Title

Flow-Mediated Susceptibility and Molecular Response of Cerebral Endothelia to SARS-CoV-2 Infection.

Journal

Stroke, 52(1)

ISSN

0039-2499

Authors

Kaneko, Naoki
Satta, Sandro
Komuro, Yutaro
et al.

Publication Date

2021

DOI

10.1161/strokeaha.120.032764

Peer reviewed

HHS Public Access

Author manuscript

Stroke. Author manuscript; available in PMC 2022 January 01.

Published in final edited form as:

Stroke. 2021 January ; 52(1): 260–270. doi:10.1161/STROKEAHA.120.032764.

Flow-mediated Susceptibility and Molecular Response of Cerebral Endothelia to Sars-CoV-2 Infection

Naoki Kaneko, MD., Ph.D.[1,*], **Sandro Satta, Ph.D.**[2,*], **Yutaro Komuro, Ph.D.**[3], **Sree Deepthi Muthukrishnan, Ph.D.**[4], **Visesha Kakarla, B.S.**[5], **Lea Guo, B.S.**[1], **Jennifer An, B.S.**[3], **Fanny Elahi, M.D., Ph.D.**[6], **Harley I. Kornblum, M.D., PhD.**[4], **David S. Liebeskind, M.D.**[3], **Tzung Hsiai, M.D, Ph.D**[2,7], **Jason D. Hinman, M.D., Ph.D.**[3,7]

[1]Department of Radiological Sciences, David Geffen School of Medicine, University of California Los Angeles

[2]Department of Medicine, David Geffen School of Medicine, University of California Los Angeles

[3]Department of Neurology, David Geffen School of Medicine, University of California Los Angeles

[4]Intellectual and Developmental Disabilities Research Center, Semel Institute of Neuroscience, University of California Los Angeles

[5]School of Medicine, University of California San Diego

[6]Memory and Aging Center, University of California San Francisco

[7]Veterans Healthcare Administration, Greater Los Angeles Healthcare System

Abstract

BACKGROUND AND PURPOSE: Sars-CoV-2 infection is associated with an increased rate of cerebrovascular events including ischemic stroke and intracerebral hemorrhage. The mechanisms underlying cerebral endothelial susceptibility and response to Sars-CoV-2 are unknown yet critical to understanding the association of Sars-CoV-2 infection with cerebrovascular events.

METHODS: Endothelial cells were isolated from human brain and analyzed by RNA-sequencing. Human umbilical vein and human brain microvascular cells were used in both monolayer culture and endothelialized within a 3D-printed vascular model of the middle cerebral artery. Gene expression levels were measured by qPCR and direct RNA hybridization. Recombinant Sars-CoV-2 S-protein and S-protein containing liposomes were used to measure endothelial binding by immunocytochemistry.

RESULTS: ACE2 mRNA levels were low in human brain and monolayer endothelial cell culture. Within the 3D-printed vascular model, ACE2 gene expression and protein levels were progressively increased by vessel size and flow rates. Sars-CoV-2 S-protein containing liposomes

Corresponding Author: Jason D. Hinman, M.D., Ph.D., Department of Neurology, David Geffen School of Medicine, University of California Los Angeles, Neuroscience Research Building, Rm 415, 635 Charles E. Young, Dr. South, Los Angeles, CA 90095, jhinman@mednet.ucla.edu, Phone: 310-825-6761.

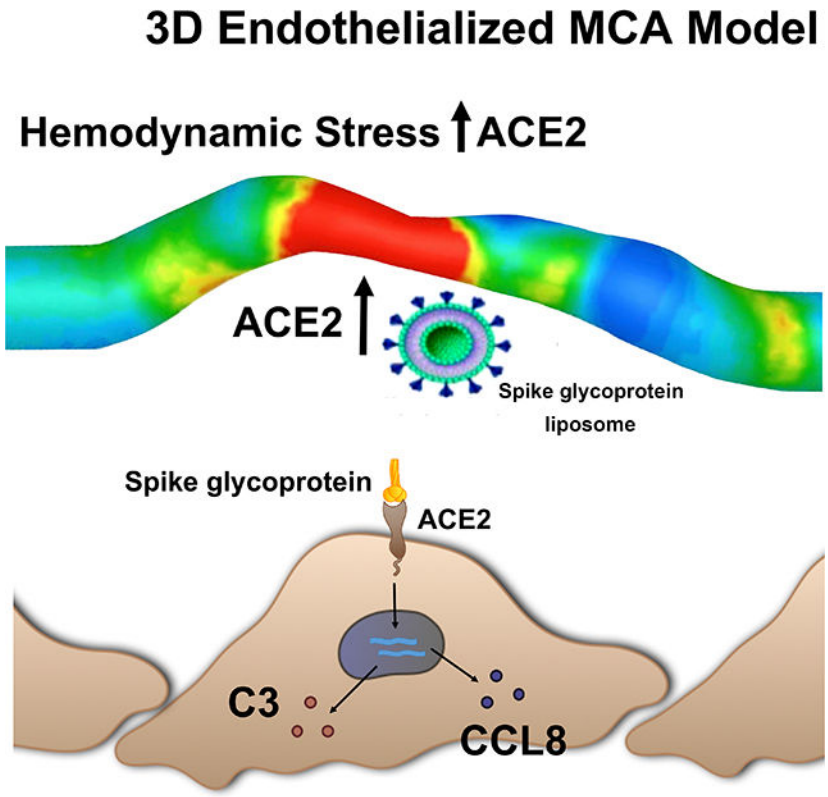
*These authors contributed equally to this work

Disclosures: On behalf of SS and TH, the UC Regents intends to submit a provisional patent for the generation of liposomes as detailed in the manuscript. DSL receives support for acting as an imaging core lab for Cerenovus, Genentech, Medtronic, and Stryker.

were detected in HUVECs and HBMECs in 3D-MCA models but not in monolayer culture consistent with flow-dependency of ACE2 expression. Binding of Sars-CoV-2 S-protein triggered 83 unique genes in human brain endothelial cells including up-regulation of complement component C3.

CONCLUSIONS: Brain endothelial cells are susceptible to direct Sars-CoV-2 infection through flow-dependent expression of ACE2. Viral S-protein binding triggers a unique gene expression profile in brain endothelia that may explain the association of Sars-CoV-2 infection with cerebrovascular events.

Graphical Abstract



Keywords

cerebral blood flow; vascular endothelium; vascular response; virus; experimental models; Sars-CoV-2; Ischemic Stroke; Cerebrovascular Disease/Stroke; Blood-brain Barrier

INTRODUCTION:

Infection with Sars-CoV-2 is associated with an increased rate of cerebrovascular events including ischemic stroke and intracerebral hemorrhage [1–3]. Comparisons of ischemic rates in Sars-CoV-2 infected individuals with historical averages of ischemic stroke rates combine with case series of atypical stroke patients to support an estimated seven-fold increase of stroke in Sars-CoV-2 infection [4, 5]. The mechanisms underlying this cerebrovascular risk are unknown. With infection initiated in the nasopharynx and lung, widespread inflammation

activated by this novel coronavirus may trigger a systemic pro-thrombotic state and circulating cytokines may act on blood-brain-barrier permeability [6], thereby increasing the risk of ischemic and/or hemorrhagic events. While these pathophysiologic mechanisms fit broadly with our understanding of cerebrovascular injury, direct infection of the brain endothelia by Sars-CoV-2 may trigger localized phenomena including thrombosis and cellular permeability. However, the factors that could mediate tropism of Sars-CoV-2 for the cerebral endothelia may help to identify individuals at high risk for cerebrovascular complications associated with Sars-CoV-2 and lessen the burden of stroke associated with this global pandemic.

In the lung, the cellular tropism of Sars-CoV-2 is mediated by viral S-protein binding to the human angiotensin converting enzyme-2 (ACE-2), followed by S-protein cleavage by the transmembrane protease, serine 2 (TMPRSS2) allowing viral entry [7]. ACE-2 expression is reported in both murine cerebral endothelia and pericytes [8–10] but there are important molecular differences in murine and human ACE-2 that limit the value of this observation. ACE-2 expression has been reported in human brain and Sars-CoV-2 RNA can be detected in post-mortem brain specimens [11]. However, the primary data supporting ACE-2 expression and Sars-CoV-2 susceptibility by the cerebral endothelia remain limited. Similarly, the important viral entry co-factor, TMPRSS2, is generally absent from human brain [10], making the true mechanisms of viral susceptibility unknown.

Here, we utilized a novel 3D-printed endothelialized model system to identify the factors that modulate human cerebrovascular expression of ACE2, how these factors drive susceptibility of brain endothelium to Sars-CoV-2 infection, and the effect of S-protein binding to the cerebral vasculature. We compared expression of ACE-2 and TMPRSS2 in human brain microvascular cells with that of human umbilical vein endothelial cells under conditions of shear stress in a 3D-printed model of the human middle cerebral artery. We tested the hypothesis that viral S-protein binding to brain microvascular cells is dependent not only on molecular interaction with ACE2 but also requires a flow-mediated stimulus. We further sought to identify the brain-specific effect on endothelial cell gene expression triggered by viral S-protein binding.

MATERIALS AND METHODS:

The authors declare that all supporting data are available within the article and its online supplementary files.

Patient samples:

We obtained fresh human brain tissue samples from children and adolescents (5–19 years old) undergoing brain surgeries (temporal or frontal lobe craniotomy) under an approved University of California, Los Angeles (UCLA) Institutional Review Board (IRB) protocol. The resected tissue consisted of small sections of healthy cortex or white matter, and were considered normal according to MRI, and electroencephalogram studies. Samples were collected immediately following surgery.

Magnetic activated cell sorting (MACS) for cell type enrichment:

We performed MACS using anti-CD31 Dynabeads (ThermoFisher scientific) according to manufacturer's protocol to enrich for normal vascular endothelial cells. 300–700mg of unsorted portions of normal brain white matter was used as controls. RNA extraction was performed immediately followed enzymatic dissociation or cell type isolation using Qiagen RNeasy Microkit. Total RNA integrity was examined using the Agilent Bioanalyzer 2000 (Agilent, Santa Clara, CA) and quantified with NanoDrop® (Thermo-Fischer, Waltham, MA).

RNA sequencing and analysis:

100ng of cDNA was used for library preparation using Ovation® Ultralow Library Systems. All samples were multiplexed into a single pool and sequenced with 50bp paired-end to get ~45 million reads per sample using Illumina HiSeq 2000 sequencer (Illumina, San Diego, CA). Quality control and alignment to the H. sapiens (Hg38) refSeq and gene annotation was performed using STAR. Total counts of read-fragments aligned to candidate gene regions were derived using HTSeq program with Human Hg38 as a reference and used as a basis for the quantification of gene expression. FPKM values were reported as measure of relative expression units.

Modeling—A human-specific intracranial artery stenosis model was created by modifying a prior method to build a brain aneurysm model.[12, 13] Digital Imaging and Communication in Medicine (DICOM) data acquired from CT angiography were exported, and the 3D vessel image was converted to a stereolithography format (.stl). The wall shear stress distribution, streamlines, and flow velocity were calculated and visualized for the model. Patient-specific vascular molds were fabricated using a Mojo 3D printer (Stratasys, Eden Prairie, Minnesota, USA). The vascular molds made of acrylonitrile butadiene styrene (ABS) were soaked in ABS solvent and chemically smoothed to remove the stair-like layers of the printed objects.[13] After drying, degassed polydimethylsiloxanes (PDMS) were coated and cured on the cast, and hollow stenosis models acquired by removing the mold.

Cell culture, endothelialization, and perfusion culture—Cell culture: Human umbilical vein endothelial cells (HUVECs) and human brain microvascular endothelial cells were cultured in Endothelial Cell Growth Medium 2, (PromoCell) and Complete Classic Medium With Serum and CultureBoost (Cell Cystems), respectively with gentamicin/amphotericin in an CO_2 incubator at 37°C, and used between passages 6 and 10. To enhance cell adhesion to silicone, silicone tubing and created vascular models were soaked in 10% (3-aminopropyl) trimethoxysilane in 100% ethanol overnight for hydrophiliazation, and in 0.1mM sulfosuccinimidyl-6-(4′-azido-2′-ni- trophenylamino)-hexanoate (sulfo-SANPAH) in water with the aid of UV activation (10min x two treatments; 365nm, 36 W, at a distance of 5cm) for cross-linking.[13] The models were washed with phosphate-buffered saline (PBS), and coated with fibronectin in PBS at 40 μg/mL. The tubing and vascular models were again washed with PBS and then exposed to UV light for sterilization before cell seeding. HUVECs or HBMECs were attached on the tubing and vascular models using a 3D rotating machine with two axes for 48 hours. The tubing or vessel models with endothelial lining were connected to silicone tubes and a perfusion pump, and then culture medium with the

viscosity similar to human blood (3.9 cP by dextran), was perfused for 24 hours in a CO_2 incubator.

qPCR—Triplicate samples of endothelial cell RNA were extracted using Qiagen RNeasy plus micro kit and reverse transcribed to cDNA using SuperScript VILO cDNA Synthesis Kit. qPCR performed using TaqMan Gene Expression Assays (hACE2 #4331182; TMPRSS2 #4331182; GAPDH #4453320) and relative expression determined using ΔΔCt method and results compared by two-way ANOVA (p<0.05).

Liposome Spike S1 Preparation—Rhodamine containing liposomes (Encapsula Nanoscience) prepared with Immunosome-Biotinyl Cap were coupled to Sars-CoV-2 S1 Protein, His, Avitag (Acrobiosystem #S1N-C82E8) in a 1:2 ratio and excess S1 protein dialyzed out from the liposome prep. Control liposome preparations were prepared identically in the absence of Sars-CoV-2 S1 protein. Binding of S-protein liposomes to ACE2 was verified by modified ELISA and in ACE2-transfected HEK293T cells. Liposome preparations were diluted to 1:500 prior to use, added to cultures or perfusion culture media for 24 hrs. Additional details available in Supplemental Material.

Immunocytochemistry—Endothelial cells were rinsed with PBS, fixed with 2% paraformaldehyde and multi-stained with target proteins and DAPI (nucleus). To observe the ECs on the curved PDMS surface, z stacks were taken every 10 μm to approximately 800 μm thickness and 2D maximum intensity projection images were rendered. Mean levels of ACE2 immunostaining in a 1x1 mm field of view (n=2) were measured and normalized to phalloidin signal.

Nanostring nCounter assay—Monolayer cultures of HBMECs and HUVECs were treated with 5 μg/mL of recombinant Sars-CoV-2 Spike (Active Trimer) His Protein (R&D Systems #10549-CV) for 24 hrs. Cells were lysed, RNA collected as above, and 100 ng of total RNA was hybridized with probes and run on the nCounter preparation station for removal of excess probes per manufacturer protocol. The number of the target mRNA transcripts was directly counted by digital analyzer. The data was normalized to the geometric means of spiked-in positive controls and spiked-in negative controls, and the housekeeping genes. The nSolver software from NanoString was used to normalize count numbers by geometric mean and calculate the fold change and statistical significance, with p<0.05 considered significant. Data were visualized using R.

RESULTS:

While ACE2 expression has been reported in the human brain, the cell-specific expression pattern of ACE2 is unknown. We isolated CD31+ human endothelial cells from normal brain tissue resected during surgery and performed RNA-seq (n=2). ACE2 levels were below the threshold of detection in both isolated endothelial cells and whole brain white matter indicating low transcriptional expression (Fig. 1A). Similarly, recognized Sars-CoV-2 viral entry co-factor, TMPRSS2, demonstrated very low transcript detection in isolated CD31+ endothelial cells. Other Sars-CoV-2 viral entry co-factors including plasminogen (PLG) and furin were differentially detected with furin robustly expressed in CD31+ isolated

endothelial cells. Existing databases of cultured endothelial cell gene expression demonstrate low levels of ACE2 [14].

To determine the effect of vessel size and flow on ACE2 expression, we created a series of 3D vessel models of varying diameter (2–4 mm), endothelialized the luminal surface with human umbilical vein endothelial cells (HUVECs) and subjected these to 24 hr of perfusion culture (3.9 cP/15 dynes/cm^2). Compared to monolayer HUVECs, vessel size drove a significant increase in ACE2 gene expression (p<0.0001, F=55.79, one-way ANOVA, Fig. 1B). Shear stress in addition to 3D rotational culture further increased ACE2 mRNA levels. To identify the differential effect of shear stress on ACE2 expression, we used a 4 mm vessel endothelialized with HUVECs or human brain microvascular endothelial cells (HBMECs) and subjected these models to normal (15 dynes/cm^2) and high (22.5 dynes/cm^2) shear stress for 24 hrs. Compared to monolayer cells, shear stress induced ACE2 gene expression in both HUVECs and HBMECs (p<0.0001, F=153.6, two-way ANOVA, Fig. 1C). The application of any shear stress triggered ACE2 expression in HUVECS (adjusted p<0.0001), while in HBMECs the effect of higher shear stress significantly increased ACE2 expression (adjusted p=0.012). Both monolayer HUVECs and HBMECs demonstrated low levels of ACE2 protein expression in cytoplasmic and perinuclear space by immunocytochemistry (Fig. 1D). As in the 3D model system, stimulation of monolayer HUVECs and HBMECs with horizontal shear stress increased ACE2 protein expression by western blot (Supplemental Fig. I). Consistent with human brain RNA-seq, expression of TMPRSS2 could not be reliably detected at the RNA or protein level in HBMECs across multiple experiments.

Based on the observation that ACE2 expression is triggered by vessel structure and shear stress, we used a CTA image of a partially stenosed middle cerebral artery to 3D-print a vessel replica and create a silicone model with the luminal surface lined with HBMECs (Fig. 2A). Immunocytochemistry for ACE2 was performed in proximal, stenotic, and distal segments of the MCA model corresponding to regions of normal (~15 dynes/cm^2), high (~30 dynes/cm^2), and low (<4 dynes/cm^2) wall shear stress determined by computational fluid dynamics (Fig. 2B). Normalized levels of ACE2 protein expression were determined and found to be significantly increased in the 3D model compared to monolayer cells (p=0.043, F=7.27, one-way ANOVA). ACE2 expression levels within the stenotic portion of the MCA model were notably higher when compared to monolayer cells (adjusted p=0.046; Fig. 2C).

The spike protein (S-protein) of Sars-CoV-2 binds directly to ACE2 and mediates viral attachment and cellular entry. To determine cerebral endothelial susceptibility to Sars-CoV-2 infection, we prepared S-protein coated liposomes containing fluorescent rhodamine measuring approximately 100 nm in size (Supplemental Fig. II & III). S-protein liposomes bind to hACE2 and can be detected by rhodamine liposome signal in an ACE2 ELISA (Fig. 3A). Control and S-protein liposomes were added to monolayer cultures of HUVECs and HBMECs. We observed no measurable cellular detection of S-protein liposomes in either cell type (Fig. 3B). Forty-eight hours after endothelialization, we exposed 3D MCA models to 24 hrs of flow culture with control and S-protein liposome. Primarily within the stenotic portion of the 3D MCA model, S-protein liposomes were found bound to HBMECs while flow culture with control liposomes were not detected (Fig. 3C).

To determine the brain endothelial-specific response of S-protein binding, we used a direct RNA hybridization assay measuring mRNA expression levels of pre-specified 785 host response genes involved in pathways of homeostasis, adaptive immune response, host susceptibility, and interferon response. HBMEC exposure to recombinant S-protein trimer for 24 hrs resulted in differential expression of 24 genes (14 up-; 10 down-regulated) (Fig. 4A), while the response to recombinant S-protein in HUVECs was less robust (17 genes: 4 up-; 13 down-regulated) (Supplemental Data). After normalizing across cell types, a distinct brain endothelial specific gene expression profile was identified containing 80 genes (12 up-; 68 down-regulated) (Fig. 4B). Selected genes (C3, CTSW) differentially expressed in HBMECs were confirmed as up-regulated after both S-protein and liposome exposure by qPCR (Supplemental Fig. IV). The key up-regulated genes by human brain endothelial cells and their association with Sars-CoV-2 infection are presented in Table 1 [15–19].

DISCUSSION:

This study establishes two critical observations about the potential relationship between Sars-CoV-2 and cerebrovascular susceptibility to infection and the brain endothelia-specific response triggered by infection. First, we show that normal ACE2 gene expression by endothelial cells in human brain is very low. In a related fashion, we show that ACE2 expression is increased by shear stress and that this flow-mediated increase in ACE2 facilitates susceptibility to Sars-CoV-2 Spike Protein binding to brain endothelia. Secondly, we identified a unique gene expression profile within brain endothelia in response to Spike Protein binding. While prior work has sought to use gene and protein expression levels of ACE2 in mouse and human vascular cell types as a surrogate for viral susceptibility, they have not utilized a dynamic flow-dependent model to directly demonstrate viral-endothelial interactions, which we achieved using a novel S-protein coated liposome. With a multiplicity of cerebrovascular pathologies associated with severe Sars-CoV-2 infection including ischemic stroke affecting large and small caliber vessels, microhemorrhages, and hemorrhagic encephalitis [1, 5, 20–24], there is an urgent need to identify the mechanisms that may mediate this increased risk of cerebrovascular events [25265]. A key discriminating factor in these distinct pathologies are differences in arterial pulsatile or venous steady flow in the brain that may play critical roles in driving viral susceptibility. Thus, because ACE2 levels are relatively low in the brain, we hypothesized that viral susceptibility in the brain may be dependent on additional factors such as fluid shear stress that are difficult to equilibrate with human physiology in monolayer cell culture and even in animal models. Furthermore, we anticipated that brain endothelia may harbor a distinct response to Sars-CoV-2 infection that may be useful in determining individual risk for cerebrovascular complications.

Similar to other coronaviruses, Sars-CoV-2 appears to use the human ACE2 protein to gain entry to cells [7]. In the lung, this viral internalization process also requires the enzymatic activity of TMPRSS2 to cleave the viral spike protein enabling viral entry [7]. Using human post-mortem tissue expression and antibody binding, a single previous study has suggested that ACE2 is expressed in human endothelial cells including those in brain [11]; however this study lacked specific images demonstrating ACE2 expression in the brain nor did it specify the vessel size or pattern of ACE2 expression. Gene expression databases from mouse and human provide conflicting data on ACE2 expression but do suggest vascular or perivascular

cell expression of ACE2, making the brain endothelia a likely target of circulating virus. In contrast, no data exist on TMPRSS2 expression in vascular endothelium. Variable expression of ACE2 in cerebrovascular endothelia may be one key factor driving the association of Sars-CoV-2 infection and stroke. Here, we show that in MACS isolated endothelial cells from normal human brain, moderate depth RNA-sequencing did not identify robust gene expression of ACE2 nor TMPRSS2. Alternative viral co-factors including furin appear enriched in brain endothelia. Using qPCR from cultured brain endothelial cells, ACE2 mRNA was detectable, though at low levels and TMPRSS2 was not detected. At the protein level, ACE2 is detectable in cultured brain endothelial cells while TMPRSS2 is absent. Together, these findings suggest that the basal gene expression of ACE2 in the human brain vasculature is low and that alternative mechanisms for viral co-entry to those utilized by Sars-CoV-2 in the lung must be present in the brain.

Critically, using a 3D-printed vessel replica system, we demonstrate that ACE2 gene expression is regulated by fluid shear stress in both undifferentiated endothelial cells and brain microvascular endothelia. A recent report observed that pulsatile shear stress in vascular endothelial cells promotes ACE2 expression and can reduce vascular inflammatory pathways [27]. The present findings are consistent with this prior observation and demonstrate that this response is conserved in brain endothelia. In animal models of systemic atherosclerosis, over-expression of ACE2 is associated with a reduction in atherogenic processes and a reduction in the progression of atherosclerotic plaque thickness [28, 29]. In part, this may be due to the local metabolism of angiotensin-II by ACE2 generating Angiotensin-1,7 which exerts action on the Mas receptor to inhibit NF-κB inflammatory cascades [30]. Our findings of pulsatile shear stress triggering an increase in ACE2 expression by brain endothelia suggest that local increases in ACE2 levels as a result of high blood pressure or intracranial atherosclerosis may increase the susceptibility to Sars-CoV-2 infection of brain endothelia.

Direct viral infection of the endothelium has been reported in multiple organs in Sars-CoV-2 infected patients [31] but data are lacking to support direct viral invasion in the cerebral endothelium. Using the 3D-printed vessel replica model, we demonstrated that S-protein-containing liposomes designed to be approximately the size of viral particles (100 nm) and demonstrated to bind to ACE2 can interact with brain endothelia during pulsatile flow. This interaction was not present in monolayer endothelial cells for either HUVECs or HBMECs despite low but detectable levels of ACE2. This suggests that viral-endothelial interactions are at least partially regulated by the dynamic process of blood flow perhaps through a threshold level of ACE2 expression, which we showed is increased in stenotic regions of blood vessels. Using immunocytochemistry, we could not reliably distinguish ACE2 levels between liposome positive cells as opposed to those without liposome binding, however this deserves additional study. While not precisely comparable to *in vivo* viral-cell interactions, because this *ex vivo* model uses patient-derived imaging sequences to replicate vessel segments, it is a useful tool to determine what variant cerebrovascular anatomies (stenosis, aneurysms, etc) can increase or decrease viral-endothelial interactions and can provide a moderate through-put platform for therapeutic testing. In addition, we anticipate being able to use this system to determine the enzymatic co-factors needed for viral entry that appear unique in brain endothelia in contrast with other organs.

After establishing the flow-dependency of S-protein binding to brain endothelia, we sought to identify the differential response of brain endothelia to Sars-CoV-2 infection. Viral infection with Sars-CoV-2 has been associated with a marked cytokine surge that can potentially increase local and distant thrombotic events [32]. Here, we show that simple engagement of ACE2 by recombinant S-protein can regulate distinct gene expression patterns in brain endothelia. While this study pre-specified a number of immune regulatory genes, S-protein exposure triggers up-regulation of a number of genes already implicated in Sars-CoV-2 infection including IL-4 and IL-10 signaling [33–35]. S-protein binding also appears to down-regulate several important pathways in brain endothelia including the inflammasome and apoptotic pathways, potentially facilitating infected cell survival and viral replication. Identification of a brain endothelia-specific gene expression profile in response to Sars-CoV-2 S-protein exposure is critical to understanding the response of the cerebral vasculature to viral infection. This brain endothelial-specific profile includes several important up-regulated pathways. Notably, the most significant up-regulated gene by HBMECs compared to HUVECs is C3. The C3 factor plays a central role in the complement cascade, involved in both alternative and classical pathways. When C3 is cleaved into C3a and C3b, the C3a moiety serves as a potent cytokine amplifying the inflammatory signaling, while the binding of C3b to cellular surfaces in combination with other complement factors can lead to cellular destruction. This signal for complement activation already has direct relevance to stroke with C3-null mice demonstrating reduced neuroinflammation in response to infection[36], infarct volumes[37], post-stroke inflammation[38], and human subject evidence for plasma exosomes enriched with C3b as a biomarker of cerebrovascular disease [39]. IL-27 is induced after intracerebral hemorrhage and hones neutrophil signaling to promote brain repair [40]. Up-regulation of EBI3, a component of functional IL-27, suggests this neuroprotective signaling cascade is activated by S-protein binding. Circulating levels of CCL8 are increased in the ferret model of Sars-CoV-2 infection and persist after the viral infection resolves[15]. IL-15 is an immunoregulatory cytokine with strong anti-viral properties[41], for which anti-IL-15 superagonists are currently in clinical trial in Sars-CoV-2 infected individuals (NCT04324996). That viral S-protein alone can trigger a brain endothelial-specific response is significant in that it implies unique properties of the S-protein:ACE2 interaction that go beyond activation of the renin-angiotensin system. Secondly, because many of these differentially regulated genes are likely secreted by brain endothelial cells, it may allow for identification of Sars-CoV-2 infected individuals at risk for cerebrovascular complications through serum/plasma monitoring.

Overall, a small number of genes were differentially regulated in response to S-protein exposure. This may be a result of a relatively short incubation period, the use of a targeted gene expression array, and the relatively low basal expression of ACE2 in monolayer HBMECs. Despite this, gene expression differences were detectable and generally fit with the current understanding of how brain endothelial cells respond to other viral infections. Presumably, cellular infection by encapsulated Sars-CoV-2 virus in the presence of shear stress would trigger a more robust gene expression profile that may further identify brain-specific pathways activated by Sars-CoV-2 that can inform our understanding of the cerebrovascular tropism and response to this novel and unique virus.

This study has several important limitations. Firstly, binding of S-protein liposomes to the vessel wall in our 3D model could be driven by mechanisms other than S-protein:ACE2 interactions. While this is the primary molecular mechanism for cellular entry of Sars-CoV-2, other mechanisms may mediate cellular binding under shear stress. Though the S-protein liposomes we generated for this study have high affinity for ACE2 by ELISA, we did not definitively demonstrate that S-protein liposomes require ACE2 for binding in the 3D MCA model, as might be demonstrated by ACE2 knockdown or blocking. Proposed alternative mechanisms including basigin/CD147 have not been convincingly shown to mediate viral:cell interactions [42]. Despite this, other molecular mechanisms may mediate the interaction between Sars-CoV-2 and brain endothelia. This could be clarified by specific S-protein binding experiments targeting the brain endothelia. Secondly, ACE2 expression is increased by culturing in 3D using fibronectin as a binding agent even in the absence of flow. While we used monolayer endothelial cultures for comparison in many of these studies, realistic vessel modeling comparing flow vs. no flow paradigms may create a more biologically meaningful system for the study of Sars-CoV-2:ACE2 interactions in endothelial cells. Nonetheless, our work highlights the need to appropriately model the organ-specific complications of Sars-CoV-2 infection. In the context of stroke, the effects of blood flow on brain endothelia are significant and functionally alter biologic properties including the susceptibility to Sars-CoV-2 infection. Recognizing this along with the known unique biologic properties of brain endothelia compared to endothelia in other organs, we were able to identify a distinct gene expression profile in brain endothelia that may be useful in determining individualized risk for stroke and other cerebrovascular complications increasingly common during the Sars-CoV-2 pandemic.

SUMMARY/CONCLUSIONS:

The Sars-CoV-2 cellular receptor ACE2 is poorly expressed by human brain endothelia but can be triggered in a flow-dependent manner. Because of flow-dependent ACE2 expression, cerebral vessels are susceptible to Sars-CoV-2 infection and bind viral S protein under shear stress but likely require unique co-factors for viral entry. Binding of viral S protein triggers a unique gene expression profile in brain endothelia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

The authors are indebted to the UCLA COVID-19 Basic, Translational, and Clinical Science Team for intellectual stimulation.

Sources of Funding: This project was supported by a Rapid COVID-19 Response Grant from the American Heart Association #20203858 (NK, TH, JDH), the UCLA W. M. Keck Foundation COVID 19 Research Award Program (NK, JDH), the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation (SDM, HIK), NINDS NS112799 (NK, DSL, JDH), NHLBI HL118650 and HL149808 (SS, TH), and the Veterans Healthcare Administration I01 BX004356 (TH) (JDH).

Non-standard Abbreviations and Acronyms

ACE2	Angiotensin converting enzyme-2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HBMEC	Human brain microvascular endothelial cells
HUVEC	Human umbilical vein endothelial cells
TMPRSS2	Transmembrane protease, serine 2
MACS	Magnetic activated cell sorting
MCA	Middle cerebral artery

References

1. Varatharaj A, Thomas N, Ellul MA, Davies NWS, Pollak TA, Tenorio EL, Sultan M, Easton A, Breen G, Zandi M, et al. Neurological and neuropsychiatric complications of covid-19 in 153 patients: A uk-wide surveillance study. Lancet Psychiatry 2020
2. Tian J, Yuan X, Xiao J, Zhong Q, Yang C, Liu B, Cai Y, Lu Z, Wang J, Wang Y, et al. Clinical characteristics and risk factors associated with covid-19 disease severity in patients with cancer in wuhan, china: A multicentre, retrospective, cohort study. Lancet Oncol 2020
3. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, Xiang J, Wang Y, Song B, Gu X, et al. Clinical course and risk factors for mortality of adult inpatients with covid-19 in wuhan, china: A retrospective cohort study. Lancet 2020;395:1054–1062 [PubMed: 32171076]
4. Merkler AE, Parikh NS, Mir S, Gupta A, Kamel H, Lin E, Lantos J, Schenck EJ, Goyal P, Bruce SS, et al. Risk of ischemic stroke in patients with coronavirus disease 2019 (covid-19) vs patients with influenza. JAMA Neurol 2020
5. Oxley TJ, Mocco J, Majidi S, Kellner CP, Shoirah H, Singh IP, De Leacy RA, Shigematsu T, Ladner TR, Yaeger KA, et al. Large-vessel stroke as a presenting feature of covid-19 in the young. N Engl J Med 2020;382:e60 [PubMed: 32343504]
6. Teuwen LA, Geldhof V, Pasut A, Carmeliet P. Covid-19: The vasculature unleashed. Nat Rev Immunol 2020
7. Hoffmann M, Kleine-Weber H, Schroeder S, Kruger N, Herrler T, Erichsen S, Schiergens TS, Herrler G, Wu NH, Nitsche A, et al. Sars-cov-2 cell entry depends on ace2 and tmprss2 and is blocked by a clinically proven protease inhibitor. Cell 2020;181:271–280 e278 [PubMed: 32142651]
8. Qi J, Zhou Y, Hua J, Zhang L, Bian J, Liu B, Zhao Z, Jin S. The scrna-seq expression profiling of the receptor ace2 and the cellular protease tmprss2 reveals human organs susceptible to covid-19 infection. bioRxiv 2020
9. He L, Mäe MA, Sun Y, Muhl L, Nahar K, Liébanas EV, Fagerlund MJ, Oldner A, Liu J, Genové G, et al. Pericyte-specific vascular expression of sars-cov-2 receptor ace2 – implications for microvascular inflammation and hypercoagulopathy in covid-19 patients. bioRxiv 2020
10. Zhang Y, Chen K, Sloan SA, Bennett ML, Scholze AR, O'Keeffe S, Phatnani HP, Guarnieri P, Caneda C, Ruderisch N, et al. An rna-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. J Neurosci 2014;34:11929–11947 [PubMed: 25186741]
11. Hamming I, Timens W, Bulthuis ML, Lely AT, Navis G, van Goor H. Tissue distribution of ace2 protein, the functional receptor for sars coronavirus. A first step in understanding sars pathogenesis. J Pathol 2004;203:631–637 [PubMed: 15141377]
12. Kaneko N, Mashiko T, Ohnishi T, Ohta M, Namba K, Watanabe E, Kawai K. Manufacture of patient-specific vascular replicas for endovascular simulation using fast, low-cost method. Scientific reports 2016;6:39168 [PubMed: 27976687]

13. Kaneko N, Mashiko T, Namba K, Tateshima S, Watanabe E, Kawai K. A patient-specific intracranial aneurysm model with endothelial lining: A novel in vitro approach to bridge the gap between biology and flow dynamics. J Neurointerv Surg 2018;10:306–309 [PubMed: 28652298]
14. Khan S, Taverna F, Rohlenova K, Treps L, Geldhof V, de Rooij L, Sokol L, Pircher A, Conradi LC, Kalucka J, et al. Endodb: A database of endothelial cell transcriptomics data. Nucleic Acids Res 2019;47:D736–D744 [PubMed: 30357379]
15. Blanco-Melo D, Nilsson-Payant BE, Liu WC, Uhl S, Hoagland D, Moller R, Jordan TX, Oishi K, Panis M, Sachs D, et al. Imbalanced host response to sars-cov-2 drives development of covid-19. Cell 2020;181:1036–1045 e1039 [PubMed: 32416070]
16. Gralinski LE, Sheahan TP, Morrison TE, Menachery VD, Jensen K, Leist SR, Whitmore A, Heise MT, Baric RS. Complement activation contributes to severe acute respiratory syndrome coronavirus pathogenesis. mBio 2018;9
17. Schulte-Schrepping J, Reusch N, Paclik D, Bassler K, Schlickeiser S, Zhang B, Kramer B, Krammer T, Brumhard S, Bonaguro L, et al. Severe covid-19 is marked by a dysregulated myeloid cell compartment. Cell 2020;182:1419–1440 e1423 [PubMed: 32810438]
18. Di Giorgio S, Martignano F, Torcia MG, Mattiuz G, Conticello SG. Evidence for host-dependent rna editing in the transcriptome of sars-cov-2. Sci Adv 2020;6
19. de Aquino MT, Kapil P, Hinton DR, Phares TW, Puntambekar SS, Savarin C, Bergmann CC, Stohlman SA. Il-27 limits central nervous system viral clearance by promoting il-10 and enhances demyelination. J Immunol 2014;193:285–294 [PubMed: 24890725]
20. Ghosh R, Dubey S, Finsterer J, Chatterjee S, Ray BK. Sars-cov-2-associated acute hemorrhagic, necrotizing encephalitis (ahne) presenting with cognitive impairment in a 44-year-old woman without comorbidities: A case report. Am J Case Rep 2020;21:e925641 [PubMed: 32799213]
21. Morassi M, Bagatto D, Cobelli M, D'Agostini S, Gigli GL, Bna C, Vogrig A. Stroke in patients with sars-cov-2 infection: Case series. J Neurol 2020;267:2185–2192 [PubMed: 32436105]
22. Katal S, Balakrishnan S, Gholamrezanezhad A. Neuroimaging and neurologic findings in covid-19 and other coronavirus infections: A systematic review in 116 patients. J Neuroradiol 2020
23. Delamarre L, Gollion C, Grouteau G, Rousset D, Jimena G, Roustan J, Gaussiat F, Aldige E, Gaffard C, Duplantier J, et al. Covid-19-associated acute necrotising encephalopathy successfully treated with steroids and polyvalent immunoglobulin with unusual igg targeting the cerebral fibre network. J Neurol Neurosurg Psychiatry 2020;91:1004–1006 [PubMed: 32651243]
24. Bridwell R, Long B, Gottlieb M. Neurologic complications of covid-19. Am J Emerg Med 2020;38:1549 e1543–1549 e1547
25. Aggarwal G, Lippi G, Michael Henry B. Cerebrovascular disease is associated with an increased disease severity in patients with coronavirus disease 2019 (covid-19): A pooled analysis of published literature. Int J Stroke 2020:1747493020921664
26. Merkler AE, Parikh NS, Mir S, Gupta A, Kamel H, Lin E, Lantos J, Schenck EJ, Goyal P, Bruce SS, et al. Risk of ischemic stroke in patients with covid-19 versus patients with influenza. medRxiv 2020
27. Song J, Hu B, Qu H, Wang L, Huang X, Li M, Zhang M. Upregulation of angiotensin converting enzyme 2 by shear stress reduced inflammation and proliferation in vascular endothelial cells. Biochem Biophys Res Commun 2020;525:812–818 [PubMed: 32169277]
28. Zhang YH, Hao QQ, Wang XY, Chen X, Wang N, Zhu L, Li SY, Yu QT, Dong B. Ace2 activity was increased in atherosclerotic plaque by losartan: Possible relation to anti-atherosclerosis. J Renin Angiotensin Aldosterone Syst 2015;16:292–300 [PubMed: 25070352]
29. Dong B, Zhang C, Feng JB, Zhao YX, Li SY, Yang YP, Dong QL, Deng BP, Zhu L, Yu QT, et al. Overexpression of ace2 enhances plaque stability in a rabbit model of atherosclerosis. Arterioscler Thromb Vasc Biol 2008;28:1270–1276 [PubMed: 18403726]
30. Ingraham NE, Lotfi-Emran S, Thielen BK, Techar K, Morris RS, Holtan SG, Dudley RA, Tignanelli CJ. Immunomodulation in covid-19. Lancet Respir Med 2020
31. Varga Z, Flammer AJ, Steiger P, Haberecker M, Andermatt R, Zinkernagel AS, Mehra MR, Schuepbach RA, Ruschitzka F, Moch H. Endothelial cell infection and endotheliitis in covid-19. Lancet 2020;395:1417–1418 [PubMed: 32325026]

32. Sun X, Wang T, Cai D, Hu Z, Chen J, Liao H, Zhi L, Wei H, Zhang Z, Qiu Y, et al. Cytokine storm intervention in the early stages of covid-19 pneumonia. Cytokine Growth Factor Rev 2020
33. Wen W, Su W, Tang H, Le W, Zhang X, Zheng Y, Liu X, Xie L, Li J, Ye J, et al. Immune cell profiling of covid-19 patients in the recovery stage by single-cell sequencing. Cell Discov 2020;6:31 [PubMed: 32377375]
34. Han H, Ma Q, Li C, Liu R, Zhao L, Wang W, Zhang P, Liu X, Gao G, Liu F, et al. Profiling serum cytokines in covid-19 patients reveals il-6 and il-10 are disease severity predictors. Emerg Microbes Infect 2020;9:1123–1130 [PubMed: 32475230]
35. Akbari H, Tabrizi R, Lankarani KB, Aria H, Vakili S, Asadian F, Noroozi S, Keshavarz P, Faramarz S. The role of cytokine profile and lymphocyte subsets in the severity of coronavirus disease 2019 (covid-19): A systematic review and meta-analysis. Life Sci 2020;258:118167 [PubMed: 32735885]
36. Wu F, Zou Q, Ding X, Shi D, Zhu X, Hu W, Liu L, Zhou H. Complement component c3a plays a critical role in endothelial activation and leukocyte recruitment into the brain. J Neuroinflammation 2016;13:23 [PubMed: 26822321]
37. Mocco J, Mack WJ, Ducruet AF, Sosunov SA, Sughrue ME, Hassid BG, Nair MN, Laufer I, Komotar RJ, Claire M, et al. Complement component c3 mediates inflammatory injury following focal cerebral ischemia. Circ Res 2006;99:209–217 [PubMed: 16778128]
38. Yang J, Ahn HN, Chang M, Narasimhan P, Chan PH, Song YS. Complement component 3 inhibition by an antioxidant is neuroprotective after cerebral ischemia and reperfusion in mice. J Neurochem 2013;124:523–535 [PubMed: 23199288]
39. Elahi FM, Harvey D, Altendahl M, Casaletto KB, Fernandes N, Staffaroni AM, Maillard P, Hinman JD, Miller BL, DeCarli C, et al. Endothelial-derived exosomes demonstrate a link between endothelial innate inflammation and brain dysfunction and injury in aging. bioRxiv 2019
40. Zhao X, Ting SM, Liu CH, Sun G, Kruzel M, Roy-O'Reilly M, Aronowski J. Neutrophil polarization by il-27 as a therapeutic target for intracerebral hemorrhage. Nat Commun 2017;8:602 [PubMed: 28928459]
41. Verbist KC, Klonowski KD. Functions of il-15 in anti-viral immunity: Multiplicity and variety. Cytokine 2012;59:467–478 [PubMed: 22704694]
42. Shilts J, Wright GJ. No evidence for basigin/cd147 as a direct sars-cov-2 spike binding receptor. bioRxiv 2020

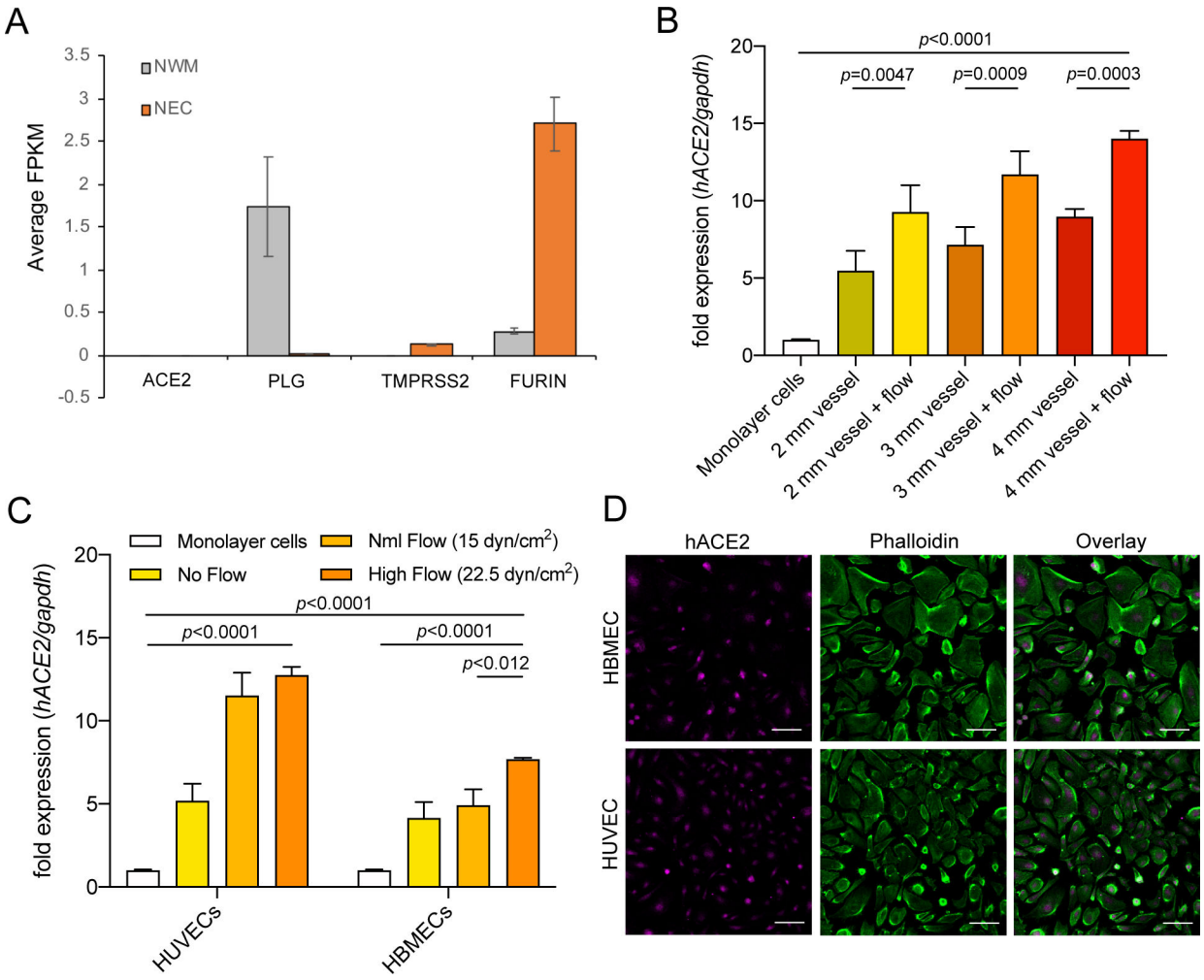


Figure 1. Flow-dependent ACE2 expression in cerebral endothelial cells.

Average FPKM of gene expression derived from RNA-sequencing of normal white matter (NWM; gray) and normal CD31+ endothelial cells (NEC; orange) (n=2) (A). Fold-expression by qPCR for human ACE2 (hACE2) relative to GAPDH in HUVEC cells by vessel size and shear stress (15 dynes/cm^2) (p<0.0001 by ANOVA; B). Fold-expression levels of HUVECs and HBMECs low shear stress vs. high shear stress perfusion culture (p<0.0001 by two-way ANOVA; adjusted p-values provided Tukey's post-hoc; C). Immunocytochemistry for ACE2 in monolayer HBMECs and HUVECs (D). Error bars represent S.D. Scale bars = 100 μm.

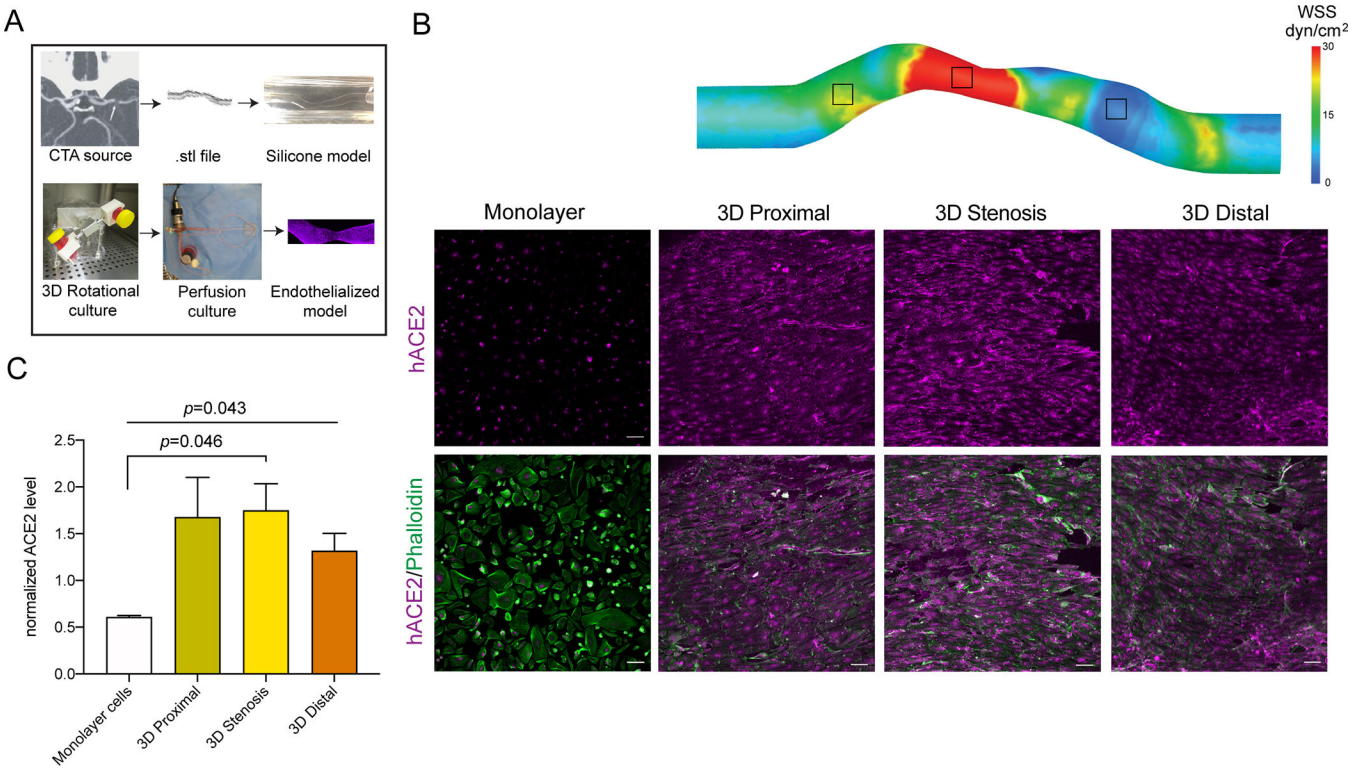


Figure 2. Regional variation in ACE2 levels using a 3D-printed MCA model.

Workflow to generate 3D-printed MCA stenosis model (A). Representative immunocytochemistry for ACE2 in monolayer HBMECs as well as proximal, stenotic, and distal segments of a human brain microvascular endothelialized middle cerebral artery 3D model with computational fluid dynamic modeling of wall shear stress (WSS) across the stenotic model (B). Normalized ACE2 levels (ACE2/Phalloidin ratio) in monolayer and segments of MCA 3D model (p=0.043 by one-way ANOVA; adjusted p=0.046 by Tukey's post-hoc comparison of 2D vs. 3D culture; C). Scale bars = 100 μm.

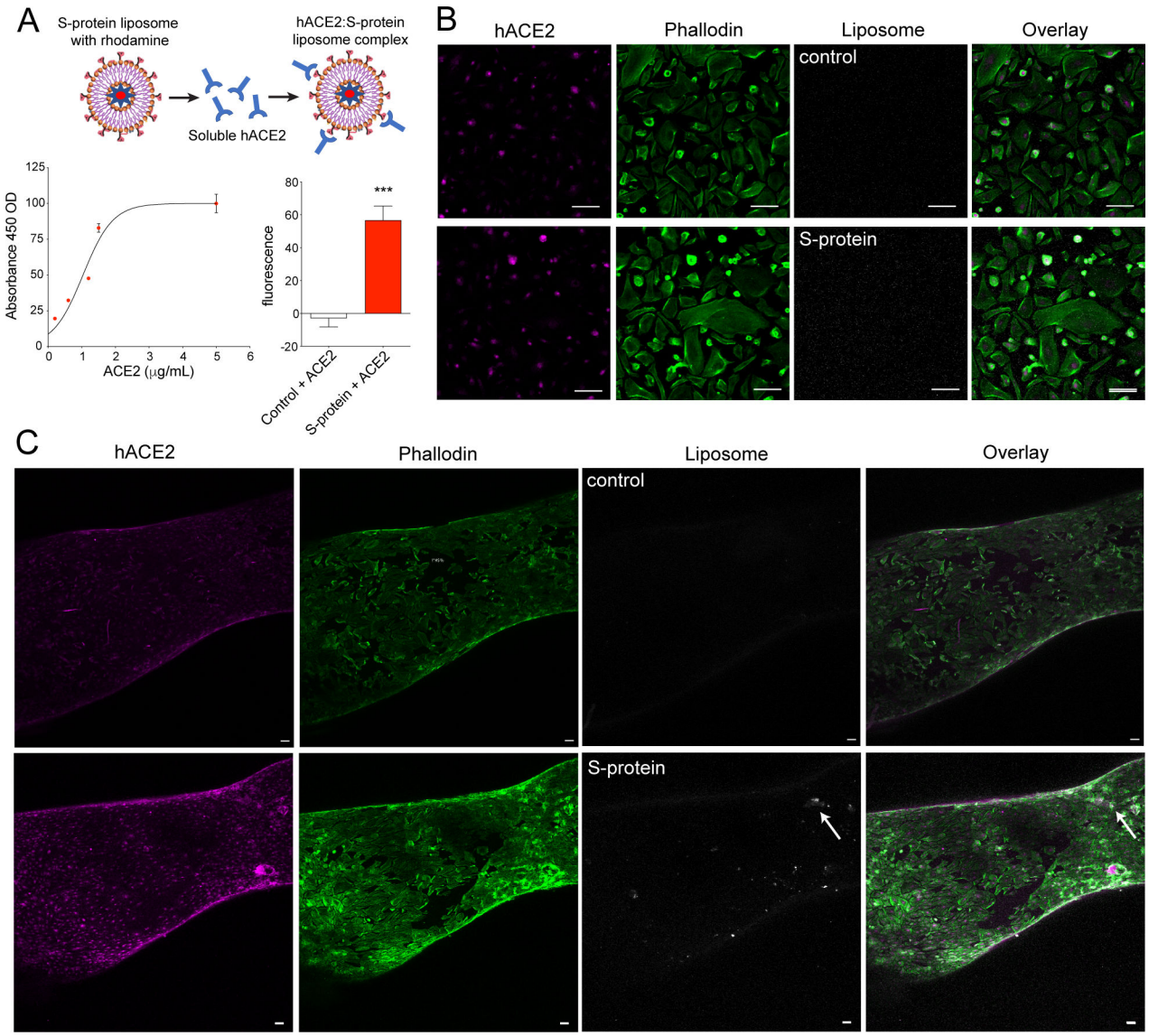


Figure 3. Flow-dependent susceptibility of brain endothelia to Sars-CoV-2 Spike Protein liposomes.

Liposomes coated with S-protein were created as described, incubated with soluble hACE2 (0–5 μg) and S-protein:ACE2 binding detected by ACE2:S-protein sandwich ELISA (left) and intrinsic rhodamine signal (right). Monolayer HBMECs were exposed to control (upper) or S-protein coated liposomes (lower) (1:100) for 24 hrs followed by immunocytochemistry for hACE2 (purple), phalloidin (green) and liposome bound rhodamine (white) (B). Maximum intensity projection of 3D MCA stenosis model endothelialized with HBMECs after 24 hr exposure to control (upper) or S-protein coated liposomes (lower) (1:500; 0.2 μg) during perfusion culture. Bound S-protein liposomes were detected within multiple luminal HBMECs (arrow, C). Scale bars = 100 μm.

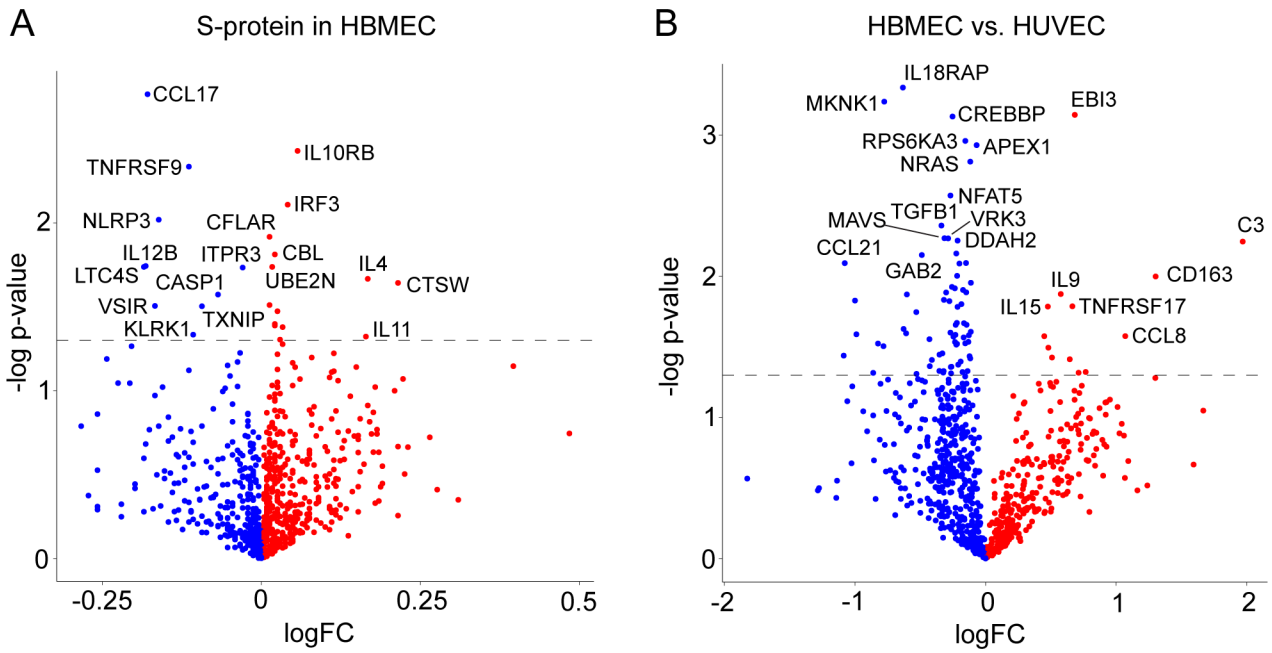


Figure 4. Brain endothelial-specific differential gene expression triggered by recombinant Sars-CoV-2 Spike Protein exposure.

Normalized differential gene expression measured by direct RNA hybridization after 24 hr exposure of HBMECs to 5 μg/mL Sars-CoV-2 Spike Protein trimer (S-protein) (A). Differential gene expression in HBMEC after S-protein exposure normalized to expression levels in HUVECs (B). Dashed line represents -log adjusted *p*-value. LogFC = log fold change.

Table 1.

Key up-regulated genes triggered by Sars-CoV-2 S-protein binding in brain endothelial cells

Gene	Signaling	Association with Sars-CoV-2
C3	Complement System; Myeloid Activation	C3 knockout mice had better prognosis and less inflammation in SARS-CoV-1 [16]
CD163	Myeloid Inflammation	*HLA-DR*^{lo} *CD163*^{hi} monocytes (cluster 2) were present mainly early in severe COVID-19 disease. [17]
CCL8	Chemokine Signaling; Mononuclear Cell Migration	CCL8 levels increased after viral infection in ferrets infected with Sars-CoV-2 and persisted even after viral load decreased. [15]
APOBEC3G	RNA Sensing	APOBECs may be involved in viral genome editing (cytosine → uracil hypermutations in virus RNA) [18]
ITGAL	Lymphocyte Trafficking; Myeloid Activation; NK Activity	Unknown
EBI3	Other interleukin signaling (IL-27)	Up-regulated during acute encephalomyelitis caused by mouse hepatitis virus and inflammation and promotes viral clearance [19]
TNFRSF17	NF-KappaB Signaling	Unknown
OSM	IL-6 Signaling; JAK-STAT Signaling	Unknown
IL9	IL-2 Signaling; JAK-STAT Signaling; TH9 Differentiation	Unknown
CXCL8	Chemokine Signaling; Myeloid Activation; NF-KappaB Signaling; NLR Signaling; RNA Sensing; Tissue Stress; TLR Signaling	Significant increase in circulating serum levels of CXCL8 in COVID-19 patients [15]
IL15	IL-2 Signaling; JAK-STAT Signaling; Myeloid Activation; TNF Signaling	Clinical trial studying the effects of IL15 superagonists in removing Sars-CoV-2 virus particles and infected cells (NCT04324996)
CCL24	Chemokine Signaling; Mononuclear Cell Migration; NF-KappaB Signaling	Unknown