Vascular inflammation and endothelial injury in SARS-CoV-2 infection: The overlooked regulatory cascades implicated by the ACE2 gene cluster

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KEY POINTS

Question: In contrast to other “respiratory” viruses, why does SARS-CoV-2 result in such multi-systemic, life-threatening complications, including the vascular inflammation, endothelial injury and pulmonary thrombotic microangiopathy for which evidence is accumulating?

Findings: With ongoing viral infection, the particularly strong interaction between SARS-CoV-2 and ACE2, followed by internalisation, operates to profoundly deplete cell surface ACE2, an essential protein under feedback regulation to restore protein expression through upregulated gene transcription. Data residing unflagged in experimental repositories indicate that the ACE2 gene lies in a co-regulated cluster with PIR and VEGFD, sharing “double-elite” enhancers, implying homeostatic host responses to restore ACE2 expression would generate damaging reactive oxygen species in the setting of impaired host defences, and separately impact on the ability of the pulmonary endothelium to respond appropriately.

Meaning: For patients who fail to suppress replication of the SARS-CoV-2 virus within a few days, the ACE2 co-regulated gene cluster predicts delayed responses that would contribute to subsequent catastrophic deteriorations, and merit further attention.
ABSTRACT

COVID-19 has presented physicians with an unprecedented number of challenges and mortality. The basic question is why, in contrast to other “respiratory” viruses, SARS-CoV-2 infection can result in such multi-systemic, life-threatening complications and a severe pulmonary vasculopathy. It is widely known that SARS-CoV-2 uses membrane-bound angiotensin-converting enzyme 2 (ACE2) as a receptor, resulting in internalisation of the complex by the host cell. We discuss the evidence that failure to suppress coronaviral replication within 5 days results in sustained downregulation of ACE2 protein expression, and that ACE2 is under negative-feedback regulation. We then expose openly-available experimental repository data that demonstrate the gene for ACE2 lies in a novel cluster of interregulated genes on the X chromosome including PIR encoding pirin (quercetin 2,3-dioxygenase), and VEGFD encoding the predominantly lung-expressed vascular endothelial growth factor D. The five double-elite enhancer/promoters that are known to be operational, and shared read-through IncRNA transcripts, imply that ongoing SARS-CoV-2 infection will reduce host defences to reactive oxygen species, directly generate superoxide $\text{O}_2^\cdot$ and $\text{H}_2\text{O}_2$ (a “ROS storm”), and impair pulmonary endothelial homeostasis. Published cellular responses to oxidative stress complete the loop to pathophysiology observed in severe COVID-19. Thus for patients who fail to rapidly suppress viral replication, the newly-appreciated ACE2 co-regulated cluster predicts delayed responses that would account for catastrophic deteriorations. We conclude that ACE2 homeostatic drives provide a unified understanding which should help optimise therapeutic approaches during the wait until safe, effective vaccines and antiviral therapies for SARS-CoV-2 are delivered.
During 2020, the world has faced unexpected health, social and economic damage due to SARS-CoV-2 infection, with over 16 million cases, more than half a million deaths, and continuing high mortality rates.\(^1\) The multifaceted severe presentations of COVID-19 have centred on viral pneumonitis/adult respiratory distress syndrome (ARDS), the “cytokine storm” of dysregulated inflammation, and multi-organ failure, with the leading cause of death now recognised as thromboembolism.\(^2,3\) There is evidence for a hypercoagulable state in excess of that observed in sepsis,\(^4\) with vascular inflammation, endothelial injury and thrombotic microangiopathy assuming prominence.\(^5-8\)

The question is why are these occurring? Many inflammatory and signalling cascades have been proposed, though as yet there are no answers to the basic question of why SARS-CoV-2 infection, as for SARS-CoV, can result in such multi-systemic, life-threatening complications, differing in individual cases, or why thrombosis plays such a pronounced role in the life-threatening phenotypes.

Here we draw attention to the physiological and homeostatic responses employed by cells in response to coronavirus infection that point towards defective endovascular defences to reactive oxygen species, a “ROS storm”, and specifically impaired pulmonary vascular homeostasis.

**The SARS-CoV-2 “receptor” angiotensin-converting enzyme 2 (ACE2)**

SARS-CoV-2, SARS-CoV and related SARS viruses are so named due to their ability to generate a severe acute respiratory syndrome (SARS) in a proportion of infected individuals. They are unusual compared to more familiar viral pathogens as they use membrane-bound angiotensin-converting enzyme 2 (ACE2) as a receptor.\(^9,10\) ACE2 is an essential component of the renin-angiotensin-aldosterone system (RAAS), normally catalysing angiotensin II degradation, with multiple cardiovascular-protective consequences.\(^11\) There is some evidence from other species that expression of the ACE2 receptor varies with age\(^12\) and sex.\(^13\)
On binding to ACE2, the SARS-CoV-2 Spike (S) envelope protein is cleaved to subunits S1 and S2 by host proteases. Specific amino acids in the S1 C-terminal domain (CTD) and host ACE2 form a network of hydrogen-bond and salt bridge interactions resulting in internalization of the complex by the host cell. The SARS-CoV-2-ACE2 interactions are 4-fold stronger than between SARS-CoV and ACE2.

The kinetics of recycling ACE2 following viral-ACE2 complex internalisation are not yet reported for SARS-CoV-2, but those of a related human coronavirus, HCoV-NL63, were examined by Dijkman and colleagues in Rhesus monkey epithelial cells. They demonstrated that cell surface expression of ACE2 starts to reduce 3 days post infection, becoming almost undetectable in infected cells from day 5 to the end of their study period, and therefore of unknown duration.

This is for an essential protein where strong evolutionary constraints have served to maintain physiological activity: The evidence is multifactorial, and most compelling ACE2 is a “loss-of-function intolerant” gene with a very low frequency of null alleles, particularly in males who only have a single copy due to its X-chromosome location. Transcription upregulation is observed within hours for many proteins sequestered in pathophysiological settings. Specifically for ACE2, mRNA transcription is regulated by an unknown negative-feedback signal attributed to ACE2 activity. Thus when ACE2 activity is reduced, homeostatic responses operate to restore the protein, apparently via upregulation of gene transcription.

The ACE2 gene enhancers

Upregulation of gene transcription is enhanced by regulatory DNA sequences that modulate distant target gene expression, often by long-range physical chromosomal interactions. Several enhancers regulate ACE2 transcription in man, and two (GH0XJ015596 and GH0XJ01557) meet what are considered to be quite stringent GeneHancer definitions for ‘double-elite’ enhancer-promoter interactions. These restrict ‘elite’ enhancer assignment to a candidate regulatory element derived...
from more than one experimental data source, and ‘double elite’ promoter-enhancer interactions to
those meeting likelihood-based score thresholds using expression quantitative trait loci, again derived
from multiple sources.\textsuperscript{21,22} Fewer than 10\% of identified enhancer-promoter interactions fulfil the
GeneHancer ‘double elite’ status.\textsuperscript{21} The validity of the stringent ‘double elite’ enhancer–gene network
is supported by the method’s capture of 38 gene pairings where each of the pair represented a
different causal gene for the same disease (hypergeometric probability P<6.0x10^{-35}).\textsuperscript{21}

\textit{GH0XJ015596} and \textit{GH0XJ015579} flank \textit{ACE2} exons 1 and 9 respectively (\textit{Supplementary Figure 1}). Both
were among \textasciitilde22,000 enhancers identified by high-resolution, high throughput chromosome
conformation capture (Hi-C) libraries from human blood cells- hematopoietic progenitors and
lymphoblastoid cell lines, with the libraries enriched for long-range promoter contacts,\textsuperscript{23} and also
identified by the ENCODE laboratories\textsuperscript{24} and Ensembl.\textsuperscript{25} While the anatomical nature of GeneHancer
enhancer interactions are defined and visible to all through tracks on the human genome browser
from the University of Santa Cruz (UCSC)\textsuperscript{21,22,26,27} mechanisms are not well studied. For instance it is
not known whether a long non-coding (lnc)RNA that overlaps \textit{GH0XJ015596} (\textit{lnc-ACE2-1, 
\textit{Supplementary Figure 2})\textsuperscript{22,29} plays a functional role in any cell type

Why stimuli to upregulate \textit{ACE2} gene transcription will have wider consequences:

The \textit{ACE2} co-regulated cluster of genes

The region on the human X-chromosome that contains the \textit{ACE2} gene is illustrated in \textit{Figure 1A}, and
annotated by position on both human genome reference sequence “build 37” (GRCh37/hg19), and
“build 38” (38/hg38). \textit{ACE2} is encoded by the reverse (-) strand, and is sited between \textit{CLTRN} and \textit{PIR},
which is adjacent to \textit{VEGF-D}.

These genes, which encode proteins with better-known names and functions, share enhancers and
other regulatory elements with \textit{ACE2} (\textit{Figure 1B}). Homeostatic responses attempting to restore
expression of cell surface ACE in the setting of ongoing SARS-CoV-2 viral replication predict upregulation and/or perturbation of these critical proteins, as discussed in the next section.

**a) ACE2, PIR and quercetin:**

The ACE2 enhancers *GH0XJ015596* and *GH0XJ015579* have double-elite interactions (confirmed by Hi-C and expression quantitative trait loci from GTEx version v6p), with the promoter of *PIR* which encodes quercetin 2,3-dioxygenase (“pirin”), and is near-ubiquitously expressed. The substrate of pirin is quercetin (3,3’,4’,5,7-pentahydroxyflavone), a flavonoid considered to be one of the most potent antioxidants of plant origin. Quercetin was identified as a potential COVID-19 therapy by supercomputer modelling of the viral S-human ACE2 receptor interface, and by genomics-guided tracing of SARS-CoV-2 targets. Quercetin has electron-donating properties due to a phenolic hydroxyl group essential for scavenging free radicals such as superoxide anion O$_2^-$; and these antioxidant activities are the reasons cited for two randomised controlled trials in COVID-19 that are currently recruiting on www.clintrials.gov.

Quercetin 2,3-dioxygenase, the protein product of *PIR*, converts the ‘antioxidant’ quercetin to o-quinone, with an o-semiquinone radical intermediate which reacts with oxygen to generate the reactive oxygen species (ROS) superoxide O$_2^-$ and H$_2$O$_2$. Hence oxidation of quercetin by up-regulated quercetin 2,3-dioxygenase is predicted to both remove an important antioxidant and directly generate ROS. Consequences will reflect the residual capacity of host anti-oxidant defences.

**b) ACE2, VEGFD, and vascular endothelial growth factor D**

The next “downstream” (3’) gene is *VEGFD* (Figure 1A). There are double-elite interactions between three *PIR* locus-encoded enhancers (*GH0XJ015492*, *GH0XJ015473*, and *GH0XJ015471*) and the promoter of *VEGFD*. Additionally, there is a long *PIR* and *VEGD* read-through RNA transcript that will not produce full length protein (Inc-VEGFD-1, Figure 2). Any change in Inc-VEGFD-1
transcription, including that mediated by the *ACE2* locus-encoded enhancers interacting with the *PIR* promoter, would alter the balance between the read-through and *VEGD* protein-coding transcripts.

This is also relevant to COVID-19 since *VEGFD* encodes vascular endothelial growth factor D which is predominantly expressed in the lungs, modifies differentiation, proliferation and permeability of vascular and lymphatic endothelial cells, is a therapeutic target for lymphangioleiomyomatosis, and independently predicts all-cause mortality in patients with suspected or known coronary artery disease.

### c) Additional cluster genes.

There are additional regulatory interactions between the *ACE2*-encoded enhancers and the preceding genes, *CLTRN* (also known as *TMEMM27*) and *CA5B* (*Figure 2B*). *CLTRN* encodes collectrin which has roles in renal proximal tubule amino acid transporter trafficking, insulin exocytosis, and neuropsychiatric stability. *CA5B* which is on the (+) strand (*Figure 2A*), encodes the mitochondrial-expressed carbonic anhydrase 5B, a metalloproteinase that generates bicarbonate for metabolic liver enzymes: defective hepatic bicarbonate production leads to biochemical findings that could be relevant to the alkalosis observed in some COVID-19 patients.

Notably, none of the major proteins or other species encoded by the contiguous genes have been linked based on curated pathways, protein–interactions, or text mining databases from over 20 million PubMed publications (*Supplementary Figure 3*).

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**Relevance of *ACE2* gene cluster to host responses following SARS-CoV-2 infection**

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https://mc.manuscriptcentral.com/qjm
The section above outlines how cellular homeostatic feedback loops, in the setting of a continued stimulus to upregulate ACE2 transcription in infected cells with severely depleted cell surface ACE2,\textsuperscript{15} will lead to the COVID-19 reminiscent scenario outlined in Figure 2.

This linking of experimentally validated data provides a compelling prediction of the delayed, life-threatening features observed following SARS-CoV-2 infection. It depends on only one assumption—relatively normal host transcription will be in operation in some SARS-CoV-2 infected cells, some of the time. This seems a reasonable assumption based on evidence from other viruses: Many interfere with host transcription, for instance, H1N1 influenza (A/WSN/33) viral infection results in a transient host transcriptional shutdown, with host RNA polymerase II gene occupancy depleted genome-wide.\textsuperscript{49} There has been no evidence to date for a similar pattern in coronaviruses, but they do have RNA-dependent RNA polymerases, and therefore might interfere with RNA transcription. However, for H1N1 influenza (A/WSN/33), escape occurs after several hours, when the virus switches to replication mode.\textsuperscript{50} Given the prolonged time-course for COVID-19 infection, it would seem reasonable to assume that relatively normal host transcription will be in operation in some to most infected cells.

**SUMMARY**

In these unprecedented times, there are substantial existing resources of experimental biological data, currently residing unflagged in major repositories, and available for interrogation, informed by first-hand knowledge of human physiology and pathology in the setting of COVID-19.

These indicate that for patients who fail to suppress replication of the SARS-CoV-2 virus within a few days, delayed consequence of essential, post-infective homeostatic responses by SARS-CoV-2-infected cells could account for subsequent catastrophic deteriorations, mediated by defective host responses.
to reactive oxygen species, augmented generation of reactive oxygen species, and ROS-induced cellular injury. Further examination is warranted.
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Competing interests:
The authors have no competing interests to declare.

Ethical approvals:
None required. All data are in open source databases.

Author contributions:
Both authors performed literature searches, and designed the work based on clinical experience, particularly from MPV, and genetic experience, particularly from CLS. CLS performed the data analysis, generated the Figures and wrote the first draft. Both authors contributed to data interpretation, and manuscript revisions before joint approval.
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FIGURE LEGENDS

Figure 1: The AKR2 Gene Cluster

A) Cartoon representation of the X chromosome region flanking AKR2, drawn to scale, with coordinates for the two human genome builds in current use, GRCh37/hg19 and Build 38/hg38. Discrete genes and other loci are encoded by either the plus (+) or the minus (-) strand, and are indicated by boxes. The four AKR2 cluster genes are denoted in darker blue, with AKR2-related enhancer elements indicated in orange, with major read-through RNA transcripts indicated in pink below: PIR-FIGF<sup>22</sup>, known also as IncVEGFD-1.2<sup>23</sup> reads from PIR exon 2 to the 3’ untranslated region of VEGFD, skipping the final exon of PIR and first exon of VEGFD. There is also a read-through transcript between CLTRN and AKR2 (AC097625.2<sup>26</sup>) that skips the final exon of CLTRN and first exon of AKR2.

B) The interacting cluster of contiguous X chromosome genes (not to scale). Circle symbols are blue for protein coding genes, orange for double-elite enhancers, and purple for non-coding RNAs. Grey background indicates overlapping loci on current resolutions. Further, generally weaker interactions have been described and are illustrated for completeness below the core cluster box. Notably however, CA5B is regulated differently to AKR2 (data not shown).

Figure 2: Conceptual Model of AKR2 homeostatic responses

Black text indicates consequences of SARS-CoV-2 binding leading to AKR2 sequestration/internalisation, in addition to subsequent consequences of viral infection, and therapies. Green text indicates homeostatic responses to increase synthesis of the full length AKR2 mRNA, and thus protein. Off target consequences are predicted to increase PIR transcription, and thus impair reactive oxygen species (ROS) scavenging and increase ROS as described in the text. VEGFD consequences are expected to be pulmonary endothelial-specific, and additional cluster genes are not illustrated for clarity.
Figure 1: The ACE2 Gene Cluster. A) Cartoon representation of the X chromosome region flanking ACE2, drawn to scale, with coordinates for the two human genome builds in current use, GRCh37/hg19 and Build 38/hg38. Discrete genes and other loci are encoded by either the plus (+) or the minus (-) strand, and are indicated by boxes. The four ACE2 cluster genes are denoted in darker blue, with ACE2-related enhancer elements indicated in orange, with major read-through RNA transcripts indicated in pink below: PIR-FIGF, known also as lncVEGFD-1.2 reads from PIR exon 2 to the 3' untranslated region of VEGFD, skipping the final exon of PIR and first exon of VEGFD. There is also a read-through transcript between CLTRN and ACE2(AC097625.226) that skips the final exon of CLTRN and first exon of ACE2. B) The interacting cluster of contiguous X chromosome genes (not to scale). Circle symbols are blue for protein coding genes, orange for double-elite enhancers, and purple for non-coding RNAs. Grey background indicates overlapping loci on current resolutions. Further, generally weaker interactions have been described and are illustrated for completeness below the core cluster box. Notably however, CA5B is regulated differently to ACE2 (data not shown).
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