



Whole Exome Sequencing Identifies a Rare Mutation in NACAD as a Possible Cause of COVID Orchitis in Brothers

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COVID orchitis (testicular pain) is reported in 10-15% of men with long COVID. We identified 2 siblings with COVID orchitis and hypothesized that genetic mutations are associated with susceptibility. Blood samples from 5 COVID-19 (+) men, three of whom had orchitis were evaluated by whole-exome-sequencing. A rare deletion on chromosome 7 was found in *NACAD* among the 3 men with orchitis. Interestingly, circulating ACE2 levels was decreased in men with COVID orchitis. This pilot study generated the hypothesis that men who develop COVID orchitis could have underlying genetic variants and altered levels in circulating ACE2 that may increase their risk. *UROLOGY* 159: 83–86, 2022. © 2021 Elsevier Inc.

Since SARS-CoV-2 was declared a pandemic, efforts have mainly focused on mitigating primary infection mortality. During prioritization of community response and vaccine production, post-acute COVID-19 syndromes silently grew in prevalence¹⁻³. Organ inflammation syndromes are now well observed and have been documented in the heart, liver, kidneys, and testes⁴. While the blood-testis barrier is known to protect testes from antigens, current evidence suggests COVID-19 can affect the testis, either by (1) hematogenous spread due to periods of heightened viral load⁵ or (2) the host's immune response leading to proinflammatory cytokine release^{6,7}. With a similar mechanism to what was seen in SARS-CoV in 2002, SARS-CoV2 binds to the ACE2 receptor to be internalized⁸. This is a common receptor found in the serum that interestingly exists in the testes in high concentrations, and when viral bound, leads to viral internalization^{9,10}.

As COVID-19 sequela outside of pulmonary symptomatology continues to grow in prevalence, causal mechanisms need to be uncovered. Particularly, in cases of COVID-19 orchitis, a genetic mechanism is very likely due to a predilection for symptom precipitation in families^{11,12}. We hypothesized that a genetic mutation may be present in individuals who developed COVID-19 orchitis that causes increased viral entry and susceptibility to orchitis. Therefore, we performed whole exome sequencing on 5 individuals, two of which were brothers with

COVID-19 orchitis and evaluated an association in serum circulating ACE2 levels.

MATERIALS AND METHODS

Patient Selection

Patients were included based on COVID-19 (+) history and presence of or lack thereof orchitis symptomatology (testicular pain). We identified 6 COVID-19 patients who all were confirmed to be COVID positive with polymerase chain reaction (PCR) tests. Patients included 3 COVID-19 (+) men without orchitis (controls) and 3 COVID (+) men with orchitis (bilateral testicular pain for at least 5 days around the time of testing PCR positive). Among the 3 men with COVID-19 orchitis symptomatology, two of them were siblings. All patients were identified at University of Miami School of Medicine Urology Clinics and provided informed consent for participation to be in accordance with the Declaration of Helsinki¹³. The study also received IRB approval (#20150740).

Whole Exome Sequencing

Peripheral venous blood samples were collected from each of the patients. DNA extraction was performed on blood using the QIAamp blood maxi kit (Qiagen, Germantown, MD) on 5 of 6 patients. Whole exome sequencing was completed at John P. Hussman Institute. Captured exomes from DNA shearing were library prepared for amplification and sequencing. 16 variants were prioritized by being shared between the 3 patients affected with orchitis, absent in controls, and introducing nonsense, frameshift, splicing or non-synonymous amino acid changes and less than 10% in population prevalence.

Phenotypic Validation

DuoSet Human ACE2 reagent kit 2 (catalog number: DY933-05) was purchased from R&D Systems, Minneapolis, USA, and enzyme-linked immunosorbent assay was used to measure duplicate levels of soluble ACE2 in the plasma samples.

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RESULTS

Case Report

Probands of this study were 3 patients who contracted COVID-19 and developed orchitis. All three subjects never experienced orchitis symptomatology in the past and reported no previous family histories. The average age of the men in the study was 25 years old. They reported an average duration of COVID-19 symptoms (fever, cough, and body aches) of 7 days, and none required hospitalization. Among the men who developed bilateral testis pain, the symptoms lasted for an average of 22 days. The median sperm concentration and sperm motility was 19 million/ml and 60% around 3 months after original infection.

Whole Exome Sequencing Results

Whole exome sequencing yielded a total of 43,857 single nucleotide variants in at least 1 of 5 participants. First, we filtered for variants shared between the 2 siblings with COVID orchitis along with the unrelated subject with COVID orchitis and for variants absent from the 2 controls, which returned 606 variants. Filtering for nonsynonymous mutations, including frameshift mutations, left 295 variants, which was reduced to 16 unique variants with a final filter of prevalence of 5% or less. Variants were individually analyzed and filtered based off criteria of (1) clear role in established COVID-19 linked orchitis ACE2 pathways and (2) read and genotype quality. Among the 16 candidate variants, we prioritized a nonsynonymous non-frameshift

deletion in *NACAD* on chromosome 7: 45084258 with a frequency of 3.9% prevalence in ExAC, which causes a deletion of 120 base pairs. This variant was present in the 3 patients with COVID-19 orchitis and absent in the controls.

PHENOTYPE RESULTS

Phenotypically, we found median circulating levels of soluble ACE2 to be 3.40 ng/ml among men who had COVID orchitis. This was lower when compared to men who developed COVID without orchitis with median ACE2 levels of 4.33 ng/mL ($P < .05$, Wilcoxon signed-rank test). These ACE2 serum levels are consistent with existing evidence from COVID infected individuals¹⁴. As ACE2 serum levels normally range from 0.68 to 2.54 ng/mL in healthy individuals¹⁵, baseline SARS-CoV2 infection is associated with elevated levels. This suggests that higher ACE2 serum levels may be protective from developing COVID-19 orchitis.

DISCUSSION

We identified a unique patient group consisting of 2 brothers and one non-related individual who all experienced a rare sequela of COVID-19 infection. Both the siblings and the non-related patient possessed a nonsynonymous non-frameshift mutation in *NACAD*. Comprehensive search through whole exome sequencing

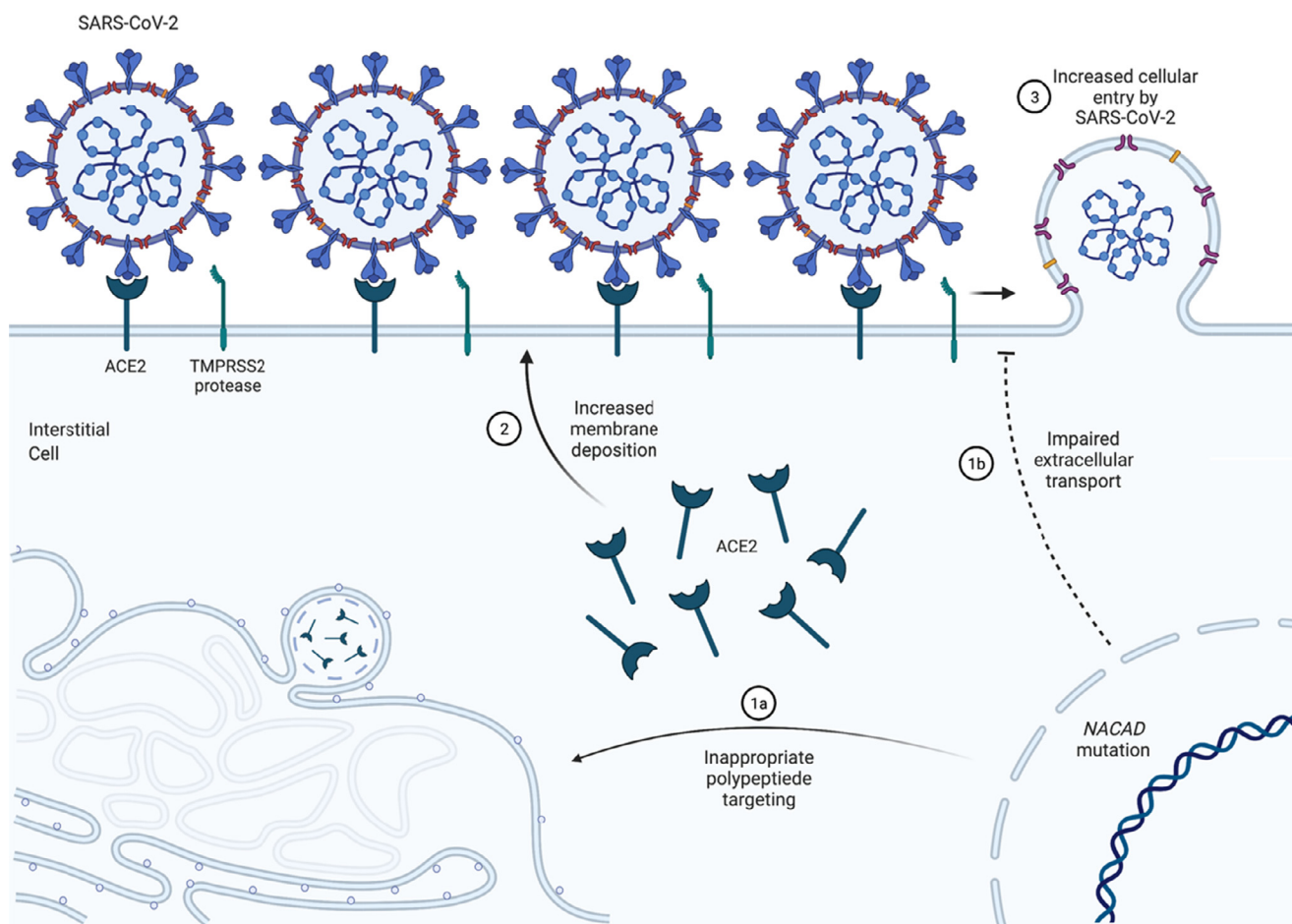


Figure 1. *NACAD* Pathway. The proposed role of *NACAD* and its interactions with polypeptide targeting via the endoplasmic reticulum (ER) and downstream changes in circulating ACE2. (Color version available online.)

studies regarding COVID-19 (+) severity showed no previous mention of this mutation¹⁶⁻¹⁸. NACAD gene products are thought to be involved with proper facilitation of polypeptide extracellular transport. Furthermore, the protein is thought to bind to nascent protein chains as translation occurs to facilitate proper interaction with the signal recognition particle occurs¹⁹. In tandem, impaired (1) polypeptide secretion and (2) endoplasmic reticulum targeting can lead to decreased extracellular protein secretion and increased cell membrane deposition of mistargeted proteins.

Non-frameshifting variants result in the gain or loss of several nucleotides divisible by three, such that the reading frame of the mRNA segment is not disrupted. The resulting mutant protein sequence differs from the wild-type with the addition and/or deletion of one or more amino acid residues. Phenotypic effects of a non-frameshift variant can result in impaired protein function and could impact biological pathways²⁰. Consequently, our findings suggest that the effect of the NACAD mutation results in modifying the extracellular and intracellular ACE2 concentrations as evident by the serology of ACE2 in COVID-19 orchitis patients compared to COVID-19 patients without orchitis. Particularly during peak viraemia, already elevated ACE2 membrane concentrations are faced with increased serum viral body concentrations to cause an increased risk of COVID-19 precipitated orchitis symptomatology^{9,21}.

We hypothesized that disrupted extracellular transport and endoplasmic reticulum interaction leads to altered intracellular levels of ACE2. Subsequently, increased intracellular ACE2 levels result in cell membrane protein deposition. We identified a unique patient group with a rare COVID-19 sequelae, of mostly similar ages, none of which reported prior orchitis symptomatology or family history. While we acknowledge limited subject verification, we have actively identified other families with similar patterns of orchitis sequelae and aim to study them. Ultimately, the combination of our genetic analysis and the difference in ACE2 levels between the groups suggests a possible mechanism for COVID-19 orchitis that warrants further investigation.

CONCLUSION

This is a pilot study that suggests a possible genetic link for increased risk of COVID-19 orchitis. We observed a mutation in NACAD in 2 brothers and one unrelated man who developed COVID-19 orchitis. Interestingly, we found lower circulating ACE2 serum levels in both brothers with orchitis and the one nonrelated orchitis subject but normal serum levels in all controls. Future studies focusing on evaluating the mechanism of how NACAD mutation will lead to changes in circulating serum ACE2 levels will uncover important mechanisms not just to understand why some men with COVID develop orchitis but also why some men develop multi-organ failure with COVID infection whereas most do not. [Figure 1](#)

References

- Nalbandian A, Sehgal K, Gupta A, et al. Post-acute COVID-19 syndrome. *Nat Med*. 2021;27:601–615. <https://doi.org/10.1038/s41591-021-01283-z>.
- Al-Jahdhmani I, Al-Naamani K, Al-Mawali A. The Post-acute COVID-19 Syndrome (Long COVID). *Oman Med J*. 2021;36:e220. <https://doi.org/10.5001/omj.2021.91>.
- Doshi P. Covid-19 vaccines: In the rush for regulatory approval, do we need more data? *BMJ*. 2021;373:n1244. <https://doi.org/10.1136/bmj.n1244>.
- Gheblawi M, Wang K, Viveiros A, et al. Angiotensin-converting Enzyme 2: SARS-CoV-2 receptor and regulator of the renin-angiotensin system: celebrating the 20th anniversary of the discovery of ACE2. *Circ Res*. 2020;126:1456–1474. <https://doi.org/10.1161/CIRCRESAHA.120.317015>.
- Dejucq N, Jegou B. Viruses in the mammalian male genital tract and their effects on the reproductive system. *Microbiol Mol Biol Rev*. 2001;65:208–231. <https://doi.org/10.1128/MMBR.65.2.208-231.2001>. first and second pages, table of contents.
- Bridwell RE, Merrill DR, Griffith SA, Wray J, Oliver JJ. A coronavirus disease 2019 (COVID-19) patient with bilateral orchitis: A case report. *Am J Emerg Med*. 2020. <https://doi.org/10.1016/j.ajem.2020.08.068>.
- National Research Project For SBGBC. [Dynamic changes of T-lymphocytes and immunoglobulins in patients with severe acute respiratory syndrome]. *Zhonghua Yi Xue Za Zhi*. 2003;83:1014–1017.
- 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. <https://doi.org/10.1002/path.1570>.
- Verma S, Saksena S, Sadri-Ardekani H. ACE2 receptor expression in testes: implications in coronavirus disease 2019 pathogenesis. *Biol Reprod*. 2020;103:449–451. <https://doi.org/10.1093/biolre/iaaa080>.
- Fan C, Lu W, Li K, Ding Y, Wang J. ACE2 Expression in kidney and testis may cause kidney and testis infection in COVID-19 patients. *Front Med (Lausanne)*. 2020;7: 563893. <https://doi.org/10.3389/fmed.2020.563893>.
- Lowe A, Chang DD, Creek G. Multiple fatalities in a family cluster of COVID-19 with acute respiratory distress syndrome. *Ochsner J*. 2020;20:134–138. <https://doi.org/10.31486/toj.20.0056>.
- van der Made CI, Simons A, Schuurs-Hoeijmakers J, et al. Presence of genetic variants among young men with severe COVID-19. *JAMA*. 2020;324:663–673. <https://doi.org/10.1001/jama.2020.13719>.
- World Medical A. World medical association declaration of helsinki: ethical principles for medical research involving human subjects. *JAMA*. 2013;310:2191–2194. <https://doi.org/10.1001/jama.2013.281053>.
- Parsa Mohammadi HAV, Seifi Arash, Miandoab Sepideh Zahak, Beiranvand Saba, Mobaraki Sahar, Mohammadi Mostafa. Investigating the relationship between serum ACE 2 level and COVID-19 patients' prognosis: a cross-sectional study. *medRxiv*. 2021.
- AlGhatrif M, Tanaka T, Moore AZ, Bandinelli S, Lakatta EG, Ferrucci L. Age-associated difference in circulating ACE2, the gateway for SARS-COV-2, in humans: results from the InCHIANTI study. *Geroscience*. 2021;43:619–627. <https://doi.org/10.1007/s11357-020-00314-w>.
- Benetti E, Giliberti A, Emiliozzi A, et al. Clinical and molecular characterization of COVID-19 hospitalized patients. *PLoS One*. 2020;15: e0242534. <https://doi.org/10.1371/journal.pone.0242534>.
- Patel DP, Punjani N, Guo J, Alukal JP, Li PS, Hotaling JM. The impact of SARS-CoV-2 and COVID-19 on male reproduction and men's health. *Fertil Steril*. 2021. <https://doi.org/10.1016/j.fertnstert.2020.12.033>.
- SeyedAlinaghi S, Mehrtak M, MohsseniPour M, et al. Genetic susceptibility of COVID-19: a systematic review of current evidence. *Eur J Med Res*. 2021;26:46. <https://doi.org/10.1186/s40001-021-00516-8>.

19. Zhang H, Zheng H, Zhu J, et al. Ubiquitin-modified proteome of SARS-CoV-2-infected host cells reveals insights into virus-host interaction and pathogenesis. *J Proteome Res.* 2021;20:2224–2239. <https://doi.org/10.1021/acs.jproteome.0c00758>.
20. Pagel KA, Antaki D, Lian A, et al. Pathogenicity and functional impact of non-frameshifting insertion/deletion variation in the human genome. *PLoS Comput Biol.* 2019;15: e1007112. <https://doi.org/10.1371/journal.pcbi.1007112>.
21. Al-Benna S. Angiotensin-converting enzyme 2 gene expression in human male urological tissues: implications for pathogenesis and virus transmission pathways. *Afr J Urol.* 2021;27:89. <https://doi.org/10.1186/s12301-021-00192-4>.