

The Role of ACE2 Receptors in the Pathogenesis of Covid-19

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Abstract The emergence of a novel pathogenic human Coronavirus first reported in China, December 2019 has attracted global attention and poses a public health concern. Scientific studies and advancements since Severe Acute Respiratory Syndrome Coronavirus-1 (SARS-CoV-1) and Middle East Respiratory Syndrome (MERS) outbreaks have accelerated the understanding of the current Coronavirus disease-19 (Covid-19) pandemic especially in drug development. Here we explore the role of angiotensin converting enzyme-2 (ACE2); receptor for SARS-CoV-1 in Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) pathogenesis. ACE2 is found to be a functional receptor for SARS-CoV-2. The receptor binding domain structure of SARS-CoV-2 is similar to SARS-CoV-1 domain that interacts with ACE2. ACE2 is highly expressed in lung type II alveolar cells and infection with SARS-CoV-2 results in respiratory condition as reported in Covid-19 patients. It is highly expressed in other human organs including heart, kidneys, and the gastrointestinal tract, thereby posing high risk in Covid-19 infection and suggesting other alternative routes of transmission. The current study predicts that ACE2 allelic variants; rs73635825, rs1299103394, rs766996587, rs961360700, rs762890235, rs1396769231 have adverse impact on the encoded ACE2 possibly influencing resistance and susceptibility to Covid-19 infection, although no functional impact has been detected to date. Furthermore, use of angiotensin converting enzyme inhibitors and angiotensin receptor blockers increases ACE2 expression levels with no associated risks in Covid-19 patients with comorbidities owing to the protective role of ACE2 in lung injury in animal disease models. The study results provide insight into the critical roles of ACE2 receptor and highlights potential targets for therapeutic interventions.

Keywords: Covid-19, SARS-CoV-2, ACE2 receptors

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1. Introduction

The Covid-19 pandemic is a global health crisis with approximately 704,192,614 confirmed cases and 7,005,699 associated deaths recorded as of this writing (March 14, 2024) (<https://www.worldometers.info/coronavirus/>). SARS-CoV-2 is a member of a family of viruses including SARS-CoV-1 and MERS and has been identified as the etiologic agent for this respiratory disease. Phylogenetic analysis reveals that SARS-CoV-2 belongs to the family *Coronaviridae*, genus *β-coronavirus*, subgenus *Sarbecovirus* [1,2]. Whole genome sequence analysis of SARS-CoV-2 reveals 89% identity to bat SARS related CoV, 80% identity to SARS-CoV-1, and 50% to MERS coronavirus [1,3,4,5].

Like other Coronaviruses, SARS-CoV-2 with a 29.9kb genome has a nucleocapsid comprising phosphorylated nucleocapsid (N) protein and positive sense single stranded genomic RNA, hemagglutinin esterase (HE), membrane (M) protein, envelope (E) protein, and spike

glycoprotein (S) [3,6,7]. Spike glycoprotein is considered a pivotal surface protein that binds to the host cell ACE2 receptor during cell entry. It facilitates fusion of viral and host cell membrane, and is a major antigenic determinant [8]. It consists of two subunits namely S1 for receptor binding; comprising N-terminal domain and C-terminal domain, and a highly conserved S2 subunit comprising the fusion peptide (FP), heptad repeat (HR1, HR2), transmembrane domain (TM), and cytoplasmic domain (Figure 1) [4].

ACE2 a transmembrane metalloprotease known to be the direct binding site for human SARS-CoV-1 and HCoV-NL63 [10] has been proposed to be the high-affinity binding site for SARS-CoV-2 [3]. ACE2 is a critical enzyme (carboxypeptidase) in regulating the renin-angiotensin- aldosterone system (RAAS) and is homologous to angiotensin converting enzyme 1 (ACE1), a target for angiotensin converting enzyme inhibitors (ACEi). ACE2 protein is encoded by *ACE2* gene positioned at Xp22.2 on X-chromosome and consists approximately 805 amino acids. It comprises two domains; N-terminal peptidase domain and a carboxy-terminal

domain [11]. ACE2 functions by proteolytically cleaving and inactivating angiotensin II, which is a peptide that induces vasoconstriction, inflammation, and fibrosis. The product of ACE2 activity is angiotensin-1-7, which is an active peptide that opposes the function of angiotensin II and which promotes vasodilation, and inhibits inflammation, and fibrosis [12,13]. ACE2 exists in two forms. These are a membrane bound form that is highly expressed in lung alveolar epithelial cells, heart, kidney, and intestinal tissues and circulatory form that is cleaved from the cell membrane by a sheddase [13,14].

Similar to SARS-CoV-1 and MERS, SARS-CoV-2 patients present with a wide range of clinical symptoms including fever, headache, rhinorrhoea, fatigue, dry cough, sneezing, nausea, diarrhoea, sore throat, dyspnoea, and pneumonia [1,15,16,17,18]. The majority of the patients have been reported to have one or more comorbidities including hypertension, diabetes, kidney failure, and coronary heart disease [19]. Initially, the disease is mild, but may accelerate causing acute respiratory distress syndrome (ARDS) and multi-organ dysfunction [20]. Currently, diverse therapeutic agents against SARS-CoV-2 are being evaluated. An antiviral drug remdesivir and glucocorticoid dexamethasone have been granted emergency use authorization by the European Medicines Agency and Food and Drug Administration for use in Covid-19 patients. Remdesivir inhibits SARS-CoV-2 [21], slowing viral replication and host damage, thereby resulting in improved clinical conditions in patients [22]. Dexamethasone regulates injurious inflammation in the lungs and lowers mortality rate in patients receiving respiratory support [23]. Understanding the pathogenesis of SARS-CoV-2 remains key in developing therapeutic drugs and vaccines. Thus, the main purpose of this study is to establish the role of ACE2 receptors in the pathogenesis of SARS-CoV-2 infection considering the available scientific literature on SARS-CoV-1 and MERS partly.

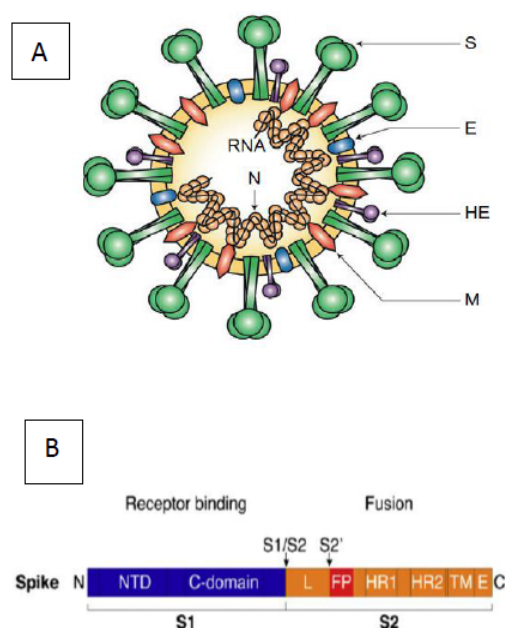


Figure 1. Basic structure of Coronavirus. A: Corona virion (9). B: Spike glycoprotein [6]

2. Methodology

2.1. Research Design

This research was entirely based on critical analysis of scientific research papers published in peer reviewed journals including; Nature, The New England Journal of Medicine, Journal of Virology, and NCBI PubMed central. I performed a PubMed search for articles using the terms; Covid-19, SARS-CoV-2, ACE2 receptors, ACE inhibitors versus ACE2 receptors, and the renin angiotensin system. The publication years were limited to 2019 and 2020. Highly cited articles were retrieved from high impact factor journals, assessed and the reference lists used to identify additional research articles. Thereafter, I selected 10 articles with relevant findings that aligned with the research aims and objectives for critical analysis. Data extracted for analysis was compared to previous study findings and conclusions were drawn.

2.2. Methods

2.2.1. Cell Cultures

Cell lines of human and animal origin included HEK293T, Vero, Caco-2, Calu-3, MDCKII, BHK-21, Huh-7, MRC-5, NIH/3T3 cells and these were cultivated for expression of SARS-CoV-1, or SARS-CoV-2, or MERS spike protein in appropriate medium, supplemented with 10% foetal bovine serum, penicillin, streptomycin. Cells were incubated at 37°C. Vesicular stomatitis virus (VSV) pseudotypes were generated as stipulated in a published protocol [24]. Selected cell lines were directly inoculated with VSV pseudotypes, while others were pre-treated with anti-ACE2 antibody to investigate receptor usage [25].

2.2.2. Immunoblotting

Pseudo virions and HEK293T cells bearing SARS-CoV-1 and SARS-CoV-2 spike protein were thawed and prepared as per standard protocols. Sodium dodecyl sulphate was used as the loading buffer. VSV- matrix protein and β -actin were used as loading controls. Samples were electrophoresed on gradient Tris-glycerine gels and thereafter transferred to polyvinylidene difluoride membrane. SARS-CoV-1 spike protein monoclonal antibody (primary) and conjugated mouse secondary antibody were used for western blotting [25,26].

2.2.3. Sequence and Structure Analysis

SARS-CoV-2 spike protein sequence (YP-009724390.1), SARS-CoV-1 sequence, and human ACE2 protein sequence (Q9BYF1) were obtained from NCBI, UniProt, and GenBank databases [27]. Their structures were identified using the BLAST program and, thereafter, retrieved from the RCSB data bank. Multiple sequence alignments of SARS-CoV-1 and SARS-CoV-2 spike protein receptor binding domain was performed using Clustal Omega program and 3D structure analysis using Cn3D program from NCBI. Protein structure simulation was undertaken using the SWISS model and molecular

docking programs including PatchDock and FireDock to analyse ACE2 and RBD interaction [25].

ACE2 genetic variants were acquired from an ensemble genome browser and gnomAD database. Impact analysis of ACE2 genetic variants was investigated using an I-mutant 2.0; predictor of effects of single point amino acid substitution based on Gibbs free energy changes. Functional impact caused by amino acid changes was predicted using SIFT (sorting intolerant from tolerant), PolyPhen-2 (polymorphism phenotyping version 2), CADD (combined annotation-dependent depletion), and REVEL (rare exome variant ensemble learner) programs. Structural models of ACE2 allelic variants were developed using atomic coordinates by using a Modeller 9.16 program. Structure analysis tools including SWISS - PdbViewer 4.1.0., PRODIGY web server and DynaMut were used specifically to predict binding affinity (between SARS-CoV-2 and human ACE2) and different types of intermolecular interactions [15,25].

2.2.4. Molecular Identification

Detection of SARS-CoV-2 from patient samples was achieved using real-time reverse transcriptase polymerase chain reaction (rRT-PCR). This is a technique where viral RNA is reverse transcribed to produce many copies of DNA for analysis. ACE2 expression pattern in various organs was established using single cell RNA-sequencing (scRNA-seq) datasets retrieved from the Gene expression omnibus database (GEO) [28].

2.2.5. Staining

For histologic staining, specific gastrointestinal tract tissues obtained using endoscopy were stained with Haematoxylin and eosin (standard stain) to assess tissue structures and ascertain damage caused to the tissues following SARS-CoV-2 invasion. Immunofluorescence staining (antigen-detection test) involved preparing samples as per the standard protocol and using an anti-ACE2 monoclonal antibody. All slides were imaged using laser scanning confocal microscopy [29].

2.2.6. Statistical Analysis

GraphPad prism 7 software package was used for statistical analyses [25]. One-way analysis of variance (ANOVA) was used to test statistical significance, where only P values lower than 0.05 were considered significant and denoted by [*] [25,30]. Logistic regression was performed on datasets with more than one independent variable to obtain odds ratios [31].

2.3. Limitations of Methods

Depending on cell culture conditions, cells tend to change their morphology, gene expression, and functionality and thereby potentially compromising the reliability of results obtained. Biological responses in cultured cells are not always recapitulated with organisms, where complex organ specific interactions occur. In animal studies, most animal models do not

always predict human health outcomes due to species differences. Human metabolic rates, pharmacodynamics, pharmacokinetics and toxicokinetics of substances differ when compared to some animal models. Bioinformatics methods used mainly rely on theoretical biology that require further laboratory tests to support or refute the hypothesis. Histological stains used can be less specific in diagnostic tests and are liable to human errors during slide preparation and analysis.

3. Results and Analysis

3.1. SARS-CoV-2 Spike Protein and ACE2 Receptor

SARS-CoV-2 entry into target cells is proposed to be dependent on viral spike protein (S) and host ACE2 receptors and is therefore a target for therapeutic intervention. To confirm this, I analysed an experimental study conducted to demonstrate that host ACE2 receptors and serine protease TMPRSS2 are involved in SARS-CoV-2 binding and entry. Comparison was made with SARS-CoV-1 and performed using human (example HEK293T cells), and animal cell lines (example Vero, MDCKII). VSV pseudotypes bearing SARS-CoV-1 spike protein (SARS-S), and SARS-CoV-2 spike protein (SARS-2-S) were used to inoculate these cell lines, which express ACE2. Immunoblot analysis was performed to analyse proteolytic processing of the spike protein at the cleavage sites (S1/S2, and S2') (Figure 2.A) and amino acid sequence analysis of spike protein was performed to establish interaction sites with the ACE2 receptors [25].

Immunoblot analysis of cell lysate of HEK293T cells to ascertain SARS-2-S expression revealed an immunoreactive protein band with molecular size ~180 kDa of unprocessed spike protein and another band with molecular size ~90 kDa equivalent to S2 subunit of the spike protein. These findings confirm successful transfection of human cells. Analysis of other cells, particularly VSV particles bearing SARS-2-S revealed a more prominent immunoreactive protein band with molecular size ~90 kDa equivalent to S2 of the spike protein (SARS-2-S) suggesting efficient proteolytic processing of SARS-2-S in human cells as observed with other Coronaviruses (Figure 2. A-C) [32]. Identical spectrum of cell lines; HEK293T, Calu-3, Caco-2, Vero, MDCKII among others were susceptible to entry driven by SARS-S and SARS-2-S, whereas Cell lines inoculated with VSV-G (G-glycoproteins) were all susceptible to entry driven by VSV glycoprotein (Figure 2C). Antiserum against human ACE2 receptors blocked SARS-2-S, SARS-S, but not MERS-S and VSV-G (results not shown). This finding strongly indicate that human ACE2 are functional receptors for both SARS-CoV-1 and SARS-CoV-2 and suggest that the ACE2 receptor is a potential therapeutic target for SARS-CoV-2 [25,26].

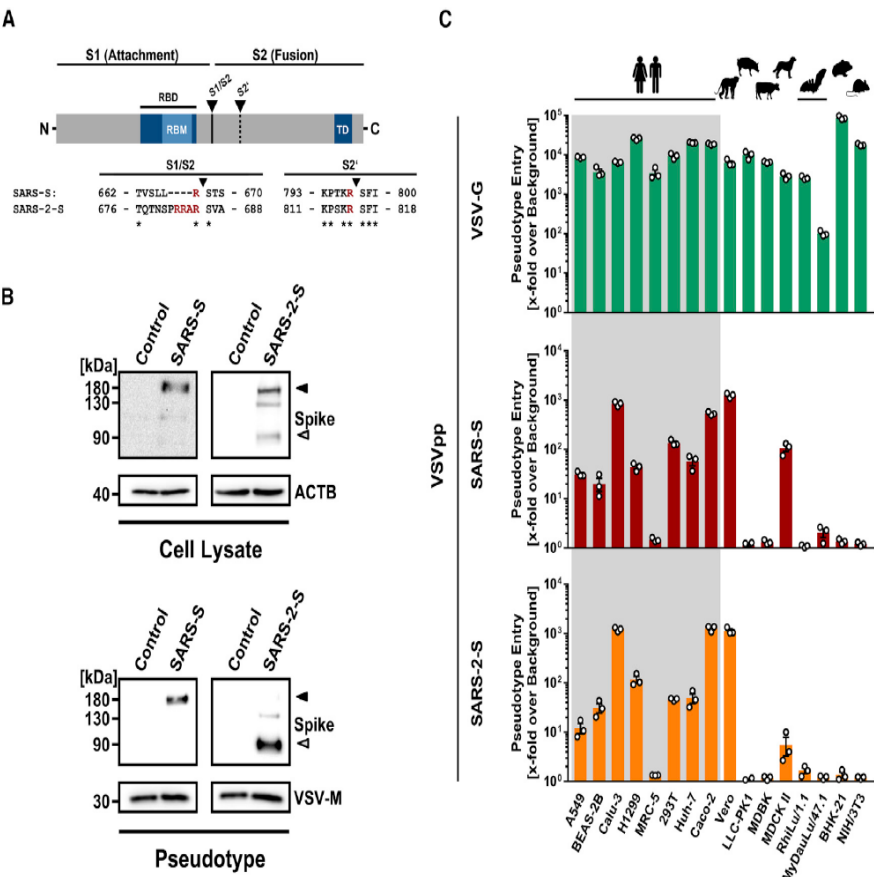
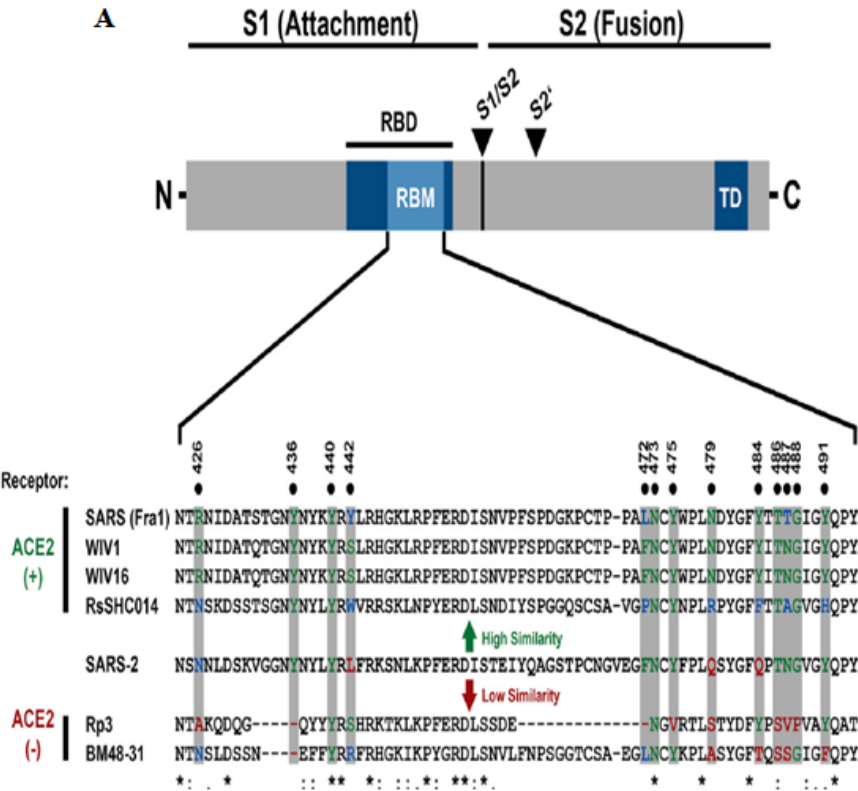


Figure 2. A. SARS-S and SARS-2-S sequence alignment. Unique PRRARS[V] furin cleavage site in red font present in SARS-2-S. Black arrow heads showing cleavage sites at S1/S2 and S2', and asterisks showing conserved amino acids. B. Western blot analysis of SARS-2-S expression (top figure) and VSV pseudotype integration (bottom figure), black arrowheads designate unprocessed spike protein and grey arrowheads designate S2 subunit of spike protein. C. VSV-G, SARS-S and SARS-2-S entry into a spectrum mammalian cell lines [25]



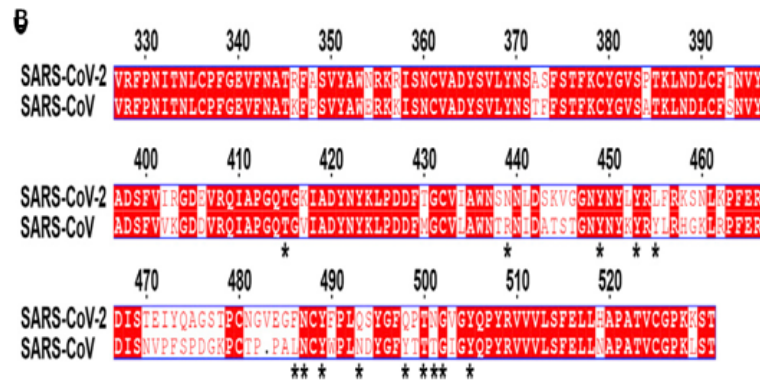


Figure 3. A. SARS-S spike protein receptor binding motif alignment with corresponding sequences of bat associated coronaviruses spike protein which are able or not to use ACE2 receptors; revealing that SARS-2-S possess amino acid residues key in ACE2 attachment process. Asterisks indicate positions with conserved residues across species, [.] site with conservative replacements, [.] semi-conservative replacements [25]. **B.** sequence alignment of SARS-CoV-1 and SARS-CoV-2 spike protein subunits with identical amino acid residues in white font, and 14 key amino acid residues signified by asterisks [15,26]

3.2. Receptor Binding Domain (RBD) Sequence Analysis and Structures

Spike protein sequence analysis revealed that SARS-CoV-2 phylogenetically clusters with SARS-CoV-1 bat related coronaviruses (SARSr-CoV), most of which utilise ACE2 receptors for cell entry. Analysis of the SARS-CoV-2 RBD revealed a unique insertion of a RRAR furin cleavage site (Figure 2A) which might have a significant role in the pathogenesis of SARS-CoV-2 [15,25], although this requires further investigation. Receptor binding motif (RBM) revealed that the most essential amino acid residues for binding ACE2 receptors in SARS-S are conserved in SARS-2-S (Figure 3A). Two similar studies involving sequence alignment of SARS-CoV-2 and SARS-CoV-1 RBD using Clustal Omega program, and BLAST program were further analysed. Results revealed 14 amino acids critical for binding SARS-S and SARS-2-S to human ACE2 receptors of which 8 are strictly conserved in both (Figure 3B) [15,26] confirming similarity in structure of the two viruses and their target cell receptors.

3.3. Molecular Simulation

To further elucidate the interactions between the viral spike protein and human ACE2, two studies exploring SARS-CoV-1 and SARS-CoV-2 structures were analysed. SARS-CoV-1 and SARS-CoV-2 spike protein sequences share 76%-78% identity, 72%-76% RBD identity, and 50-53% RBM identity [33]. Molecular simulation and 3D structure analysis using Cn3D program and Swiss model revealed superimposable structures, except for difference in loop2 linked to amino acid substitution (Figure 4. A-C). These are proposed to contribute to differences in structural flexibility and binding affinity of the two viruses for human ACE2 receptor [34]. Structural difference in SARS-CoV-1 and SARS-CoV-2 receptor binding domains identify molecular divergence between the two viruses. Similarities observed in the two viral spike protein RBD confirm their binding to the same target ACE2 (Figure 4D) thereby supporting the study hypothesis.

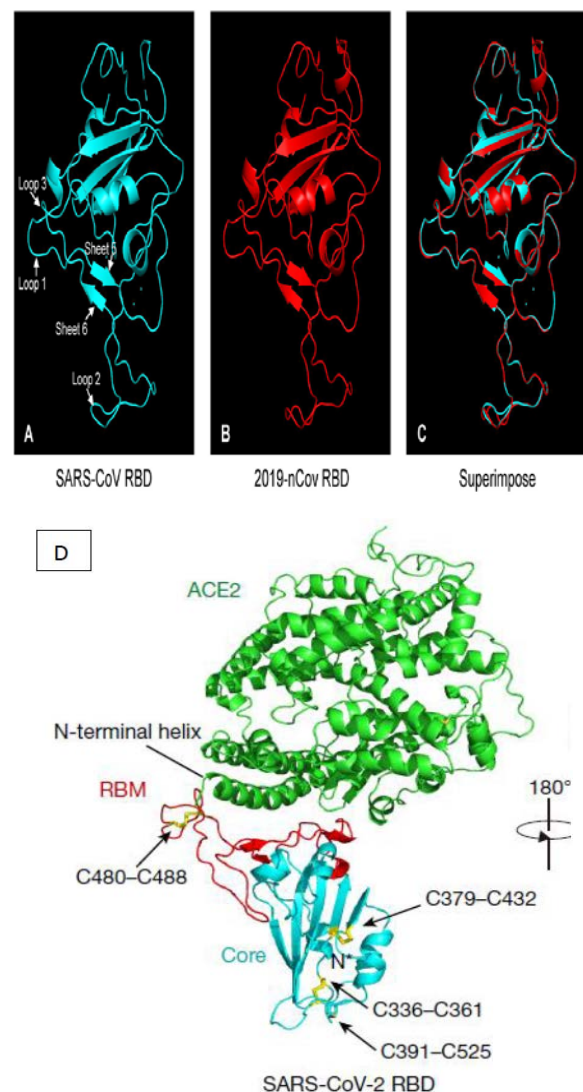
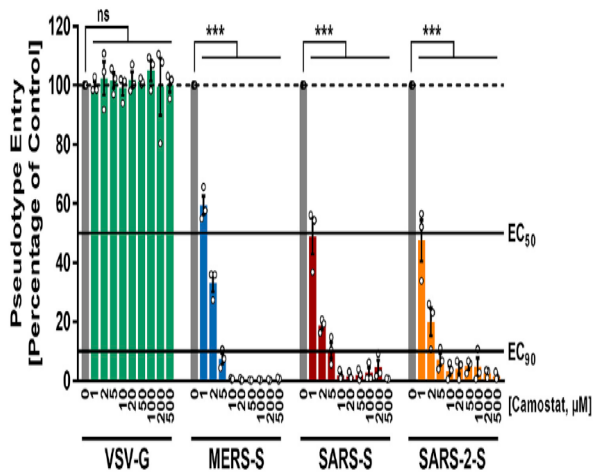


Figure 4. A-C. SARS-CoV-1 and SARS-CoV-2 Receptor binding domain structures superimposed and the difference is highlighted in loop 2 [34]. **D.** Complex structure of SARS-CoV-2 RBD bound to human ACE2 receptor. ACE2 receptor is shown in green with N-terminal helix responsible for binding. SARS-CoV-2 RBD core is shown in cyan and receptor binding motif in red. Disulfide bonds in SARS-CoV-2 RBD are shown as sticks in yellow indicated by arrows [15].

3.4. Proteases for Spike Protein Priming

SARS-CoV-1 utilizes the host proteases Cathepsin B and L (CatB/L) and TMPRSS2 for spike protein priming. Thus, I performed an analysis of the two proteases. TMPRSS2 inhibitor Camostat mesylate was used to determine whether TMPRSS2 is needed for infection of lung cells by SARS-CoV-2. It was observed that Camostat mesylate significantly reduced cell entry driven by MERS-S, SARS-S, and SARS-2-S and significantly reduced infection of Calu-3 cells (Figure 5). TMPRSS2 deficient HEK293T cells treated with ammonium chloride to block CatB/L activity revealed significantly inhibited SARS-S and SARS-2-S entry, whereas TMPRSS2 over-expressing cacao-2 cells treated with ammonium chloride were more resistant to inhibition. Thus, while there is a dependency for CatB/L the activity of the transmembrane serine protease TMPRSS2 is crucial for viral entry and immunopathology in the infected host [35]. Cultivated primary cell lines provided an insight into the spike protein, ACE2 and TMPRSS2 as potential therapeutic targets. These findings need to be recapitulated *in vivo*.



while ACE2 alleles rs73635825 (S19P) and rs1439336283 (E329G) showed lower binding affinity and exhibited differences in the intermolecular interaction with the viral spike protein (Figure 6B).

Amino acid substitutions in ACE2 variant alleles including rs73635825, rs1299103394, rs766996587,

rs961360700, rs762890235, rs1396769231 were predicted to have adverse effect on the encoded protein compared to the wild type receptor using different prediction tools; SIFT, Polyphen-2, CADD, and REVEL and estimated free energy changes (Table1). However, no functional impact of mutations was experimentally proven [36].

Table 1. Comparison of structural and functional consequences of selected ACE2 allelic variants (36)

Variants	AAC	Allele frequency	RMSD (Å)	$\Delta\Delta G$		SIFT	PolyPhen - 2	CADD	REVEL
				25°C	37°C				
rs73635825	S19P	3.13E-4	0.24	0.39	0.41	T	PD	LB	LB
rs1299103394	K26E	5.45E-6	0.29	0.67	0.66	T	B	LB	LB
rs4646116	K26R	3.88E-3	0.24	-0.34	-0.30	T	B	LB	LB
rs781255386	T27A	1.09E-5	0.19	-1.86	-1.72	T	B	LB	LB
rs778500138	E35D	N/A	0.24	-0.51	-0.49	T	B	LB	LB
rs1348114695	E35K	1.64E-5	0.24	-1.46	-1.44	T	B	LB	LB
rs146676783	E37K	3.9E-5	0.27	-1.03	-1.02	D	PD	LB	LB
rs755691167	K68E	1.09E-5	0.20	-0.67	-0.65	T	B	LB	LB
rs766996587	M82I	2.44E-5	0.27	0.73	0.77	T	B	LB	LB
rs759134032	P84T	5.47E-6	0.27	-1.40	-1.32	T	B	LB	LB
rs143936283	E329G	3.44E-5	0.17	-0.46	-0.32	T	B	LB	LB
rs9613607a00	D355N	1.17E-5	0.58	-0.86	-0.64	D	PD	LB	LB
rs1396769231	M383T	N/A	0.21	-1.57	-1.49	D	PD	LB	LDC
rs762890235	P389H	3.83E-5	0.21	-1.27	-1.19	D	PD	LB	LB
rs1238146879	P426A	5.47E-6	0.25	-1.57	-1.42	T	B	LB	LB
rs1316056737	D427Y	1.09E-5	0.21	-0.18	-0.10	D	PD	LB	LB
rs1016777825	R559S	N/A	0.21	-1.38	-1.28	T	B	LB	LB

Abbreviations: AAC: amino acid change, RMSD: root mean square deviation, D: deleterious, LB: likely benign, LDC: likely disease causing, PD: probably damaging, T: tolerated

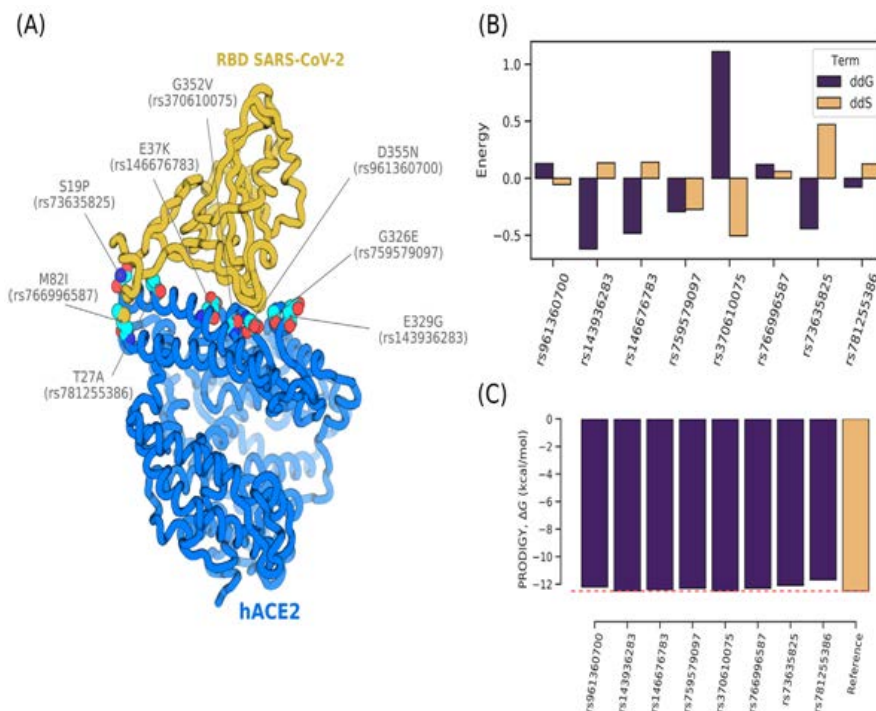


Figure 7. A. Variant human ACE2 labelled by single nucleotide polymorphism database ID (dbSNP-‘rs’) interacting with SARS-CoV-2 receptor binding domain. **B.** folding energy calculated by DynaMut (enthalpy-ddG, entropy-ddS) predicting the impact of mutations. **C:** interaction energy calculated using PRODIGY

Low binding affinity observed in ACE2 variants might be associated with potential resistance to Covid-19 infection. In addition, predicted adverse effects might be linked with different disease severity, although no

scientific evidence has been presented to date [36]. Molecular modelling and bioinformatics tools used provided crucial insight on the matter but lacked empirical means to validate the findings and make firm conclusion.

To further understand the impact of ACE2 polymorphism, I analysed an additional study investigating the impact of human ACE2 gene variants. ACE2 variants (8 in total and a subset of the previous study) that map to the protein coding region (Figure 7A) were obtained from gnomAD database and analysed using PRODIGY for predicting binding affinity and DynaMut for predicting the impact of mutations on structural aspects of the protein. Folding energy calculations enthalpy (ddG) and entropy (ddS) showed no significant changes (>1 or <1 kcal/mol) (Figure 7B). There was an observed change in the interaction energy ΔG (protein-protein) (Figure 7C) [27]. However, the findings suggest that the ACE2 variants have no significant change in binding affinity and no possible resistance to SARS-CoV-2. This contrasts with the previous study. The slight difference observed in the two studies was associated with the difference in the number of ACE2 variants and the computational methods used. Performing other comparative computational tests and *in vitro* tests to validate the findings would probably lead to a clearer conclusion.

3.6. ACE2 Expression and Multi-Organ Failure

To understand the association between ACE2 receptors and multi-organ failure, I explored a recent single cell RNA-sequencing (scRNA-seq) data analysis study on ACE2 receptor expression. This study revealed potential risk of SARS-CoV-2 infection in different human organs. Single cell RNA-seq datasets from tissues and organs of the respiratory, cardiovascular, digestive, and urinary systems were collected from the GEO database. ACE2 expression was analysed across different organs. Considering the accepted consensus that SARS-CoV-2 targets lung type II alveolar cells (AT2), ACE2 expression levels in AT2 cells were used as reference (unique molecular identifier count > 0). Single cell genomics tool Seurat V3.0 was used to differentiate cell types and Uniform Manifold approximation and Projection (UMAP) method to obtain cell scatter plots [28].

Respiratory system scRNA-seq data analysis revealed that pulmonary AT2 cells and respiratory epithelial cells express high ACE2 levels. Lung AT2 cells expressed approximately 1% ACE2 positive cells and 2% ACE2 for respiratory epithelial cells (Figure 8). Respiratory epithelial cells expressing high ACE2 levels also expressed PIGR and MUC1 genes, which are canonical markers of respiratory epithelial cells.

Cardiac system scRNA-seq data analysis revealed more than 7.5% of the myocardial cells exhibit high ACE2 expression levels (Figure 9) suggesting that the heart is possibly an organ at high risk in Covid-19 infection. High ACE2 expression levels in myocardial cells was confirmed using expression of MYL3 and MYH7 genes; canonical markers of myocardial cells.

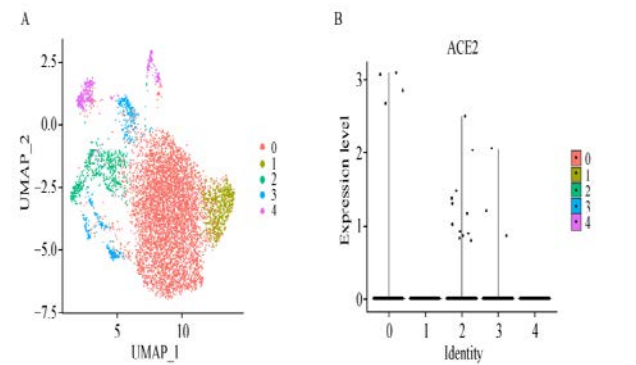


Figure 8. scRNA-seq data analysis revealed respiratory epithelial cells with high ACE2 expression. **A.** scatter plot showing respiratory tract cells categorized into five clusters. **B.** Violin plot showing ACE2 expression levels of different cell clusters [28]

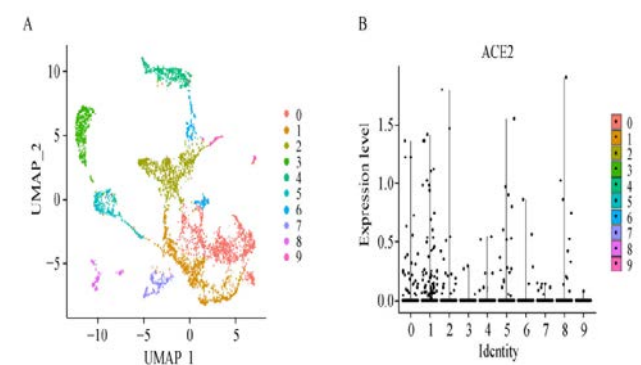


Figure 9. scRNA-seq data analysis revealed myocardial cells with high ACE2 expression. **A.** scatter plot showing respiratory tract cells categorized into 10 clusters. **B.** Violin plot showing ACE2 expression levels of different cell clusters [28]

Digestive system scRNA-seq data analysis revealed high ACE2 expression in ileum and oesophageal epithelial cells. Approximately 30% ACE2 positive cells were detected in the ileum (Figure 10) and 1% in the oesophagus (Figure 11), thereby suggesting that the ileum and oesophagus could be at high risk to infection and this is clinically evident.

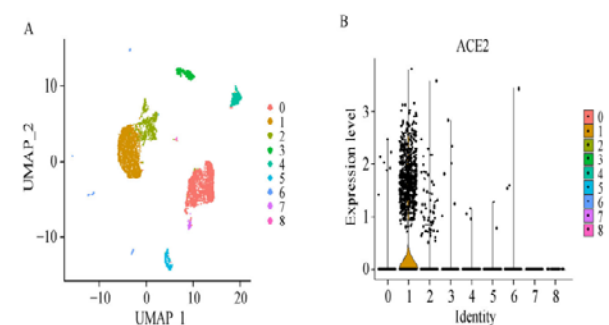


Figure 10. scRNA-seq data analysis revealed ileal epithelial cells with high ACE2 expression. **A.** scatter plot showing ileal epithelial cells categorized into nine clusters. **B.** Violin plot showing ACE2 expression levels of different cell clusters [28]

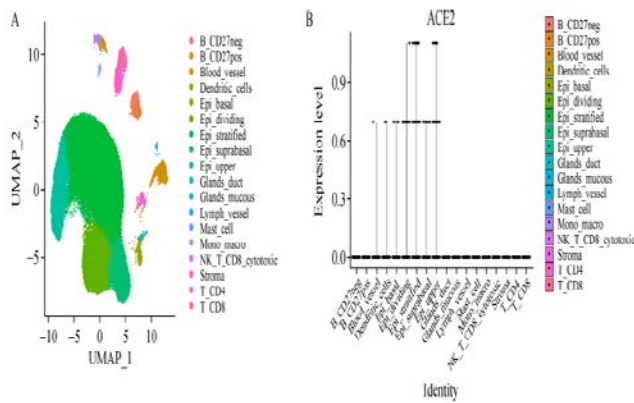


Figure 11. scRNA-seq data analysis revealed a few esophageal cells express ACE2. **A.** scatter plot showing oesophageal epithelial cells categorized into 18 clusters. **B.** Violin plot showing ACE2 expression levels of different cell clusters [28]

Urinary system scRNA-seq data analysis revealed that kidney proximal tubule cells (Figure 12) and bladder urothelial cells (Figure 13) express high ACE2 levels that is approximately 4% and 2.4% respectively of the total cell number. This suggests that the kidney and bladder are at high risk in Covid-19 infection.

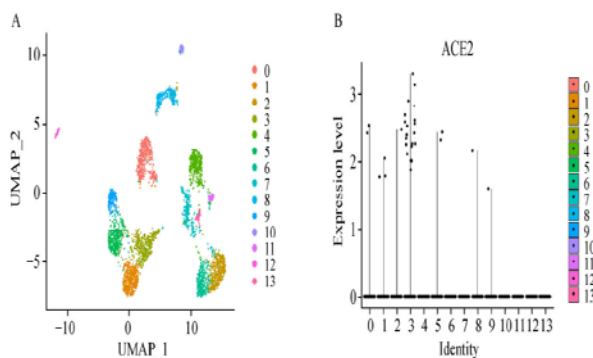


Figure 12. kidney scRNA-seq data analysis revealed high expression of ACE2 in proximal tubule (TB) cells. **A.** scatter plot showing proximal tubule cells categorized into 14 clusters. **B.** Violin plot showing ACE2 expression levels of different cell clusters [28].

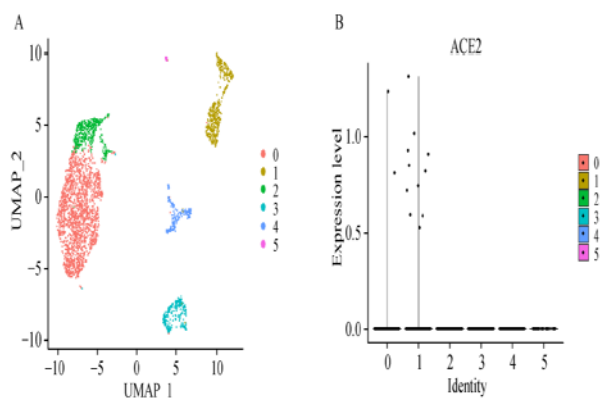


Figure 13. Bladder scRNA-seq data analysis revealed high expression of ACE2 in a few urothelial cells. **A.** scatter plot showing cells categorized into six clusters. **B.** Violin plot showing ACE2 expression levels of different cell clusters [28].

Single cell RNA-seq is essentially a robust technology providing a higher resolution of cellular differences and understanding of single cells at the genome level. In this study, the analysis provides reliable information which can be used to develop a map of high-risk organs (Figure 14) including the heart, kidney, bladder, ileum and oesophagus in addition to the lungs. This provides clinical manifestation of the infection in organ systems in Covid-19 patients and can provide a basis for multi-organ failure.

After establishing the potential risk organs based on ACE2 expression, I explored the evidence for gastrointestinal infection associated with SARS-CoV-2. In this study, stool samples were obtained from 73 hospitalized Covid-19 patients and tested for viral RNA using rRT-PCR. Gastrointestinal tissues from oesophagus, stomach, duodenum and rectum were collected from one patient using endoscopy and stained for the ACE2 receptor and the viral nucleocapsid protein (NP) using histologic (H&E; haematoxylin and eosin) and immunofluorescent staining (laser scanning confocal microscopy) [29].

Results showed that 39 patients out of 73 tested positives for viral RNA (SARS-CoV-2) in their stools. Histologic staining (H & E) of oesophagus, stomach, duodenum, and rectum tissues showed no significant damage to the epithelium. Immunofluorescent staining showed positive staining for ACE2 receptor and viral nucleocapsid protein in the cytoplasm of epithelial cells of the stomach, duodenum, and rectum (Figure 15) [29]. These results show the expression of ACE2 receptors that might enable SARS-CoV-2 entry into gastrointestinal epithelial cells. The presence of virions from infected gastrointestinal cells, shedded and detected in stools suggest the possibility of faecal oral route of transmission. Staining methods used may not provide conclusive results but were validated by rRT-PCR test results. Similar results were obtained in the previous study which employed scRNA-seq technique [28].

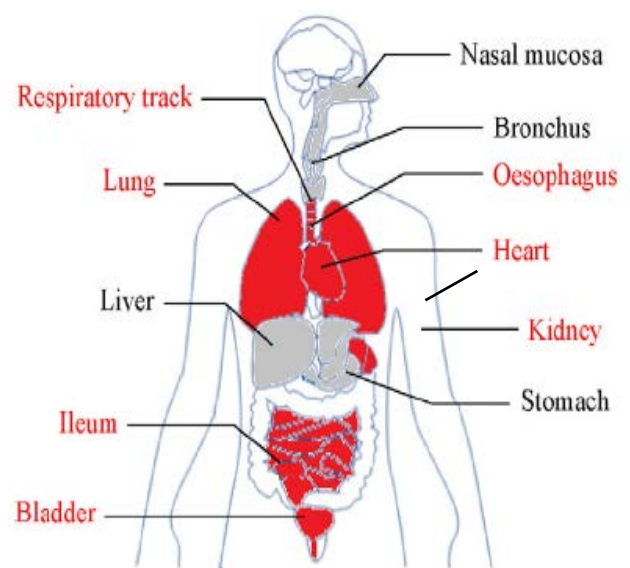


Figure 14. Human organs possibly at high risk in Covid-19 infection. Red highlights high risk organs and grey for low risk organs [28]

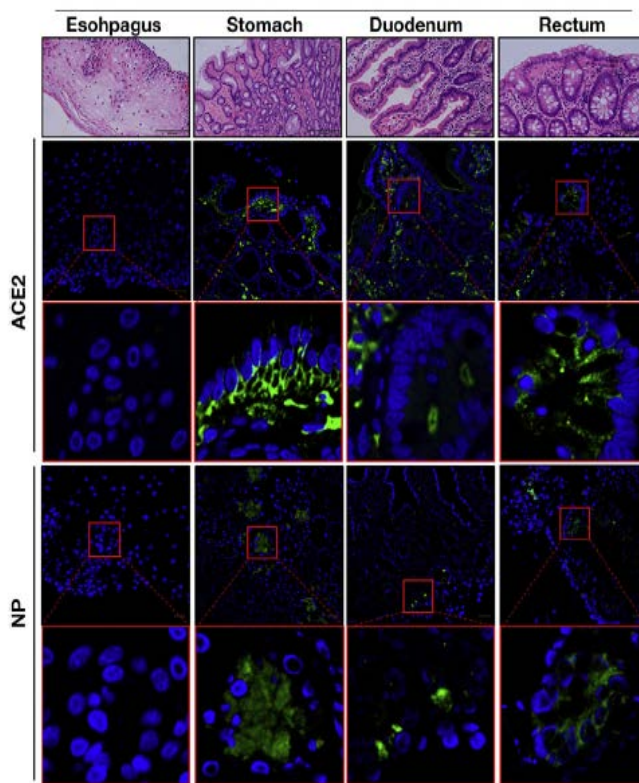


Figure 15. Histologic staining (H&E) and immunofluorescent staining images of gastrointestinal tissues; oesophagus, stomach, duodenum and rectum. Histologic image scale bar represents 100µm, and 20µm for the immunofluorescent image [29].

3.7. ACE inhibitors, Angiotensin II Receptor Blockers and the Risk in Covid-19

ACEi and ARBs are widely used in management of hypertension, heart failure, and chronic kidney disease and have been proposed to increase the expression of ACE2 receptors thereby suggesting that they might increase susceptibility to Covid-19 infection. To evaluate this possibility, I analysed the effect of ACEi and ARBs on cardiac ACE2 in rats.

Thirty-six male Lewis rats were randomly assigned to four treatment groups; vehicle (tap water), Lisinopril (ACEi), Losartan (ARB), and combined drugs (Lisinopril+Losartan). Cardiac angiotensin II (Ang II), angiotensin-1-7, and cardiac ACE2 mRNA from cardiac tissue cells were measured after 12 days of continuous administration of treatment in drinking water, in order to measure the activity of ACE2 receptors. Messenger RNA was isolated using Trizol reagent, assessed using Agilent 2100 bioanalyzer, and gene expression analysed using real time PCR. Statistical analysis was achieved using one-way ANOVA and 2-tailed student *t* test [30].

In vehicle treated rats, cardiac levels of Ang-1-7 and Ang II were comparable with a ratio of 1.07 ± 0.13 . ACE inhibition with lisinopril resulted in increased Ang-1-7 levels. The Ang-1-7/Ang II ratio increased to 1.57 ± 0.37 but this was not significant ($p > 0.05$). In ARB treated rats, Ang-1-7 and Ang II levels significantly increased with a ratio 1.21 ± 0.17 . In combined drug treatment experiments,

the levels of Ang-1-7 significantly increased compared with vehicle treated rats. In contrast, Ang II decreased to values equivalent to those observed in vehicle and lisinopril treated animals (Figure 16) [30]. This suggests use of ARB highly influences ACE and ACE2 activity compared with ACEi.

Analysis of ACE2 gene expression revealed significant increase in ACE2 mRNA in lisinopril treated rats, and 2.8fold increase in losartan treated rats, whereas combined therapy significantly reduced ACE2 mRNA to levels comparable to those in vehicle treated rats (Figure 17A). This suggests that both ACEi and ARBs increase ACE2 expression. Analysis of effects of treatments on ACE2 activity showed significant increase in ACE2 activity in losartan and combined therapy treated animals, whereas no change was seen in lisinopril treated animals (Figure 17B) [30]. This shows that both ACEi and ARBs possibly influence ACE2 expression but only ARBs affect ACE2 activity. These findings provide theoretical evidence that individuals consuming ACEi and ARBs are possibly at higher risk of infection with SARS-CoV-2. In contrast, ARDS animal model revealed that ACE2-Ang-1-7 pathway plays a protective role by antagonizing ACE-Ang-II pathway to reduce inflammation, fibrosis and acute lung injury thereby offsetting any potential increase in viral entry in acute respiratory syndrome [37]. Thus, it is arguable that ACEi and ARBs pose a risk in Covid-19 infection. Existing differences between rodent and human physiology limited the study results and therefore require more human studies.

I further analysed a population-based case-control study to evaluate the association between use of ACEi or ARBs and risks to Covid-19 infection. A total of 6,272 confirmed Covid-19 patients in Lombardy- Italy registered with the Regional Health Authority and 30,759 controls all above 40years of age were recruited for the study. Each case patient was randomly matched up to 5 controls based on age, sex and municipality of residence. The patient's clinical profiles and drug history revealed to the Regional Health Authority were extracted and processed using statistical packages, thereafter, used as data for the study. Logistic regression method was used to estimate the odds ratios at 95% confidence interval for associations between drug exposures and Covid-19 infection [31].

Data obtained from the regional repository revealed that a higher number of Covid-19 patients had comorbidities including cardiovascular disease, cancer, kidney disease and respiratory disease. More case patients frequently received prescriptions of ACEi, or ARBs compared to the controls (Table 2). Multivariable analysis of data with adjusted odds ratios at 95% confidence interval showed no evident association between use of ACEi or ARBs and susceptibility to Covid-19 infection (Table 3) [31], leading to a conclusion that ACEi and ARBs are not risk factors in Covid-19. This study included many case patients and well-matched controls with a trusted data source to produce reliable results. However, drug exposure information was limited to prescriptions and not actual consumption by case patients and controls, thereby raising concerns around the findings.

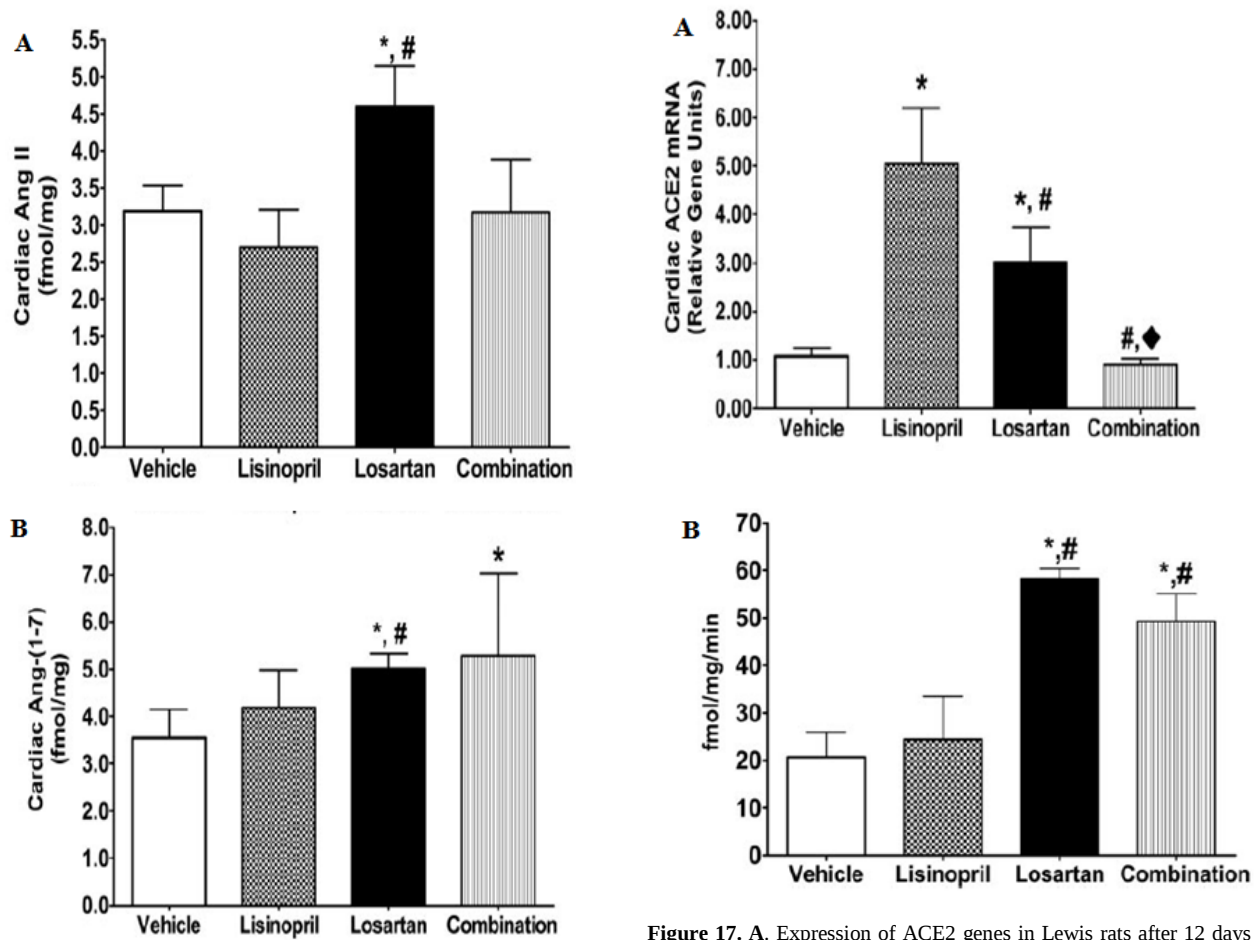


Figure 16. A-B. cardiac Ang II and Ang- (1-7) concentration in Lewis rats after 12 days of continuous treatment with vehicle, lisinopril, losartan, and combined therapy. * signify $P < 0.05$ compared with vehicle and # $P < 0.05$ compared with lisinopril. All values are mean $\pm$ SEM [30]

Figure 17. A. Expression of ACE2 genes in Lewis rats after 12 days of continuous treatment with vehicle, lisinopril, losartan, and combined therapy. B. Effect of treatments on ACE2 activity. * signify $P < 0.05$ compared with vehicle and # $P < 0.05$ compared with lisinopril. All values are mean $\pm$ SEM [30].

Table 2. Demographic and Clinical Characteristics of Patients with Covid-19 (Case Patients) and Matched Controls [31]

Characteristic	Case Patients (N = 6272) %	Controls (N = 30,759)	Relative difference
Age — yr	68 $\pm$ 13	68 $\pm$ 13	MV
Female sex — no. (%)	2303 (36.7)	11,357 (36.9)	MV
Drugs — no. (%) †			
Antihypertensive drugs overall	3632 (57.9)	15,319 (49.8)	14.0
ACE inhibitors	1502 (23.9)	6,569 (21.4)	10.5
ARBs	1394 (22.2)	5,910 (19.2)	13.3
Calcium-channel blockers	1446 (23.1)	5,926 (19.3)	13.1
Beta-blockers	1826 (29.1)	7,123 (23.2)	20.5
Diuretics	1902 (30.3)	7,420 (24.1)	20.5
Coexisting conditions and associated procedures — no. (%)			
Cardiovascular disease	1891 (30.1)	6,679 (21.7)	28.0
Coronary artery disease	473 (7.5)	1,519 (4.9)	34.6
Percutaneous coronary intervention	244 (3.9)	823 (2.7)	31.3
Heart failure	323 (5.1)	759 (2.5)	52.1
Respiratory disease	651 (10.4)	1,716 (5.6)	46.3
Chronic obstructive pulmonary disease	188 (3.0)	433 (1.4)	53.1
Asthma	18 (0.3)	35 (0.1)	60.4
Kidney disease	311 (5.0)	818 (2.7)	26.8
Chronic kidney disease	181 (2.9)	393 (1.3)	55.8
Dialysis	49 (0.8)	54 (0.2)	77.6
Cancer	1091 (17.4)	4,639 (15.1)	13.3

Table 3. Adjusted Odds Ratios for Covid-19 Associated with Use of RAAS Blockers and Other Antihypertensive Drugs [31]

Variable	Odds Ratio for Covid-19 (95% CI) *				
	ACE Inhibitors	ARBs	Calcium-Channel Blockers	Diuretics	Beta-Blockers
Severity of clinical manifestations					
Mild to moderate	0.97 (0.88–1.07)	0.96 (0.87–1.07)	1.01 (0.92–1.10)	1.07 (0.97–1.19)	0.98 (0.89–1.07)
Critical or fatal	0.91 (0.69–1.21)	0.83 (0.63–1.10)	1.15 (0.91–1.44)	0.96 (0.74–1.26)	1.07 (0.84–1.37)
Sex					
Female	0.95 (0.81–1.12)	0.89 (0.76–1.05)	1.06 (0.92–1.23)	1.12 (0.94–1.34)	1.04 (0.91–1.20)
Male	0.98 (0.87–1.11)	0.98 (0.86–1.11)	1.00 (0.90–1.11)	1.02 (0.91–1.15)	0.97 (0.87–1.08)
Age at diagnosis§					
<60 Yr	0.94 (0.71–1.25)	0.89 (0.67–1.18)	1.13 (0.88–1.46)	0.99 (0.75–1.31)	1.00 (0.78–1.29)
≥60 Yr	0.97 (0.87–1.08)	0.95 (0.85–1.06)	1.01 (0.93–1.11)	1.07 (0.97–1.19)	0.99 (0.90–1.08)

4. Discussion and Conclusion

Viral cell entry is dependent on receptor recognition and attachment which represents the initial step of viral infection [38]. Since the initial outbreak of Covid-19, understanding SARS-CoV-2 pathogenesis has been key to drug and vaccine development. The present study provides evidence that ACE2 receptor plays a critical role in SARS-CoV-2 pathogenesis. The VSV pseudotyping system revealed similar susceptibility to viral entry for HEK293T, Calu-3, Caco-2, Vero, and MDCKII expressing ACE2 receptors by SARS-CoV-1 and SARS-CoV-2 spike protein. Antiserum against human ACE2 blocked SARS-S and SARS-2-S. These findings led to a conclusion that ACE2 is a functional receptor and a potent pharmacological target although its protective efficacy is unknown and requires more elaborative human studies. To support the findings, a decade-long SARS CoV structural study predicted ACE2 as the functional receptor in SARS-CoV-2 infection [33] and SARS-CoV-2 neutralizing antibodies proved protective in animal models [39].

From this study, it is evident that ACE2 receptors are highly expressed in various organs including lungs, heart, kidneys, bladder, and the gastrointestinal tract. These findings were associated with multi-organ failure reported in Covid-19 infection. However, literature is not clear about specific levels of ACE2 needed to produce disease following viral entry. Empirical investigations on ACE2 expression and link to SARS-CoV-2 infection will help fulfil the gaps on this subject.

Exploration of ACE2 polymorphism revealed ACE2 alleles rs73635825 (S19P) and rs1439336283 (E3296) with low binding affinity to viral spike protein and were predicted to have adverse impact on encoded protein. Low binding affinity was associated with limited viral entry and replication. Genetic variants in host cell receptors have been reported to confer susceptibility and resistance against pathogenic microbes. For instance, genetic variations in human CD4 and CCR5-Δ32 receptors influence susceptibility and resistance against HIV strains [40]. Similarly, ACE2 coding variant findings may be suggestive of intrinsic resistance against SARS-CoV-2. In Brazilian patients, ACE combined with ACE2 G8790A polymorphism revealed susceptibility to hypertension [41]

thereby raising more concerns. The association of ACE2 polymorphism and associated impact in Covid-19 require further investigations.

Sequence alignment of the two viral spike protein RBDs revealed the presence of a furin cleavage site in SARS-CoV-2 which is absent in SARS-CoV-1 and was associated with the high pathogenicity observed in the new SARS virus. Furin (convertase) is widely expressed in the body tissues and functions by cleaving viral surface glycoprotein through furin cleavage site, thereby enhancing viral fusion and facilitating viral spread [42]. For instance, high pathogenicity in virulent influenza viruses (H5N1) has been associated with the presence of a furin-like cleavage site cleaved by various host cell proteases and furin [43]. Similarly, the presence of a furin cleavage sight in SARS-CoV-2 might suggest gain of function in terms of viral entry compared with SARS-CoV-1.

Further, I found that proteolytic activation of the Spike protein is necessary for membrane fusion. This involves the host cell proteases TMPRSS2, which is a critical step in SARS-CoV-2 pathogenesis and can be considered as a potential therapeutic target. Similar findings were reported in SARS-CoV-1 where Cathepsins B and L, and TMPRSS2 proteolytically activated the spike protein. Efficient Proteolytic processing of the spike protein by TMPRSS2 was reported to diminish viral sensitivity to inhibitory neutralizing antibodies, thereby promoting viral spread and pathogenesis [6,32]. Pre-activation of SARS-CoV-2 spike protein by host furin prior cell entry makes it less dependent on target cell proteases (Cathepsins B and L) unlike SARS-CoV-1.

In animal studies, use of ACEi and ARBs revealed increased cardiac ACE2 gene expression [30] which can be linked to high rate of cardiac failure in SARS-CoV-2 infections reported. SARS-CoV-1 cell entry through ACE2 receptor downregulates ACE2 gene expression [44] and is implicated in pathogenicity of the virus [45,46]. Similarly, SARS-CoV-2 utilizes ACE2 receptors and possibly downregulates ACE2 expression upon cell entry. As such, use of ACEi and ARBs resulting in increased ACE2 expression might not necessarily be a risk in Covid-19 infection, but an advantage in patients owing to the protective role of ACE2. Moreover, a double blinded clinical trial is underway to investigate the potential benefit of losartan (ARB) in Covid-19 patients. To support

this, a recent study revealed no significant association between use of ACEi or ARBs and risk in Covid-19 infection [31]. Use of ACEi and ARBs in hospitalized Covid-19 patients with coexisting hypertension was associated with reduced mortality compared to nonusers [47]. Concerns around ACEi/ARB influencing risks in Covid-19 require more detailed human studies as the currently available information is too limited to support or refute the hypotheses.

It has been postulated that diabetes and hypertension impact Covid-19 pathogenesis and prognosis [48]. Hyperglycaemia in diabetic patients impairs T-cell action, natural killer cell activity, and alters cytokine production leading to severe clinical symptoms in Covid-19 and delayed microbial clearance. [49]. A recent study involving hospitalized Covid-19 patients suggested that diabetes and hypertension resulted in delayed SARS-CoV-2 clearance. This was associated with the use of ACEi increasing ACE2 thereby facilitating viral entry and use of Cortisone an immune modulator associated with depletion of B-cells and T- cells [50]. Specific mechanisms underlying comorbidities and Covid-19 remain unclear and requires detailed studies.

The impact of Covid-19 infection ranging from high morbidity and mortality rates to social and economic challenges have led to publication of highly controversial articles on the subject, making it difficult to make conclusive remarks on the current study.

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