescent ECs is the CD31 in combination with the absence and/or presence of surface markers specific for other cells or for specific activation, dysfunction or differentiation state of the ECs (Goncharov 2017 - 10.1155/2017/9759735 Rakocevic 2017 - . 10.1016/j.yexmp.2017.02.005). A convenient list of specific endothelial cell markers and reagents for their identification can be found here together with some basic background information on each marker.

Dysregulated communication between ECs and other vascular cell types is associated with vascular **dysfunction and pathological vascular remodelling** in various pathological conditions (Rajendran et al., 2013 - 10.7150/ijbs.7502; Méndez-Barbero et al., 2021- 10.3390/ijms22147284). Rajendran et al 2013 - 10.7150/ijbs.7502, provides a good comparison of healthy and dysfunctional vasculature based on the biochemical products of (mainly) ECs including: nitric oxide (NO), prostacyclin (PGI2), reactive oxygen species (ROS), uric acid, plasminogen activator inhibitor 1 (PAI-1), von Willebrand factor (vWF), P-selectin. soluble vascular cell adhesion molecule (sVCAM.), soluble intercellular adhesion molecule (sICAM), E-selectin, C-reactive protein (CRP), tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6). Under some stress conditions (e.g. infections, cell aging and damage) vascular **dysfunction** can be triggered by specific interaction of the vascular components and the innate immune surveillance **complement** system (10.1038/nrneph.2016.70; 10.1111/cei.12952; Immunobiology: The Immune System in Health and Disease. 5th edition; 10.2340/00015555761316). Dysfunction triggered by complement activation is particularly relevant for infectious stressors initiating adoptive immunity as part of its normal host response (e.g. viruses) [10.1016/j.virol.2010.12.045; 10.3389/fimmu.2020.01450].

Given the dynamic responses of ECs to environmental signals, including these from the intimately connected perivascular cells, test systems based on ECs require careful phenotypic characterisation.

ECs structure and function exhibit significant **tissue specificity**. Singlecell RNAseq atlas of mouse endothelial cells isolated from different tissues by flow cytometry without the cell culture step, identified transcriptomic signatures of quiescent ECs and found that: arterial and venous endothelial cells from a specific tissue clustered together, showing that vascular endothelial cell heterogeneity comes mainly from tissue specificity rather than arterial, capillary or venous identity (Kalucka 2020 - 10.1016/j.cell.2020.01.015). Moreover, capillary endothelial cells that are involved in gas, ion, metabolite and hormone exchange between the blood and tissues have the highest heterogeneity among tissues (Kalucka 2020 - 10.1016/j.cell.2020.01.015). Similar results (i.e. tissue specificity) have been reported after re-analysis of independently generated single cells sequencing data Paik 2020 - 10.1161/CIRCULATIONAHA.119.041433)

Comprehensive vasculature atlas from human tissues is not available but re-analysis of scRNAseq data from control human lung cohorts has been performed (Schupp 2021 - 10.1161/CIRCULATIONAHA.120.052318). The analysis identified that broad EC categories and conserved marker genes similar to those identified the mice data (Schupp 2021 - 10.1161/CIRCULATIONAHA.120.052318; Kalucka 2020 - 10.1016/j.cell.2020.01.015).

Intercellular communication of the endothelial with the other cells of the MV appears to be an important determinant of MV (dys)function (10.1016/j.bbadis.2020.165833; 10.1007/s12012-021-09700-y; 10.1038/ncomms9024; 10.1038/s41598-018-34357-z; 10.1021/acs.molpharmaceut.8b00765). EC derived exosomes contain some unique endothelial markers, including VE-cadherin, E-selectin, P-selectin, PECAM, ICAM-1, MCAM, endoglin, and ACE. In addition, they also contain various miRNAs [10.1016/j.bbadis.2020.165833; 10.3389/fmolb.2020.619697].. All of these molecules have biological functions in both normal endothelial physiology and pathogenesis.

Glicocalix, on the lumen side the MV, generated by the endothelium is composed of a negatively charged network of GAGs and proteoglycans. It modulates interactions between the vasuclature wall and blood cell. Glicocalix represents a binding site for crucial anticoagulant mediators such as heparin cofactor II, antithrombin III, thrombomodulin and tissue factor pathway inhibitor (TFPI). [Yilmaz 2019 - 10.1093/ckj/sfz042],

Basement membrane represents the layer of complex extracellular matrix (ECM) proteins (20–200 nm) on the tissue side the endothelium. It provides a mechanical support and divide tissues into compartments, but also influence cellular behaviour Vascular basement membrane is a three-dimensional network of proteins from four major glycoprotein families: laminins, collagen IV isoforms, nidogens, and heparan sulfate proteoglycans (HSPG). Additionally, many other proteins are differentially expressed in the vascular basement membrane **depending on the developmental and physiological state** of the vasculature. These include insoluble fibronectin, fibulin 1 and 2, collagen type XVIII, thrombospondins 1, and SPARC (secreted protein acidic and rich in cysteine) (Thomsen 2017 - 10.1177/0271678X17722436, and references therein). The ECM is generated by ECs and pericytes (Thomsen 2017 - 10.1177/0271678X17722436, and references therein).

Pericytes are perivascular, mural cells that have intimate contact with the endothelial cells and together support important functions such as maintaining the physical and functional integrity of the Blood Brain Barer (BBB), regulating capillary diameter, cerebral blood flow and maintaining extracellular matrix protein levels. Their identity, ontogeny, and progeny is not characterised as well as that of endothelial cells. They express multiple markers and their origin differs by tissue, which makes their identification and understanding of their function difficult (Armulik 2011 - 10.1016/j.devcel.2011.07.001).

A recent scRNAsec analysis of microfluidic droplets of mouse tissues confirm that two previously known perycite and conserved markers (Cspg4 or Pdgfrb) are co-expressed in the mural cell clusterdefined as perycites from lung, heart, kidney, and bladder (Beek 2022 -10.3389/fcvm.2022.876591). Other potential tissue specific markers were also identified in this study.

Pericytes also have the potential to give rise to different tissues in vitro but this is not clear in vivo. (Yamazaki 2018 - 10.3389/fcvm.2018.00078).

Vascular smooth muscle cells [VSMC*l* surround the endothelium, pericytes and basal membrane in larger vessels. They contain **contractile** filaments and maintain vascular tone in response to (endocrine?, paracrine? autocrine?) action of **vasoactive** mediators and neurotransmitters (e.g. Angiotenisn II, Angiotensin 1-7, Endothelins, NO, epinephrine and norepinephrine) via their receptors or effectors e.g AGTR1, MasR, endothein receptor A and B or guanylate cyclase, and adrenoceptors, respectively.

Under different stress condition (persistant stretch, injury, inflammatory cytokines and excess oxidized lipids) and also during normal development, VSMC can undergo **phenotypic switching** or remodelling from a contractile to synthetic or proliferative phenotype wich involves a partial down regulation of the proteins that activate the contractile apparatus in favour of the synthetic and proliferative cellular machinery [10.5772/intechopen.77115; 10.1152/physrev.00041.2003].

Proliferative smooth muscle cells have an attenuated response to vasoconstrictors and vasodilators, probably due to the down regulation of the contractile apparatus and certain elements of the subcellular signalling machinery that is involved in vasoconstriction. Notably, many of the vasoactive modulators (e.g. angiotensin II endothelin and noradrenaline) also function to promote smooth muscle proliferation. Chronically elevated levels platelet derived growth factor (PDGF), for example, generated from unstable thrombus, can also contribute to proliferative vascular disorders. On the other hand, nitric oxide, limits smooth muscle hyperplasia and hypertrophy. ACE2, Angotenisn 1-7 and Mas receptor also appear to play important role in the modulation of the prliferative phenotypic switching of VSMC [10.1155/2012/121740; 10.1161/HYPERTENSIONAHA.114.03388; 10.1042/BSR20192012; 10.26355/eurrev_202004_20867].

Similar to the endothelial cells, VSMCs produce exosomes containing components of the Extracellular Matrix (ECM) such as collagens, proteoglycans, hyaluronan and laminin as well as matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteases (TIMPs) which are particularly important for repair and remodelling of growing or damaged vessels. miRNAs released from VSMC also , are increasingly recognised as intercellular signalling molecules important for MV (dys)function [10.1186/s12964-022-00949-6; 10.1016/j.ymthe.2017.03.031]

Progenitor cells - [*to complete*]

Fig. 1: Cellular structure of small vasculature from Jin et al. 2020

MV is a dynamic organ and proper cellular differentiation, renewal and intercellular interactions mediated by various signalling molecules govern its functional and structural integrity. These interactions also govern the return to **homeostasis** under some stress conditions, restoring basal structure/function of the vasculature and ultimately well oxygenated tissue/organ.

In addition to the markers for specific MV cell lineage, **other more ubiquitously expressed proteins** (such as angiotensin converting enzyme 2 – ACE2) may, under some circumstances, represent markers for differentiation state or (dys)function of the MV at cellular or tissue level. For example, ACE2 expression in HBMEC & HUVEC perfusion culture is stimulated by flow (HBMEC < HUVEC) (qRT-PCR); also it is increased by flow intensity and vessel shape in the MCA 3D model of stenosis (immunostaining cells) [10.1161/STROKEAHA.120.032764]. Given that ACE2 is implicated as the main receptor for viral entry of SARS-CoV2 associated with COVID19

disease, the level and the dynamics of ACE2 expression is likely to be important for driving COVID19-associated vascular dysfunctions. Notably, in mature human tissues, ACE2 is not expressed at significant levels in the vascular compared to other cells evaluated (Human Protein Atlas version 22.0). Within the human vascular tissue it appears that expression in smooth muscle cells is significantly higher than that in endothelial cells and comparable to that in fibroblasts (Human Protein Atlas version 22 – single cell type-vascular). Pericytes are not specifically identified in this project.

Excessive **disruption of the structural integrity of the** MV or **interference with** the normal balanced **function of molecular mediators** leads to inability of the MV to maintain homeostasis i.e. (micro)vascular **dysfunction.**

How it is Measured or Detected

MV dysfunction can be evaluated and/or quantified by:

• **Macroscopic and microscopic** observations of the **structural integrity** of the structure of the MV (basic histochemical staining or immunostaining for specific cellular markers) [e.g. PMID 17974127; 10.1101/2020.08.19.251249; 10.1016/S2352-3026(20)30216-7, 10.1007/s00428-020-02886-6; 10.1038/s41379-021-00793-y; 10.1161/CIRCRESAHA.120.317447; 10.3390/diagnostics10080575; 10.1002/path.5549]

• Evaluation of the **functional integrity** of the barrier: Electric Cell-Substrate Impedance Sensing (ECIS), trans-endothelial electrical resistance (TEER), FITC-dextran permeability assays [e.g. [10.1016/j.nbd.2020.105131; 10.1152/ajplung.00223.2021; 10.3389/fcvm.2021.687783]

• Differential expression of **surface adhesion molecules** (eg. ICAM-1, VCAM-1) by FACS-SCAN assay [e.g. 10.1186/s13054-021-03631-4] or Western blotting [e.g. 110.3389/fcvm.2021.687783]

• Differential expression of **pro-inflammatory and or anti-inflammatory cytokines** by various RNA measurement assays, various immune based assays using specific antibodies) [10.1016/j.nbd.2020.105131; 10.1161/STROKEAHA.120.032764; 10.1172/JCI148635], including proteomic approaches [10.1172/JCI148635]

• Differential expression of **matrix metalloproteinases** (e.g. MMP2, MMP3, MMP9, MMP12) by various RNA measurement assays or various immune based assays using specific antibodies or fluorescent tags) [e.g [10.1016/j.nbd.2020.105131]

• Differential expression of **coagulation and/or fibrinolytic factors** (e.g. plasminogen activator inhibitor 1(PAI-1), plasminogen activator (tPA), urokinase (uPA)) by various RNA measurement assays, various immune based assays using specific antibodies, or various assays for their specific enzymatic activity. [e.g. [10.1165/rcmb.2020-0544OC] including proteomic approaches [10.1172/JCI148635]

• Detection of tissue/cell stress markers: (e.g. reactive oxygen species (ROS); prostaglandins (PG); vasoactive peptides, such as angiotensin II (Ang II), angiotensin (1-7) (Ang 1-7) or activity of their receptors

• Detection of contractile factors, including endothelin (ET), thromboxane A2 (TXA2)

• Analysis and quantification of exosomal markers

Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2311: Binding to ACE2 leads to ACE2 dysregulation

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Viral spike protein interaction with ACE2 leads to microvascular dysfunction, via ACE2 dysregulation	adjacent		
Binding of S-protein to ACE2 in enterocytes induces ACE2 dysregulation leading to gut dysbiosis	adjacent	Moderate	

Key Event Relationship Description

This KER summarises the evidence for dysregulation of ACE2 (KE 1854) as a result of its interaction with the viral spike (S) protein of SARS-CoV and SARS-CoV2 (KE1739). This interaction is likely an important aspect of COVID-19 pathogenesis. As the initial step of SARS-CoV2 infection, it is a potential target for intervention at the point of viral entry on cellular level, but also latter for treatment of the disease during viral replication at organism level. In addition, it is important to summarise exiting evidence for this KER and evaluate the WoE for the prevailing hypotheses that COVID-19 pathogenesis (and hence relevant treatments) is largely governed by the binding of the S protein to ACE2 leading to down-regulation of its physiological function as a protease that converts Angotensin II to Angiotensin 1-7 and hence disturbs the balance within the RAS system. Evidence summary and evaluation should also help identify key data/evidence gaps and inform critical tests and approaches to fill them.

Evidence Supporting this KER

Aspect of ACE2 dysregulation after S-binding	WoE	Rationale	Empirical evidence Ref.
Molecular level view **ACE2 enzymatic function is dysregulated**	**Moderate** ↑	**Plausibility - Moderate**: Enzymatic function of ACE2 as a protease can be affected by allosteric interaction with S-protein by analogy to many other enzymes (Liu & Nussinov 2016; Dutta 2022; Wang DS 2022; Trozzi 2022). However, empirical evidence for ACE2 and other stressors/allosteric modulators is limited, also because not many small molecule inhibitors or activators have been identified. (Important knowledge gap and high priority future research and development] **Empirical evidence - Moderate**: There is empirical	

		evidence that the S proteins (FL or RBD) from both SARS-CoV and SARS-Cov2 S virus which are known to bind to ACE2 with different affinity affect ACE2 enzymatic function. Most evidence shows increase in enzymatic activity (Kcat and/or K_M) of ACE2 for different substrates in chemico and in vivo (plasma from infected patients or even recovered COBID19 patients). Relationship to higher effect depending on the S-binding activity is demonstrated (Kiseleva (2021)). Dose dependence is demonstrated (Kiseleva (2021); Jinghua). Not all substrates are affected equally. Most affected is DBAK and not AngII. (Future research need on the structural aspects of ACE2 activity for different substrates, role of ACE2 in KKS vs RAS system, identification and characterisation of ACE2 modulating agents). One study (Daniell et., al (2022) is inconsistent with the majority (finds decreased ACE2 activity with commonly used pseudo substrate). Further analysis of the assays used (conditions, calculations of activity, substrate concentration/availability) is need to assess the reasons of the inconsistency. Interestingly this study finds increase of the level ACE2 protein itself in the plasma testing system.	Kiseleva (2021) [10.1093/jb/mvab041] Jinghua Lu (2020) [10.1074/jbc.RA120.015303] §Reindl-Schwaighofer (2021) [10.1164/rccm.202101-0142LE] Patel (2021) [10.1183/13993003.03730-2020] Daniell (2022) [10.1016/j.omtm.2022.07.003]
Cellular/Tissue-Plasma level view **ACE2 protein level is dysregulated**	**High** Dysregulation of ACE2 level for the cellular and tissue compartments is dysregulated in a complementary manner **↓ cell membrane** **↑ extracellular/interstitium/plasma** Together with the dysregulated enzymatic activity (see section above): important implications for effects on higher organisational levels remain to be explored	**Plausibility - High**: Cell membrane bound ACE2 is known to be cleaved/shed from the cell membrane by cellular proteases ADAM17/TACE (Liu & Nussinov 2016; Dutta 2022; Wang DS 2022; Trozzi 2022). This shedding effectively downregulates the protein level of enzymatically active ACE2 on the cell surface while releasing the catalytically active ectodomain in the tissue interstitium and plasma. [knowledge gap: significance of ACE2 shedding and cell/tissue distribution of the catalytic activity for function. (ii) role of endocrine, paracrine, hormonal activity of ACE2 catalytic substrates and products] **Empirical evidence - High**: There is extensive and consistent empirical evidence that the S proteins (FL or RBD) from both SARS-CoV and SARS-Cov2 S virus, stimulate ACE2 shedding by its known proteases ADAM17/TACE, but also another protease that also cleaves the S protein itself (TMPRASS). There is also evidence that ACE2 is internalised into the cell following interaction with recombinant S proteins (FL or RBD) or non-replicating spike protein containing pseudo-SARS-CoV. It is assumed that the ectodomain and its enzymatic activity is also internalised. However there is also evidence which shows that the S-protein is internalised without the ACE2 codomain (Heurich, 2014). The differences in the studies can be explained by closer examination of the experimental conditions, labels and antibodies used (as ACE2	Haga 2008 10.1073/pnas.0711241105 Haga 2010 10.1016/j.antiviral.2009.12.001 **Glowacka 2010 10.1128/JVI.01248-09** **Patra 2020 10.1371/journal.ppat.1009128** **Lei (2021) [10.1161/CIRCRESAHA.121.318902]** **Gao X (2022) [10.1016/j.jinf.2022.06.030]** **Taglauer (2022) [10.1016/j.ajpath.2021.12.011]** **Ackermann (2020) [10.1056/NEJMoa2015432]** **Yijia Li (2021) [10.1172/JCI148635]** **Reindl-Schwaighofer (2021) [10.1164/rccm.202101-0142LE]** **Lundström (2021) [10.1002/jmv.27144]** **Wank K. (2022) [10.1161/HYPERTENSIONAHA.121.18295]** **El-Shennawy (2022) [10.1038/s41467-021-27893-2]** **Mariappan (2022) [10.1016/j.biochi.2022.06.005]** **Taglauer (2022) [10.1016/j.ajpath.2021.12.011]** **Daniell (2022) [10.1016/j.omtm.2022.07.003]** **Patel 2021 10.1183/13993003.03730-2020**

		ectodomain is cleaved after interaction with S protein it is important which antibody is used to detect it and where is the label placed when recombinant GFP chimeras are used). Overall, the evidence demonstrates that S protein (and virus by analogy) can enter cells by endocytosis, but it remains to be elucidated how prevalent and pathophysiologically significant this entry mechanism is compared to the membrane fusion mechanism. From the point of view of ACE2 regulation, the latter mechanism together with the consistently observed ACE2 shedding, would imply externalisation and not internalisation of the ACE2 activity following interaction with the virus.	**Raghavan (2021) - 10.3389/fcvm.2021.687783** **Gorshkov 2022 - 10.1021/acsnano.0c05975** **Inoue, 2007 - 10.1128/JVI.00253-07** **Wang H., 2008 -10.1038/cr.2008.15** **Wang S., 2008 - 10.1016/j.virusres.2008.03.004** **Heurich, 2014 -10.1128/JVI.02202-13**
Cellular level view **Ace2 mRNA level is dysregulated**	**Moderate** ↓ ? ↑	**Plausibility - Moderate**: ACE2 is an ISG and is differentially expressed in may systems relevant to viral replication and innate immune response. However there is no evidence (to my knowledge) that ACE2 itself, after binding to its substrates can initiate intracellular signalling that would lead to differential mRNA expression. It is plausible however, that ACE2 dysregulation can modulate mRNA expression from number of genes (including the ace2 gene itself) indirectly via the resulting dysregulation of levels of its substrates, products and their effect on their receptors. Hence the “moderate” call. It is not highly plausible, although not impossible that direct S-binding (independent of replication/ persistency of the stress) can lead to Ace2 mRNA dysregulation. **Empirical evidence – Moderate** (related to direct S-binding)**:** There are number of studies reporting that exposure to the Spike protein, non-replicating pseudoviruses or replicating viruses can be associated with differential expression of Ace2 mRNA in different tissues or interstitial fluids (e,g BALF, saliva, nasopharyngeal swabs). However, the evidence appears conflicting in terms of the direction of the dyregulation. Both up and down regulation are reported and no obvious pattern emerges yet as to the critical factor for the two types of regulation (tissue or fluid type; in vitro/in vivo; replicating/non-replicating stressor; time of measurement after stress; presence of dead vs infected v.s bystander cells etc.). Further analysis of the details of the test systems is needed to elucidate the underlying aspects of the apparent inconsistences and conflicting evidence in test systems other than the GI tract (see Uncertainties and Inconsistences section for the relevant analysis in the GI tract).	Li (2020) [10.1016/j.bbrc.2020.04.010] Lieberman (2020) [10.1371/journal.pbio.3000849] Feng (2020) [10.3389/fmolb.2020.568954] Garvin (2020) [10.7554/eLife.59177] Lee (2020) [10.1080/22221751.2020.1827985] Heuberger (2021) [0.15252/emmm.202013191] Lamers (2020) [10.1126/science.abc1669] ¥Taglauer (2022) [10.1016/j.ajpath.2021.12.011] *Khan (2022) [10.1093/cvr/cvac097] *Pistollato (2022) [10.1016/j.reprotox.2022.04.011] Nataf & Pays (2021) [10.3390/ijms221910440] Triana (2021) [10.15252/msb.202110232] @Volbeda (2021) [10.1186/s13054-021-03631-4] ^Gao X (2022) [10.1016/j.jinf.2022.06.030]

Biological Plausibility

Angiotensin-converting enzyme 2 (ACE2) is a transmembrne protein which under normal physiological conditions acts as a carboxypeptidase with broad substrate specificity. Angiotensin II is recognised as the main substrate of ACE2, but ACE2 hydrolyses a number of other biologically active peptides including DABK, apelin-13,

neurotensin(1–11), dynorphin A-1–13), β-casomorphin-(1–7), and ghrelin (Vickers 2002, Humming, 2007). Hence, ACE2 is one of the major regulating enzymes in the local tissues and systemic plasma RAS and KKs systems, mainly balancing the action of its homologue ACE.

In the gut, ACE2 interacts with the Broad Neutral (0) Amino Acid Transporter 1 (B^0AT1) (Slc6a19) and chaperons its membrane expression. As B^0AT1 mediates the uptake of neutral dietary amino acids, such as tryptophan and glutamine, into intestinal cells (Camargo, 2009), ACE2 is indirectly involved in regulating intestinal amino acid homeostasis, expression of antimicrobial peptides, and the gut microbiome (Camargo, 2020, Hashimoto, 2012).

Considering the above, it is plausible that interference with any aspect of the ACE2 expression, it's enzymatic or B^0AT1s-shaperone function represents a potential target for dysregulation with a wide range of downstream effects.

ACE2 is not known to act as a classical receptor that would itself initiate intracellular signalling cascades after binding of ligands or substrates and very few selective inhibitors or stimulators of its activity are available to study the effects of potential stressors and modulators of ACE2. However, binding of SARS-CoV and SARS-CoV2 spike protein to ACE2 has been demonstrated (refs from Event: 1739) and this interaction represents an essential step for viral entry into the cells (refs from Event: 1738).

How interaction of ACE2 with the spike protein (KE upstream) perturbs ACE2 function (KEdownstream) is evaluated in the empirical evidence section of this KER.

At a molecular level, it is plausible that binding of the spike protein to the extracellular domain of ACE2 may perturb the catalytic activity of both, the cell membrane mACE2 or the cleaved catalytically active ectodomain sACE2, by allosteric interaction with the catalytic site (Liu & Nussinov 2016; Dutta 2022; Wang DS 2022; Trozzi 2022).

Interaction with spike protein may also perturb the shedding of ACE2 catalytic ectodomain. ACE2 is constitutively cleaved (shed) mainly by another membrane bound metalloprotease ADAM17, also known as TACE, TNFalpha Converting Enzyme (Zunke 2017) in a number of cells and tissues (Guy 2008 in human cardiac myofibroblasts; Peng Jia, 2009 in human lung epithelial cells and BALF; Werner, 2005 in canine epithelial polarised kidney MDCKII cells stabilly expressing ACE2; Iwata, 2009 in Chinese Hamster Ovary Cells). The exact role of constitutive ACE2 shedding is not well understood, but proteolytc ectodomain shedding of membrane proteins is a fundamental post-translational regulatory mechanism of the activity/function of a wide variety of proteins, including growth factors, cytokines, receptors and cell adhesion proteins (Lichtenthaler et al., 2018).

Binding of the spike (S) protein from both SARS-CoV has been associated with cleavage of the ACE2 ecodomain by TACE/ ADAM17 (Haga 2008, Haga 2010, Jocher, 2022), but also by another host protease TMPRASS2 (Heurich, 2014; Shula 2011) which also cleaves the Spike protein itself thus mediating viral entry by fusion of the cellular and viral membranes. Spike protein mediated ADAM17 shedding of ACE2 has not been demonstrated for SARS-CoV2 (to my knowledge). However, the interplay between spike and these proteases has been suggested as critical and linked events based on analogy and also some structure-function considerations (Schreiber 2020; Heasly 2022; Zipeto 2020). It is plausible then, that the spike induced shedding of ACE2 (via ADAM17 or other proteases) perturbs the distribution of the catalytically active mACE2/sACE2 and consequently differentially dysregulates ACE2 function/activity locally and systematically (Wysocki 2010). This in turn would have significant implications for the development of therapies that would target ACE2 activity and the RAS system appropriately in terms of delivery and timing.

Additionally, it is plausible that membrane bound ACE2 is down-regulated due to endocytosis of viral particles as suggested by earlier studies with SARS-CoV spike protein (Inoue, 2007; Wang H., 2008; Wang S., 2008) but also with SARS-CoV2 spike protein (Raghavan 2021, Gorshkov 2022). The relative contribution of the membrane fusion versus endocyitic route varies for the two SARS-CoV viruses (CoV1 and CoV2). This difference has been suggested as an important aspect of the differential infectivity and transmissibility of the two viruses (Zhu, 2021). It may also affect the relative magnitude and direction of mACE2 and sACE2 dyregulation.

Finally, transcriptional dysregulation of ACE2 mRNA synthesis is also plausible, but the process is not likely to be an immediate consequence of spike binding to ACE2, but rather requires some downstream signalling (potentially other intermediate KEs)and time for the biosynthesis of the mRNA and protein. Consistent with this, there is evidence that Ace2 (human but not the mouse), is an Interferon Stimulated Gene (ISG) activated also by some other viruses, inflammation, hypoxia and Synthetic dsRNA, poly(I:C) (Ziegler, 2020, Smith, 2020; Salka 2021, Zhuang 2020). However, it emerges that interferons and viruses stimulate a particular truncated form of ACE2, the dACE2 and not the full length ACE2 (Onabajo 2020; Scagnolari, 2021; Blume, 2021; Oliveto, 2022). The significance of this (post?)transcriptional modulation of Ace2 mRNA is not clear, and the enzymatic activity of the product of the alternatively spliced or, Ace2 mRNA initiated from a different promotor (which contains the catalytic domain but not the spike protein binding site) has not been examined. In addition, there is some evidence that S-protein binding can also lead to (direct?) suppression of ACE2 and Type I Interferon synthesis. To facilitate the analysis of the WoE for relevant perturbation of ACE2 as related to mRNA synthesis following the interaction with SARS-CoV and SARS-CoV2 spike protein, existing empirical evidence is outlined in the KER highlighting considerations for stressor type and time concordance between the two KEs.

Empirical Evidence

This KER summarises the evidence for dysregulation of ACE2 (KE 1854) as a result of binding of the viral spike (S) protein of SARS-COV and SARS-COV2 (KE1739). This is likely an important aspect of COVID19 pathogenesis as an initial step it is a potential target for intervention at (re)infection but also for treatment of the disease. In addition, it is important to summarise the exiting evidence for this KER as the majority of hypotheses on the pathogenesis of COVID19 and potential treatments consider S protein binding leads to down-regulation of ACE2.

The evidence included in this KER was identified in the period between October 2020 and March 2021 with a Pubmed search syntax……, and additional targeted searches for specific aspects to ACE2 regulation. The searches were aimed to identify the evidence for the effect of SARS-COV or SARS-COV2 on any aspect ofACE2 regulation i.e. effect on expression of ACE2 at mRNA or protein level, ACE2 shedding and enzymatic activity, all described in KE 1854.

The studies generally use SARS-COV or SARS-COV2 Virus like Particles (pseudovirus), recombinant or transiently expressed S protein from SARS-COV and SARS-COV2 (in Table 1 designated S protein (1) and S-protein (2), respectively) in various cell lines e.g. Vero E6 and Huh7-that express endogenous ACE2 or HEK293T transfected with ACE2 expressing vectors. Concurrent increase of the soluble form of ACE2 (sACE2) and/or enzymatic (peptidase) activity is monitored in some of the studies (Table).

The evidence mostly relates to evaluation of direct binding of S protein to ACE2, but some evidence is indirect and can potentially relate to dysregulation resulting from infection progression/viral replication and reinfection of new cells in the context of the parallel inflammatory processes.

Details of the evidence for dysregulation of ACE2 at mRNA and protein level (including enzymatic activity) as a result of interaction with SARS-COV 1 and 2 S proteins: : https://aopwiki.org/system/dragonfly/production/2022/02/12/8oiauj0w7m_Empirical_evidence_ACE2_dysregulation_120222.pdf

GI tract specific evidence for dysregulation at mRNA level

Stressor	"dose"	ACE2 mesuement	dysregultion type	time point	test system	tissue	species	note	note2	ref
SARS-COV2	1 MOI	protein - cell lysate	up	72hpi	cell ine	GI - colorectal adenocarcinoma	human			Lee at all (2020) 10.1080/22221751.2020.1827985
SARS-COV2	1 MOI	mRNA -qRT-PCR	up	72hpi	cell ine	GI - colorectal adenocarcinoma	human			
SARS-COV2	1 MOI	mRNA - qRT-PCR	up	48hpi	organoid -3D	GI - colon	human			Heuberger et al., 2021 10.15252/emmm.202013191
SARS-COV2	1 MOI	mRNA - bulk transcriptomics	up	48hpi	organoid -3D	GI - colon	human			
SARS-COV2	1 MOI	mRNA - bulk RNAsec	up*	60hpi	organoid -3D	GI- illeum	human	*modest? (24h not disscussed. See Nataf & Pays (2021)	in DIF organoids	Lamers et al., 2020 10.1126/science.abc1669
SARS-COV1	1 MOI	mRNA - bulk RNAsec	up*	24hpi 60hpi	organoid -3D	GI- illeum	human	*modest?	in EXP organoids	
								re-	Concomitant down-reg of B0AT transporter.	

SARS-COV2	1 MOI	mRNA - bulk RNAsec	down	24hpi	organoid -3D	GI- illeum	human	analysed data by Lamers et al., 2020	But note all genes that they looked at were down at 24 and recovered by 60hpi.	Nataf & Pays (2021) [10.3390/ijms221910440
SARS-COV2	1000K pfu	mRNA - scRNAseq	down	12hpi 24hpi	organoid -2D	GI - illeum	human	down-reg effect in both infected and bystanders compared to mock		Triana et al., (2021) [10.15252/msb.202110232]
SARS-COV2	1000K pfu	mRNA - scRNAseq	down	12hpi 24hpi	organoid -2D	GI - colon	human	down-reg effect only in infected cells, no effect in bystanders compared to mock		

Uncertainties and Inconsistencies

Evidence Collection Strategy

The literature search is not comprehensive or systematic and needs continuous improvement.

In all of the studies reviewed here, ACE2 dysregulation following exposure to recombinant S-protein and replicating virus is considered as equivalent for the prpose of evaluationg ACE2 dysregulation. However, it should be noted that the literature screening step showed that inflammation and exposure to other stressors (nanomaterials and other viruses, like influenza) were also associated with ACE2 dysregulation, indicating that aspects of the stressor multiplication and/or persistency, additional to the S-protein binding, may be responsible for the observed ACE2 dyregulation. These references were tagged but not further analysed and not included in this KER that is focused on S-protein binding. They can be informative if this KER is eventually modified and potentially split into two: (i) direct/adjacent KER (studies examining ACE2 dysregulation after direct and short term exposure to S-protein binding or non-replicating pseudoviruses) and (ii) indirect/nonadjacent KER (studies with replication virus and persistent infection).

Focus on GI tract relevant evidence (published in https://doi.org/10.3390/jcm11185400**)**

Both ACE2 down and up regulation are observed in the gut as in the other test systems (see gut unreated evidence table). The apparent inconsistences regarding the direction and magnitude of ACE2 dysregulation in the different studies may reflect the dynamic, temporal components of the dysregulation driven not only by the interaction of ACE2 with the surface viral components i.e. S-protein binding, but also by the interaction of the replicating viral components with the innate immunity response elements, particularly in the test systems with replicating viruses used with the GI-tract-derived organoids.

ACE2 mRNA down-regulation in SARS-COV2 treated GI-derived organoids is reported in one study using scRANseq analysis (Triana et al., (2021). The down-regulation was specific to enterocytes actively replication the virus. Another study Nataf & Pays (2021) also reports profound but transient ACE mRNA down regulation.

Evidence for SARS-COV2 mediated up-regulation of ACE2 mRNA in GI-tract derived organoids is also available (Lamers et al., 2020; Lee at all (2020); Heuberger et al., 2021) and consistent with the similar studies in many other tissue/organ systems (see evidence table for other organs above). This is also consistent with the finding that ace2 is an Interferon Stimulated Gene (IFG) in airway epithelial cells (Ziegler, 2020) and also in colon enterocytes (Heuberger et al., 2021). In fact, all there studies also demonstrate a time concordance of ACE2 mRNA up-regulation with stimulation of ISG response in the infected organoids (Lamers et al., 2020; Lee at all (2020); Heuberger et al., 2021). Interestingly, even the scRNAseq study by Triana et al., 2021 which zoomed on specific cells within the organoid found that SARS-COV2 treatment induced distinct proinflammatory and ISG expression profiles in infected and bystander cells in the organoid. Namely, expression of interferon-stimulated genes was pronounced in bystander cells, while the infected cells showed strong NFkB/TNF-mediated pro-inflammatory response but a limited production of ISGs, suggesting that while SARS-CoV-2 may activate ISG by paracrine signaling, it may suppress the autocrine action of interferon i.e inducton of IGS including ACE2 in infected cells. This would be consistent with down-regulation of ACE2 in the infected cells observed in this study. In addition, this may explain why in some studies down-regulation of ACE2 mRNA can be observed under certain conditions (e.g. bulk mRNA measurements) and at some (earlier) time points of replication. Furthermore, the relationship of an observed increase of ACE2 mRNA to dysregulation at protein and enzymatic level remains to be elucidated. Indeed, most recently Harnik et al. 2021 (10.1038/s42255-021-00504-6) examined the spatial discordances between mRNAs and proteins in the intestinal epithelium and their significance for interpretation of transcriptomic data. Such apparent discordances have also been reported in the heart and lung tissue in mice and human (KE1854).

Identification of alternative forms of ACE2 mRNA and protein, an N-terminus truncated dACE2, which appears to have distinct transcriptional regulation profile compared to flACE2 (Onabajo et al, 2020, Janakowski et al (2021) - 0.1016/ j.isci.2021.102928) may also account for some of the observed inconsistences. However, a detailed analysis of experimental conditions in past, and careful design of probes and primers in future studies is necessary. Interestingly, concomitant down and up regulation of 97kD and 80kD anti-ACE2 polyclonal Ab-reacting proteins has been detected in differentiating human colon adenocarcinoma cell line HT29 (Bártová et al. (2020) 10.18632/aging.202221). Considering only one form of ACE2 relevant (97KD the only form detected in HEK293 and A549), the authors conclude that ACE2 is down-regulated in mature differntiatiatiated enterocytes compared to undifferentiated ones. This is in contrast to all mRNA and even cytoimmunostaining studies described above which demonstrate that a highest level of ACE2, both mRNA and protein is detected in the mature enterocytes and at the brush borders of the intestine and 3D organoids (eg. Lamers et all., 2020, Lee et al., 2020; Hauberge et al., 2021; Triana et al., 2021). Notably, the initial analysis by Onabajo et al, 2020 found that dACE2 mRNA is enriched in in squamous tumors of the respiratory, gastrointestinal and urogenital tracts.

The uncertainties and uncosnstances disused above illustrate clearly the need for careful characterization of the test systems to facilitate robust interpretation of the results.

In addition, we note that to date (to our knowledge), the majority of studies related to SARS-COV2-mediated ACE2 dysregulation, focus on ACE2 mRNA expression while structural/protein and functional studies are lacking, particularly in the gastro intestinal system. The novel gut-derived organoid systems can help address this gap by monitoring level and cell distribution of ACE2 protein as well as its function as B^0AT1chaperon, via monitoring the membrane expression and/or the transporter function of B^0AT1 itself. In addition, treatment with S-protein and/or non-replicating SARS-COV2 pseudo-viruses [Minghai & Zhang (2021) 10.7150/ijbs.59184], may help address better any potential direct effect of S-binding on ACE2 dysregulation. Finally, a development of more complex organoid systems that would also include microbiota or elements of the immune and/or vascular system are needed to better examine ACE2 dysregulation by SARS-COV2 but also the effects of such dysreulation at higher organizational level and in conjunction with the other elements of the RAS system.

Finally, evidence on up- or down-regulation of ACE2 in the GI tract of SARS-COV infected patients is not available to our knowledge. Examining the GI specific transcriptomic, proteomic and biomarker databases of COVID19 patents may help address some of these uncertainties.

Quantitative Understanding of the Linkage

Known modulating factors

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)
Sex	both male and female sex (XX and XY chromosomes)	ACE2 localizes to the X sex chromosome, displaying higher expression in female than in male tissues [1], contributing to explain why women have milder disease progression. Lower levels of ACE2 in SARS-CoV-2 patients has been associated with higher rates of severe outcomes [2]. In particular, ACE2 is involved in the protection of acute lung injury [3], as reduction in ACE2 levels after infection has been associated with severe lung injury [4]. Because females have higher ACE2 levels, presumably more ACE2 remains available after viral entry and impairs severe lung and cardiac manifestation.	1. doi: 10.1177/1933719115597760 2. doi: 10.3390/ijms21082948 3. doi: 10.1038/nature03712 4. doi: 10.1038/nm1267
Chemicals (weak evidence)	PFAS (PFOA)	One study showed that PFOA upregulates ACE2 expression in lungs [1].	1. doi: 10.1016/j.toxrep.2021.11.014
Vitamin D moderate evidence)	Vit D deficiency	ACE2 is expressed in the human vascular endothelium and the respiratory epithelium [1]. VDR is also highly expressed in the lung tissue [2]. The effect of vitamin D and VDR on RAS occurs via both induction of ACE2/Ang (1-7) and the vasoactive Mas Receptor axis activity and inhibition of renin and of the ACE/Ang II/AT1R axis, thereby increasing expression and concentration of ACE2, MasR and Ang (1–7) [3]. Thus, vitamin D and VDR exert a vasorelaxant, anti-hypertensive modulation of the axis. Supportive evidence is provided by a VDR agonist, calcitriol, that down-regulated RAS activation in a rat model of acute lung injury [2]. The association of low vitamin D status with overactivation of RAS regulated by ACE2 has been observed also in non-infectious diseases [4]. Therefore, low vitamin D status, through a reduced VDR ligand, supports ACE2 dysregulation and ACE2/ACE imbalance, directly impacting the endothelium of lung vessels [5].	1. doi: 10.3390/cells9071652 2. doi: 10.3892/mmr.2017.7546 3. doi: 10.1002/rmv.2119 4. doi: 10.1016/j.jsbmb.2021.105965 5. doi: 10.1007/s10456-021-09805-6
Genetic factors		Currently, many studies focus on the impact of ACE2 SNPs that alter its expression level. However, SNPs that facilitate binding to S protein have not been inspected in a systematic and genome-wide manner. Many authors have postulated that SNPs in the ACE2 gene (Xp22.2) could affect the binding affinity of SARS-CoV-2 [1]. Altered binding between ACE2 and the S protein is expected to affect the RAS cascade, but no conclusive evidence has been identified so far.	1. doi: 10.1097/FPC.0000000000000436
Pre-existing heart failure		The dysregulation of ACE2 and of the RAS system is a characteristic of several cardiovascular pathologies having detrimental inflammatory effects, both locally (in the heart) and systematically [1]. Recently, evidence showed that the S protein itself has profound effects on the normal functioning of the cardiac pericytes also by non-infective mechanisms, e.g., by stimulating the pericyte-mediated release of pro-inflammatory factors that can lead to endothelial cell death [2].	1: https://doi.org/10.1016/j.cbi.2021.109738 2: https://doi.org/10.1042/CS20210735
Diet	Several dietary compounds impact the ACE axis	Many proteins found in seaweed have ACE-inhibiting properties and are thought to shift the balance of RAS towards the less inflammatory ACE2/Ang (1-7)/MAS axis [215]. Resveratrol, a stilbene compound found in several plant foods, appears to be able to promote this pathway as well, as it was found in multiple in vitro and in vivo studies to decrease the expression of angiotensinogen, ACE, and AT1R, and increase the expression of the AT2R and Mas receptor [206,216].	• 206: http://doi.org/10.1007/s11010-021-04275-2 • 215: http://doi.org/10.1093/nutrit/nuaa126 • 216: http://doi.org/10.1080/21623945.2021.1965315

Relationship: 2312: Binding to ACE2 leads to Downregulation of ACE2

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Viral spike protein interaction with ACE2 leads to microvascular dysfunction, via ACE2 dysregulation	adjacent		
Binding of viral S-glycoprotein to ACE2 receptor leading to dysgeusia	adjacent	Moderate	Not Specified

Key Event Relationship Description

The current KER relates to evidence for downregulation of the ACE2 following the binding of the viral spike S protein of SARS-COV (1 or 2).

According to the evidence (mostly from the study of SARS-CoV-1 in the years preceding the SARS-CoV-2 emergence and the COVID-19 pandemic) the infection of cells by SARS-CoV leads to downregulation of ACE2, either:

- in the form of shedding of the ACE2 (which has other repercussions from a RAAS point of view)

- internalization / endocytosis of the ACE2

- downregulation of mRNA / protein expression.

The literature focusing on SARS-CoV-2 is quite scarce, especially in relation to animal work (only 5 studies have been identified) up to November 2021.

Relationship: 2831: ACE2 dysregulation leads to (Micro)vascular dysfunction

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Viral spike protein interaction with ACE2 leads to microvascular dysfunction, via ACE2 dysregulation	adjacent		

Key Event Relationship Description

ACE2, is a carboxypeptidase with broad substrate specificity that deactivates or generates active biological peptides such as Angiotensin II (Ang II), Angiotensin 1-7 (Ang1-7), des-Arg9-bradykinin (DABK) but also others (10.1042/BJ20040634; 10.1074/jbc.M200581200; 10.1002/path.2162). These bioactive peptides and their receptors constitute the Renin Angiotensin (RAS) andKinin Kallikrein Sytem (KKS) which represent balancing regulatory networks of the vascular tone, inflammation, cell activation/proliferation, tissue remodelling and pain (10.1152/ajpheart.00723.2018; 10.3389/fnins.2020.586314).

This KER aims to summarise the evidence that addresses the relationship between ACE2 dysregulation and microvascular dysfunction as the vasculature represents one of the main targets of the RAS system (10.1152/ajpheart.00723.2018).

This KER was developed as part of the CIAO project (www.ciao-covid.net) and is therefore it focused mainly on one stressor of ACE2 dysregulation (SARS-COV2 and its spike protein which is the viral protein that binds to ACE2). Additional relevant evidence on the same or other stressors are welcomed.

Evidence Supporting this KER

Biological Plausibility

Microvascular dysfunction manifesting as vasculitis of the small blood vessels in different organs has been identified in COVID19 patients in a number of studies (e.g. 10.1016/j.ebiom.2020.10318 10.1056/NEJMoa2015432; 10.1016/j.anndiagpath.2020.151645).

Angiotensin Converting Enzyme 2 (ACE2) is recognised as the main receptor for the COVID19 causing SARS-CoV2 virus. In particular the viral spike (S) protein binds to ACE2 mediating viral entry (10.1016/j.cell.2020.02.052; 10.1128/JVI.00127-20) although other receptors or less specific (recognizing glycans on the virion surface of many viruses rather than specific viral protein) to mediators of viral entry have also been suggested (10.1002/rmv.2207; 10.3390/v14112535).

ACE2 is also known to be a cell receptor for SARS-CoV (10.1038/nature02145) although its spike protein shows ~10- to 20-fold lower binding affinity for ACE2 (10.1126/science.abb2507). Evidence from SARS patients is nowhere near that for COVID19, but some evidence of multiorgan injury, including to systemic vacuities has been reported (10.1002/path.1440).

ACE2, is a carboxypeptidase with broad substrate specificity that deactivates or generates active biological peptides such as Angiotensin II (Ang II), Angiotensin 1-7 (Ang1-7), des-Arg9-bradykinin (DABK) but also others (10.1042/BJ20040634; 10.1074/jbc.M200581200; 10.1002/path.2162). These bioactive peptides are involved in regulating vascular tone, inflammation, cell activation/proliferation and tissue remodelling via their receptors Angiotensin II receptor 1 (AT1R), Angiotensin II receptor 2 (AT2R), Mas-Related G Protein-Coupled Receptor (Mas) (10.1152/ajpheart.00723.2018; 10.3389/fnins.2020.586314).

Therefore it is plausible to explore the evidence examining spike protein-mediated dysregulation of ACE2 on aspects of (micro)vascular dysfunction.

Empirical Evidence

The protein profile of blood plasma represents a good indicator of (micro)vascular dysfunction at tissue level.

Increase of soluble ACE2 protein (sACE2) in plasma of SARS-CoV-2 positive patients was correlated with increase of the level of von Willebrand factor (vWF) and coagulation factor VIII comparing COVID19 to healthy controls by ELISA assay (10.1002/jmv.27144). Another study using proteomic/OLING approach and comparing viremic and aviremic COVD19 patients (10.1172/JCI148635) found increase of a larger panel of **angiogenesis** and **endothelial damage related** markers, including the vWF, and also **coagulation/fibrinolysis related factors:** tissue factor III and SERPINE1/Plasminogen activator inhibitor-1 (PAI-1), as well as **complement related pathway** proteins (PTX3, C1QA). In this study PROC was decreased in the viremic versus aviremic SARS-CoV-2 patients. Importantly, the proteomic study (10.1172/JCI148635) find that the dysregulation of specific endovascular damage markers (ANGPT2, ESM1), coagulation pathway markers (F3/tissue factor, vWF and PROC) and also the complement related pathway proteins (PTX3, C1QA) was specifically associated with SARS-CoV-2 viremia and not with generalised inflammation comparing SARS-CoV2 negative participants with respiratory dysfunction and an inflammatory profile (CRP >10 mg/dL). However, it is not clear whether ACE2 level is also significantly increased specifically in the SARS-CoV2 viremic patients compared to non COVID-19 controls (the supplementary data is not detailed -see sup fig 7). ACE2 mRNA is known to be upregulated by other infections, inflammation, hypoxia and synthetic dsRNA, poly(I:C) (Ziegler, 2020, Smith, 2020; Salka 2021, Zhuang 2020).

Unlike the proteomic study, the (10.1002/jmv.27144) did not find evidence for dysregulation of SERPINE1/PAI-1 in COVID-19 vs healthy controls. In fact, SERPINE1/PAI-1 protein levels were unaffected as were the level of ACE in plasma in COVID-19 patients compared to healthy controls.

Macroscopic indicators of dysfunction of the vasculature have also been reported in hACE2 transgenic mice infected with SARS-CoV (PMID 17974127) and SARS-CoV2 (10.1101/2020.08.19.251249 – bioRxiv; 10.1172/jci.insight.142032). (PMID 17974127) examined different organs and found: in LUNG: oedema, focal haemorrhage, degenerated blood vessels surrounded by monocytic and lymphocytic infiltrates; KIDNY: glomerular capillaries dilated markedly and engorged with lymphocytic infiltrate present in the renal interstitial; BRAIN - vasculitis, and haemorrhage. With SARS-CoV2 multiple intra-alveolar and intra-arteriolar microthrombi adherent to the endovasculature were observed that stained positive for the platelet marker CD61 (glycoprotein IIIa), as well as intra-bronchiolar blood clots and interstitial hemorrhagic patches (10.1101/2020.08.19.251249). Another study with SARS-CoV2 (10.1172/jci.insight.142032) report that vasculitis was the most prominent finding in the lungs of infected transgenic mice.

Wild type mice are resistant to SARS-CoV-2 infection as mouse ACE2 is not binding efficiently to the viral S protein, supporting the role of S-binding to ACE2 (direct or by the virtue of amplification of the stressor) in mediating the observed vascular damage and dysfunction. The findings are also consistent with autopsy findings in COVID-19 patients that showed widespread microthrombi and microangiopathy (/10.1016/S2352-3026(20)30216-7, 10.1007/s00428-020-02886-6, 10.1038/s41379-021-00793-y 10.1161/CIRCRESAHA.120.317447 and references therein).

There is also supporting evidence in lung tissue from autopsies of SARS-CoV2 infected humans compared to controls that died from unrelated causes including car accidents, that indicate increase of ACE2 protein in the lings (by immunostaining) correlated with increase of clotting factor VIII (FVIII) by immunostaining (10.3390/diagnostics10080575; 10.1002/path.5549). Another study identifies gross changes to the vascular component in both the interstitial capillaries and the mid-size vessels, with capillary fibrin micro-thrombi, as well as organized thrombi even in medium-sized arteries, in most cases not related to sources of embolism.

Similarly, increased ACE2 expression associated with multiple fibrin trombi in medium size pulmonary vessels without signs of inflammation is reported in COVID-19 autopsies compared to control [10.1002/path.5549]. Based on CD34 staining authors observe preserved but desquamation of the endothelial cells from the vascular walls, indicating endothelial damage and perturbed intercellular communication 10.1002/path.5549]. These case studies are based on very small number of patients and are not quantitative, but appear to converge on the finding that ACE2 immunohystochemical staining is increased in the lungs of patients with terminal COVID-19, particularly in the regions of small vasculature and alveolar damage. In lung autopsies from COVID-19 patients [10.1093/cvr/cvac097] S-protein collocalised (immunostaining) with the pirocyte marker Neural-glial antigen 2(NG2)/ Chondroitin sulfate proteoglycan 4 (CSP4). Collocalisaton staining was more pronounced in patients with thrombotic complications. Endothelial marker CD144/Cadhirin was also decreased in COVID-19 autopsies compared to age matched controls, and pronounced in patients with thrombotic complications. At the same time WWF factor was increased. Authors note that NG2+ Spike+ cells (actiated pirocytes phenotype) were not lining the endothelium, in particular, cells with high spike staining was associated with ICAM-1 staining suggesting pericyte activation and detachment in terminal cases of COVID-19. Together with the decrease of CD144 these finding strongly suggest dysregulation of the pirocyte-endothelial communication. ACE2 was not evaluated in vovo, but the same study included analisis in human iPCS derived 3D vascular organoids (see section on in vitro data).

10.1016/j.anndiagpath.2020.151645 examined biopsies form different tissues of patients with severe COVID19 (24 out of 26 with fatal outcome). Different degrees of (micro)vascular damage was observed in the different tissues, also without signs of observable inflammation. Small (micro) and bigger thrombi were observed in all tissues in the different size vasculature examined. Endothelial and sub-endothelial microvascular deposition of C3d, C4d and/or C5b-9 was detected by Immunohystochemical staining in the lung, liver, heart and brain autopsies. However, viral RNA was not detected (by in situ hybridisation) in endothelial cells but in cells with the morphology of macrophages. Furthermore, endothelial cells of the microvasculature showed no, or low (~10% of cells), immuno staining for ACE2 in most tissues, except the subcutaneous fat and in the brain where it collocalised with spike protein. Authors suggest spike-containing abortive pseudovirions can be released from infected cells and they can dock on ACE2 positive endothelial cells most prevalent in the skin/subcutaneous fat and brain, leading to activation of the complement pathway/coagulation cascade resulting in thrombogenic vascular dysfunction. In an earlier study focused on skin biopsies of COVID19 patients, [10.1016/j.humpath.2020.10.002] found evidence of endothelial cell injury also largely unaccompanied by significant infiltration of inflammatory cells, where the affected microvasculature in the skin showed significant immunohistochemical staining for C5b-9, C3d, and C4d. C5b-9 colocalized with ACE2 and spike protein.

A number of in vitro studies are available that use particularly recombinant spike protein (or its S1 or S2 subdomains) to explore the mechanistic drivers of the relationship between ACE2 dysregulation elicited by the binding of the spike protein and any aspect of the observed (micro)vascular disfunction at tissue/organ level. Mainly, different endothelial cell cultures (primary and immortalised) are used, but also 2D and 3D co-cultures.

Different types of dysregulation are explored and reported depending on the focus on the researchers, methods used for detection of specific markers of dysregulation and cell systems available:

- **Loss of the barrier integrity** based on: Electric Cell-Substrate Impedance sensing (ECIS), trans-endothelial electrical resistance (TEER), FITC-dextran or or FITC-BSA permeability assays. [10.1016/j.nbd.2020.105131; 10.1152/ajplung.00223.2021; 10.3389/fcvm.2021.687783; 10.4081/monaldi.2022.2213].

- Cell death and apoptosis preferentially of pericytes and to a lesser extent of ECs [10.1093/cvr/cvac097] – 72hpt

- Surface expression of **adhesion proteins** (↑ICAM-1, ↑VCAM-1) based on FACS-SCAN assay [10.1016/j.nbd.2020.105131 – significant outcome detected as early as 4hpt; (↓JAM-A, ↓Connexin-43, ↓PECAM based on Western blotting, time not specified) - 10.3389/fcvm.2021.687783]. No effect on mRNA for VCAM and E-selection have also been reported monitored 24hpt [10.4081/monaldi.2022.2213].

- Up-regulation of **pro-inflammatory cytokines** (IL6, IL1, CCL5, CXCL10) (qRT-PCR) [10.1016/j.nbd.2020.105131 significant outcome detected as early as 4hpt]. IL8, CXCL10, TGFbeta, MCP-1/CCL2 (qRTPCR) [10.4081/monaldi.2022.2213 measured only at 24hpt, CCL5 and IL6 not significantly changed in this study].

- Up-regulation of **matrix metalloproteinases** (e.g.MMP2, MMP3, MMP9, MMP1) and their regulators (e.g. MMP inhibitor TIMP1 (qRT-PCR) [10.1016/j.nbd.2020.105131 significant outcome detected as early as 4hpt];

• Up-regulation of **anti-inflammatory** cytokines (e.g. IL4, IL11, IL10RB) and down-regulation of inflammasome (NLRP3) and apoptotic pathways (CCL17) (Nanostring nCounter Assay and qRT-PCR for selected mRNAs to confirm) [10.1161/STROKEAHA.120.032764 – study monitored outcomes at 24hpt]

• Production of **coagulation and/or fibrinolytic factors** such as the plasminogen activator inhibitor 1(PAI-1), the **inhibitor of the pro-fibrinolytic factors** plasminogen activator (tPA) and urokinase (uPA). [10.1165/rcmb.2020-0544OC – study monitored outcome 24hpt at protein level by western blotting of cell lysates with anti PAI-1 antibody). Time course study in a co-culture test system showed increase of synthesis and secretion of coagulation factors: tissue factor (TF), factor (F)-V, thrombin, and fibrinogen with decrease in tissue factor pathway inhibitor (TFPI). mRNA, but also protein synthesis and secretion was measured) [10.3390/ijms231810436 – effect seen as early as 6hpt with two variants of S-protein].

The above findings have been obtained with different cell systems:

10.1016/j.nbd.2020.105131 - 2D&3D primary culture models with human brain microvascular endothelial cells (hBMVECs/D2) and immortalised hCMEC (telomerase-immortalized human brain endothelial cell line) 3D perfused cultures. Assays done 4hpt.

10.1093/cvr/cvac097 – vascular organoids derived from human-induced pluripotent stem cells consisting of endothelial cells and pirocytes

10.1152/ajplung.00223.2021; 10.3390/ijms231810436 - human lung microvascular endothelial cells (HLMEC), alone or in co culture with neutrophils

10.1161/STROKEAHA.120.032764 -human brain microvascular endothelial cells (HBMECs) & human umbilical vascular endothelial cells HUVECs. This study also identified some differences between the activation profiles of HBMECs and HUVECs. Characteristically different in HBMEC was the observed ↑ in C3, CCL8, IL15, IL9 that was not observed in HUVECs.

10.4081/monaldi.2022.2213 - human umbilical vascular endothelial cells HUVEC

10.1165/rcmb.2020-0544OC - Human pulmonary microvascular endothelial cell (HPMECs)

In vitro 3D vascular organoid system [10.1093/cvr/cvac097] also supported studies with replicating virus. Organoids infected with SARS-CoV2 (0.5 MOI for 48h), spike protein immunofluoresecence (imaging) co-localised 2x more with pyrocyte markers (CD140b(PFGFR)+/NG2+ cells) than with UAE1-positive ECs. Pirocyte apploptosis was also significantly higher (TUNEL assay). Presumably, UAE1 rather than CD144 was chosen as a "housekeeping" EC marker as ACE2 (spike receptor) co-localised 2x less with EC (CD144+) cells than with perycites (CD140b(PFGFR)+/NG2+ cells) in uninfected organoids. Taken together, the results strongly suggest that that SARS-COV2 is preferentially taken up by pirocytes via the ACE2. Viral entry was associated with increased cell apoptosis (TUNEL assay) which was significantly higher in pericytes (markers) compared to ECs (markers).

Given the preferencial infection and death of pericytes, the relatively low EC death (TUNEL) and the observed CD144 decrease (immunostaining) suggest that ACE2-dependent virus entry into pyrocites could lead to cell death and further to vascular dysfunction due to loss of functional pirocyte-EC communication.

In the same organoid test system [10.1093/cvr/cvac097], treatment with recombinant viral S or N proteins (72h) decreased the percentage of live pirocytes and ECs (FACS scan for live/total CD140b(PFGFR)/NG2+ and CD144+/CD31+ cells, respectively). In the case of ECs the % decrease of live cells appears comparable after treatment with S, N and combination of S+N protein. In the case of perycites, effect of S alone and in combination with N appears much more pronounced. For both cell types, treatment with proinflmatory IL1-beta togather with S, N or both antigens, did not have *additional* cytotoxic effect. IL1beta alone, had greater cytotoxic effect on ECs compared to pirocytes. However, neither EC nor pirocytes appeared to be activated by the S or N protein treatment as judged by the lack of significant change of CD31 or CD140b(PFGFR)/NG2+/ICAM-1+ cells, respectively. Similarly expression of WVF, IL6 or IL8 (ELISA of supernatants) was not affected by S or N treatment of ECs. In contrast, IL1-beta alone activated both cell types (as judged by increase of ICAM-1+ cells of both types) and also induced of WVF, IL6 or IL8 production by ECs.There was no difference between the teratemnt with IL1-beta alone or with the recombinant viral proteins, suggesting that viral replication rather than interaction with the viral spike (or N) protein may be driver of cell activation observed in the patients lung authopsies . At mRNA level, joined S + N treatment induced ACE2 expression (qRT-PCR) as early as 4hpt, peak at 24hpt, maintained up to 72hpt. Individual antigen treatment is not tested (or shown?).

Taken together thehe lines of evidence from testing systems of different complexity [10.1093/cvr/cvac097] suggests that in vivo the most likely primary target for viral infection or uptake of S-containing abortive pseudoviral particles in he (micro)vasculature are the pericytes. Infection/antigen uptake induce pericyte damage which dysregulates the intercellular crosstalk with ECs which in turn dysregulates overall vascular permeability (one potential aspect by interference with CD144 expression/function). In a parallel process EC may be also be activated by the inflammatory response in vivo, leading to significant increase in vascular permeability and microvasuclar dysfunction. Neutrophils are not present in this test system where apparently different results were obtained [10.3390/ijms231810436], where on the other hand pericytes are not present.

Uncertainties and Inconsistencies

Uncertainties:

It is difficult to decipher or generalise which if any particular cell type in the (micro)vasculature represent a key factor in the putative spike-protein mediated (micro)vasculature dysfunction, particularly because the expression of ACE2 varies significantly in the cell systems used, (see Table below). Closer analysis is needed regarding the reagents/antibodies used in these studies and comparative characterisation of the experimental systems, as it appears that ACE2 expression may depend on the dimensionality (2D/3D, co-cultures) but also the dynamics (medium flow or stasis) of the test system.

Notably, ACE2 expression in HBMEC & HUVEC perfusion culture is stimulated by flow (HBMEC < HUVEC) (qRT-PCR); also it is increased by flow intensity and vessel shape in the MCA 3D model of stenosis (immunostaining cells) [10.1161/STROKEAHA.120.032764].

Singlecell RNAseq analysis of quiescent mouse endothelial cells isolated from different tissues by flow cytometry without the cell culture step, does not identify ace2 enrichment in the ECs from any of the mouse tissues while ace is one of the top-50 marker genes in ECs from arteries in the brain, testes and small intestine. scRNAseq of primary cell cultures from EC from human lung identified similar cell subpopulations and marker genes in mice and human, but interestingly, scRNAseq of commercially available primary lung ECs demonstrated a loss of their native lung phenotype in culture . (Schupp 2021 - 10.1161/CIRCULATIONAHA.120.052318). 10.1038/s41419-020-03252-9

There are also some apparently conflicting results. For example although [10.1016/j.nbd.2020.105131] shows S-mediated increase of many markers of MV dysfunction, without evidence for any change in ACE2 levels as measured by Western blot of cell culture lysates. This suggests that although the effect is spike protein mediated it may not necessarily be ACE2 mediated and that other receptors for S protein may be involved in MV.

Cell lines	Type of cells	From	ACE2 expressed	ACE2 detection assay	ref
hBMVEC	microvascular endothelial	Brian/human	+	Cell lysate (Ab)	10.1016/j.nbd.2020.105131
hCMEC	microvascular endothelial	Brian/human/immortalised	+	Cell lysate (Ab)	
HBMECs	microvascular endothelial	Brian/human	-/+	ACE2 expression in HBMEC & HUVEC perfusion culture is induced by flow (HBMEC < HUVEC) [qRT-PCR]; also it is increased by	10.1161/STROKEAHA.120.032764
HUVEC	vascular endothelium	Umbilical cord/human	basal level of ACE2 in CD31+ endothelial brain cells in monocultures was **very low** but still visible by		

Additional uncertainty in this KER evidence analysis is that most of the studies that use various cultured endothelial cell models, do not specifically measure/identfy ACE2 dysregulation aspects in the same time and binding of S-protein to ACE2 is often assumed. In fact, infection of endothelial cells by SARS-CoV2 is still debated. [10.1021/acscentsci.0c01537] show that ACE2 is expressed at very low level in HUVEC-TERT, immortalised umbilical endothelial cell culture. In contrast two C-type lectin receptors, CD209L (also known as L-SIGN) and CD209 (also known as DC-SIGN), are highly expressed. CD209L and CD209 bind spike RBD domain and mediate SARS-CoV-2 entry into HUVEC-TERT (demonstrated by interference with CD209L). Thus, while endothelial cells may be permissive to SARS-CoV-2 entry/replication, the involvement of ACE2 in these undifferentiated cells in monolayer culture, is

			immunocitostaining in both cell types. Interestingly expression was perinuclear not at the cell membrane.	flow intensity and vessel shape in the 3D printed model of middle cerebral artery, particularly of stenotic parts (immunostaining cells)	
HUVEC-TERT	vascular endothelium	Umbilical cord/human	-	Cell lysate (Ab)	10.1021/acscentsci.0c01537
A549	epithelium	Lung/carcinoma	+	Cell lysate (Ab)	
HPMEC	Microvascular Endothelial	Lung/human	-/+ **almost undetectable** in quiescent HPMEC lysates so they did the studies in HeLa transfected with hACE2	Cell lysate (Ab)	10.1165/rcmb.2020-0544OC
HULEC-5a	microvascular endothelial	Lung/human	-/+?	Cell lysate (Ab) (Ab in this study is not detecting ACE2 in A549, to check if ACE2 in these carcinoma cells is dACE2 and the Ab is against the ectodomain?)	10.1038/s41419-020-03252-9
HUVEC	vascular endothelium	Umbilical cord/human	-		
A549	epithelium	Lung/carcinoma	-		
HPAEpiC	epithelial cell line - AT2 cell-derived	Lung/human	+		

Cell culture	Type of cells	Organ origin	ACE2 expressed	ACE2 detection assay	ref
Primary culture	microvascular endothelial	Lung/human	Not measured but TER affected after S1 subunit-treatment (less by flS)	n/a	10.1152/ajplung.00223.2021
Primary culture	Endothelial hPMECs hBMECs hCMECs hGMECs HUVECs	Multiple pulmonary Brain Cardiac Glomerular Umbilical	- (only HUVEC show a staining at 80K not at control 130K of Vero-E6 and CAlU3)	[protein-Wstern and mRNA-qRT-PCR]	10.1128/mBio.03185-20
Primary culture	HUVECs	Note: HUVECS were compared with several placental cell lines (JAR –epithelial carcinoma, BeWo - Oligodendroglioma and HTR-8/SVneo – vili SV40 transfomed)	HUVEC had highest mRNA but low whole cell and surface expression of ACE2 compared to the other cells, particularly the epithelial JAR	Nice comparison of mRNA (rqRT-PCR), whole cell protein (Western) and surface protein (FACS-SCAN) in several cell tipes in the same experiment.	10.4081/monaldi.2022.2213

Organoids	Type of cells	Organ origin	ACE2 expressed	ACE2 detection assay	ref
	endothelial (CD31+), also characterised as UAE1+, or CD144+ in some experiments pericytes (CD140b+NG2+) fibroblast (CD140b+NG2-)	Induced pluripotent stem cells	pirocutes >> ECs	Immunocytostaining and microscopic fluoresecence analysis of organoid sections. collocalisation with cell specific markers analysis.	10.1093/cvr/cvac097

uncertain. The same study demonstrates that, when expressed, ACE2 mediates pseudovirus entry more efficiently and in vitro binds S-protein RBD with higher affinity than CD209L and CD209 [10.1021/acscentsci.0c01537].

Sumilarly, [10.3390/ijms231810436] that demonstrates effect of spike treatment on a number of markers of endothelial dysfunction in HLMEC alone, or in co-culture with neutrophils also does not characterise the test system for ACE2 status or time concordance with any potential ACE2 dysregulation in either of the cell types in the co-culture. Such characterisation would be helpful in understanding the primary target of the viral or abortive spike-containing particles, as well as the intercellular paracrine interactions driving adversity at tissue level.

Therefore, although in some studies the dependence of aspects of (micro)vacular dysfunction on ACE2 is demonstrated [10.1128/mBio.03185-20], the type of ACE2 dysregulation/effect (upregulation/downregulation) that could be related to the observed aspect of (micro)vascular dysregulation is not clear.

Other plausible mechanisms:

Viral replication and inflammation-related oxidative stress are also explored as significant factors in coagulation and DIC following SARS-CoV-2 infection/stress and may be more direct drivers of the (micro)vascular dysfunction compared to ACE2 dysregulation. Consistent with this, [10.1128/mBio.03185-20] find that primary cultures of scattered endothelial cells derived from microvascular tissue from different organs, express very low levels of ACE2 (if any) and cannot be infected by SARS-CoV2. However, when transduced to overexpress hACE2 they could be infected and the interaction with the replicating virus led to stimulation of mRNA synthesis for **coagulation and pro-inflammatory mediators**: tissue factor (TF), thrombomodulin (TM), tumour necrosis factor alpha (TNFa), interleukin 6 (IL-6), IL-1b, and E-selectin (qRT-PCR assay following 6h interval time course from 0-24hpi showing significant stimulation for TF and TNFa, as early as 6hpi and for the rest of the mediators at latter time points). E selectin was also stimulated early with pick at 12hpi. In human heart tissue of COVID-19 infected patients ACE2 immuno-histological staining showed increasing expression towards the small vessels: capillaries >> arterioles/venules. The main coronary arteries were virtually devoid of ACE2 receptor [10.1016/j.ebiom.2020.103182]. In the same study, staining of harts from influenza infected patients showed much lower expression of ACE2 even in capillaries, and in small vessels, practically the same in coronary artery.

ACE2 is known to be upregulated by other inflammation, hypoxia and synthetic dsRNA, poly(I:C) (Ziegler, 2020, Smith, 2020; Salka 2021, Zhuang 2020, indicating that inflammation, low oxygen levels and viral replication ma precede or in parallel potentiate ACE2 mediated MV dysfunction by increasing the ACE2 levels in the MV cells which then maintain viral infection cycle but also govern MV dysfunction via spike-protein binding mechanism in the surviving cells.

ACE2 independent (though spike-mediated) mechanisms leading to microvasuclar disfunction (TEER disruption), have also been suggested [10.1038/s41467-022-34910-5]. In this study human pulmonary microvascular endothelial cells (HPMEC) were used as a representative endothelial cell relevant to COVID-19 pathology that do not endogenously express ACE2 and are not permissive to SARS-CoV-2 infection (as demonstrated by the authors in their study). That allowed them to separate viral replication from S-mediated dysfunction and compare them to transfected ACE2 overexpressing line of HPMEC and also HPMEC in which ace2 gene was disrupted by CRISPR-Cas9. The alternate mechanism appears to involve perturbation of surface levels of key endothelial glycocalyx layer (EGL) components, including sialic acid (SIA), heparan sulfate (HS), hyaluronic acid (HA), and chondroitin sulfate (CS) (immunofluorescence staining) and EGL-degrading enzymes such as hyaluronidase and neuraminidase. Integrins and TGFbeta sugnaling also appar essencial for tis mechanism. Intradermal and intranasal administration of recombinant spike protein also appeared to disrupt endothelial barrier function in vivo in leak models in WT C57BL/6 mice which do not express human ACE2 and are not permissive to infection by most SARS-CoV-2 variants. Binding of spike protein to alternative (co)receptors including integrins and heparin sulfate has been implicated as well [10.3390/v13040645; 10.1016/j.cell.2020.09.033]. It should be noted that in some test systems ACE2 mRNA was up-regulated as a result of spike treatment in vitro (eg. 80% confluent HUVEC cell [10.1016/j.jbc.2022.101695s]) even though the initial spike interaction was likely to an alternative receptors leading to activation of the quiescent endothelium. Resulting ACE2 up-regulation and/or activation of quiescent endothelium could initiate additional and parallel ACE2 dependent perturbations leading to vascular dysfunction [10.1021/acscentsci.0c01537].Contribution of the effects of the activation of the **adaptive immune response** to spike and other viral components or endogenous host prpteins relevant to (micro)vascular dysfunction is possible but not explored sufficiently by this evidence collection strategy, although it has been indicated [10.3390/pathophysiology29020021; 10.3389/fimmu.2022.906063]

Gaps:

Overall, the studies with more differentiated 2D/3D cultures and recombinant S protein as a stressor, strongly suggest that spike-mediated ACE2 dysregulation could also,

in part lead to aspects of (micro)vascular dysfunction. The initial target cells still remain to be elucidated. This may vary in different organs. Notably, a very recent cell atlas of adult human myocardium made by single nuclear transcriptome analysis found that the highest level of ACE2 mRNA in the heart was found in the pericytes a type of perivascular mural cells [10.1093/cvr/cvaa078]. Therefore, more complex and better characterised in vitro, optimally microfluidic dynamic systems, are needed to better understand molecular and cellular aspects of SARS-CoV-2, spike protein-mediated or more general toxicity (including general inflammation) -mediated (micro)vascular dysfunction. One potentially very useful system has already been described. [10.1016/j.cell.2020.04.004] developed human capillary organoids from induced pluripotent stem cells (iPSCs). They resembled human capillaries with a lumen, CD31+ endothelial lining, PDGFR+ pericyte coverage, as well as formation of a basal membrane. Consistent with [10.1021/acscentsci.0c01537} these organoids were permissive to SARS-CoV-2 infection and replication that could be significantly but only partially blocked by soluble human recombinant ACE2 in a dose dependent manner. However, the target cell and the ACE2 status of the different cells in the organoids are not reported and authors discuss the possibility that there might be other co-receptors/auxiliary proteins or even other mechanisms by which viruses can enter cell.

However, in another iPCS derived organoid system with characterised ACE2 expression, authors show that perycites express significantly higher levels of ACE2 also show preferential uptake of viral particles. In addition productive infection of perycites has been found in primary cultures of cardiac perycites in vitro and in hart tissue from COVID-19 patents [10.1016/j.jacbts.2022.09.001].

In addition, the role of microparticles (MPs) and microvesicles (MV) in mediating intercellular communication or its perturbation need to be examined in more detail. [10.1152/ajpheart.00409.2022] show that plasma MV from platelet and white blood cell origin contain high amounts of ACE2 that binds the spike protein of SARS-CoV-2. MVs also contain other adhesion moolecules (eg. Tissue Factor- CD152) mediating EC adhesion and alternative entry pathway by endocytosis for viral or abortive spike-containing particles with affinity for ACE2, ultimately leading to activation and dysfunction of the EC and micro(vsculature). It is interesting that [10.1152/ajpheart.00409.2022] found difference in the ACE2 containing MVs between normal and diabetic patients which have increased incidence of severe and fulminant COVID-19.

Extracellular vesicles (EVs) including microvesicles (MVs) and exosomes can also mediate cell–cell signalling in a paracrine and/or even endocrine mechanism via their protein and RNA cargo (10.1093/nar/gky985; 10.1016/j.cell.2016.01.043). Non coding RNAs such as microRNAs (miRNA or miR) have been implicated in various biological processes including angiogenesis and vascular dysfunction (10.1530/VB-19-0009). A number of miRs (eg miR122, miR143, miR155, miR181a and miR1246, miR421) have also been implicated in regulation of ACE2 in different tissues and plasma (10.1016/j.ncrna.2020.09.001; 10.1016/j.yjmcc.2020.08.017; 10.1016/j.omtn.2022.06.006; 10.1042/CS20130420). Therefore interference with the expression of relevant miRs by exogenous stressors, including RNA viruses, LPS or dysregulated endogenous signalling molecules (eg. vasoactive peptides) can potentially lead to ACE2 dysregulation and (micro)vascular dysfunction and need to be examined in greater detail.

Finally, the distribution and role in (micro)vascular dysfunction, of the receptors for the substrates and products of ACE2 need to be investigated in these systems to better elucidate a potential link with up- or down-regulation of ACE2 function locally and/or systemically. Test systems that could monitor activation of the complement system proteins would also be valuable.