

A Crispr-Cas9 system designed to introduce point mutations into the human ACE2 gene to weaken the interaction of the ACE2 receptor with the SARS-CoV-2 S protein

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ABSTRACT

The human angiotensin-converting enzyme 2 (ACE2) has a crucial role on blood pressure control; however, ACE2 is also the primary SARS-CoV-2 (S domain) virus receptor. Inhibiting or even reducing the expression of the native ACE2 might diminish the viral entry into the cells, but may cause a failure of ACE2 biological activity, primarily in patients with comorbidities, including diabetes mellitus or hypertension. Since the ACE2 catalytic site and the SARS-CoV-2 receptor are distinct, we designed a Crispr-Cas9 model system, predicting the respective sequences for a guide RNA (gRNA) and a single-stranded oligo dideoxy nucleotide (ssODN), to introduce point mutations into the exon 1 of the human *ACE2* gene, which encodes the alpha-helix, implicated on the binding of the SARS-CoV-2 envelope S protein. Protein modeling predicted that the specific substitutions of residues Phe28, Lys31, and Tyr41 for Ala at the ACE2 alpha-helix do not significantly alter ACE2 native conformation. The analysis of the impact of these mutations on ACE2 receptor function predicted a weakening of the binding of the SARS-CoV-2 protein S. An experimental genome editing of cells based on these Crispr-Cas9 elements might reduce the SARS-CoV-2 ability to enter the epithelial cell, preserving the biological activity of ACE2 enzyme.

INTRODUCTION

New coronavirus 2019 (SARS-CoV-2), the agent of COVID-19, belongs to a family of four Coronaviridae genera of probable bat origin (CIOTTI et al., 2020; ZHOU et al., 2020). CoVs are single-stranded positive RNA viruses with a 5'-cap structure and a 3'-poly-A tail, surrounded by an envelope. Additionally, CoVs exhibit a spike glycoprotein (S), composed of the S1 and S2 subunits, which permit virus to enter the host cell, fusing into viral and cell membranes, respectively (CIOTTI et al., 2020). The interaction of the SARS-CoV-2 spike S1 subunit with the human angiotensin-converting enzyme 2 (ACE2), present on many epithelial cell surfaces, is believed to be the major mechanism for cell invasion (CIOTTI et al. 2020; LAN et al., 2020).

Diabetes mellitus (DM) and hypertension have been pointed as major comorbidity factors associated with mortality due to SARS-CoV-2 infection. Therefore, concern and interest have preoccupied physicians and researchers regarding the relationship between these comorbidities and COVID-19 fatalities (GUAN et al., 2020; DANSER et al., 2020; MUNIYAPPA and GUBBI, 2020).

The treatment of DM has been performed with hypoglycemic agents; however, whether or not inadequate drug treatment or deregulated plasma glucose levels are related to the increased COVID-19 morbidity, are unanswered questions. Several mechanisms have been proposed to explain the increased susceptibility to COVID-19 in DM patients, including: i) higher virus cell binding and affinity, ii) active entry of the virus into cells; ii) decreased T cell function, impairing virus clearance; iii) increased

susceptibility to hyperactive inflammation, iv) cytokine storm; and iv) the presence of underlying cardiovascular disorders (MUNIYAPPA and GUBBI, 2020).

Blockade of the enzymatic conversion of angiotensin 2 into angiotensin 1-7 represents the key mechanism for the treatment of hypertension (TURNER 2015), propitiating an overexpression of ACE2 enzyme in lung, kidney and heart cells; however, this effect may favor the attachment of SARS-CoV-2 to epithelial cells (DANSER et al. 2020). Nevertheless, there is no reason for hypertensive patients to abandon the use of antihypertensive treatment due to concerns about the susceptibility of contracting COVID-19, since the damage to the body without these drugs may be accrued (MUNIYAPPA and GUBBI, 2020).

Literature data mining on the comorbidities associated with severe COVID-19 (e.g., cardiovascular disease, familial pulmonary hypertension, chronic obstructive pulmonary disease, diabetes, lung cancer, chronic renal failure, asthma, autoimmune diseases) revealed a broad set of shared genes, stressing ACE2 that was induced in four or more of these comorbidities (PINTO et al., 2020). To reduce infection by SARS-CoV-2, researchers have proposed pharmacological strategies to inhibit ACE2 (Sanders et al. 2020). However, the ACE2 enzyme plays a crucial role in the conversion of angiotensin 2 by controlling blood pressure, and a reduction or inhibition of its expression may have deleterious consequences. Considering that the catalytic site of ACE2 and the domain associated with SARS-CoV-2 entry into the cell present distinct locations, we addressed the possibility of developing a strategy to decrease the potential binding of the SARS-CoV-2 virus S protein to the ACE2 alpha-helix, preserving the catalytic site of the enzyme.

Researchers are also concerned about devising pharmacological strategies to inhibit ACE2 in an attempt to reduce infection by SARS-CoV-2 (SANDERS et al., 2020). However, the ACE2 enzyme plays a crucial role on the conversion of angiotensin 2 by controlling blood pressure, and a reduction or inhibition of its expression may have deleterious consequences. Considering that the catalytic site of ACE2 and the domain associated with SARS-CoV-2 entry into the cell present distinct locations, we addressed the possibility of developing a strategy to decrease the potential binding of the SARS-CoV-2 virus S protein to the ACE2 alpha-helix, preserving the catalytic site of the enzyme.

MATERIAL AND METHODS

Protein interaction of the ACE2 alpha-helix with the SARS-CoV-2 S1 protein

To evaluate the possible effect of point mutations on: i) the conformation of the ACE2 alpha-helix, ii) the viral protein S - ACE2, protein-protein interaction complex and iii) the identification of amino acid residues present at the virus/receptor interface, we used the Protein Data Bank (PDB, available at <https://www.rcsb.org/>), using the code 6m0j (LAN et al., 2020). The 3D structure visualization was performed using the PyMOL 2.1.1 software (available at <https://pymol.org/2/>). The impact of amino acid mutations

was predicted using Mutabind2 (ZHANG et al., 2020) (available at <https://lilab.jysw.suda.edu.cn/research/mutabind2/>).

The destabilization of the ACE2 N-terminal alpha-helix was analyzed using SwissModel homology modeling and *ab initio* protein software (<https://swissmodel.expasy.org/>) and PEP-FOLD3 (<https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/>). For the analysis, a sequence of 25 amino acid residues that constitute the ACE2 N-terminal alpha-helix was considered.

Crispr-Cas9 gRNA and ssODN design directed to ACE2 gene

The single guide RNA (gRNA) was defined using the pipeline offered by the Benchling platform (available at <https://www.benchling.com>). For setting a gRNA to be used together with a single-stranded oligo dideoxy nucleotide (ssODN), we used the following ACE2 genomic coordinates, GRCh38, hg38, *Homo sapiens*, gene ACE2 ENSG00000130234, chromosomal localization chrX 15.561.033-15.602.148 (-), exon 1, transcript ACE2-201 ENST000004274411, CCDS14169.

Since spCas9 recognizes the protospacer adjacent motif (PAM) NGG sequence, this system was selected as a major guide variable for a 20 nucleotide gRNA. We focused on a region of the ACE2 gene that encodes the protein alpha-helix, close to the target for the desired mutation. The different gRNAs designed were then visualized with their respective on-target and off-target scores, their nucleotide positions at ACE2 exon 1, and their respective PAM sequences.

Genetic polymorphism of ACE2

To evaluate the worldwide nucleotide variability at the ACE2 gene segment, we used data from the International Genome Sample Resource that includes data from the 1000 Genomes Project among other projects (<https://www.internationalgenome.org/data>).

RESULTS

Protein analyses of wild type and Crispr-Cas9 edited ACE-2 alpha-helix

The evaluation of the interaction surface of the complex formed by ACE2 N-terminal alpha-helix and viral S protein (available from crystallographic data in the Protein Data Bank) enabled us to identify more than 20 amino acid residues at the interface (Figures 1a and 1b). We observed that residue substitution at the ACE2 side (Phe 28, Lys 31 and Tyr 41) affected the potential interaction with the viral protein S.

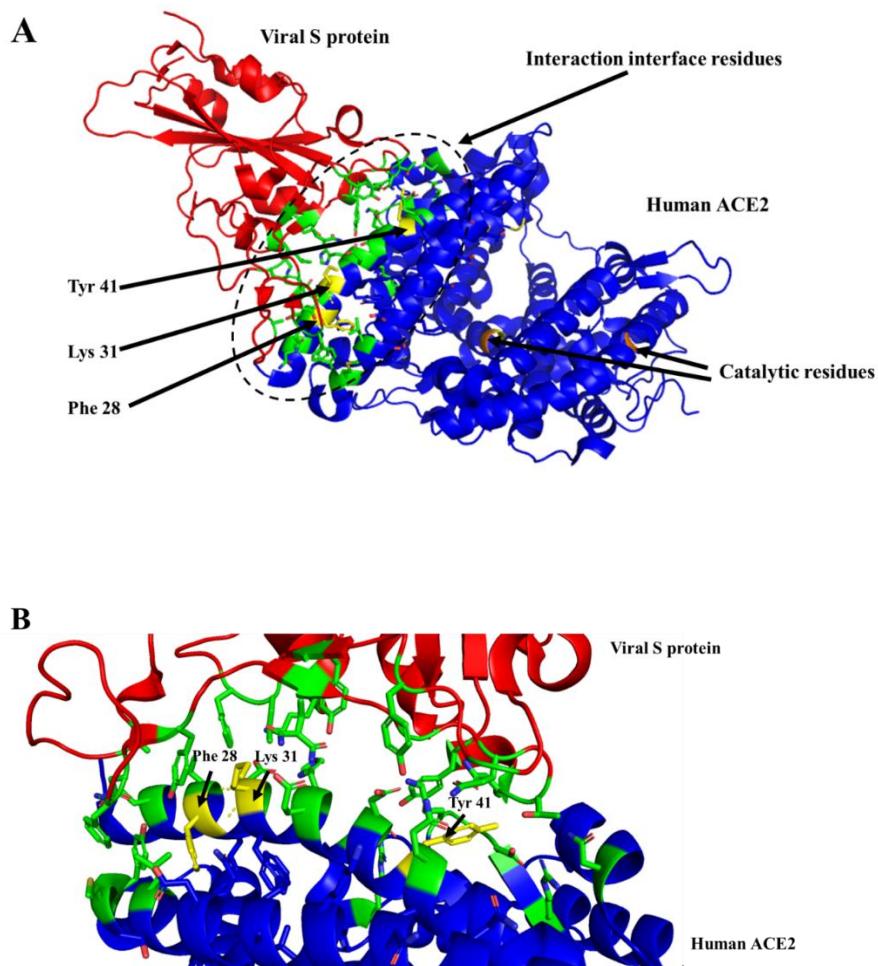


Figure 1. Modeling of the interaction of SARS-CoV-2 protein S (red) with ACE2 receptor (blue). In green is represented the interaction interface and in yellow the position of the amino acid residues (Phe28, Lys31, Tyr41), which when substituted by Ala affected the interaction (A). A detailed view showing the interaction of the amino acid residues (Phe28, Lys31, Tyr41 in yellow) from the ACE2 side (blue) with SARS-CoV-2 protein S (red) (B).

Analysis of the impact of mutations introduced in each of these residues demonstrated a high potential effect on destabilizing the ACE2-viral protein S interaction interface when a non-synonymous mutation changed the Phe 28, Lys 31 and Tyr 41 residues to Ala in each of these positions. Together, these three mutations represent an increase in free energy of $5.26 \text{ kcal mol}^{-1}$, a value predicted to destabilize the interaction interface of ACE2 with viral S protein.

Modeling of the ACE2 N-terminal alpha-helix demonstrated a conservation of the helicoidally structure when substitutions for Ala were introduced at the positions Phe 28, Lys 31, and Tyr 41 (Figures 2a, 2b, 2c).

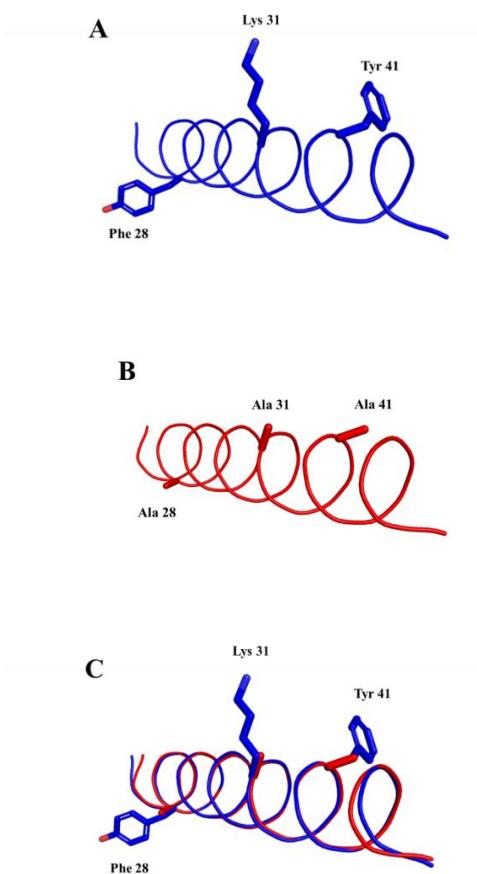


Figure 2. Modeling of the ACE2 N-terminal alpha-helix. In blue is represented the wild type ACE2 N-terminal alpha-helix (A), in red the edited ACE2 N-terminal alpha-helix (B) and the superposition of the two structures (wild type and edited) (C).

Designing gRNA and ssODN directed to exon 1 of ACE2 gene

We designed the respective sequences for gRNA and ssODN directed at the exon 1 of the *ACE2* gene, encoding the N-terminal alpha helix, defining the gRNA (ACTTTGATAGAACAGGTCTT) and the PAM (CGG) sequences, which presented specificity = 40 and efficiency = 59.6, accordingly to the Benchling scores.

The ssODN was designed with equal arms presenting 60 nucleotides in length, positioned to the left of the first base pair (bp) and to the right of the last altered bp to be edited in the target gene region. The total length of the ssODN was 161 bp, and the full ssODN sequence was defined as: (CTTCTCAGCCTTGGCTGTAAGTGCTGCTCAGTCCACCATTGAGGAACAGGCCAAGACAGCT TTGGACGCGTTAACACACGAAGCTGAAGACCTGTTGCTCAAAGTTCACTTGCTTGGATT ATAACACCAATATTACTGAAGAGAATGTCCAAAAA).

The gRNA, PAM and ssODN sequences were positioned in the antisense chain within ACE2 exon 1 (Figures 3 and 4) and, therefore, at the time of use, they should be written as a complementary reverse sequence. The prediction of off-targets for this system is shown in the Supplemental Table 1 available at (www.rge.fmrp.usp.br/passos/off_targets_gRNA_ACE2).

ENSG00000130234	Homo	sapiens	ACE2	gene
ATGTCAGCTCTGGCTCTCAGCCTGTTGCTGTAAGTGTGCTCAGTCCACCATGAGGAACAGGCCAAGACATTT GGACAACTTAACCACGAAGCCGAAAGCTGTTCTATCAAAGTTCACTTGCTCTTGGATTATAACCCAATATTACTGAAGAG ATATGTCACAAACATGAATAATGCTGGGGACAATGGTCTGCCTTTAAAGGAACAGTCACACTGCCAAATGTATCCACTAC AAGAAATTCAAATCTCACAGTCAGCTCAGCTGCAGGCTCTCAGCAAAATGGGCTTCAGTGCTCTCAGAAGACAAGAGCA AACGGTTGAACACAATTCTAAATACAATGAGCACCATCTACAGTACTGGAAAAGTTGTAACCCAGATAATCCACAAGAATGCTT ATTACTGAACCAAGGTTGAATGAAATAATGGCAAACAGTTAGACTACATGAGAGGCTCTGGGCTTGGAAAGCTGGAGAT CTGAGGTGGCAAGCAGCTGAGGCCATTATATGAAGAGTATGTGGCTTGAAAATGAGATGGCAAGAGCAAATCATTAG GACTATGGGATTATTGGAGAGGAGACTATGAAGTAAATGGGTAGATGGCTATGACTACAGCCGCGGCCAGTTGATTGAAG ATGTGGAACATACCTTGAAGAGATTAAACCATTATATGAACATCTCATGCCTATGTGAGGGCAAAGTTGATGAATGCCTATCC TTCTATATCAGTCCAATTGGATGCCCTCTGCTCATTTGCTTGGTATATGTTGGGTAGATTTGGACAAATCTGACTCTTGA CAGTCCCTTGGACAGAAACCAAACATAGATGTTACTGATGCAATGGTGGACCAGGCCGGATGCAAGAGAAATATTCAAG GAGGCCGAGAAGTTCTTGATCTGTTGGCTTCTTAATATGACTCAAGGATTCTGGAAAATCCATGCTAACGGACCCAGGA AATGTTAGAAAGCAGTCTGCCATCCCACAGCTGGGACCTGGGAAGGGCAGTCAGGATCCTATGTGCACAAAGGTGAC AATGGACGACTTCCTGACAGCTCATGAGATGGGCATATCCAGTATGATATGGCATATGCTGCACAACTTTCTGCTAAGA AATGGAGCTAATGAAGGATTCCATGAAGCTGTTGGGAAATCATGTCACCTCTGCTCAACAAAGCAGTACGATTGTTGGACTC GGCTTCTGTCACCGATTTCAGAAAGACAATGAAACAGAAATAACTTCTGCTCAACAAAGCAGTACGATTGTTGGACTC TGCCATTACTACATGTTAGAGAAGTGGAGGTGGATGGCTTAAAGGGAAATCCAAAGACCAGTGGATGAAAAGTGG TGGGAGATGAAGCGAGAGATAGTTGGGGTGGGAACCTGTGCCCATGATGAAACATACTGTGACCCCGCATCTGTTCCA TGTGTTCTGATGATTACTCATTGATATTACACAAGGACCTTACCAATTCCAGTTCAAGAAGCACTTGTCAAGCAGCTA AACATGAAGGCCCTGCACAAATGTGACATCTCAAACTCTACAGAAGCTGGACAGAAACTGTTGAA				

Figure 3. FASTA sequence of the genomic region that encodes the human ACE2 [chrX 15.561.033-15.602.148 (-), gene ENSG00000130234, transcript ENST000004274411, CCDS14169]. In green, the respective codons for Phe (position 28), Lys (position 31), and Tyr (position 41 at the N-terminal alpha-helix of ACE2). In yellow, the PAM sequence. In purple, the gRNA sequence. The gRNA and PAM sequences were found in the antisense chain and, therefore, should be written as a complementary reverse sequence.

ENSG00000130234 Homo sapiens ACE2 gene

ATGTCAAGCTCTCTGGCTCTTCTCAGCCTTGTGCTGTAACGTGCTCAGTCCACCATTGAGGAACAGGCCAAGACAGCT
TTGGACGCGTTAACCACGAAGCTGAAGACCTGTCGCTCAAAGTCACCTGCTCTTGAATTATAACACCAATATTACTGAA
 GAGAATGTCCAAAACATGAATAATGCTGGGGACAAATGGCTGCCTTTAAAGGAACAGTCCACACTGCCAAATGTATCCA
 CTACAAGAAATTCAAAGTCAAGCTCAGCTCAGGCTCTCAGCAAATGGCTTCAGTGAACAGATAATCCACAAGAA
 AGCAACAGGTGAACACAATTCTAAATACAATGAGCACCATCTACAGTACTGGAAAAGTTGTAAACCCAGATAATCCACAAGAA
 TGCTTATTACTGAACCAGGTTGAATGAAATAATGCAACAGTTAGACTACAATGAGAGGTCTGGGCTTGGGAAAGCTGG
 AGATCTGAGGTGCGCAAGCAGCTGAGGCCATTATATGAAGAGTATGTGGCTTGGGAAAGCTGG
 TGAGGACTATGGGATTATTGGAGAGGAGACTATGAAGTAATGGGTAGATGGCTATGACTACAGCCGCGGCAGTTGATT
 GAAGATGTGGAACATACCTTGAAGAGATTAACCAATTATGAACATCTCATGCCTATGTGAGGGCAAAGTTGATGAATGCC
 TATCCTCTTATCAGTCCAATTGGATGCCTCCCTGCTCATTTGCTTGGTATATGTGGGAGTTGGACAATCTGACTC
 TTTGACAGTCCCTTGGACAGAAACCAAACATAGATGTTACTGATGCAATGGTGACCAGGCCTGGGATGCACAGAGAAATT
 CAAGGAGGCCGAGAAGTCTTGTATGTTGGCTCTTAATATGACTCAAGGATTCTGGAAAATTCCATGCTAACGGACCCA
 GGAAATGTTAGAAAGCAGTCTGCATCCACAGCTTGGGACCTGGGGAAAGGGCGACTTCAGGATCCTATGTGCACAAAGGT
 GACAATGGACGACTTCTGACAGCTCATGAGATGGGCATATCCAGTATGATGGCATATGCTGCACAACCTTCTGCTA
 AGAAATGGAGCTAATGAAGGATTCCATGAAGCTGTTGGGAAATCATGTCATTTCTGCAGGCCACACTAACGATTAAATCC
 ATTGGTCTCTGTCACCGATTCAAGAAGACAATGAAACAGAAATAACTCCTGCTCAAACAAGCAACTCAGATTGTTGGG
 CTCTGCCATTACTACATGTTAGAGAAGTGGAGGTGGATGGCTTAAAGGGGAAATCCCAAAGGACAGTGGATGAAAAAG
 TGGTGGGAGATGAAGCGAGAGATGTTGGGGGGAACTGTGCCCCATGATGAAACATACTGTGACCCGCATCTGTT
 CCATGTTCTGATGATTACTCATTCACTGATATTACACAAGGACCTTACCAATTCAAGACTTCAAGAAGCACTTGTCAAGCAG
 CTAACATGAAGGCCCTTGACAAATGTGACATCTCAAACACTACAGAAGCTGGACAGAAACTGTTGAA

Figure 4. FASTA sequence of the genomic region that encodes the human ACE2 [chrX 15.561.033-15.602.148 (-), gene ENSG00000130234, transcript ENST000004274411, CCDS14169] after Crispr-Cas9 editing. The respective codon changes are shown in green. TTT (Phe) to GCT (Ala) at position 28, AAG (Lys) to GCG (Ala) at position 31 and TAT (Tyr) to GCT (Ala) at position 41 in the N-terminal alpha-helix of ACE2. In blue is shown the silent mutation introduced in the PAM sequence, exon 1 of the ACE2 gene is underlined and the ssODN sequence is in bold.

Genetic polymorphism of ACE2

To date, the human ACE2 gene presents 317 non-synonymous single-nucleotide polymorphisms (SNPs) across its 18 coding exons, exhibiting population frequencies below 0.1%. None of these variations has been associated with any clinical phenotype registered in the ClinVar database (<https://www.ncbi.nlm.nih.gov/clinvar/>) (LANDRUM, 2014).

DISCUSSION

The intrinsic characteristic of SARS-CoV-2, which emerged in late 2019, as a highly infectious COVID-19 pandemic has been defying worldwide scientists to scrutinize new antiviral drugs, take advantage of the ones already in use or propose new strategies to lessen the burden of the pandemics (SANDERS et al., 2020).

In this study, protein-modeling tools were used to evaluate the effect of replacement of specific amino acid residues in the ACE2 alpha-helix, which are implicated on SARS-CoV-2 virus binding. Since Crispr-Cas9 emerged as a new possibility for treatment of human diseases, including those caused by viruses, for instance the disruption of HIV-1 CCR-5 receptor gene (XU et al., 2017), we used this strategy as a model system for design a gRNA and an ssODN directed to ACE2 alpha-helix genomic region to introduce desired knockin point mutations.

We predicted the elements of the Crispr-Cas9 system, i.e., the gRNA and ssODN, to selectively introduce destabilizing point mutations in specific amino acid residues of the N-terminal alpha-helix of the human ACE2 gene, the main SARS-CoV-2 receptor. The destabilizing effect of the edited mutations weakened the interface interaction between ACE2 alpha-helix and SARS-CoV-2 S protein, while preserving the helicoidally structure at the ACE2 side.

Since the binding site between CoVs and ACE2 has been identified (LI et al., 2005; HOFFMANN et al., 2020; ZHANG et al., 2020), several strategies have been proposed directed to ACE2, including ACE inhibition or ACE2 administration. The blockage of human ACE2 with small molecules or even with antibodies (ZHANG et al., 2020) could be an alternative for COVID-19 treatment. The antiviral umifenovir acts as an inhibitor of the fusion between the SARS-CoV-2 S protein and ACE2; however, this drug has not yet been approved for use outside of Russia and China (KADAM and WILSON, 2017; SANDERS et al., 2020). On the other hand, the use of ACE2 blockers could worsen COVID-19 (SANDERS et al., 2020; FANG et al., 2020; SOMMERSTEIN et al., 2020; ESLER et al., 2020; DIAZ, 2020; VADUGANATHAN et al., 2020), since the increased expression of ACE2 may facilitate the entry of SARS-CoV-2 into cells. In addition, ACE2 blockade *per se* can complicate the condition of patients with arterial hypertension.

Administration of soluble ACE2 could compete with membrane ACE2 and reduce SARS-CoV-2 entry into the cells. Indeed, the excess of ACE2 could competitively bind to SARS-CoV-2, not only preventing the virus to enter the cells, but also rescuing ACE2's cellular activity, which negatively regulates the renin-angiotensin system and protects the lung from injury (IMAI et al., 2005; YU et al., 2020). Therefore, treatment with a soluble form of ACE2 could inhibit viral entry into cells (LI et al., 2003; LI et al., 2005) and protect the lung from injury (KUBA et al., 2005; IMAI et al., 2005; WOSTEN-VAN ASPEREN et al., 2011).

Epidemiological, clinical, laboratory and systems biology approaches have corroborated the role of ACE2 in the pathogenesis of COVI-19, including: i) increased disease morbidity and mortality in the presence of comorbidities (CHEN et al. 2020; ZHOU et al., 2020), ii) association of comorbidities with ACE2, required for the SARS-CoV-2 virus to bind and enter host cells (KUBA et al., 2005; YAN et al., 2020; ZHOU et al., 2020), iii) increased ACE2 levels in the pulmonary endothelium arteries of patients with pulmonary hypertension (ORTE et al., 2000), and iv) *in silico* studies encompassing co-expression analyzes, meta-analyzes and construction of gene networks have indicated the gene networks associated with increased expression of ACE2 in the lung of patients with comorbidities (PINTO et al., 2020).

ACE2 exhibits a double role on COVID-19, it is an important enzyme to control blood pressure and, at the same time, is the main receptor for SARS-CoV-2. ACE2 neutralization may inhibit its biological role on blood pressure control and

administration of ACE2 may impede virus entry to the cell, but may produce unforeseen collateral effects.

In this context, we designed a Crispr-Cas9 model system that destabilizes part of the ACE2 protein that interacts with the virus (amino acid residues Phe28, Lys31 and Tyr41 of the N-terminal alpha-helix). At the same time, this system preserves the structural conformation and the most critical amino acid residues (Arg273, His505, His345, Arg169, Arg514 and Tyr515) responsible for ACE2 catalytic function (GUY et al., 2005).

We further verified whether mutations in the *ACE2* gene have been described in human populations that could be associated with any disease. The genetic variations in this gene are below 0.1% in worldwide populations and at the time of writing this paper, no clinical condition thing has been associated.

To the best of our knowledge, this is the first research work that shows a perspective for the use of the Crispr-Cas9 system to edit the human *ACE2* gene within the context of COVID-19, which focuses on reduction the SARS-CoV-2 interaction, while preserving the physiological function of the ACE2 enzyme.

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