Research paper

Identification of COVID-19 prognostic markers and therapeutic targets through meta-analysis and validation of Omics data from nasopharyngeal samples

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ABSTRACT

Background: While our battle with the COVID-19 pandemic continues, a multitude of Omics data have been generated from patient samples in various studies. Translation of these data into clinical interventions against COVID-19 remains to be accomplished. Exploring host response to COVID-19 in the upper respiratory tract can unveil prognostic markers and therapeutic targets.

Methods: We conducted a meta-analysis of published transcriptome and proteome proﬁles of respiratory samples of COVID-19 patients to shortlist high conﬁdence upregulated host factors. Subsequently, mRNA overexpression of selected genes was validated in nasal swabs from a cohort of COVID-19 positive/negative, symptomatic/asymptomatic individuals. Guided by this analysis, we sought to check for potential drug targets. An FDA-approved drug, Auranoﬁn, was tested against SARS-CoV-2 replication in cell culture and Syrian hamster challenge model.

Findings: The meta-analysis and validation in the COVID-19 cohort revealed S100 family genes (S100A6, S100A8, S100A9, and S100P) as prognostic markers of severe COVID-19. Furthermore, Thioredoxin (TXN) was found to be consistently upregulated. Auranoﬁn, which targets Thioredoxin reductase, was found to mitigate SARS-CoV-2 replication in vitro. Furthermore, oral administration of Auranoﬁn in Syrian hamsters in therapeutic as well as prophylactic regimen reduced viral replication, IL-6 production, and inﬂammation in the lungs.

Interpretation: Elevated mRNA level of S100s in the nasal swabs indicate severe COVID-19 disease, and FDA-approved drug Auranoﬁn mitigated SARS-CoV-2 replication in preclinical hamster model.

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1. Introduction

The COVID-19 pandemic has emerged as the biggest global public health crisis of this century. As of August 4, 2021, more than 200 million infections and 4.2 million deaths have been reported (https://www.worldometers.info/coronavirus/). The causative agent SARS-CoV-2 contains a single-stranded positive-sense RNA genome that encodes 29 proteins [1]. COVID-19 disease is quite heterogeneous, and its manifestation ranges from asymptomatic, mild, severe to lethal, depending on a variety of host, virus, and environmental factors [2]. Age, sex, ethnicity, and co-morbidities have all been implicated in determining disease outcomes [2, 3]. An effective and early interferon (IFN) response is critical in resolving viral infections [4], however, SARS-CoV-2 has multiple strategies to suppress host immune responses [5]. Disruption of immune homeostasis and
induction of cytokine storm has been recognized as one of the underlying causes of severe COVID-19 [6], yet the molecular mechanisms underlying immune dysregulations are yet to be defined.

Several research groups have applied the force high-throughput methodologies to profile the host responses upon viral infections [7-14]. This has resulted in a wealth of virus-host interaction Big Data, which holds the key to novel therapeutic strategies and molecular markers of infection and disease progression. Examining host response at the primary site of infection in the upper respiratory tract is crucial to understanding viral pathogenesis. Various studies have utilized BALF and nasopharyngeal swabs to characterize the changes in transcripts and proteins during infection to understand COVID-19 pathogenesis [7-13], which have highlighted significantly upregulated genes and biological pathways altered during infection. While proinflammatory cytokines, chemokines, enzymes in neutrophil-mediated immunity, and several IFN stimulated genes (ISGs) have consistently shown up in their analysis, experimental validation and mechanistic studies are generally lacking [8-13]. A detailed characterization of antiviral responses in the upper respiratory tract of patients, its variation with age and sex, and association with progression of disease severity remains to be accomplished.

The goal of our study was to identify genes that are consistently upregulated during SARS-CoV-2 infection in the upper respiratory tract of patients and understand their role in viral infection and disease progression. More specifically, we were interested in secreted signalling mediators which can serve as markers of disease progression or druggable proteins that can serve as therapeutic targets. For this, we surveyed the literature for Omics data from COVID-19 positive patient’s nasal swab and BALF samples and selected 4 transcriptional and 3 proteomic datasets. We performed a hypergeometric distribution-based overlap analysis followed by cumulative fold-change score-based prioritization to shortlist genes. This was followed by an examination of selected gene expression levels in nasal swab/BALF samples from a cohort of COVID positive, negative, symptomatic, and asymptomatic individuals, ranging from 30-60 years in age and of mixed gender. Receiver operating characteristic (ROC) curve analysis of gene expression data in nasal swabs revealed S100 family genes (S100A6, S100A8, S100A9, and S100P) as high confidence markers of disease severity. Among other shortlisted genes, Thioredoxin (TXN) emerged as a significantly upregulated factor supported by multiple datasets. Thioredoxin is a proinflammatory protein that requires to be reduced by Thioredoxin reductase enzyme, which itself can be targeted by an FDA-approved gold drug Auranofin [15]. We tested the antiviral efficacy of Auranofin in cell culture and preclinical Syrian hamster challenge model and found that it can reduce SAR-CoV-2 replication over one order of magnitude at a well-tolerated non-toxic dosage. We also establish its mechanism of protection, which is through suppressing the expression of proinflammatory cytokine IL-6 expression. This drug is already in clinical use for inflammatory diseases and can have clinical implications in COVID-19 treatment based on our data.

Through collective global efforts, several COVID-19 vaccines have become available in an astonishingly short period, although new virus variants have emerged, some of which can escape vaccine-mediated immunity [16, 17]. Progress on the development of antivirals and disease prognostic markers has been lagging. Repurposing clinically approved drugs for use against SARS-CoV-2 has been an attractive option and has been explored by many research groups through different approaches [18]. Our study translates COVID-19 virus-host interaction and response Big Data into potential actionable clinical interventions, including the use of S100 genes as a prognostic marker in nasal swabs and repurposing the clinically approved drug, Auranofin for COVID-19 treatment.

2. Methods

2.1. Ethics statement

This study was conducted after approval from Institutional Human Ethics Committee (Approval Number: IHEC No. 13-11092020), Institutional Bio-Safety Committee (Approval Number: IBSIC/IISC/ST/17(2020) and Institutional Animal Ethics Committee (Approval Number: IAEC/IISC/ST/784(2020), following the Indian Council of Medical Research and Department of Biotechnology recommendations. For use of human samples, informed consent was obtained from each participant, before the study. All experiments involving infectious SARS-CoV-2 were conducted in the Viral Bio-safety level-3 facility at the Indian Institute of Science.

2.2. Cells and viruses

Authenticated (relevant documentation regarding authentication by suppliers is available in Supplemental Data) HEK 293T cells expressing human ACE2 (NR-52511, BEI Resources, NIAID, NIH, RRID: CVCL_A7UK) and VeroE6 cells (CRL-1586, ATCC, RRID:CVCL_0574) were cultured in complete media containing Dulbecco’s modified Eagle medium (12100-038, Gibco) with 10% Hi-FBS (16140-071, Gibco), 100 IU/ml Penicillin, 100 μg/ml Streptomycin and 0.25%μg/ml Amphotericin-B (Penicillin-Streptomycin-Amphotericin B, ICN1674049, MP Biomedicals) supplemented with Glutamax(TM) (35050-061, Gibco). SARS-CoV2 (Isolate Hong Kong/VM20001061/2020, NR-52282, BEI Resources, NIAID, NIH) was propagated and titered by plaque assay in Vero E6 cells as described before [19].
2.3. Omics data collection and processing

Transcriptomics and protein abundance data from COVID-19 patient's naso- and oropharyngeal swabs, bronchoalveolar lavage fluid (BALF), and other respiratory specimens were chosen from PubMed, BioRxiv, and MedRxiv using different combinations of keywords like "COVID-19, SARS-CoV-2, Transcriptomics, Proteomics, BALF, swab". Studies dealing with gene expression profiles of SARS-CoV-2 infected non-human cell lines and tissues were not considered. The SARS-CoV-2 and COVID-19 collections in the EMBL-EBI PRIDE proteomes database [20] were retrieved and used without any modification. In the NCBI GEO database [21], the following combination of terms was used to collect relevant datasets: ((covid-19 OR SARS-CoV-2) AND gse [entry type]) AND "Homo sapiens"[porgn: _txid9606]. The retrieved datasets were then filtered by their date of publication to collect the studies published between the 1st of January 2020 and the 15th of September 2020. The filtration of datasets was carried out using two parameters, fold-change, and its significance value. Genes and proteins with a fold-change value of $\geq 1.5$ and q-value $\leq 0.05$ were chosen for the overlap analysis. The raw p-value was used for filtering in cases where the adjusted p-value was not provided, albeit with a more stringent cut-off of $\leq 0.01$. The UniProt IDs in filtered protein abundance datasets were converted to their corresponding primary Gene Symbols using UniProt [22].

2.4. Gene set overlap analysis

The GeneOverlap class of R package "GeneOverlap" [23] was used for testing whether two lists of genes are independent, which is represented as a contingency table, and then Fisher's exact test was used to find the statistical significance. Genes with less than 0.01 overlap p-value were selected for further analysis. The number of background genes for proteome-proteome pairwise study and the transcriptome-proteome pairwise study was 25,000, i.e., the number of protein-coding genes in Hg19. For the transcriptome-transcriptome overlap study, the number of background genes was taken to be the union of total expressed genes in both the datasets considered.

2.5. Gene ontology, Interferome, cellular and tissue localization analysis

Enriched GO terms were obtained by express analysis on MetaScape [24] and plotted using ggplot2 [25]. The database Interferome v2.01 [26] was queried using gene symbols for identifying interferon-regulated genes (IRGs) in normal samples of the respiratory system from both in vitro and in vivo experiments in humans. For cellular localization, each gene was queried on UniProt annotation [27] and Human Protein Atlas ver20.0 [28, 29] and then manually annotated. The single-cell expression data of transcripts was also obtained from Human Protein Atlas ver20.0 (Available from http://www.proteintatlas.org/). They were further filtered to obtain cells that are associated with the immune system or respiratory tract.

2.6. Virus-Host protein-protein interaction network analysis

The interaction data for the selected 46 genes were retrieved from publicly available interaction datasets [14]. The retrieved information was then used to generate a network map. Cytoscape v3.8.0 [30] was used to construct the interaction network for virus-host protein-protein interaction (PPI). STRING database within the Cytoscape store was used to query the proteins to elucidate the interactions between the proteins significantly altered during SARS-CoV-2 infection. The resulting STRING interaction network (confidence $\geq 0.999$ for all the proteins and confidence $\geq 0.90$ for NAMPT; max number of interactions $= 10$) was merged with the virus-host PPI on Cytoscape.

2.7. qRT-PCR based measurement of cellular gene expression for patient samples

Nasopharyngeal swabs were collected from COVID-19 patients and healthy individuals for diagnostic purposes by hospitals from Bengaluru Urban city and brought to COVID-19 Diagnostic Facility at the Indian Institute of Science in viral transport media (VTM). RNA from patients was isolated using kits recommended and provided by the Indian Council of Medical Research. Samples were chosen to have an almost equal number of patients falling into categories of age, sex, COVID-19 status, and symptomatic status (Table 1). Demographic information was not used as an inclusion criterion. Although a priori sample size determination was not conducted, the number of samples were chosen based on technical constraints and previous publications [31, 32]. The de-identified patient data is available upon request (see Data Sharing statement).

Equal amounts of RNA were converted into cDNA using PrimeScript™ RT Reagent Kit with gDNA Eraser (Perfect Real Time) (RR047A, Takara-Bio) and then diluted with 80 $\mu$l nuclease-free water. The gene expression study was conducted using PowerUp™ SYBR™ Green Master Mix (A25778, Applied Biosystems™) with 18s rRNA as the internal control and appropriate primers for the genes (Supplementary Table 3).

2.8. Cytotoxicity assay

HEK-ACE2 cells were seeded in a 96-well cell culture dish pre-coated with 0.1 mg/mL poly-L-lysine (P9155-5MG, Sigma-Aldrich) and 24 hr later, treated with 0, 1, 2, and 4 $\mu$M Auranofin (A6733, Sigma-Aldrich) in triplicates. Cells were incubated at 37°C, 5% CO2, and 48 hr later, cytotoxicity was measured using AlamarBlue™ Cell Viability Reagent (DAL 1025, Thermo Fisher) as per manufacturer’s instructions.

2.9. Infection in HEK-ACE2 and VeroE6 cells

Cells were seeded in a 24-well cell culture dish (pre-coated with 0.1 mg/mL poly-L-lysine for HEK-ACE2) and 24 hr later, used for infection.

**HEK-ACE2:** Cells were first pretreated for 3 hr with 0, 0.125, 0.25, 0.5, and 1 $\mu$M Auranofin in quadruplicates. Infection was done with 0.1 MOI SARS-CoV-2 in 100 $\mu$l inoculum in DMEM supplemented with 10% FBS for 1 hr at 37°C.

**VeroE6:** Cells were pretreated for 3 hr with 0 and 1 $\mu$M Auranofin in quadruplicates. Cells were infected with 0.001 MOI SARS-CoV-2 in 100 $\mu$l inoculum in DMEM supplemented with 2% FBS for 1 hr at 37°C.

For both cell lines, complete medium (DMEM with 2% FBS for VeroE6) restoring the initial dose of the drug was added to the cells. After 48 hr, cells were processed separately for plaque assay, western blot analysis, and RNA extraction using TRIZol™ Reagent (15596018, Thermo Fisher).

2.10. Western blot

Cells were washed with 1X PBS (162528, MP Biomedicals) and lysed with 1X Laemmli buffer (1610747, BIO-RAD). Cell lysates were loaded and resolved using a 10% SDS-PAGE gel, and the separated proteins were transferred onto a PVDF membrane (IPVH00010, Merck). Blocking was performed using 5% Skimmed milk (70166, Sigma-Aldrich) in 1X PBS containing 0.05% Tween 20 (P1379, Sigma-Aldrich) (1X PBST) for 2 hr at room temperature with slow rocking. Primary antibody incubation was performed overnight (12 hr) at 4°C using SARS-CoV / SARS-CoV-2 (COVID-19) spike antibody (180 KDa) (GTX632604, GeneTex, RRID: AB_2864418 or NR-52947, BEI Resources, NIAID, NIH). Secondary antibody incubation was performed for 2 hr at room temperature with slow rocking using
Goat Anti-Mouse IgG H&L (ab6789, Abcam, RRID: AB_955439) or Goat Anti-Rabbit IgG H&L (ab6721, Abcam, RRID: AB_955447). The blots were developed using Clarity Western ECL Substrate (1705061, BIO-RAD). Blots were probed for beta-actin (42 KDa) using mouse monoclonal antibody to beta Actin [AC-15] (HRP) (ab49900, Abcam, RRID: AB_887494). All antibodies were authenticated by the respective companies and relevant documentation is available in Supplementary Data.

2.11. Plaque assay

Plaque assay to measure infectious virus counts were performed as described before [19]. VeroE6 cells were seeded in 6-well cell culture dishes to reach complete confluence the next day. Cells were washed once with 2 mL warm PBS and incubated with dilutions of cell culture supernatants in 200 µL complete MEM for 1 hr at 37°C. The virus inoculum was then removed, and cells were overlaid with DMEM containing 2% FBS and 0.8% agarose (MB002, Himedia). After 48 hr incubation, cells were fixed with 4% formalin, and plaques were visualized by crystal violet (CG158, Merck) staining.

2.12. Tissue-culture infectious dose 50 (TCID₅₀)

HEK-ACE2 cells were seeded in a 96-well cell culture dish pre-coated with 0.1 mg/mL poly-L-lysine and 24 hr later, used for infection. Cells were first pretreated for 3 hr with 1 µM Auranofin and subsequently infected with two-fold serial dilutions of SARS-CoV-2 starting at 0.1 MOI. Each condition was performed in ten wells. Plates were incubated for 48 hr, and the presence or absence of cytopathic effects were recorded. TCID₅₀ was estimated using methods described by Reed and Muench [33].

2.13. Cytopathic Effect (CPE) reduction

HEK-ACE2 cells were seeded in a 24-well cell culture dish pre-coated with 0.1 mg/mL poly-L-lysine and 24 hr later, used for infection. Cells were first pretreated for 3 hr with 1 µM Auranofin in triplicates and subsequently incubated with 0.1 MOI SARS CoV-2 in 100 µL inoculum for 1 hr at 37°C. Subsequently, 400 µL complete medium restoring the prior dose of the drug was added to the cells. After 48 hr, the percentage of viable cells was measured by Trypan blue (93595, Sigma-Aldrich) dye exclusion method.

2.14. Animal experiments

**Animal Handling:** All animal experiments were performed using 10 to 12-week-old male and female Syrian golden hamsters purchased from Biogen Laboratory Animal Facility (Karnataka, India). The animals were allowed to acclimatize for 3 days at the experimental location, and given access to pellet feed and water ad libitum. Males and females were housed separately and maintained on a 12-hr day/night light cycle at the Viral Biosafety level-3 facility at the Indian Institute of Science. Hamsters were euthanized by an overdose of Ketamine (Bharat Parenterals Limited) and Xylazine (21, Indian Immunologicals Ltd).

**Toxicity and Infection assays:** Toxicity of 1 and 5 mg/kg bodyweight Auranofin was tested on Syrian golden hamsters by once-daily oral administration of the drug in 200 µL PBS. This corresponds to a dosage of 1 mg/kg (Hamster) x 0.13 (conversion factor) = 0.13 mg/kg (Human equivalent dose) and 5 mg/kg (Hamster) x 0.13 (conversion factor) = 0.65 mg/kg (Human equivalent dose) Auranofin per day (conversions as described in [https://www.fda.gov/media/72309/download](https://www.fda.gov/media/72309/download)). The total bodyweight of hamsters was monitored for up to 7 days (see Supplementary Fig 8). Infection experiments were performed by intranasal inoculation of animals with 10⁵ PFU SARS-CoV-2 in 100 µL PBS. The animals were anesthetized using intraperitoneal injections of Ketamine (150 mg/kg) (Bharat Parenterals Limited) and Xylazine (10 mg/kg) (21, Indian Immunologicals Ltd) cocktail before infection. Prophylactic treatment involved oral administration of Auranofin (5 mg/kg/day) 3-, 2-, and 1-day before infection and followed by virus challenge at day 0. The therapeutic treatment regimen used oral administration of Auranofin (5 mg/kg/day) starting at 24-hr post-infection (hpi), followed by 2- and 3-days post-infection (dpi). Total body weight was recorded each day during the entire course of the experiment until the animals were sacrificed at 4 dpi. Viral RNA load in lung tissue specimens was detected by qRT-PCR. Sample size for hamster experiments were chosen based on previous studies [34, 35].

2.15. RT PCR for viral copy number calculation

For qRT-PCR, total RNA was isolated using TRIzol™ Reagent (15596018, Thermo Fisher) as per manufacturer’s instructions and equal amounts of RNA was used to determine viral load using AgPath-ID™ One-Step RT-PCR kit (AM1005, Applied Biosystems) using primers and probes targeting the SARS CoV-2 N-1 gene (Forward primer: 5’ GACCCAAAAATCAGCGAAAAT3’ and Reverse primer: 5’TCTGTGTTACTGCGTTATCTG3’; Probe: (6-FAM / BHQ-1) ACCCCGATTACGTTGTGGGACC). Viral copy number was estimated by generating a standard curve using SARS-CoV-2 genomic RNA standard.

2.16. Histopathology of Lung tissue

Lung tissue specimens of hamsters were fixed in 4% paraformaldehyde PBS, and embedded in paraffin blocks. Tissue sections of 4-6 µm thickness were stained with Hematoxylin and Eosin (H&E) for examination by light microscopy as previously described [36].

2.17. Graphical representations and statistical analysis

Statistical analyses and overlaps were performed in the R statistical environment version 4.0.3 via RStudio version 1.3.1093. All statistical tests were two-sided unless specified otherwise. Plots were made using the ggplot2 package in R [25] and GraphPad Prism v8.0.2. In boxplots, the hinges of boxes represent the first and third quartiles. The whiskers of the boxplot extend to the value which is 1.5 times the distance between the first and third quartiles. Each data point in the boxplot represents one of the triplicates in qRT-PCR for a particular gene in a particular patient sample. Heatmaps were generated using the R package Complex Heatmap with Euclidean method used for clustering [37]. Receiver Operating Characteristic (ROC) curve analysis and Optimal cut-off determination were performed using the online tool easyROC (ver. 1.3.1) [38].

2.18. Role of funding source

Only financial support was provided by the funders. They have not participated in study design, data collection, data analyses, interpretation, or writing of the report.

3. Results

3.1. Compilation and overlap analysis of published transcriptomics and proteomics data from COVID-19 patient samples revealed 566 upregulated host factors

We started the study by compiling the host factors that are consistently and significantly upregulated in the upper respiratory tract of COVID-19 patients. For this ‘top-down’ approach to narrow down
severity markers and drug targets from genome-wide data, we decided to use published transcriptomics and proteomics datasets derived from nasal swab or BALF samples of COVID-19 patients. We chose four transcriptomics (T), and three proteomics (P) datasets, and further analyses were performed according to a rationally designed workflow (Fig. 1a). All datasets included differentially expressed genes in infected patients with healthy individuals as control (see Supplementary Table 1). The selection criteria (described in materials and methods) included at least 1.5-fold gene upregulation at both mRNA and protein levels. The filtration of data was carried out to sort only significantly upregulated genes from all the datasets (see Supplementary Table 2). A pairwise overlap analysis was performed on the filtered genes/proteins from each study and significantly overlapping genes (p-value < 0.01 calculated using Fisher’s exact test) between T1-T3 (14), T1-T4 (9), T1-P3 (2), T3-T4 (504), T3-P1 (10), T3-P2 (8), T3-P3 (17), T4-P1 (8), T4-P3 (15) and P1-P3 (3) were determined (Fig. 1b, Supplementary File 1). This method was adapted from similar overlap analysis conducted previously to compare...
multiple virus-host interaction datasets and to obtain the significance of intersections [39]. Union of significant intersections after the overlap analysis results in 566 genes (Fig. 1b). To reiterate the functional characteristics of the differentially expressed genes (DEGs), we examined the biological processes and signaling pathways they are involved in. Pathway enrichment of 566 genes from the union of all significant intersections from overlap analysis showed enrichment of biological processes like protein elongation, interferon (IFN) signaling, chemotaxis of granulocytes, and inflammatory pathways (Fig. 1c). The antiviral response to respiratory viral infections, including SARS-CoV-2, is driven by interferons (IFNs) [40]. Hence, we examined the shortlisted set of genes for their potential regulation by different categories of IFNs, using the Interferome tool [26]. We found that out of 566 genes, 76 were regulated by type I IFN, 148 genes by type II IFN, 190 genes were regulated by both type I and type II IFN, while 16 genes were commonly regulated by all the three classes of IFNs (Fig. 1d). These 16 genes are well-characterized interferon-stimulated genes (ISGs), which include direct antiviral effector ISGs (IFITs, MX1, OAS3, and OAS1), as well as positive regulators (STAT1) of IFN response [41]. This indicated an active IFN-mediated innate antiviral response in the upper respiratory tract cells during SARS-CoV-2 infection and highlighted potential antiviral factors.
3.2. Rank ordering and shortlisting of upregulated host factors highlighted host factors regulating the antiviral and inflammatory immune response in COVID-19 patients

Since proteome dictates the outcome inside a cell, soluble factors are key in shaping the antiviral response. We focused on genes supported by orthogonal transcript (T) and protein (P) abundance data. For this, we chose genes from the union of intersections of T-T, T-P, and P-P overlaps, which was reported at least in one of the proteomics studies. This narrowed down the list to a total of 46 genes that were intersecting in T-P (26), P-P (2), T-T (16), T-P-P (1), and T-T-P-P (1) overlaps (Fig. 2a and 2b, Supplementary File 1). A cumulative score for the 46 selected significantly upregulated genes was calculated using the sum of their log2 fold-change values in the parent datasets and ranked (Fig. 2c). The enrichment of these 46 genes in each of the datasets, where the expression is reported, is shown in Fig. 2b. Many of these genes are directly regulated by different classes of interferons. 15 genes are regulated by IFN-I, while 8 genes by IFN-II. 20 genes are regulated by both type-I and type-II IFNs, while only 2 genes by all the three types of IFNs (Fig. 2d). Most of the IFITs and other ISGs that were earlier determined in our analysis to be regulated by all the three types IFNs are no more in the list since those ISGs were only reported upregulated at transcriptome level (only in T-T overlap) and hence were lost when the genes were filtered for their upregulation at the protein level, leaving behind only MX1 and OAS3 (Fig. 1c and 2d). The biological functions of the selected 46 genes were also investigated to understand their roles in COVID-19 pathophysiology. The enriched pathways were mainly related to innate immune response and defense against microbes along with inflammatory and immune signaling, neutrophil degranulation, and cellular response to TNF and interferon-gamma (Fig. 2e).

Further, to understand the potential role of shortlisted genes in COVID-19 pathophysiology, their interactions with SARS-CoV-2 proteins were inspected by analyzing the publicly available SARS-CoV-2 cellular interactome data [14]. For this, host protein-protein interactions were retrieved from the STRING database [42] and merged with the virus-host protein-protein interactions giving a discrete picture of how the viral proteins target various cellular processes during infection. Other than NAMPT, UQCRC2, and RAB5C, it was mainly proteins associated with ribosomes that were primary interactors to the SARS-CoV-2 proteins (Fig. 2f and 2g). We also examined the intracellular, cellular, tissue,
and organ-specific expression for shortlisted genes using publicly available data [28, 29]. Many upregulated proteins were predicted to localize in the intracellular organelles like endoplasmic reticulum, mitochondria, Golgi complex, and endosomes (see Supplementary Fig 1a), while 19 genes were predicted to be secretory. A thorough analysis of the list of 46 selected genes using Human Tissue Atlas revealed that they are expressed in the respiratory tract and in immune effector cells known to survey infection sites (see Supplementary Fig 1b). The relative expression levels show that genes associated with protein synthesis (ribosomal proteins and elongation factors) are highly expressed compared to any other genes and are enriched across all the tissues in the map (see Supplementary Fig 1b).

3.3. qRT-PCR based validation in a cohort of COVID-19 positive/negative, symptomatic/asymptomatic individuals reveals differential upregulation of selected genes in a disease-specific manner

For validation using qRT-PCR and further analysis, we selected genes with a cumulative score greater than 10, except for IGHM due to the lack of compatible primers (Fig. 2c). Also, we considered genes belonging to the S100 family that came up within 46 shortlisted genes, since they are known regulators of inflammation [43, 44]. Furthermore, we also selected TXN since it was supported by multiple lines of evidence and appeared in the TT-TP-PP overlap in our study (Fig. 2a). The COVID-19 patient cohort used for qRT-PCR of genes included 63 individuals (both males and females, aged 30-60 years), out of which 16 each were COVID-19 positive symptomatic (PS), COVID-19 negative asymptomatic (NA), COVID-19 negative symptomatic (NS), and 15 were COVID-19 positive asymptomatic (PA) healthy category (Table 1). Total mRNA from the nasal swab was isolated and the upregulation of 14 selected genes was verified by qRT-PCR. The log2 fold-change expression with respect to the average of the negative asymptomatic group (Fig. 3a) was calculated and plotted on a heatmap (Fig. 3a), which depicts the mRNA enrichment of the selected genes in different patient samples and categories. Next, we determined the correlation between the viral RNA load in COVID-19 patients (qRT-PCR of viral envelope (E) gene) and log2 fold-change of selected host genes in the patient samples. It was observed that the threshold cycle (Ct) value for the E gene

![Fig. 4. ROC analysis of genes in COVID-19 positive patients to identify prognostic markers. a) ROC curve for Ct value of genes in COVID-19 positive patients. The black dashed line corresponds to no prognostic potential where True positive rate (Sensitivity) and False positive rate (1-Specificity) are equal. b) The AUC value for each ROC curve along with the p-value (calculated non-parametrically (DeLong’s estimate) using the Wald test statistic). c) Boxplot of Ct values (technical replicates) for significant S100 family of genes in Positive asymptomatic (PA) and Positive symptomatic (PS) patients. The red dashed line shows the optimal Ct cut-off determined by the ROC01 method (also shown in the label in each graph). d) Optimal Ct cut-off, sensitivity, and specificity values for significant S100 family of genes.](image-url)
was negatively correlated with log₂ fold-change of genes showing that viral load and expression levels of the selected genes are positively correlated (see Supplementary Fig 3). Furthermore, the upregulation of selected host genes was more pronounced in positive symptomatic patients than positive asymptomatic individuals (Fig. 3a, see Supplementary Fig. 3). A comparative heatmap in Fig. 3b gives an insight into the genes that can be considered as COVID-19 disease and/or severity marker. While all the upregulated genes except SERPINB3 indicate infection (Fig. 3b; NA-PS), only a few genes showed significant upregulation in a COVID-19 specific manner (Fig. 3b; NS-PS).

Multiple genes from the S100 family, including S100A8, S100A9, S100A6, and S100P, were significantly upregulated in positive symptomatic patients when compared to other three categories (NA, NS, PA), suggesting their potential diagnostic and prognostic value (Fig. 3b, NS-PS). Expression of neutrophil defensin alpha 3 (DEF3A) was upregulated in some of the positive symptomatic patients but remained undetermined in many cases. Furthermore, we examined the influence of age and sex on the upregulation of selected genes in patient samples by categorizing them based on age groups [30-40, 41-50 and 51-60] and gender (male and female) (Fig. 3c, Fig. 3d, see Supplementary Fig 4 and Supplementary Fig 5). The qRT-PCR data revealed that all the selected genes were induced in positive symptomatic patients, irrespective of age or gender. However, closer examination of the heatmap reveals S100 family genes (S100A8, S100A9, and S100P) being upregulated to a higher level in the 30–40-year age group and male individuals (Fig. 3c, 3d).

Fig. 5. Auranofin inhibits SARS-CoV-2 replication in cell culture. a-c) HEK-ACE2 cells were pre-treated with the indicated amount of the drug for 3 hr, infected with 0.1 MOI SARS-CoV-2, and incubated for 48 hr. a) Viral RNA copy number in cells was determined by qRT-PCR. b) Cell lysates were analyzed by western blot, probed for spike (180 kDa) and beta-actin (42 kDa). c) Cell culture supernatant was collected from virus control and 1 μM drug-treated, and infectivity titers were measured using plaque assay. d) HEK-ACE2 cells were infected with serial dilutions of SARS-CoV-2 in the presence or absence of 1 μM Auranofin and TCID₅₀ was estimated 48 hr post-infection. e-f) HEK-ACE2 cells were pre-treated with 1 μM drug for 3 hr and then infected with SARS-CoV-2 at 0.1 MOI for 48 hr. e) Fold change of viable cells was measured by trypan blue dye exclusion and data was analyzed by normalizing the values to uninfected cell control. f) Brightfield images of representative images (scale bar - 200 μm). g-i) VeroE6 cells were pre-treated with 1 μM of Auranofin for 3 hr and then infected with SARS-CoV-2 at 0.001 MOI for 48 hr. g) Viral RNA copy number was measured by qRT-PCR. h) Cell lysates were analyzed by western blot and probed for spike (180 kDa) and beta-actin (42 kDa). i) Infectivity titers were measured by plaque assay with cell culture supernatant. For all comparisons, *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns – not significant (using t-test with Bonferroni corrections for multiple comparisons wherever necessary). Error bars represent mean ± standard error.
3.4. ROC analysis of mRNA expression of shortlisted significant genes in the COVID-19 cohort unveils the prognostic potential of the S100 family of genes

The COVID-19 symptomatic group of patients included individuals with breathing difficulty, fever, hospitalization, and SARI (severe acute respiratory infections), whereas asymptomatic patients had none of these features (Table 1). To evaluate the prognostic value of selected genes in differentiating asymptomatic vs symptomatic COVID-19 cases, we conducted a non-parametric ROC curve analysis [38] for the 11 genes that were significant after comparison between positive symptomatic and asymptomatic groups (Fig. 3b, PA-PS). For this, we used their Ct values for COVID-19 positive cases to plot the curve and the area under the curve (AUC) was computed (Fig. 4a). All genes were found to significantly differ (AUC > 0.5) from the line where True positive rate = False positive rate, indicating their potential to differentiate between asymptomatic and symptomatic individuals (Fig. 4b). The optimal Ct value cut-off was determined for significant genes using the ROC01 method which finds the point in the ROC curve closest to (0,1) corresponding to 100% specificity and sensitivity. Since the prognostic marker should correctly identify symptomatic patients from asymptomatic ones, we looked at the genes with maximum sensitivity while not compromising on specificity at the optimal cut-off. S100A8 (Cut-off = 9.964663, Sensitivity = 0.938, Specificity = 0.688) had the highest sensitivity at the optimal cut-off. Other S100 family members like S100A9 (Cut-off = 8.533607, Sensitivity = 0.854, Specificity = 0.729), S100A6 (Cut-off = 8.472503, Sensitivity = 0.745, Specificity = 0.718) and S100P (Cut-off = 11.23458, Sensitivity = 0.812, Specificity = 0.622) also showed good prognostic potential (Fig. 4c and 4d). Genes like LCN2 (Cut-off = 11.23362, Sensitivity = 0.744, Specificity = 0.756), AGR2 (Cut-off = 11.19266, Sensitivity = 0.775, Specificity = 0.708) and ASS1 (Cut-off = 12.70913, Sensitivity = 0.7, Specificity = 0.771) were also found to have desired sensitivity and specificity values (see Supplementary Fig 6).
3.5. Thioredoxin reductase inhibitor drug Auranofin significantly mitigates SARS-CoV-2 replication in vitro, and in vivo in the hamster challenge model

Thioredoxin (TXN) was a hit that appeared in the TT-TP-PP overlap in our study and remained in the shortest list gene set at the end of the meta-analysis. Although its expression upregulation or the prognostic value was not the highest, it is part of a druggable pathway. Thioredoxin is known to promote inflammatory cytokine induction, apoptosis, and regulate redox status, for which it switches between oxidized and reduced forms through the action of thioredoxin reductase, which can be inhibited by an FDA approved orphan drug Auranofin (2,3,4,6-tetra-o-acetyl-L-thio-β-D-glycopyran-sato-S-{triethyl-phosphine}-gold) [15, 45, 46]. We sought to check the effect of Auranofin on SARS-CoV-2 infection and replication in cell culture and animal models. To begin, cell viability assay performed in HEK-ACE2 and VeroE6 cells using increasing doses of Auranofin showed minimal cytotoxicity at the lowest concentration (1 μM) and had predicted CC50 of 9.659 μM (see Supplementary Fig. 7a, 7b). The effects of increasing doses of Auranofin, up to 1 μM (~0.67 mg/L of media), was then tested on SARS-CoV-2 replication in vitro. For this, HEK ACE2 cells were pretreated with the drug, which remained present during the entire course of infection. Analysis of viral RNA 48 hr post-infection showed a reduction of more than one order of magnitude, starting at treatment with 0.25 μM Auranofin (Fig. 5a). With a calculated EC50 = 0.29 μM, the selectivity index (CC50/EC50) of Auranofin was determined to be 33.3. The potent antiviral effect of Auranofin was confirmed by western blot for the full-length viral spike protein (Fig. 5b). Treatment with 1 μM Auranofin showed a significant reduction of infectious virus titers in cell culture supernatants at 48 hr post-infection (Fig. 5c) and this was supported by a ∼2-log reduction by TCID50 assay (Fig. 5d). Virus-induced cytopathic effects (CPE) was also mitigated significantly and cell viability increased by ∼6 fold in the presence of Auranofin (Fig. 5e and 5f), as observed by microscopy and measured by trypan-blue exclusion assay. Furthermore, the anti-viral effect of Auranofin was also demonstrated in VeroE6 cells, wherein, analysis of viral RNA 48 hr post-infection revealed a 3-log reduction in drug-treated cells (Fig. 5g). This was reflected in western blot analysis of infected cells, where we observed almost complete inhibition of viral spike protein expression (Fig. 5h). Plaque assay quantification of infectious virus particles from cell culture supernatants revealed >1 log reduction in the presence of Auranofin (Fig. 5i).

Next, we proceeded to confirm the antiviral activity of Auranofin in Syrian golden hamsters, which are currently considered as the animal model of choice to evaluate vaccines and antivirals [36]. Auranofin (PubChem CID 6333901) toxicity and bioavailability in rodents have been described before [47], based on which we first tested its oral toxicity in hamsters at 1mg/kg and 5mg/kg body weight, which showed the drug was well tolerated at the tested doses (see Supplementary Fig 8). For infection studies, the drug was orally administered in prophylactic and therapeutic formats; before and after infection, respectively (Fig. 6a). The viral titers in lungs of animals at Day 4 revealed that both prophylactic and therapeutic administration of Auranofin with a non-toxic concentration of 5mg/kg body weight was more effective at mitigating virus replication in lung tissue, compared to the vehicle control group (Fig. 6b). Bodyweight loss results were also indicative of the same when compared to the virus challenge group (Fig. 6c). Also, we found that the TXN gene was upregulated in cell culture as well as in the lungs of infected animals compared to the mock group, which correlates to our findings from patient sample gene expression data (Fig. S9). Examination of H&E stained histological sections of the lung showed evident damage of alveolar epithelial lining and cellular infiltration in infected animal lungs. The lung damage and inflammation were clearly reduced in the case of both therapeutic as well as prophylactically treated animals (Fig. 6d). Furthermore, TXN has been reported to increase proinflammatory cytokine induction [48], especially IL-6, which is a well-established mediator of COVID-19 severity [49]. We tested the effect of Auranofin on IL-6 production in infected hamster lungs and found it to be significantly diminished (Fig. 6e). This is likely to be a mechanism of action of Auranofin against SARS-CoV-2 infection and disease.

4. DISCUSSION

Several studies have analysed changes in global transcriptome and proteome in COVID-19 patient samples of various kinds [7-13]. These studies have given an overview of the biological processes that are modulated during SARS-CoV-2 infection; however, translation of this knowledge into antiviral interventions requires validation and mechanistic studies. Meta-analysis of virus-host interaction Big Data is a useful approach to narrow down key host factors and processes involved in viral replication and pathogenesis [39, 50]. In our study, we focussed on transcriptomics and proteomics data from COVID-19 positive nasal swab and BALF samples and performed an integrative analysis to identify host factors involved in SARS-CoV-2 infection and disease progression. We reasoned that changes at mRNA levels must also be manifested at the protein level to bring out phenotypic differences in the infected individuals. Hence, we designed our meta-analysis pipeline to shortlist genes that were represented in orthogonal transcriptomics as well as proteomics datasets. Expression of the genes selected through meta-analysis was examined in nasal swab/BALF samples collected for COVID-19 diagnosis from a cohort of individuals that were COVID-19 negative or positive and within those two categories either asymptomatic or symptomatic. The cohort design was to ensure the identification of genes that are overexpressed in a COVID-19 specific manner and those which indicate disease severity. The initial compilation of upregulated factors had 566 genes, of which 46 genes passed through the selection pipeline (Fig. 2b). Most of these genes turned out to be IFN regulated and among them, the major category was ribosomal proteins (RPs), including RSP3A, RPL4, RPL5, RPL18, RPL13A, RPS4X, RPL7A, RPS9, and RPS3 (Fig. 2b). RPs have been reported to be hijacked by different

### Table 1

| Patient Status | Number of patients | Average age | Number of males | Number of females | Number in the age group 30-40 | Number in the age group 41-50 | Number in the age group 51-60 |
|----------------|--------------------|-------------|----------------|-------------------|-----------------------------|-----------------------------|-----------------------------|
| Negative Asymptomatic | 16 | 43.9 | 8 | 8 | 6 | 5 | 5 |
| Negative | 16 | 41.7 | 12 | 4 | 9 | 4 | 3 |
| Symptomatic | 15 | 44.3 | 7 | 8 | 6 | 5 | 4 |
| Positive Asymptomatic | 16 | 45 | 8 | 8 | 5 | 5 | 6 |
| Positive | 16 | 45 | 8 | 8 | 5 | 5 | 6 |
viruses, including SARS-CoV-2. During infection it shut off host translation and facilitate IRES-mediated translation of viral proteins [51-53]. Inspection for reported interactions between shortlisted RPs with the SARS-CoV-2 proteins revealed that nsp1, nsp8, nsp9, and nucleocapsid (N) proteins of SARS-CoV-2 are potential interactors (Fig. 2f). This suggests extensive targeting of host translational machinery by multiple SARS-CoV-2 proteins in the upper respiratory tract cells. Other shortlisted cellular proteins with reported interactions with viral proteins were NAMPT, UQRC2C, and RABSC (Fig. 2g). These are involved in cellular processes like ATP production, NAD synthesis, and vesicular fusion respectively, all of which have been reported to be influenced during viral infections [54-58].

Subsequent ranking of genes based on cumulative upregulation score across different datasets, with dual support from transcriptomic and proteomic evidences, shortlisted 14 high confidence upregulated genes (Fig 2b). To confirm their upregulation during SARS-CoV-2 infection and the effect of patient age, sex, disease severity on the same, their expression was measured in a cohort of patients described earlier (Table 1). The data revealed that 11 genes were upregulated significantly in the PS category when compared to PA and hence had prognostic value. Whereas, 8 genes were upregulated when compared to the NS category, hence had diagnostic value (Fig. 3b). The data indicated higher levels of selected gene expression in younger male patients, which is consistent with previous reports of age and sex-dependent differences in COVID-19 induced gene expression and disease severity [7, 59]. Among host factors that appeared at the end of meta-analysis and validation in the COVID-19 cohort, the S100 family of genes (S100A6, S100A8, S100A9, S100A12, and S100P) emerged as a major group. An upregulation of S100 proteins is reported previously as an indication of viral or bacterial infections [43]. The extracellularly secreted S100 proteins include S100A12, S100A8, and S100A9 (see Supplementary Fig 1a), all of which have been shown to serve as a danger signal and in regulation of immune response [44]. They activate NF-kB signalling through RAGE and TLR4 pathways stimulating the cells to produce proinflammatory cytokines at the site of infection [44]. Several studies have explored serum diagnostic and prognostic markers by evaluating transcriptomic and proteomic changes in mild, severe, and fatal cases of COVID-19 [60, 61]. An increase in S100A8/A9 (calprotectin) levels in serum have been correlated with severe forms of the disease [62]. Transcriptomic studies on lung tissue of fatal COVID-19 cases have also reported an upregulation in S100A12, S100A8, S100A9, and S100P in patients [63]. In our study, the ROC curve analysis of the PA and PS group qRT-PCR data showed that all shortlisted S100s (except S100A12) had significant sensitivity as a prognostic marker of symptomatic COVID-19 (Fig. 4c, d). Overall, taking our data and published information together, the S100 family of genes can be considered as reliable prognostic markers of COVID-19 infection and disease progression. Another host factor LCN2, which came up in our study was previously shown to be an important biomarker for viral infection [64], and was also reported to be upregulated in transcriptomic and proteomic studies in COVID-19 patients [65, 66]. Furthermore, Serine protease inhibitor (SERPIN) family genes SERPINB3 and SERPINB1 were present among the initially selected 46 upregulated genes. SERPINB3 was at the top of cumulative upregulation ranking (Fig. 2c) and in the COVID-19 cohort, it was significantly upregulated in the PS category. It is an inhibitor of papain-like cysteine proteases such as cathepsin [67], which is required for Spike cleavage during SARS-CoV-2 entry [68]. Interestingly SERPIN1A deficiencies or mutations in populations were found to be associated with severe forms of COVID-19 [69]. Taken together, this indicates a potential antiviral role for SERPINS against SARS-CoV-2, which needs further exploration.

Finally, one gene of interest which passed the rigor of meta-analysis was TXN. Although its cumulative upregulation or prognostic values were not very high, we explored its potential as a therapeutic target. Thioredoxin is a small redox protein that plays an active role in keeping the intracellular compartment in a reduced state, which is important to prevent protein aggregation [70]. The thioredoxin system consists of three components, namely thioredoxin, thioredoxin reductase, and the reducing agent nicotinamide adenine dinucleotide phosphate (NADPH). Thioredoxin reductase is a homeostatic redox enzyme that can be inhibited by FDA-approved, gold-containing triethylphosphine drug Aurano [15]. This drug has been shown to have inhibitory activity against rheumatoid arthritis, cancer, HIV/AIDS, parasitic, and bacterial infections [71], albeit with side effects like diarrhea (45-50%), rashes (24%), abdominal cramping (14%), stomatitis (13%) and nausea (10%) [47]. The drug is sold under the brand name RIDAURA® in the USA and Goldar in India, where it cost INR 112 (~1.5 USD) for 10 tablets of 3 mg, making it an economically viable option. Aurano is a metallodrug, which may have implications in its mechanism of action. Another metalodrug, ranitidine bismuth citrate, was found to exert SARS-CoV-2 antiviral activity by sequestering Zinc ions, necessary for helicase function [72]. A recent study by Rothen et al. showed Aurano to inhibit SARS-CoV-2 in Huh-7 cells at an EC50 of 1.4 µM [73]. In comparison, our data in HEK-ACE2 cells showed improved antiviral activity at much lower concentrations of the drug (EC50 = 0.29 µM or 197 µg/L of the medium; selectivity index - 33.3, versus 4.07), as evidenced by decrease in viral infectious counts, viral RNA, protein, and cytopathic effects (Fig. 5a-f). This effect was also confirmed in VeroE6 cells. In term of concentration, the EC50 value would be 13.3 µg/L which is dominated by steady-state serum gold concentrations of 300 µg/L after oral administration of 3 mg dose of aurano and 500-700 µg/L after oral administration of 6 mg in humans [47]. Since the EC50 value can be easily achieved in humans, it hints at the translational potential of this study. Furthermore, we went on to validate the antiviral activity of Aurano in the first time for the preclinical hamster challenge model. Results showed a significant reduction in the lung viral load and rescue of animal body weight, when the drug was orally administered, which may be attributed to the anti-inflammatory activity of the compound [74]. Notably, Aurano has been shown to decrease proinflammatory cytokines IL-6, IL-1β, and TNFα mRNA levels during SARS-CoV-2 infection in vitro, which are known mediators of disease severity [73]. In Aurano-treated animals, lung tissue damage, cellular infiltration, and inflammation, as well as IL-6 expression, was significantly reduced compared to control infected animals. This substantiates our proposed mechanism of action of Aurano against SARS-CoV-2. Similar immunosuppressive medications like dexamethasone and IL-6 receptor inhibitor tocilizumab are being used for COVID-19, which could aid in reducing the inflammation that leads to poor prognosis in severe COVID-19 cases [75, 76]. Furthermore, TNFα mRNA levels were upregulated in cell culture, hamsters as well as nasal swabs of COVID-19 patients, which confirms it as a reliable phenotype of infection and target for therapy. Aurano also has inhibitory effects on the PI3K/AKT/mTOR pathway [77], which is required for SARS-CoV-2 viral protein translation [78, 79]. This may also contribute to its mechanism of action, however, that needs to be further investigated.

There are a few limitations to our study, which can be alleviated with follow-up experiments. This includes a small sample size of human nasal swabs samples and limited experimentation in the hamster model. With a greater number of human samples in a larger cohort, a detailed categorization of patients (such as hospitalized vs. non-hospitalized, ICU vs. non-ICU, survived vs. deceased) can confirm the utility of S100 markers in predicting the diseases severity with high confidence. Also, with detailed experimentation in hamsters, especially with changing the Aurano dosage and treatment intervals, a more effective regimen can be identified. Furthermore, a detailed characterization of the mode of action of Aurano against SARS-CoV-2 needs to be conducted. Nonetheless, this study highlights the value of comprehensive analyses of Omics datasets to gain insight into infection biology and identify avenues for potential therapeutic targeting. The selected gene expression data obtained with the COVID-19 cohort reaffirmed the heterogeneity of individual immune response, the role of age, sex, and the effect of viral load, all
of which are in coherence with observations made by other research groups. We especially uncover the prognostic value of S100 family genes in nasal swabs, many of which are soluble secretory factors. They can be easily tested by RT-PCR or ELISA-based methods in nasal swabs that are routinely collected for diagnostic purposes. Finally, the identification of Auranofin (already in clinical use for other medical conditions) as a drug that can be further explored as a potential COVID-19 treatment option culminates the importance of our study and meta-analysis approach in translating virus-host interaction Big Data into clinical interventions.

CONTRIBUTORS

ST conceived the study. AB, OK, RN, RS performed the experiments. ST, AB, OK, RN, SS, RS, DS, DG analysed the data. SM, HB, MJ, DKS, AS provided patient samples. SS, AB, ST, OK, RN wrote the manuscript. AB, OK and ST have verified the underlying data. All authors read and approved the final version of the manuscript.

Declaration of Competing Interest

Dr. Tripathi and Ms. Oyahida have a patent, Indian Patent ‘A gene expression signature in nasopharyngeal swab samples for highly sensitive and specific COVID-19 prognosis’ pending, and a patent, Indian Patent ‘Use of Auranofin and its combination with other antiviral agents for COVID-19 treatment’ pending. Dr. Biji has a patent, Indian Patent ‘A gene expression signature in nasopharyngeal swab samples for highly sensitive and specific COVID-19 prognosis’ pending. Dr. Narayan has a patent, Indian Patent ‘Use of Auranofin and its combination with other antiviral agents for COVID-19 treatment’ pending. The other authors have nothing to disclose.

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Data sharing statement

Data that support the findings of this study (deidentified participant data, study protocol, raw data etc.) will be available from the corresponding author, Dr Shashank Tripathi (shashankt@iisc.ac.in) upon request following publication of this study.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2021.103525.

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Quantitative proteomics of hamster lung tissues infected with SARS-CoV-2 reveal host factors having implication in the disease pathogenesis and severity

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Abstract
Syrian golden hamsters (Mesocricetus auratus) infected by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) manifests lung pathology. In this study, efforts were made to check the infectivity of a local SARS-CoV-2 isolate in a self-limiting and non-lethal hamster model and evaluate the differential expression of lung proteins during acute infection and convalescence. The findings of this study confirm the infectivity of this isolate in vivo. Analysis of clinical parameters and tissue samples show the pathophysiological manifestation of SARS-CoV-2 infection similar to that reported earlier in COVID-19 patients and hamsters infected with other isolates. However, diffuse alveolar damage (DAD), a common histopathological feature of human COVID-19 was only occasionally noticed. The lung-associated pathological changes were very prominent on the 4th day post-infection (dpi), mostly resolved by 14 dpi. Here, we carried out the quantitative proteomic analysis of the lung tissues from SARS-CoV-2-infected hamsters on day 4 and day 14 post-infection. This resulted in the identification of 1585 proteins of which 68 proteins were significantly altered between both the infected groups. Pathway analysis revealed complement and coagulation cascade, platelet activation, ferroptosis, and focal adhesion as the top enriched pathways. In addition, we also identified altered expression of two pulmonary surfactant-associated proteins (Sftpd and Sftpb), known for their protective role in lung function. Together, these findings will aid in understanding the mechanism(s) involved in SARS-CoV-2 pathogenesis and progression of the disease.

Abbreviations: ABSL3, animal biosafety level 3; AERD, aspirin exacerbated respiratory disease; ARDS, acute respiratory distress syndrome; BAL, bronchoalveolar lavage; BCA, bicinchoninic acid assay; CF, cystic fibrosis; DAD, diffuse alveolar damage; DAPI, 4′, 6-diamidino-2-phenylindole; DTT, dithiothreitol; HPLC, high performance liquid chromatography; IAA, iodoacetamide; PANTHER, protein analysis through evolutionary relationships; PBST, phosphate-buffered saline with tween detergent; PCA, principal component analysis; TEABC, triethylammonium bicarbonate.

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1 | INTRODUCTION

The recent outbreak of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has emerged as a global public health crisis affecting millions of people worldwide. SARS-CoV-2, the causative agent of the coronavirus disease 2019 (COVID-19), primarily infects the respiratory tract resulting in respiratory failure consistent with the acute respiratory distress syndrome (ARDS). The aggressive inflammatory response associated with infection leads to tissue damage and fatal lung injury. The death of the virus affected epithelial cells and endothelial cells, and activation of resident dendritic cells, monocytes, and macrophages result in the dysregulated inflammatory response. This triggers further activation and recruitment of immune cells leading to tissue damage and exacerbation of respiratory distress and disease severity. However, the levels of circulating cytokines in COVID-19 patients are found to be lower in comparison to patients experiencing ARDS due to other reasons. Post-mortem investigation of COVID-19 patients revealed severe vascular injury including alveolar microthrombi. Virus-induced coagulopathy has also been implicated to trigger COVID-19 associated pneumonia and ARDS. Currently, our understanding of COVID-19 associated lung injury is limited and requires further studies to elucidate the intricate and specific mechanisms.

Severe clinical manifestations involving multi-organ failure have resulted in high morbidity and mortality, thus demanding immediate therapeutic measures and disease management. In this regard, a better understanding of viral and host factors involved in this disease’s pathogenesis has paramount significance. After identifying the SARS-CoV-2 virus, multiple genomic and proteomics-based approaches have been adapted to understand the host response to this viral infection. In the recent past, different studies have been conducted using proteomics technology to understand the virus-host protein interactome, changes in host protein expression upon virus infection, and for diagnosis of SARS-CoV-2 infection. Most of these studies have been carried out with patients’ liquid specimens like serum, plasma, sputum, and bronchoalveolar lavage. Clinical manifestations of COVID-19 are primarily related to the lung; hence clinical proteomics of infected lung tissues are essential to understand and combat this disease. The information obtained from such studies will provide the rationale for designing novel diagnostic and therapeutic interventions. Using lung tissues obtained from a small number of deceased COVID-19 patients, an earlier study has identified differentially expressed proteins in biological processes like blood coagulation, metabolism, immune response, angiogenesis and cell microenvironment regulation. Another group has extracted proteins from Formalin-Fixed Paraffin-Embedded (FFPE) lung tissues of COVID-19 deceased patients and identified high expression of proteins associated with SARS-CoV-2 entry (cathepsins B and L) and inflammatory response modulator (S100A8/A9). In a recent study, proteomic analysis of autopsy samples of nineteen COVID-19 patients showed the elevation of cathepsin L1, rather than ACE2 in infected lung tissues and highlighted the dysregulation of biological processes like angiogenesis, coagulation, and fibrosis in different organs including lungs. Most of these studies report the upregulation of proteins predominantly implicated in the hyperinflammatory state, repair state, and lung fibrosis.

However, so far no experimental controlled lung proteomics study is available to characterize the molecular mechanisms underlying COVID-19 pathogenesis at different stages of the disease progression. Preclinical animal models that recapitulate multiple sequential events associated with human diseases are precious tools to understand the mechanistic aspects of human disease progression. In human patients, the COVID-19 associated lung pathology is a major clinical concern.

At present, no animal model recapitulates all aspects of COVID-19 in humans. Among different available animal models, so far hamsters have been widely utilized in SARS-CoV-2 infection studies. Hamster model of COVID-19 mimics a mild pattern of human disease with full recovery. Although SARS-CoV-2 infection of hamsters induces lung pathologies like pulmonary edema, consolidation, and interstitial pneumonia, but failed to develop diffuse alveolar disease, a prominent clinical feature noticed in COVID-19 patients experiencing severe disease. Further characterization of this model is essential for the development of effective therapeutics and vaccines against this virus. An in-depth understanding of host response to SARS-CoV-2 infection in hamsters will elucidate this model’s similarity or dissimilarity with human patients. Although efforts to identify differentially expressed proteins in diverse body fluids of COVID-19 patients were made, there is a dearth of evidence related to differentially expressed proteins in human lung tissues at acute and convalescent stages of SARS-CoV-2 infection. Fresh lung tissues of COVID-19 patients during infection or recovery are ethically impossible to obtain. Hence, so far, data obtained
from tissues of deceased COVID-19 patients are only available. In this regard, tissues obtained from animals infected with SARS-CoV-2 at different days post-infection will prove beneficial. In this study, efforts have been made to quantitatively compare the lung proteome in SARS-CoV-2-infected hamsters at various days post-infection. The differentially expressed proteins identified in this study will provide information on various host proteins that might have a significant role in the pathogenesis of SARS-CoV-2 infection or disease manifestation. Some of the differentially expressed proteins identified in this study can also be validated in easily accessible patient samples such as body fluids as potential biomarkers for predicting the disease course.

2 | MATERIALS AND METHODS

2.1 | Animal ethics

In this study, attempts were made to evaluate the infectivity of one of the local isolates of SARS-CoV-2, IND-ILS01/2020 (GenBank accession ID-MW559533.2) in the Syrian Golden Hamster model. All the experiments were performed with prior approval of the Institutional Biosafety Committee (IBSC) and Institutional Animal Ethical Committee (IAEC). The study was carried out adhering to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India.

2.2 | Animal studies

For this study, eleven hamsters of the age group 6-7 months were acclimatized at the ILS ABSL3 facility for 4-6 days prior to the experiments. As shown in the schematic (Figure 1A), on day zero, six animals were infected intranasally with SARS-CoV-2 (10^5 TCID50)\(^{18}\) and five animals were mock-infected (only PBS) under intraperitoneal ketamine (200 mg/kg) and xylazine (10 mg/kg) anesthesia. The SARS-CoV-2 virus used in this study was isolated from a clinically confirmed local COVID-19 patient (IND-ILS01/2020; GenBank accession ID-MW559533.2). Virus stock from the 10th passage was titrated by plaque and TCID50 (Median Tissue Culture Infectious Dose) assays and used for this animal challenge study. Out of the six infected animals, three animals were sacrificed on 4 dpi (day post-infection), and the other three were sacrificed on 14 dpi. Out of the five mock-infected animals, three were sacrificed on 4 dpi, and the other two were sacrificed on 14 dpi. Throughout the experiment, all the animals were monitored daily, and body weights were recorded on alternate days. On the day of sacrifice, tissues from all the vital organs and other organs like the pancreas, spleen, and gastrointestinal tracts were harvested, preserved, and further processed for histopathological analysis and viral load estimation. All the groups had two male and one female animal, except the 14 dpi mock-infected group, which had one male and one female animal in it.

2.3 | Sample collection, storage, and processing

Tissues harvested from different organs, including lungs, were divided into three parts. One part was stored in buffered formalin and further processed for histopathological analysis. The second part was immediately put into TRIzol RNA isolation buffer, and the third part was snap-frozen and stored at −80°C until further use. Formalin-fixed tissues were further processed and sectioned for Hematoxylin and Eosin (H&E), Immunohistochemistry (IHC), and Immunofluorescence analysis (IF) as reported earlier.\(^{22}\)

2.4 | Hematoxylin and Eosin (H&E) staining and lung pathology scoring

Tissues from multiple lobes of all the animals were processed and stained with H&E staining. Stained lung sections were assigned with different numbers and evaluated in a blinded fashion. Lung pathology scoring was performed similarly as reported by Li et al.\(^{23}\) The scoring was performed in a blinded fashion and cross-validated by two experienced evaluators. Briefly, the sections were scored for three major pulmonary pathological features associated with SARS-CoV-2 infection (bronchiolitis, alveolitis, and vasculitis/endotheliitis) (Supplementary Figure S1). Each feature was scored on a scale of 0-4 and the total score was obtained by adding values for each feature. The total score ranged between 0 and 12.

2.5 | Immunohistochemistry

Collected tissues were processed and sectioned as reported previously.\(^{22,24}\) Sections were deparaffinized, rehydrated, and subjected to antigen retrieval (Vector Laboratories) treatment for 20 minutes followed by blocking the endogenous peroxidase with 3% hydrogen peroxide in methanol for 20 minutes. Horse serum (Vector Laboratories) was used for blocking the sections for 30 minutes at room temperature and incubated with Ki-67 antibody (#VP-RM04; Vector Laboratories, 1:100) or rabbit polyclonal anti-human Sftpd antibody (1:500) antibody overnight at 4°C.\(^{25}\) Sections were washed twice
with 1× PBS and treated with biotinylated anti-rabbit/mouse IgG secondary antibody (Vector Laboratories) for 45 minutes, followed by ABC reagent for 30 minutes. Diaminobenzidine (Vector Laboratories) was used as a substrate to develop the stain. Hematoxylin was used as a counterstain followed by dehydration with alcohol, clearing with xylene, and mounting with permanent mounting media (Vector Laboratories). Stained sections were observed under the microscope (Leica DM500), and images were taken at different magnifications.

**FIGURE 1** Syrian Golden Hamster model of SARS-CoV-2 infection by a local SARS-CoV-2 isolate. A, Study design to evaluate the infectivity of local-isolate in Syrian Golden Hamster infection model. B, Graph showing percent body weight change in hamsters after mock-infection or SARS-CoV-2 infection. C, Digital images of lungs harvested from mock-infected or infected animals. At 4 dpi, infected lungs have massive congestions visible from the surface (highlighted with white border). No gross changes were noticed in mock-infected (4 dpi) and infected (14 dpi) lungs. Images showing H&E stained lung tissues harvested from mock-infected or infected (4 or 14 dpi) hamsters (scale bar = 200 µm). D, Immunofluorescence images of mock-infected or infected lung tissue sections showing presence of Nucleocapsid protein (N) in bronchial and alveolar epithelial cells. E, Immunoblot analysis showing the Nucleocapsid protein expression in the lungs previously archived lysate (normal), mock-infected (4 dpi), infected 4 dpi or 14 dpi lung tissues. F, Graph showing the viral RNA quantification by RT-qPCR in hamster lung tissues. G, Immunohistochemistry staining with Ki67 showing cell proliferation of bronchial and alveolar cells (scale bar = 50 µm). H, Graph showing pathology score of the lung tissues after the infection (4 and 14 dpi).
2.6 | **Immunofluorescence**

Sections were deparaffinized, rehydrated, and subjected to antigen retrieval treatment and serum blocking as reported earlier.\(^22\) Sections were incubated with SARS-CoV-2 N protein (Nucleocapsid) (#11-2003; Abgenex, 1:200) or SOD2/Mn-SOD (#NB100-1992; Novus biologicals, 1:100) in a humidified chamber overnight at 4°C. Sections were washed twice with PBST for 5 minutes each and incubated with anti-Rabbit Alexa Fluor 594 (#A-11037; Life technologies, 1:500) or anti-Mouse Alexa Fluor 594 (#A-11005; Life technologies, 1:500) for 45 minutes under dark conditions at room temperature. Sections were washed with PBST twice and mounted with ProLong Gold Antifade reagent with DAPI (#P36935; Invitrogen) and visualized using Leica TCS SP8STED confocal microscope.

2.7 | **Western blot analysis**

For Western blot analysis, cells were re-suspended in Radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50 mM NaF, 1 mM Sodium orthovanadate (Na\(_3\)VO\(_4\)), 0.1% SDS, and 0.5% TritonX-100) containing the protease inhibitor cocktail (Thermo Scientific). The whole-cell lysates (WCL) were subjected to SDS-PAGE and transferred to nitrocellulose membrane (Thermo Scientific), followed by blocking and immunoblotting with antibodies specific for SARS-CoV-2 Nucleocapsid protein (#11-2003, Abgenex) or β-actin (#4970, CST).\(^26\)

2.8 | **qRT-PCR**

RNA isolation was carried out from hamster tissue samples using TRizol reagent (#10296010, Invitrogen). The isolated RNA was subjected to qRT-PCR for determining the viral load. We performed one-step multiplex real-time PCR using TaqPath I-Step Multiplex Master Mix (#A28526, Thermo Fisher Scientific), targeting SARS-CoV-2 gene with primer and probe set specific for nucleocapsid (N). The standard curve for absolute quantification of viral genome copies was generated using log-fold dilutions of plasmid harboring the SARS-CoV-2 nucleocapsid gene.\(^27\)

2.9 | **Sample preparation for proteomics analysis**

The lung tissue samples of SARS-CoV-2-infected on 4 and 14 dpi along with mock-infected (4 dpi) were processed and homogenized using liquid nitrogen and lysed in lysis buffer containing 4% sodium dodecyl sulfate (SDS) and 50 mM triethylammonium bicarbonate (TEABC). The samples were subjected to sonication three times for 10 seconds storing on ice to prevent overheating between the sonication and were followed by heating at 90°C for 5 minutes. The lysates were then incubated at room temperature for cooling and centrifuged at 12 000 rpm for 10 minutes. The protein concentration present in the supernatant was determined using a bicinchoninic acid assay (BCA) kit (Thermo Scientific Pierce) and an equal amount of protein from each group was pooled for further analysis. LC-MS/MS approach was used using isotopomer labels, “tandem mass tags” (TMTs), to determine the relative quantification of proteins.\(^28\)

2.10 | **In-solution digestion and TMT labeling**

Protein lysate of 300 µg from pooled samples of each group was reduced by incubating in 10mM Dithiothreitol (DTT) at 60°C for 20 minutes. Alkylation was carried out in the dark with 20 mM iodoacetamide (IAA) at room temperature for 10 minutes. The lysate was further subjected to acetone precipitation, and the pellet was dissolved in 50 mM TEABC. Digestion was carried out at 37°C for 16 hours using trypsin (Sciex, #4326682) at a final concentration of 1:20 (w/w). The reaction was acidified using 0.1% formic acid, and the peptides were lyophilized and stored at −80°C until further use.

2.11 | **LC-MS/MS acquisition**

The digested peptides were fractionated into six fractions using StageTip fractionation. Fractionated peptides (6 fractions in triplicate, total of 18 runs) were analyzed on Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) interfaced with EASY-nLC 1000 nanoflow liquid chromatography system (Thermo Scientific, Odense, Southern Denmark). Each fraction was reconstituted in solvent A (0.1% formic acid) and loaded onto trap column (75 µm × 2 cm) Thermo Scientific Acclaim PepMap 100 C18 (#164535; Thermo Scientific) (3 µm particle size, pore size 100 Å) at a flow rate of 5 µl/min with solvent A (0.1% formic acid in water).

The peptides were resolved on an analytical column (EASY-Spray C18 Reversed Phase HPLC Column, 2 µm, 75 µm × 500 mm; Thermo Scientific) using a linear gradient of 7%-30% solvent B (0.1% formic acid in 95% acetonitrile) over 100 minutes at a flow rate of 300 nl/min. Data-dependent Mass Spectrometry acquisition was carried out at top speed mode with full scans (350-1500 m/z) acquired using an Orbitrap mass analyzer at a mass resolution of 120 000 at 200 m/z. For MS/MS, top intense precursor ions from a 3-second duty cycle were selected and subjected to higher-energy collision dissociation (HCD) with 35% normalized collision energy. The
fragment ions were detected at a mass resolution of 30 000 at m/z of 200. Dynamic exclusion was set for 30 seconds. Lock-mass from ambient air (m/z 445.1200025) was enabled for internal calibration as described previously.29

2.12 | Bioinformatics and statistical analysis

The Proteome Discoverer 2.3 (Thermo Scientific, Bremen, Germany) was used to carry out protein identification and quantitation. All raw files were searched against a Mesocricetus auratus (Syrian Golden hamster) protein database in Universal Proteins Resource Knowledgebase (UniProt) (32,336 entries) supplemented with common contaminants (116 entries) using SequestHT as a search algorithm. The search parameters included trypsin as the proteolytic enzyme with a maximum allowed missed cleavages to two. Oxidation of methionine and acetylation of protein N-terminus were set as dynamic modifications. In contrast, static modifications included cysteine carbamidomethylation and TMT modification at the N-terminus of the peptide and lysine residue. Precursor mass tolerance was set to 10 ppm, and fragment mass tolerance was set to 0.05 Da. Peptide Spectrum Matches were identified at 1% False Discovery rate. The differential expression ratios between the groups were calculated. Proteins with differential expression ratios ≥1.5 (upregulated) or ≤0.67 (downregulated) were considered as differentially expressed. The significance of differences between groups was calculated using Student’s t test (two-tailed), and a P-value ≤ .05 was considered statistically significant. All the differentially expressed proteins across the two groups (Infected 4 dpi and infected 14 dpi) were compared using a Venn diagram (Venny 2.1, https://www.bioinfogp.cnb.csic.es/tools/venny/) and further analyzed with PANTHER classification system, version 16.0 (http://www.pantherdb.org). Gene Ontology (GO) and pathway analysis using Kyoto Encyclopedia of Genes and Genomes (KEGG) were performed using the Enrichr online tool (http://www.amp.pharm.mssm.edu/Enrichr/).30

Principal component analysis was performed using sample-wise scaled unfiltered normalized protein abundance data using PCAtools. Heatmaps were generated using protein-wise scaled and filtered protein abundance (1.5 and 1.3 up- or down-regulation; P-value ≤ .05 in any comparison) and k-means clustering (k = 3).

3 | RESULTS

3.1 | Clinical features, viral load, and histopathological changes in lungs

To investigate the pathogenicity of SARS-CoV-2, Syrian hamsters were infected with the virus, and harvested tissue samples were collected at two different time points (4 & 14 dpi) for viral load and pathological analysis (Figure 1A). Corroborating earlier reports, a significant weight loss was noticed in all the infected animals at 4 dpi. On 4 dpi, all the animals lost around 15% of their initial body weight (Figure 1B). After 6 dpi, the infected animals started regaining their body weight. During the course of the experiments, no mortality was found in both infected and mock-infected animals. At the time of organ isolation, congestion of lungs was grossly visible only in infected animals at 4 dpi (Figure 1C). Further histopathological analysis of the 4 dpi SARS-CoV-2-infected tissue samples showed the presence of severe pathological lesions in the lungs (Figure 1C and Supplementary Figure S1A-G). Multifocal necrosis and the desquamation of bronchial epithelial cells and infiltration of inflammatory cells were present in the SARS-CoV-2-infected lung tissues (Figure 1C). Around 50% lung area was affected in all the infected animals, and the lesions were patchy throughout the lungs. Necrosuppurative bronchitis and interstitial pneumonia were evident in all the infected animals at 4 dpi (Supplementary Figure S1D,E). Different areas of the infected lung tissues showed consolidation of lungs and hemorrhage (Supplementary Figure S1C). These hamsters exhibited severe interstitial pneumonia, as evidenced by the thickening of the alveolar wall, altered alveolar structure, and immune cells’ infiltration (Supplementary Figure S1D). At 4 dpi, infected lungs have occasional features of diffuse alveolar damage (DAD) including necrosis of alveolar epithelial cells, presence of intra-alveolar immune cells, cellular debris, and protein exudates (Supplementary Figure S1F). However, hyaline membrane formation was very rarely noticed, which indicates a less severe form of DAD.31 Endothelium near the damaged areas was reactive, as evidenced by mononuclear cells’ adhesion to the endothelium (Supplementary Figure S1A). In certain instances, the immune cells have invaded the vessel wall and caused endotheliitis (Supplementary Figure S1B). In one of the infected animals (4 dpi), visceral pleural invasion of immune cells was also noticed (Supplementary Figure S1G). However, no noticeable histopathological changes were observed in mock-infected hamster lung at any time point. After 14 dpi, hamsters infected with SARS-CoV-2 exhibited only mild inflammatory infiltration and tissue damage suggestive of the resolution of disease manifestation (Figure 1C). Histopathological evaluation of tracheal tissues from all the animals also showed severe tracheal epithelial and endothelial damage in 4 dpi-infected tissues compared to 14 dpi-infected or mock-infected tissues (Supplementary Figure S2). The significantly higher lung pathology score at 4 dpi-infected tissues than 14 dpi corroborates the earlier reports and indicates the self-limiting nature of this disease in the hamsters (Figure 1H).
Immunofluorescence (IF) staining of lung tissue sections with SARS-CoV-2 nucleocapsid (N) protein showed the viral antigen-positive bronchial and alveolar epithelial cells at 4 dpi, which were not detected at 14 dpi tissues (Figure 1D). Immunoblot analysis of the lung tissue protein lysates also corroborated the IF findings (Figure 1E). The viral genome copy number estimation showed the presence of viral genome in both 4 dpi and 14 dpi-infected lung tissues; however, the copy number was significantly low in 14 dpi tissues compared to 4 dpi tissues (Figure 1F). Immunohistochemical staining for Ki67, a marker of cell proliferation, showed marked cellular proliferation (hyperplasia) of bronchial and alveolar cells at 4 dpi (Figure 1G). This finding corroborates with the earlier report.20

3.2 Quantitative proteomics analysis

3.2.1 Molecular alterations in pulmonary pathology induced by SARS-CoV-2 infection

Analysis of all the animals’ body weights, lung pathology, and status of viral protein in lung tissues clearly demonstrated normal animal health in both the 4 dpi and 14 dpi mock-infected animals. At the same time, lung tissues from the 4 dpi and 14 dpi-infected hamsters manifested features of acute and convalescent stages of the infection, respectively. One of the major objectives of the current study was to compare the lung proteome of SARS-CoV-2-infected hamster lung tissues at acute and convalescent stages of the infection. Hence, we employed a quantitative proteomics approach to identify the proteomic alterations in hamster lung tissue induced by SARS-CoV-2 at different time points (4 dpi and 14 dpi) compared with only 4 dpi mock-infected control tissues (as a representative of mock-infected groups). The quantitative proteomics data were analyzed using high-resolution LC-MS/MS in triplicates where the raw files were searched using Proteome Discoverer 2.3. The search resulted in the identification of 1585 proteins expressed across all the samples. Of these, 50 and 18 proteins were differentially expressed (cut off 1.5-fold, \( P \leq .05 \)) at 4 dpi and 14 dpi, respectively. The 50 differentially expressed proteins in 4 dpi included 33 upregulated and 17 downregulated proteins whereas 18 differentially expressed proteins in 14 dpi included nine upregulated and nine downregulated proteins. A complete list of the proteins identified is provided in Supplementary Table S1. All the differentially expressed proteins were further represented as a heat map using hierarchical clustering analysis comparing mock-infected with the infected samples (Figure 2A). Interestingly, the principal component analysis (PCA) demonstrated distinct protein expression patterns among the mock-infected 4 dpi and 14 dpi-infected samples, depicting variation among them (Figure 2B). In PC1, we observed distinct clusters of data representing the 14 dpi and 4 dpi lung tissue samples, and separate clusters representing the mock-infected (4 dpi) and 14 dpi samples in PC2. Technical triplicate samples of the same groups are clustered together.

We compared all the identified proteins across the two groups (Infected 4 dpi and infected 14 dpi) to identify the number of common and differentially expressed proteins. This comparison was carried out using a Venn diagram (Venny 2.1, https://www.bioinfogp.cnb.csic.es/tools/venny/). Upon comparison, we identified 41 proteins exclusive to 4 dpi tissue samples and nine exclusive to 14 dpi tissue samples when compared with mock-infected (4 dpi) tissue samples, while nine proteins were shared between the groups (Supplementary Figure S3). Of the total identified proteins, 50 proteins were differentially expressed (33 upregulated and 17 downregulated; \( P \leq .05 \)) in 4 dpi-infected groups. Among these proteins, majority were involved in the blood coagulation, integrin signaling pathway, alternative complement activation signaling pathway, and plasminogen activating cascade (PANTHER classification system, version 16.0).32 Similarly, 18 proteins were differentially expressed (nine upregulated and nine downregulated; \( P \leq .05 \)) in the infected 14 dpi group. This included proteins belonging to the plasminogen activating cascade and PI3K-Akt signaling pathway. The differentially expressed proteins are graphically represented as a heat map and volcano plots representing distinct proteomic patterns between the two groups compared to mock-infected (Supplementary Figure S4A-C). We also evaluated the altered protein expression during the acute and convalescent stages of infection (14 dpi vs 4 dpi) (Supplementary Figure S4D).

We further compared the differentially expressed proteins of statistical significance across the SAR-CoV2-infected (4 dpi and 14 dpi) and mock-infected (4 dpi) groups. A comparison of the downregulated proteins and upregulated proteins from both groups did not result in any common protein. However, among the 33 proteins upregulated in the 4 dpi tissue samples, three proteins such as superoxide dismutase (Sod2), myosin-2 (Myh2), and calgranulin-B (S100a9) were downregulated in the 14 dpi group. This included proteins belonging to the plasminogen activating cascade and PI3K-Akt signaling pathway. The differentially expressed proteins are graphically represented as a heat map and volcano plots representing distinct proteomic patterns between the two groups compared to mock-infected (Supplementary Figure S4A-C). We also evaluated the altered protein expression during the acute and convalescent stages of infection (14 dpi vs 4 dpi) (Supplementary Figure S4D).

We further compared the differentially expressed proteins of statistical significance across the SAR-CoV2-infected (4 dpi and 14 dpi) and mock-infected (4 dpi) groups. A comparison of the downregulated proteins and upregulated proteins from both groups did not result in any common protein. However, among the 33 proteins upregulated in the 4 dpi tissue samples, three proteins such as superoxide dismutase (Sod2), myosin-2 (Myh2), and calgranulin-B (S100a9) were downregulated in the 14 dpi tissue samples. These proteins are known to regulate various cellular processes such as antioxidant defense, cellular localization, cell adhesion, and cellular migration. Similarly, among the 33 proteins upregulated in the 4 dpi lung tissue, four proteins, including Fibronectin beta chain (Fgb), Ferritin (Fth1), Calpactin I (S100a10), Thymosin (Tmsb4x) were found to be upregulated in a 14 dpi sample. These proteins were involved in the regulation of biological processes and exhibit catalytic activity. On further comparison of downregulated proteins identified across
FIGURE 2  Lung tissue proteome of hamsters infected with SARS-CoV-2 at different time points. A, Heatmap of the differential expressed proteins ($P \leq .05$; fold change cut off of 1.5) were plotted sample wise suggesting the proteomic alterations across the infected samples (4 and 14 dpi) compared to mock-infected (4 dpi). B, Principal Component Analysis (PCA) was performed and plotted using PCAtools with technical replicates marked by R1, R2, and R3. The PCA plot represents the variance explained by the principal components (denoted by PC) indicating a clear separation of the samples among the three groups, ie, mock-infected (4 dpi), infected (4 dpi), and infected (14 dpi). C, Pathway analysis was performed using Enrichr online tool using the KEGG database. The enrichment analysis was performed for upregulated and downregulated differentially expressed proteins for all the three groups infected (4 dpi) versus mock-infected (4 dpi); Infected (14 dpi) versus mock-infected (4 dpi); and infected (14 dpi) versus infected (4 dpi). The horizontal axis represents the enrichment score $-\log_{10} (P$-value) of the pathway and the vertical axis represents the pathway category. The red color represents upregulated proteins and the blue bar represents downregulated proteins.
both 4 dpi- and 14 dpi-infected tissues, two proteins were common in both groups, the serine/arginine-rich splicing factor 1 (Srsf1) and Guanine nucleotide-binding protein G (q) subunit alpha (Gnaq).

3.2.2 | Functional characteristic of significantly altered proteins

To systematically investigate the molecular differences in the hamster lung owing to SARS-CoV-2 infection, we carried out a Gene Ontology (GO) analysis of the differentially expressed proteins. The biological process such as “platelet degranulation,” “regulated exocytosis,” and “fibrinolysis” were enriched mainly in the proteins upregulated in infected (4 dpi) lung tissues, whereas “wound healing,” “collagen fibril organization,” and “actin cytoskeleton reorganization” were enriched in downregulated proteins (Supplementary Table S2). The proteins associated with these pathways include fibrinogens (Fga, Fgb, and Fgg); complement factors (C4a, C3); alpha-2-antiplasmin (Serpinf2); Alpha-1-B Glycoprotein (A1bg), Apolipoprotein A1 (Apoa1), and Alpha-1-acid glycoprotein 1 (Orm1). Notably, the terms such as “regulation of substrate adhesion-dependent cell spreading” and “endothelial cell migration” were mostly observed in upregulated and downregulated proteins in 14 dpi (Supplementary Table S3).

3.2.3 | Pathway analysis

KEGG pathway analyses of the differentially expressed proteins in lung tissue at 4 dpi compared to mock-infected (4 dpi) tissue revealed that SARS-CoV-2-triggered the activation of complement and coagulation cascade and ferroptosis. The analysis also resulted in the identification of other aberrant pathways such as platelet activation, focal adhesion, and tight junction. Interestingly, we also observed an aberrant expression of proteins associated with necrosis, cholesterol metabolism, ferroptosis, and interleukin-17 signaling pathway in the 14 dpi tissue samples suggestive of the underlying mechanisms that lead to tissue damage and lung injury during SARS-CoV-2 infection (Figure 2C).

3.3 | Secretory proteins

To investigate the amount of secretory proteins among the pool of differentially expressed proteins in the infected tissue samples, we compared our data with the proteins annotated as “secretory proteins (2640 proteins),” “Secreted in the blood (729 proteins),” “Lung enriched (13 proteins),” “Lung proteome (19 649 proteins),” and “Group enriched (61 proteins expressed in the lung)” (Supplementary Figure S4E). We found four proteins, Advanced Glycosylation End-Product-Specific Receptor (Ager), secretoglobin family 1A member 1 (Scgb1a1), Surfactant associated protein B (Sftpb), and Surfactant associated protein D (Sftpd) identified in the current study were specific to lung proteome.

3.4 | Validation of selected proteins’ expression status

To validate the hamster lung proteome data identified in this study, we randomly picked two proteins, SOD2 and Sftpd, and checked their expression through IF and IHC, respectively. We found a significantly higher level of SOD2 expression in the infected 4 dpi tissues than 4 dpi mock-infected or 14 dpi-infected tissues (Figure 3A). Similarly, analysis of Sftpd expression showed lower expression of this protein in bronchial epithelial cells of 4 dpi and 14-dpi infected lung tissues than 4 dpi mock-infected samples (Figure 3B). Together, these data successfully validated the results of the mass spectrometry-based proteomics analysis.

4 | DISCUSSION

Multiple studies across the globe have demonstrated that the hamster model of SARS-CoV-2 infection mimics the milder form of COVID-19 in humans. Earlier study by using USA-WA1/2020 (NR-52281, BEI Resources) isolate showed that $5 \times 10^4$ TCID50 dose of viral infection in hamsters lead to weight loss and full recovery by 14 days. However, an infection dose of $5 \times 10^5$ TCID50 dose induced severe weight loss and partial mortality. Similarly, in a different study with an infection dose of $8 \times 10^4$ TCID50 of BetaCoV/Hong Kong/VM20001061/2020 (GISAID# EPI_ISL_412028) virus, the investigators have reported a self-limiting model of SARS-CoV-2 with the manifestation of earlier lung tissue damage followed by recovery. All these studies also corroborate the self-limiting nature of this model as reported by others. In the recent study, Mohandas et al have used an Indian SARS-CoV-2 isolate (NIV-2020-770) at different infection doses and noticed that $10^{3.5}$ TCID50 and $10^{4.5}$ TCID50 infection dose of the virus manifested a self-limiting disease with lung pathologies at the earlier days of infection. Based on the aforementioned studies we picked a dose of $10^5$ TCID50 for the inoculation of hamsters with our isolate IND-ILS01/2020 (GenBank accession ID- MW559533.2). Similar to the earlier report, our analyses also showed a non-lethal and self-limiting model of SARS-CoV-2 infection in hamster. The histopathological and viral analysis of lung samples clearly showed that 4 dpi- and 14 dpi-infected animals represent acute and convalescent
stages of infection. The absence of the severe form of DAD features also corroborates earlier reports and indicates the limitation of this model.\textsuperscript{18}

Our quantitative proteomic analysis data showed the dysregulation of biological processes such as “collagen fibril organization” and “actin cytoskeleton reorganization” in infected 4 dpi and “regulation of substrate adhesion-dependent cell spreading” and “endothelial cell migration” in the infected 14 dpi group. Collagen network organization occurs in response to tissue damage and is a part of the wound healing process and can trigger the onset of fibrosis. The actin cytoskeleton is an important cellular component, essential for maintaining the shape and structure of the cells. Many viruses promote the rearrangement of the host cell cytoskeleton to facilitate their dissemination.\textsuperscript{36} We observed reduced expression of the proteins that contribute to dysregulation in actin polymerization at both 4 dpi and 14 dpi (Supplementary Tables S2 and S3). Reports also indicate that any alteration in the cell-matrix adhesion and extracellular matrix during injury repair can disrupt lung structure, further leading to lung damage. Our results also indicate the dysregulation of collagens in the 14 dpi lung tissues. The proteins associated with “endothelial cell migration” include Thymosin beta-4 (Tmsb4x) and High Mobility Group Box 1 (HMGB1), both of which have been reported to be potential therapeutic targets in drug discovery. Tmsb4x is a small and water-soluble peptide known for its role in angiogenesis, wound healing, and increased metastatic potential of tumor cells.\textsuperscript{37,38} Its ability to induce fibrinolysis makes it an interesting molecular drug target. Recently, HMGB1 has also emerged as a potential target for therapeutic interventions for COVID-19. It is known to play a critical role in various infections, and its elevated expression has been reported in many viral infections, including COVID-19.\textsuperscript{39,40} This association further results in receptor-dependent responses suggesting its possible role in SARS-CoV-2 infection. Further studies are warranted to uncover the functional role of HMGB1 and evaluate its inhibitors in the COVID-19 treatment.

We also observed multiple pathways such as the dysregulation of complement and coagulation cascades, platelet degranulation, and ferroptosis upon viral infection across both the groups (4 dpi and 14 dpi). Existing literature also indicates the critical role of the complement pathway in pathogenesis and disease severity of SARS-CoV-2.\textsuperscript{41,42} The complement system serves as the host systems’ first response to foreign pathogens and regulates processes such as opsonization, chemotaxis, leukocyte recruitment, activation, and phagocytosis. However, unrestricted activation of this pathway contributes to acute and chronic inflammation, coagulation, cell injury, and facilitates multiple organ failure leading to death.\textsuperscript{43}

![FIGURE 3 A, Immunofluorescence images of mock-infected or infected lung tissue sections showing the expression of Sod2 in bronchial and alveolar epithelial cells. B, Immunohistochemistry images of mock-infected or infected lung tissues showing expression of Sftpd in bronchial epithelial cells](image-url)
Significant dysregulation of complement and coagulation cascades along with elevated levels of D-dimer, fibrinogen, and von Willebrand factor have been observed in SARS-CoV-2-infected patients. In the current study, we identified higher expression levels of C3, Fgb, Fga, Fgg, Serpinf2, and Cfb in 4 dpi hamster lung tissues as compared to mock-infected (4 dpi). The higher levels of these proteins were well studied as a marker of the activation of early complement and coagulation cascade. The significant enrichment of coagulation proteins in SARS-CoV-2-infected tissues indicates the disruption of coagulation mechanisms during SARS-CoV-2 infection. In addition, the higher expression of proteins such as Fgb, Fga, and Fgg identified in the study are also known to be involved in platelet activation. Platelets are non-nucleated cell fragments derived from megakaryocytes and essential for physiological hemostasis. Additionally, they are also known for their diverse role in inflammatory and immune response by acting as inflammatory effector cells. They also serve as an indispensable element in coagulation and inflammation and their activated state is associated with cancer progression. Recently, the lung has also been proposed as a platelet biogenesis site, accounting for almost 50% of total platelets. There are reports demonstrating the role of platelets in inflammatory lung syndromes/disorders such as acute respiratory distress syndrome (ARDS), chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), aspirin-exacerbated respiratory disease (AERD), and asthma.

Our pathway analysis with significantly downregulated proteins list also identified certain proteins involved in platelet activation (Col1a2, Gnaq, Rhoa), focal adhesion (Col1a2, Parva, Rhoa), and leukocyte transendothelial migration (Cldn18, Rhoa) in day 4 infected vs mock-infected animals. Even though the pathways are commonly shared among the up and downregulated proteins, the biological properties of each protein need to be considered while concluding. The contrasting results from the pathway analysis of upregulated and downregulated proteins highlight the complex nature of these biological processes. Together, we believe that the current pathway analysis only gives a preliminary idea about the differentially expressed proteins in each group and their known biological function. Thus, more studies are required to elucidate the role of each pathway component in the pathophysiology of COVID-19.

Ferroptosis is a form of programmed cell death associated with unchecked lipid peroxidation due to the accumulation of lipid reactive oxygen species (ROS) in cells. Its role is well documented in pathophysiological processes of various diseases, such as tumors, nervous system diseases, ischemia-reperfusion injury, kidney injury, and blood diseases. Iron is a pivotal component of the ferroptosis pathway and disruption of iron metabolism has been reported in COVID-19 patients. In our study, we observed increased ferritin (Fth1) levels in 4 dpi tissues sample. Its increased expression has also been reported in COVID-19 patients. Studies on COVID-19 patients have demonstrated the usefulness of inflammatory markers such as procalcitonin, C-reactive protein, erythrocyte sedimentation rate, and serum amyloid A as an indicator of disease progression, however, little is known about the increased ferritin levels (hyperferritinemia) in these patients. In our study, we observed increased ferritin (Fth1) levels in both 4 dpi and 14 dpi lung tissues sample which corroborates with the recent findings where its increased expression has been reported in COVID-19 patients. Further studies are warranted to evaluate its role as a pathogenic mediator in COVID-19. In addition, we also report here increased levels of Fgb, and S100a10 in both groups when compared to mock-infected. Fgb is a blood-borne glycoprotein, with an active role in biological functions such as angiogenesis, wound healing, and inflammation. It serves as a marker for vascular injuries along with other pathological conditions such as colitis, lung and kidney fibrosis. Consistent with lung fibrosis observed in hamster post-infection with SARS-CoV-2 in the current study, Fgb was higher among both groups suggesting its role in blood clot formation where its higher expression is also reported in COVID-19 patients. Calpain I, belongs to the calcium-binding S100 family, and is ubiquitously expressed in the majority of cells. It is known for its role in wound healing, fibrinolysis, and angiogenesis. Binding of S100a10 with plasminogens facilitates its conversion to plasmin which in turn enhances the virulence and pathogenicity of SARS-CoV-2 by cleaving the spike protein. Moreover, we identified lower levels of Srsf1 and Gnaq among both the infected group as compared to the mock-infected. Srsf1 belongs to the family of splicing regulators and its interaction with different proteins enables it to regulate a plethora of cellular pathways. It plays a significant role in providing genomic stability and thus, viral infection results in the depletion of this protein along with other RNA binding proteins (RBPs) in the nucleus. Such association of the viral genome with host RBPs initiates apoptosis, further resulting in the release of viral particles during infection by compromising the host machinery. Gnaq, constitute the family of largest cell surface membrane receptors, expressed ubiquitously in mammalian cells, and known to be involved in multiple ways during viral infection. Studies have also demonstrated its role in regulating both innate and adaptive immunity. Here we report the reduced expression of Gnaq in hamster lung tissues infected with SARS-CoV-2. Similar data were also observed in virus-infected macrophages implicating its role as a negative regulator of antiviral immune responses.

Pulmonary surfactant proteins constitute a type of lipoprotein complex comprising 90% lipids and 10% surfactant proteins (Sftpa, Sftpb, Sftpc, and Sftpd). These surfactant proteins contribute to providing defense against pathogens and play a critical role in efficient gaseous exchange at the air-liquid interface in the alveoli and provide lung stability.
We observed aberrant expression of the surfactant proteins (Sftpb, and Sftpd) across the 4 and 14 dpi-infected samples compared to the mock-infected samples suggesting the decline in the normal functionality of lung respiratory gaseous exchange owing to virus infection. The previous report has suggested the role of viral proteins in modulating the surfactant metabolism and thus, resulting in host immune compromise.

As reported earlier, we also noticed distinct damage to the epithelial-endothelial barrier in 4 dpi-infected lung tissues. This barrier system’s damage is believed to be the major mediator of ARDS in different respiratory viral infections, including SARS-CoV-2. The loss of the epithelial-endothelial barrier allows leakage of blood components into the alveolar lumen and lung interstitium. At the same time, it also allows the leakage of lung proteins into circulation. In the recent past, it is shown that COVID-19 patients who developed ARDS have significantly higher IL-6 and Sftpd circulatory levels compared to patients who did not have ARDS. These findings further indicate that the identification of pneumo-proteins in circulation is an indicator of the severity of lung pathology. In our lung tissue proteome analysis, we noticed a significant downregulation of Sftpd protein in tissues with high lung pathology score (4 dpi), which suggests that certain proteins might have a differential pattern of expression level in circulation and at the primary site of infection (lungs). Based on several features of pulmonary surfactants, they are believed to have importance in COVID-19 pathogenesis, diagnosis or therapy. It has been proposed that a lower concentration of pulmonary surfactant is a critical risk factor for COVID-19. Studies have also reported that concentrations of SP-A and SP-B were low in bronchoalveolar lavage (BAL) of patients before and after the onset of ARDS. The low level of pulmonary surfactant proteins detected in 4 dpi lung tissues underscores their possible role in the COVID-19 pathology. Altogether, these pulmonary lung surfactants might be considered as potential therapeutics to aid in COVID-19 treatment. Secretoglobin Family 1A Member 1 (Scgb1a1) encodes a member of the secretoglobin family of small secreted proteins, a component of pulmonary surfactant, which is expressed in mucus-secreting cells. This protein is known for its anti-inflammatory/ immunomodulatory and anti-fibrotic functions. We identified severe downregulation of Scgb1a1 in the lung tissue post-infection (4 dpi), suggesting respiratory distress owing to virus-mediated lung injury. Some literature have also reported its altered expression following lung injury and where its absence is marked with the greater inflammatory response. It is also known to regulate alveolar macrophage-mediated inflammatory response upon virus invasion. However, further studies are required to study its effectiveness and the underlying mechanism(s) associated with virus infection. Further, it suggests the usefulness of the hamster model of SARS-CoV-2 infection in evaluating the therapeutic efficacies of these proteins for COVID-19.

Through an ultra-high-throughput clinical proteomics approach, Messner et al. identified protein expression signatures in serum/plasma samples including complement factors, components of coagulation systems, immunomodulators, and proinflammatory factors that can classify COVID-19 patients based on WHO grading. The authors have proposed 27 protein groups (23 upregulated and 4 downregulated) as potential biomarkers of disease severity. Out of these upregulated proteins, we noticed six proteins Complement factor B (Cfb), Fibrinogens (Fga, Fgb, Fgg), Haptoglobin (Hp), and Galectin-3-binding protein (Lgals3bp) are also present in the list of upregulated proteins at 4 dpi vs mock-infected groups of our study. However, albumin (Alb), and transferrin (Tf) whose downregulation correlated with COVID-19 severity are found to be upregulated at the 4 dpi infection group of our study. There are multiple possibilities for this discrepancy like (a) blood proteome and lung proteome might be different in SARS-CoV-2-infected humans or animals, (b) difference in techniques or methods used to analyze samples, and (c) species-dependent differential (hamsters or human) host response to SARS-CoV-2 infection. Similarly, proteomic and metabolomic profiling of serum samples obtained from 46 COVID-19 and 53 control individuals showed deregulation of three major pathways namely complement system, macrophage function, and platelet degranulation in severe COVID-19 patients. A study by Park et al. employed an in-depth proteome profiling of undepleted plasma revealed signatures of proteins involved in neutrophil activation, platelet function, and T cell suppression. Based on their findings, the authors have proposed specific plasma proteins as predictive biomarkers of COVID-19. In a different study, proteomic analysis of serum samples from early COVID-19 patients also identified differentially expressed proteins known to have a function in SARS-CoV-2 infection-associated inflammation and immune signaling. The findings of our study also corroborate the aforementioned findings.

Efforts to investigate the alteration in bronchoalveolar lavage fluid (BALF) proteome in COVID-19 patients compared to the non-COVID-19 controls demonstrated that SARS-CoV-2 infection induces alteration in BALF proteome with enrichment of proteins involved in proinflammatory cytokine-mediated signaling and oxidative stress response. Superoxide dismutase (SOD) is vital for human health and upregulation of SOD2 expression upon challenge with human pathogens suggests its role in immune response. Antioxidant enzymes such as SOD2 are pivotal to protect from free superoxide anion, which can damage epithelial cells and impair their function. Thus, enhanced expression of Sod2 in SARS-CoV-2-infected hamster lungs, suggests the upregulation of antioxidant response...
to prevent oxidative stress-induced tissue damage, lung injury, and respiratory distress.

Taken together, the current study highlights the proteomic alterations caused owing to SARS-CoV-2 infection during the course of infection and provides an insight on the molecular pathogenesis and possible therapeutic targets of COVID-19. Importantly, the current study provides strong molecular evidence that shows the similarities between SARS-CoV-2 infection in humans and hamsters and supports the clinical relevance of this model in COVID-19 research. However, the present study has some limitations of the small sample size considered for the analysis and use of limited experimental conditions (single viral dose and strain). This demands similar extensive research in a large number of animals and different experimental conditions for further validation. Moreover, due to the small number of animals, the effects of age, sex, and comorbidities await further investigation. The unavailability of complete genome information of Syrian golden hamsters has also substantially restricted our findings. We hope in the future with the availability of complete genome sequence and hamster-specific reagents like antibodies will help in obtaining more relevant information from our current findings.

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CONFLICT OF INTEREST
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS
S. Senapati and G. H. Syed, conceived the idea, and supervised the overall experiments. V. Suresh, and K. Avula contributed to animal inoculation and tissue analysis. B. Singh, K. Avula, and G. H. Syed contributed to viral copy number estimation and viral culture. V. Mohanty and R. K. Reddy carried out sample preparation for proteomics analysis. V. Mohanty, A. Ghosh, and V. Suresh were involved in proteomics data analysis, data interpretation, prepared figures and tables. A. R. Suryawanshi and S. K. Raghav crosschecked the bioinformatics data analysis. V. Suresh and D. Parida did tissue processing and staining. S. Senapati carried out the pathological evaluation of tissue sections as one of the evaluators. S. Chattopadhyay, P. Prasad, R. K. Swain, R. Dash, and A. Parida facilitated in establishing Standard Operating Procedures (SOPs) for ABSL3-related activities and intellectually contributed in manuscript writing and interpretation of the data. All authors were involved in manuscript writing and editing.

DATA AVAILABILITY STATEMENT
The raw data files and the MSF files were submitted to the PRIDE partner repository[76] with data set identifier PXD024547.

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SUPPORTING INFORMATION
Additional Supporting Information may be found in the Supporting Information section.

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Immunogenic potential of DNA vaccine candidate, ZyCoV-D against SARS-CoV-2 in animal models

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ABSTRACT
Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), initially originated in China in year 2019 and spread rapidly across the globe within 5 months, causing over 96 million cases of infection and over 2 million deaths. Huge efforts were undertaken to bring the COVID-19 vaccines in clinical development, so that it can be made available at the earliest, if found to be efficacious in the trials. We developed a candidate vaccine ZyCoV-D comprising of a DNA plasmid vector carrying the gene encoding the spike protein (S) of the SARS-CoV-2 virus. The S protein of the virus includes the receptor binding domain (RBD), responsible for binding to the human angiotensin converting enzyme (ACE-2) receptor. The DNA plasmid construct was transformed into E. coli cells for large scale production. The immunogenicity potential of the plasmid DNA has been evaluated in mice, guinea pig, and rabbit models by intradermal route at 25, 100 and 500 μg dose. Based on the animal studies proof-of-concept has been established and preclinical toxicology (PCT) studies were conducted in rat and rabbit model. Preliminary animal study demonstrates that the candidate DNA vaccine induces antibody response including neutralizing antibodies against SARS-CoV-2 and also elicited Th-1 response as evidenced by elevated IFN-γ levels.

1. Introduction

Three highly pathogenic human coronaviruses (CoVs) have been identified so far, including Middle East respiratory syndrome coronavirus (MERS-CoV), severe acute respiratory syndrome coronavirus (SARS-CoV) and severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). Among them, SARS-CoV was first reported in Guangdong, China in 2002 [1]. SARS-CoV caused human-to-human transmission and resulted in the 2003 outbreak with about 10% case fatality rate (CFR), while MERS-CoV was reported in Saudi Arabia in June 2012 [2]. Even though with its limited human-to-human transmission, MERS-CoV showed a CFR of about 34.0% [3]. The SARS-CoV-2 was first reported in Wuhan, China in December 2019 from patients with pneumonia, and it has exceeded both SARS-CoV and MERS-CoV in its rate of transmission among humans [4,5]. The outbreak of a novel coronavirus (SARS-CoV-2) represents a pandemic threat that has been declared a public health emergency of international concern (PHEIC). Currently, the intermediate host of SARS-CoV-2 is still unknown, and no effective prophylactics or therapeutics are available, though various drugs have shown mild to moderate protection but none of them have shown conclusive evidence.

Several pre-clinical or clinical trials are going on, which include repurposing of already approved drugs but with different indications such as anti-malarial, anti-viral, anti-parasitic drugs, or monoclonal antibodies, etc [6,7]. However, these drugs may help to prevent worsening of the coronavirus infection only and there is still an unmet need of a vaccine against novel coronavirus SARS-CoV-2.

Huge progresses were made in last one year for bringing effective vaccines against SARS-CoV-2. As per WHO draft landscape of vaccines, currently 172 vaccine candidates are in pre-clinical development, 63 are in clinical development. Among the candidates in clinical development, 6 are based on plasmid DNA technology including our vaccine candidate. Other 5 candidates are from Inovio Pharmaceuticals/Beijing Advaccine Biotechnology/
Three candidates including two mRNA based candidate from Pfizer and Moderna and Chimpanzee adenovirus vector based candidate from AstraZeneca achieved emergency use approval globally. The emergency use was approved based on Phase-3 efficacy data. The mRNA vaccines from Pfizer reported 95% efficacy [9], whereas Moderna and AstraZeneca reported 94.5% and 70.4% efficacy respectively for their vaccine candidate [10].

The conventional active vaccines are made of a killed or attenuated form of the infectious agent. Vaccination with live attenuated and killed vaccines in most cases results in generation of humoral but not a cell-mediated immune response. What is required in such cases, but not available, are antigens that are safe to use, that can be processed by the endogenous pathway and eventually activating both B and T cell response. The activated lymphocytes generated would destroy the pathogen-infected cell. For these reasons, a new approach of vaccination that involves the injection of a piece of DNA that contains the genes for the antigens of interest are under investigation. DNA vaccines are attractive because they ensure appropriate folding of the polypeptide, produce the antigen over long periods, and do not require adjuvants. These host-synthesized antigens then can become the subject of immune surveillance in the context of both major histocompatibility complex class I (MHC I) and MHC II proteins of the vaccinated individual [11]. By contrast, standard vaccine antigens are taken up into cells by phagocytosis or endocytosis and are processed through the MHC class II system, which primarily stimulates antibody response. In addition to these properties, the plasmid vector contains immunostimulatory nucleotide sequences- unmethylated cytidine phosphate guanosine (CpG) motifs- that induce strong cellular immunity [12]. Finally, DNA vaccines have been shown to persist and stimulate sustained immune responses. Other advantages are that the technology for producing the vaccine is very simple and rapid, secondly the DNA molecule is stable, has a long shelf life, and does not require a strict cold chain for distribution. DNA vaccines are also safer than certain live-virus vaccines, especially in immunocompromised patients. It also circumvents the numerous problems associated with other vaccines, such as immune responses against the delivery vector and concern about safety related to the use of any viral vector [13].

Prior studies have demonstrated that a DNA vaccine approach for SARS and MERS can induce immune response including neutralizing antibody (nAb) responses in clinical trials and provide protection in challenge models. Previous studies indicated immunization in animal models with DNA vaccines encoding MERS-CoV spike (S) protein provided protection against disease challenge with the wild type virus. In subjects immunized with MERS-CoV DNA vaccine durable neutralizing antibodies (nAbs) and T cell immune responses were measured, and a seroconversion rate of 96% was observed and immunity was followed for 60 weeks in most study volunteers [14]. Similarly, NIH completed Phase 1 clinical trial for SARS-DNA vaccine. Dose of 4.0 mg was tested in healthy adults who were vaccinated on days 0, 28, and 56. The vaccine was found to be well-tolerated and induced antibody responses against the SARS-CoV in 80% of subjects after 3 doses [15]. More recently, Inovio pharmaceuticals developed DNA vaccine INO-4800 against SARS-CoV-2, which was found to be safe and immunogenic in Phase-1 trial, eliciting either or both humoral or cellular immune responses [16].

The spike proteins of SARS-CoV-2 and SARS-CoV were reported to have identical 3-D structures in the receptor-binding domain. SARS-CoV spike protein has a strong binding affinity to human Angiotensin converting enzyme 2 (ACE-2) receptor, based on biochemical interaction studies and crystal structure analysis. SARS-CoV-2 and SARS-CoV spike proteins have high degree of homology and they share more than 70% identity in amino acid sequences [17]. Further, Wan et al., reported that glutamine residue at position 394 (E394) in the SARS-CoV-2 receptor-binding domain (RBD), corresponding to E479 in SARS-CoV, which is recognized by the critical lysine residue at pos-31 (K31) on the human ACE-2 receptor. Further analysis suggests that SARS-CoV-2 recognizes human ACE-2 receptor more efficiently than SARS-CoV increasing the ability of SARS-CoV-2 to transmit from person to person [18]. Thus, the SARS-CoV-2 spike protein was predicted to also have a strong binding affinity to human ACE-2 receptor.

ACE-2 is demonstrated as a functional SARS-CoV-2 spike (S) protein receptor in-vitro and in-vivo. It is required for host cell entry and subsequent viral replication. Zhou et al., demonstrated that overexpressing ACE-2 receptor from different species in HeLa cells with human ACE-2, pig ACE-2, and civet ACE-2 receptor allowed SARS-CoV-2 infection and replication, thereby establishing that SARS-CoV-2 uses ACE-2 as a cellular entry receptor. In transgenic mice model with overexpression of human ACE-2 receptor, SARS-CoV infection enhanced disease severity and lung injury, demonstrating that viral entry into cells through ACE-2 receptor is a critical step [19,20]. Thus for SARS-CoV-2 pathogenesis, spike (S) protein play a critical role by mediating entry of virus into the cell through human ACE-2 receptor and is an important target for vaccine development.

Here we report, design, production and pre-clinical testing of our DNA vaccine candidate. The proposed Coronavirus vaccine candidate comprises of a DNA plasmid Vector carrying spike (S) gene region of SARS-CoV-2 spike (S) protein along with gene coding for IgE signal peptide. The spike gene region was selected from submitted Wuhan Hu-1 isolate sequence (Genebank Accession No. MN908947.3). It’s expected that the plasmid construct with desired gene of interest will enter host cells, where it remains in the nucleus as an episome; without getting integrated into the host cell DNA. Thus using the host cell’s protein translation machinery, the inserted cloned DNA in the episome will direct the synthesis of the antigen it encodes. The approach involving the synthesis of antigen within the cells has several potential advantages. The protein produced by plasmid-transfected cells is likely to be expressed within the cell and folded in its native conformation. Further the signal peptide will prompt cells to translocate the protein, usually to the cellular membrane. The antigen is recognized by antigen presenting cells (APCs) and further induces antibodies including neutralizing antibodies through major histocompatibility complex (MHC) class pathway [13]. The DNA vaccine candidate induces antibody response against SARS-CoV-2 spike (S) protein, following immunization with just a single dose. Neutralizing antibody response was also demonstrated against wild type SARS-CoV-2 strain, which may play a substantial role in viral clearance and mitigation of human clinical disease. Immunogenicity of this DNA vaccine candidate targeting the SARS-CoV-2 S protein in animal model supports further clinical development of this candidate in response to the current COVID-19 pandemic situation.

2. Material and methods

2.1. Selection of spike (S) gene region based on in-silico analysis

For our DNA vaccine candidate, the target antigen amino acid sequence of SARS-CoV-2 spike(S) from Wuhan Hu-1 isolate (Gene-
bank Accession No. MN908947.3) was analysed in-silico by National Centre for Biotechnology Information (NCBI) blast tool and Clustal W multiple sequence alignment software to predict homology to other circulating SARS-CoV-2 spike(S) protein. In order to develop a vaccine candidate which provides broad protection against all circulating strains of SARS-CoV-2, the whole spike region of Wuhan Hu-1 strain having some conserved region and induce robust immune response is selected for clone development.

2.2. Generation of ZyCoV-D vaccine construct

Gene sequence was submitted to GeneArt, Thermo Fisher Scientific and codon optimized full length Spike (S) region of SARS-CoV-2 virus with IgE signal sequence was synthesized. The chemically synthesized Spike (S) gene region preceded by IgE signal sequence was inserted into pVAX-1 plasmid DNA vaccine vector (Thermo Fisher Scientific). Subsequently, the plasmid DNA construct was transformed in DH5-α chemistry competent cells (Thermo Fisher Scientific). After heat shock transformation step, E. coli clones carrying the plasmid DNA constructs were isolated by plating on LB agar plate containing Kanamycin antibiotic.

Single colonies were picked and inoculated in flasks containing LB broth from Hi-Media with Kanamycin. Flasks were incubated in 37 °C incubator shaker at 225 rpm for 20 Hrs. Culture from each clone was used for plasmid isolation using miniprep plasmid isolation kit. Restriction digestion was carried out with BamH1, Nhe1 and Aap1 for all constructs to check expected band releases of inserts to select the positive clones. Positive clones were selected for preparation of glycerol stocks and stored at −70 °C.

2.3. In-vitro expression analysis of the constructs

In-vitro expression of DNA vaccine candidate was checked by transfection of the same in vero cell line. For transfection experiments, vero cells were seeded at density of 3 × 10^5 cells/ml in 6 well plates and kept in CO2 incubator to attain 80–90% confluency. After 24Hrs, once the cells reached the desired confluency, transfection was carried out in OptiMEM serum free medium with Lipofectamine 2000 reagent (Thermo Fisher). Two different concentrations (4 µg and 8 µg) of DNA construct was used for transfection experiments. We keep the amount of transfection reagent constant while plasmid DNA concentration varied to achieve 1:1 and 1:2 ratios of volume to mass. After transfection, media was replenished with fresh DMEM medium (Biowest) containing FBS. After 72Hrs, plates were fixed with 1:1 acetone and methanol. Anti-S1 rabbit polyclonal antibody (NB100-56048) from Novus Biologicals was added to each well and incubated for 1Hr followed by incubation with FITC labelled anti- rabbit antibody (Merck). Cells were washed three times with PBS and stained with DAPI counter stain. Fluorescence images were captured using an inverted microscope (Zeiss AX10) at 20X magnification.

2.4. Animal immunization

The immunogenicity study for the ZyCoV-D vaccine was carried out in inbred BALB/c mouse, guinea pig, and New Zealand white rabbit model after having ethical approval from Institutional Animal Ethics Committee, CPCSEA Reg. No.: 335/PO/RcBi/S/01/ CPCSEA, with IAEC approved application numbers: VAC/010/2020 and VAC/013/2020. BALB/c mouse (five to seven-week-old), guinea pigs (five to seven-week-old) and New Zealand White rabbits (six to twelve-week-old) were used in this study. For mouse intradermal immunization, on day 0; 25 and 100 µg of DNA vaccine was administered to the skin by using 31 gauge needle. Animals injected with empty plasmid served as vehicle control. Two weeks after immunization, animals were given first booster dose. Similarly all mice were given second booster dose two weeks after first booster dose. For guinea pig study, intradermal immunization was carried out using same dosing and schedule. In rabbits, DNA vaccine was administered to the skin by using Pharmajeot R Tropis R needle free injection system (NFIS) at 500 µg dose at same 3 dose regimen and schedule. The Pharmajeot R Tropis R delivers vaccines intradermally by means of a narrow, precise fluid stream that delivers the vaccine into the skin.

Blood was collected from animals on day 0 (before immunization) & 28 (after 2 dose) and on day 42 (after 3 dose) for immunological assessments from sera samples. In mouse model long term immunogenicity of the vaccine was assessed for up to day 126. Further, IFN-γ response from splenocytes at day 0, 28, and 42 were assessed.

2.5. Measurement of antibody titres by ELISA

ELISA was performed to determine antibody titres in different animal sera samples. In brief, Maxisorp ELISA plates (Nunc) were coated with 50 ng/well of recombinant S1 spike protein of SARS-CoV-2 (Acro, USA Cat no. S1N-CS2H3) in phosphate-buffered saline (PBS) overnight at 4 °C. Plates were washed three times with PBS then blocked with 5% skimmed milk (BD Difco) in PBS for 1 Hr at 37 °C. After blocking plates were then washed thrice with PBS and incubated with serial dilutions of mouse, guinea pig and rabbit sera and incubated for 2 Hrs at 37 °C. After that, plates were again washed thrice followed by incubation with 1:5,000 dilution of horse radish peroxidase (HRP) conjugated anti-guinea pig IgG secondary antibody (Sigma-Aldrich) or 1:2,000 dilution of HRP conjugated anti-mouse IgG secondary antibody (Sigma-Aldrich) or 1:5,000 dilution of HRP conjugated anti rabbit IgG secondary antibody (Sigma-Aldrich) for 1 Hr at 37 °C. Plates were washed again thrice with PBS and then developed using TMB Peroxidase Substrate (KPL). Reaction was stopped by Stop Solution (1 N H2SO4). Plates were read at 450 nm wavelength within 30 min using a multimode reader (Molecular Devices, USA).

2.6. Virus neutralization assays using wildtype SARS-CoV-2

Micro-neutralization test (MNT) was performed at Translational Health Science and Technology Institute (THSTI), NCR Biotech Science Cluster, Faridabad – 121001 Haryana, India. The virus was obtained from the BEI resources, USA (Isolate USA-WA1/2020), passaged and titrated in Vero-E6 cells. The sera samples collected from immunized animals were heat-inactivated at 56 °C for 30 min followed by ten fold serial dilution with cell culture medium. The diluted sera samples were mixed with a virus suspension of 100 TCID50 in 96-well plates at a ratio of 1:1 followed by 1 Hr incubation. This is followed by 1 Hr adsorption on Vero-E6 cells seeded 24 Hrs prior to experiment in 96 well tissue culture plate (1 × 10^5 cells/well in 150 µl of DMEM + 10% FBS). The cells were subsequently washed with 150 µl of serum free media and 150 µl of DMEM media supplemented with 2% FBS, followed by incubation for 3–5 days at 37 °C in a 5% CO2 incubator. Cytopathic effect (CPE) was recorded under microscopes in each well. Neutralization was defined as absence of CPE compared to virus controls. Reporting for the virus neutralization titres was done as dilution factor of serum sample at which no cytopathic effect (CPE) was observed.

2.7. Detection of neutralizing antibodies by competitive inhibition ELISA

Competitive inhibition ELISA was performed using SARS-CoV-2 neutralization antibody detection kit (GenScript, USA). The kit detects circulating neutralizing antibodies against SARS-CoV-2 that
block the interaction between the receptor binding domains of the viral spike glycoprotein (RBD) with the ACE-2 cell surface receptor.

Different animal sera samples serially diluted with dilution buffer provided in the kit. The diluted sera samples were incubated with HRP conjugated RBD at 1:1 ratio for 30 min at 37 °C along with positive and negative controls. The sera and HRP conjugated RBD mix was then added to the ELISA plate pre-coated with the ACE-2 protein. After that, plates were incubated for 15 min at 37 °C followed by washing four times with wash solution provided in the kit. After washing steps, TMB solution was added to the well and incubated in dark for 15 min at room temperature, followed by addition of stop solution. Plates were read at 450 nm. Inhibition concentration (IC50) of sera sample was calculated by plotting the percentage competition value obtained for each dilution versus serum dilution in a non-linear regression curve fit using Graph pad Prism 8.0.1 software.

2.8. IFN-γ ELISPOT assay

For IFN-γ ELISPOT assay, spleens from immunized mice were collected in sterile tubes containing RPMI-1640 (Thermo Fisher Scientific) media supplemented with 2X Antibiotic-Antimycotic (Thermo Fisher Scientific). Cell suspensions were prepared by crushing the spleen with disk bottom of the plunger of 10 ml syringe (BD) in sterile petri plates. Then 5–10 ml of RPMI-1640 medium supplemented with 1X Antibiotic-Antimycotic was added to it and the contents were mixed for homogeneity. Dishes were kept undisturbed for 2 min and the clear supernatant was pipetted out slowly into cell strainer (BD). The filtrate was collected in sterile tubes and the cells were pelleted by centrifugation at 4 °C for 10 min at 250 × g in a centrifuge (Thermo Fisher Scientific). The pellet containing red blood cells (RBCs) and splenocytes were collected. 2–3 ml RBC Lysing Buffer (Invitrogen) was added to the pellet containing splenocytes and incubated at room temperature for 5–7 min. After incubation RPMI1640 supplemented with 10% FBS (Biowest) and 1X Antibiotic-Antimycotic solution was added thrice with HRP conjugated RBD at 1:1 ratio for 30 min at 37 °C along with positive and negative controls. The sera sample was calculated by plotting the percentage competition value obtained for each dilution versus serum dilution in a non-linear regression curve fit using Graph pad Prism 8.0.1 software.

2.9. Biodistribution study

Biodistribution of DNA vaccine candidate was studied in Wistar rat model. Two groups of Wistar rats received either a single bilateral, intradermal administration of 1.0 mg of plasmid or a single bilateral, intradermal administration of 0.5 mg of plasmid per animal in each group respectively. At different time points (2 Hrs, 24 Hrs, 168 Hrs, and 336 Hrs) animals from each group were sacrificed and brain, lungs, intestine, kidney, heart, spleen, skin, and blood samples were harvested from the animals. All animal procedures were approved by the institutional animal ethics committee.

Animals were carefully dissected using a separate set of tools for each individual organ in order to avoid contamination and organs were snap-frozen after the collection until further use. Still frozen, the organs were sliced with scalpel and extraction of total DNA was performed on blood and frozen tissue samples using the DNeasy 96 Blood and Tissue kit (Qiagen, Germany) and according to the manufacturer's instructions. Quantitative PCR was carried out with the PowerUp™ SYBR™ Green Master Mix (Applied Biosystems by Thermo Fisher Scientific, Lithuania) using plasmid specific primers on StepOne™ Real-Time PCR System (Applied Biosystems by Thermo Fisher Scientific, Lithuania). Plasmid DNA at different concentrations (1 × 10² to 1 × 10⁷ copies/mL) were used to construct the standard curves. The concentrations of samples were calculated by using standard curve with Applied Biosystems StepOnePlus™ software Ver. 2.2.2 (Applied Biosystems by Thermo Fisher Scientific, Lithuania).

2.10. Statistical analysis

Statistical analysis of the results and graph creation were done with the Graph Pad Prism (version 8.0.1) and Microsoft Excel (version 7.0) for general statistical calculations, such as arithmetic mean and standard deviation. p values of < 0.05 were considered significant.

3. Results

3.1. In-silico analysis of SARS-CoV-2 spike (S) protein

In-silico analysis confirmed more than 99% homology of the spike protein amino acid sequence from Wuhan strain with other circulating strains around the world including India. Although cross-neutralization studies against different circulating strains would be needed to evaluate broader protective ability of the vaccine candidate.

3.2. Generation of DNA vaccine constructs

Synthesis of SARS-CoV-2 spike (S) protein gene containing IgE signal peptide gene region and further cloning into pVAX-1® vector results in generation of SARS-CoV-2 DNA vaccine construct. Restriction digestion with BamHI resulting in linearized DNA fragments of ~6.78 kb and restriction digestion analysis with Nhel and Apal resulting in generation of fragments of ~2.89 kb of vector and ~3.89 kb of spike protein (S) gene was used to confirm the insertion of spike(S) into the vector as shown in the Figs. 1A and 1B. Gene sequencing analysis further confirmed the insertion of appropriate sequence in the desired orientation.

3.3. In-vitro expression of DNA vaccine candidate

In Immunofluorescent studies were carried out to confirm the expression of S protein on candidate DNA vaccine transfected Vero cells (Fig. 2A and 2B). Immunostaining with FITC- labeled secondary antibody revealed the expression of the Spike protein after transfection of Vero cells with the candidate vaccine constructs. Further cell nuclei were observed by DAPI counter staining.

3.4. Humoral immune response to DNA vaccine candidate

Immunization with DNA vaccine candidate by intradermal route elicited significant serum IgG responses against the S protein.
in doses-dependent manner in BALB/c mice, guinea pigs and rabbits with mean end point titres reaching ~28000 in BALB/C mice, ~140000 in guinea pigs and ~17000 in rabbits respectively on day 42 after 3 doses (Figs. 3, 4 and Fig. 5). Long term antibody response was studied in mice almost 3.5 months after the last dose and a mean end point IgG titres of ~18000 was detected (Fig. 3) suggesting sustainable immune response was generated by DNA vaccine candidate.

Neutralizing antibody titres were evaluated in BALB/C mice, guinea pigs and rabbits following immunization by using micro-neutralization assay and GenScript neutralizing antibody detection kit. Neutralizing antibodies were elicited by DNA vaccine candidate in mice, guinea pigs and rabbits. Sera from DNA vaccine candidate immunized BALB/c mice could neutralize wild SARS-CoV-2 virus strains with average MNT titres of 40 and 160 at day 42 with 25 and 100 µg dose regimens respectively (Table 1). Using GenScript neutralizing antibody detection kit average IC50 titres of 82 and 168 were obtained at day 42 with 25 and 100 µg dose regimens respectively. Further, neutralizing antibodies were also detected in long term immunogenicity studies in BALB/c mice. Significant rise in neutralizing antibodies levels were also observed in guinea pigs and rabbits (Table 1).

3.5. Cellular immune response to DNA vaccine candidate

T cell response against SARS-CoV-2 spike antigen was studied by IFN-γ ELISpot assay. Groups of BALB/c mice were sacrificed at day 14, 28, 42 post-DNA vaccine administration (25 and 100 µg dose). Splenocytes were harvested, and a single-cell suspension was stimulated for 24 h with the peptide pool covering the entire Spike glycoprotein region (10-15mers with 11 aa overlap). Significant increase in IFN-γ expression, indicative of a strong Th1 response, of 200–300 SFC per 10⁶ splenocytes against SARS-CoV-2 spike peptide pool was observed for both the 25 and 100 µg dose in post 42 day immunized mice splenocytes (Fig. 6).

3.6. Bio-distribution

Biodistribution of DNA vaccine candidate was evaluated in Wistar Rats at single dose of 0.5 mg and 1.0 mg administered intradermally. DNA was extracted from various tissue samples including the site of injection, brain, blood, lungs, intestine, kidney, heart and spleen at different time points post injection as described above. RT-PCR was performed by plasmid specific primers to detect copy numbers. Following injection of maximum ~10¹⁴ plasmid DNA copies in Wistar Rats, maximum local concentration of 10³–10⁷ plasmid copies at the site of injection were detected two hours post injection. (Figs. 7A and 7B). We also observed biodistribution of plasmid molecule in blood, lungs, intestine, kidney, heart, spleen, skin post 24 Hrs of injection which got cleared by 336 Hrs (day 14) in most of the organs except skin (site of injection) where only 10²–10³ copies were detected. Although by 672 Hrs (day 28) post injection, no plasmid copies were detected at site of injection as well.

Fig 1A. Schematic diagram of ZyCoV-D synthetic DNA vaccine constructs. pVAX-1 vector containing SARS-CoV-2 spike gene insert.

Fig 1B. Restriction analysis of DNA vaccine constructs. Restriction digestion with BamHI resulting in generation of single fragments of ~6.78 kb and restriction digestion of vector with Nhel and Apal resulting in generation of fragments of ~2.89 kb of vector and ~3.89 kb of spike protein (S) gene was used to confirm the insertion of spike(S) sequence into the vector.
Fig. 2. Analysis of in-vitro expression of Spike protein after transfection of Vero cells with DNA construct or empty plasmid (Control) by immunofluorescence. Expression of Spike protein was measured with polyclonal rabbit anti-SARS Spike Protein IgG and FITC anti-IgG secondary (green) and cell nuclei was observed by DAPI counter staining (blue). Images were captured using inverted fluorescence microscope at 20X Magnification.

Fig. 3. Antibody response after DNA vaccination in BALB/c mice and long term immunogenicity. BALB/c mice were immunized at week 0, 2 and 4 with DNA vaccine construct or empty control vector as described in the methods. Sera were collected at day 28 (black), day 42 (grey) and day 126 (dark grey) evaluated for SARS-CoV-2 S1-specific IgG antibodies. Antibody response were presented as end point titre and calculated considering day 0 optical density (OD) value of ELISA as end point. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. Antibody response after DNA vaccination in Guinea Pigs. Guinea pigs were immunized at week 0, 2 and 4 with DNA vaccine construct or empty control vector as described in the methods. Sera were collected at day 28 (checks) and day 42 (stripes) and evaluated for SARS-CoV-2 S1-specific IgG antibodies. Antibody response were presented as end point titre and calculated considering day 0 optical density (OD) value of ELISA as end point. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Development of safe and effective vaccine against SARS-CoV-2 is needed to curb the global pandemic. DNA vaccine platform has several advantages, which positions it well to respond to disease outbreaks, such as COVID-19. The ability to design and immediately synthesize candidate vaccine constructs allow us to carry out in-vitro and in-vivo testing within days of receiving the viral sequence. The expression and localization of S protein expressed by ZyCoV-D were investigated using an immunofluorescence assay. The immunofluorescence assay with rabbit anti–S1 antibody by ZyCoV-D were investigated using an immunofluorescence assay. The expression and localization of S protein expressed in different animal models and has demonstrated ability to elicit immunogenic response against SARS-CoV-2. S-antigen in animal species. Primary antibody response starts mounting in serum two weeks after two doses and reaches peak two weeks after third immunization. The serum IgG levels against spike antigen in mice were maintained even after three months post last dosing suggesting a long-term immune response generated by the DNA vaccine candidate. This also indicates that ZyCoV-D can possibly induce robust secondary anamnestic immune response upon re-exposure, generated by balanced memory B and helper T cells expression and has been reported for other DNA vaccine candidates [22].

We reported serum neutralizing (Nab) titres following DNA vaccination, which was tested by micro-neutralization assay and GenScript neutralizing antibody detection kit. The Nab titre values tested by both methods demonstrated that the DNA vaccine candidate generates robust response and neutralizes the SARS-CoV-2 virus conferring protective immunity against infection. In future if these Nab titres will be established as correlate of protection virus conferring protective immunity against infection. In future if these Nab titres will be established as correlate of protection across multiple vaccine studies in both animals and humans, then this parameter can be utilized as a benchmark for clinical development of SARS-CoV-2 vaccines.

We also observed that ZyCoV-D vaccine is capable of inducing T-cell response complementary to antibody response in mice model as demonstrated by IFN-γ ELISPOT. This is very important as successful DNA vaccination is known to induce both humoral and cellular responses in both animals and human [14,15,16,23]. The mechanism of action for DNA vaccine candidate includes both class-I antigen-processing pathways (i.e., intracellular processing of viral proteins and subsequent loading onto MHC class-I molecules) and class-II antigen-processing pathways (i.e., endosomal loading of peptides generated from endocytosed viral antigens).

### Table 1

Sera neutralizing antibody titres after DNA vaccine administration to BALB/c mice, Guinea pigs and New Zealand White Rabbits.

| Species               | Immunization regimen | Neutralization Assay                  | Neutralization Titre Day 28 | Neutralization Titre Day 42 | Neutralization Titre Day 126 |
|-----------------------|----------------------|--------------------------------------|----------------------------|-----------------------------|-----------------------------|
| BALB/c Mice           | 25 µg Days 0, 14, 28 | Micro-neutralization (SARS-CoV-2 USA-WA1/2020)-MNT<sub>100</sub> | 20                         | 40                         | –                           |
| Days 0, 14, 28        | 100 µg               | Micro-neutralization (SARS-CoV-2 USA-WA1/2020)-MNT<sub>100</sub> | 40                         | 160                        | –                           |
| Days 0, 14, 28        | 25 µg Days 0, 14, 28 | GenScript<sup>®</sup> Neutralization Assay-IC<sub>50</sub> | 14                         | 82                         | 23                          |
| Days 0, 14, 28        | 100 µg               | GenScript<sup>®</sup> Neutralization Assay-IC<sub>50</sub> | 71                         | 168                        | 91                          |
| Guinea Pigs           | 25 µg Days 0, 14, 28 | Micro-neutralization (SARS-CoV-2 USA-WA1/2020)-MNT<sub>100</sub> | 20                         | 80                         | –                           |
| Days 0, 14, 28        | 100 µg               | Micro-neutralization (SARS-CoV-2 USA-WA1/2020)-MNT<sub>100</sub> | 40                         | 320                        | –                           |
| Days 0, 14, 28        | 25 µg Days 0, 14, 28 | GenScript<sup>®</sup> Neutralization Assay-IC<sub>50</sub> | 14                         | 129                        | –                           |
| Days 0, 14, 28        | 100 µg               | GenScript<sup>®</sup> Neutralization Assay-IC<sub>50</sub> | 21                         | 371                        | –                           |
| New Zealand White     | 500 µg injected by   | GenScript<sup>®</sup> Neutralization Assay-IC<sub>50</sub> | 30                         | 108                        | –                           |
| Rabbits               | NFS Days 0, 14, 28   |                                      |                            |                            |                             |
secreted from cells using IgE signal peptide onto MHC class II molecules). Among T cell responses, Th1 response is important because vaccine-associated enhanced respiratory disease (VAERD) is associated with Th2-biased immune response. Indeed, immunopathologic complications characterized by Th2-biased immune responses have been reported in animal model of the SARS-CoV or MERS-CoV challenge [24,25,26] and similar phenomena have been reported in clinic trials vaccinated with whole-inactivated virus vaccines against RSV and measles virus [27,28]. In addition, the importance of Th1 cell responses has been highlighted by recent study of asymptomatic and mild SARS-CoV-2 convalescent samples [29]. These results collectively suggest that vaccines capable of generating balanced antibody responses and Th1 cell responses may be important in providing protection against SARS-CoV-2 diseases.

The usefulness and efficiency of a spring-powered, needle-free Injection System (NFIS) for delivering ZyCoV-D vaccine in rabbits was also demonstrated in the study. Similar observation was reported earlier with application of NFIS for DNA vaccines against Hantavirus and Zika virus [30,31]. The use of NFIS eliminates use of needles during vaccine administration thus eliminates the costs and risk associated with sharp-needle waste. Further, NFIS doesn’t required external energy sources such as gas cartridges or electricity and spring provides the power for the device. These injector create a stream of pressurized fluid that penetrates upto 2 mm in skin at high velocity resulting in uniform dispersion and higher uptake of DNA molecules in cells compare to needle and syringe where the intradermal accumulation is inconsistent across individuals (as measured by bleb size) and varies among animal species [30].

We have also demonstrated the protective efficacy of our vaccine candidate delivered by NFIS in Rhesus macaques and manuscript was available online at BioRixiv [32]. The ZyCoV-D vaccine candidate injected at dose of 2 mg by NFIS elicited significant SARS CoV-2 specific IgG, Nab titers and lower viral loads in animals post challenge.

Bio-distribution pattern for ZyCoV-D was also evaluated and level of plasmid DNA was measured at different intervals in various tissues in Wistar rats post intradermal injection.

Post intradermal injection, the plasmid was found to clear off from most of the organs by 14 days post injection except site of injection, which also cleared off by 28 days post injection. Our outcome was very similar to other DNA vaccine candidate including HIV-1, Ebola, Severe Acute Respiratory Syndrome (SARS), and a West Nile Virus candidate developed [33]. Biodistribution and plasmid copy number detection studies for the vaccine candidates were done in different animal models [33]. It was observed that animals injected with 2 mg (equivalent to $10^{14}$ plasmid copies) by both intramuscular and subcutaneous route have detectable plasmid copies in first one week of vaccination at the site of injection with copies in order of $10^4$-$10^6$. Over the period of 2 months, the plasmid clears from the site of injection with only a small percentage of animals in group (generally 10–20%) retaining few copies (around 100 copies) at the injection site. Directly after injection into skin or muscle, low levels of plasmids are transported via the blood stream and detected in various organs at early time points. However, the plasmids are eventually, cleared from the organs and are normally found exclusively at the site of injection at later time points.

In summary, these initial results demonstrate the immunogenicity of our ZyCoV-D DNA vaccine candidate in multiple animal models. These studies strongly support the clinical evaluation as a vaccine candidate for COVID-19 infection.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
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Authors Contribution
KM conceptualized, designed, developed the vaccine candidate, provided guidance on data analysis and manuscript preparation. AD designed, developed vaccine candidate, performed data analysis for ELISPOT assay and prepare manuscript. CRTM and HC developed analytical procedures for testing of the vaccine and performed data analysis for ELISA, neutralization and biodistribution assay. HPRP, HSC, GS, PD, SV, SP, SR developed process for vaccine production, MB performed animal experiments, AS performed ELISPOT assay, NL and MAR performed ELISA assay, RDD performed biodistribution assay, SK, AS, VS and AP helped in construct design.

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Research Paper

Safety and Immunogenicity of a DNA SARS-CoV-2 vaccine (ZyCoV-D): Results of an open-label, non-randomized phase I part of phase I/II clinical study by intradermal route in healthy subjects in India

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ABSTRACT

Background: ZyCoV-D is a DNA vaccine candidate, which comprises a plasmid DNA carrying spike-S gene of SARS-CoV-2 virus along with gene coding for signal peptide. The spike(S) region includes the receptor-binding domain (RBD), which binds to the human angiotensin converting Enzyme (ACE)-2 receptor and mediates the entry of virus inside the cell.

Methods: We conducted a single-center, open-label, non-randomized, Phase 1 trial in India between July 2020 and October 2020. Healthy adults aged between 18 and 55 years were sequentially enrolled and allocated to one of four treatment arms in a dose escalation manner. Three doses of vaccine were administered 28 days apart and each subject was followed up for 28 days post third dose to evaluate safety and immunogenicity.

Findings: Out of 126 individuals screened for eligibility. Forty-eight subjects (mean age 34.9 years) were enrolled and vaccinated in the Phase 1 study Overall, 12/48 (25%) subjects reported at least one AE (i.e. combined solicited and unsolicited) during the study. There were no deaths or serious adverse events reported in Phase 1 of the study. The proportion of subjects who seroconverted based on IgG titers on day 84 was 4/11 (36.36%), 4/12 (33.33%), 10/10 (100.00%) and 8/10 (80.00%) in the treatment Arm 1 (1 mg: Needle), Arm 2 (1 mg: NFIS), Arm 3 (2 mg: Needle) and Arm 4 (2 mg: NFIS), respectively.

Interpretation: ZyCoV-D vaccine is found to be safe, well-tolerated and immunogenic in the Phase 1 trial. Our findings suggest that the DNA vaccine warrants further investigation.

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1. Introduction

Severe Acute Respiratory Syndrome coronavirus 2019 (COVID-19), emerged in December 2019 in Wuhan, China [1]. A novel coronavirus was identified as the etiologic agent in January 2020. The genetic sequence of the virus became available (MN908947.3) in January 2020. Within months of emergence, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections and the resulting disease, COVID-19, spread worldwide. On 11 March 2020, the World Health Organization (WHO) declared the COVID-19 outbreak a pandemic [2].

COVID-19 disease is rapidly transmitted from human to human, with influenza-like symptoms ranging from mild disease to severe disease and multi-organ failure, eventually resulting in death, especially in aged patients with co-morbid conditions [3,4]. Vaccines are considered to be the most effective treatment to control the pandemic and help to restore the global economy [5,6]. There are currently more than 63 COVID-19 candidate vaccines undergoing clinical trials and more than 172 COVID-19 candidate vaccines undergoing pre-clinical development worldwide, including mRNA vaccines, replicating or non-replicating viral vectored vaccines, DNA vaccines,
Research in context

Evidence before this study

We searched PubMed on March 23, 2021, using the search terms “COVID-19”, “SARS-CoV-2”, “vaccine”, and “clinical trial”. Cadila Healthcare Limited, India developed a candidate vaccine ZyCoV-D comprising of a DNA plasmid vector carrying the gene encoding the spike protein (S) of the SARS-CoV-2 virus. Preliminary animal study demonstrates that the candidate DNA vaccine induces antibody response including neutralizing antibodies against SARS-CoV-2 and provided Th-1 response as evidenced by elevated IFN-γ levels.

Added value of this study

This first-in-human trial showed that the DNA vaccine was tolerable and immunogenic in healthy adults. The DNA vaccine candidate induces antibody response against SARS-CoV-2 spike (S) protein, following immunization with three doses administered 28 days apart. Neutralizing antibody response was also demonstrated against wild type SARS-CoV-2 strain, which may play a substantial role in viral clearance and mitigation of human clinical disease. This study has also evaluated safety and immunogenicity of DNA vaccine administered by two different methods of administration.

Implications of all the available evidence

Implications of all the available evidence Many vaccine candidates are in rapid development, including recombinant-protein based vaccines, replicating or non-replicating viral vector-based vaccines, DNA vaccines, and mRNA vaccines (which mostly have focused on the spike glycoprotein or receptor binding domain), live attenuated vaccines, and inactivated virus vaccines. Our findings indicate that DNA vaccine is safe and immunogenic in healthy adults.

autologous dendritic cell-based vaccine, and inactive virus vaccines [7]. The results of the Phase 1 and 2 trials of several vaccines, such as a chimpanzee adenovirus-vectored vaccine, recombinant adenovirus type-5 (Ad5)—vectored vaccine, inactivated vaccines and mRNA vaccines, have been published. The results of few phase 3 trials have also been published now [8,9].

In December 2020, Pfizer Inc. and BioNTech SE received a temporary authorization for emergency use of COVID-19 mRNA vaccine against COVID-19 from the USFDA. The Regulatory Agencies approved this vaccine in the UK, Canada, Saudi Arabia and Bahrain as well [10]. Moderna, Pfizer, Johnson & Johnson (USA and EU-approved vaccines) as well as AstraZeneca and others (EU-approved vaccines) also received a temporary authorization for emergency use. On 16th January, 2021, the first COVID-19 vaccine received a temporary authorization for emergency use by the Drug Controller General of India (DCGI) in India and many more remain in development.

As of May 16, 2021, SARS-CoV-2 had infected more than 162 million people and killed more than 3.3 million since the start of the pandemic worldwide [11]. The number of reported SARS-CoV-2 cases in India till May 19, 2021 is also on an increase with ~25 million confirmed cases and ~283,248 deaths [12]. The worldwide impact of this pandemic on human society calls for the rapid development of safe and effective therapeutics and vaccines.

Here, we report the safety and immunogenicity of DNA based SARS-CoV-2 vaccine data from the Phase 1 clinical trial of an ongoing, Phase 1/2 clinical study, which commenced in July 2020 to evaluate the impact of ZyCoV-D vaccine in preventing Covid-19 in healthy adult subjects, 18–55 years of age. The data includes evaluation of the 1 mg and 2 mg dose levels of ZyCoV-D vaccinated healthy adult subjects. Collection of phase 2 data on vaccine immunogenicity and the durability of the immune response following vaccination is ongoing, and those data are not reported here.

We have developed a DNA vaccine candidate for prevention of COVID-19. It is comprised of a plasmid DNA carrying spike-S gene of SARS-CoV-2 virus along with gene coding for signal peptide. The spike(S) region includes the receptor binding domain (RBD), which binds to the human angiotensin converting Enzyme (ACE)-2 receptor and mediates the entry of virus inside the cell. The DNA construct was produced on large scale by transformation in E. coli [13]. The immunogenicity potential of the plasmid DNA has been evaluated in mice, guinea pig, and rabbit models by intraderal route at 25, 100 and 500 μg dose. Preliminary studies have demonstrated that the DNA vaccine induces antibody response including neutralizing antibodies (NAB) against SARS-CoV-2 and also provides Th-1 response as evidenced by elevated IFN-γ levels [13].

In fact, a similar approach has already been used in the past for development of Middle East Respiratory Syndrome (MERS) and SARS coronavirus vaccines [14,15]. The MERS DNA vaccine was found to be well-tolerated in humans with a seroconversion rate of 94% in vaccinated volunteers, whereas, the SARS DNA vaccine induced antibody response in 80% subjects. Based on the earlier published literature of similar vaccines, the expected human dose of 2019-nCoV vaccine by intradermal administration will be 1 mg or 2 mg of the 2019-nCoV DNA vaccine candidate.

2. Methods

2.1. Study design and participants

We conducted a single-center Phase 1 trial of the DNA plasmid spike protein COVID-19 vaccine candidate at a Clinical Unit of Zydus Research Center, Cadila Healthcare Limited in Ahmedabad, Gujarat, India.

This trial was initiated after obtaining the approvals of the Ethics Committee (EC) and DCGI (dated 08 July 2020) and registering the trial with the Clinical Trial Registry of India (CTRI) (Identifier: CTRI/2020/07/026352). The study was performed in accordance with the Declaration of Helsinki, Good Clinical Practice and applicable local regulations. An independent data safety monitoring board was established before the start of the study to provide oversight of the safety data during the study. The authors had full access to all the data in the study.

Eligible participants were healthy adults aged between 18 and 55 years; body weight >50 kg; body-mass index of between 18.5 and 29.9 kg/m². For inclusion in the trial, participants needed to be able to understand the content of informed consent and be willing to sign the informed consent document; and be able and willing to complete all the scheduled study visits. Exclusion criteria included SARS-CoV-2 infection, confirmed by presence of serum-specific antibody against SARS-CoV-2 detected by enzyme-linked immunosorbent assay (ELISA) or chemiluminescence technique; positive results for COVID-19 as detected by qualitative reverse transcription polymerase chain reaction; history of SARS/ MERS infection; history of contact with a confirmed active SARS-CoV-2 positive patient within 14 days; participation in another clinical study of a SARS-CoV-2 candidate vaccine. Pregnant or breastfeeding women were also excluded. A comprehensive list of eligibility criteria is provided (Supplementary Table S1). Written informed consent was obtained from each participant before screening for eligibility.

2.2. Vaccine

The ZyCoV-D vaccine was developed by Cadila Healthcare Limited, Ahmedabad, India. The DNA vaccine candidate against SARS-CoV-2 is
This study, Pharmajet Tropis were scheduled after each vaccination until day 84 (End-of-study). In via syringe and needle or NFIS on days 0, 28 and 56. Follow-up visits cine (1 mg: Needle or NFIS; 2 mg: Needle or NFIS) four weeks apart. Participants were immunized intradermally with three doses of vac-
mant arm 3 [2 mg; Needle] and treatment arm 4 [2 mg; NFIS]. The next 12 subjects were allocated to treatment arm 1 [1 mg: Needle]; the first 12 subjects were allocated to treatment arm 1 [1 mg; Needle]; the next 12 subjects were allocated to treatment arm 2 [1 mg; Needle-Free Injection System (NFIS)] once safety was es-
blished in the previous treatment arm; same was followed for treat-
ment arm 3 [2 mg; Needle] and treatment arm 4 [2 mg; NFIS]). Participants were immunized intradermally with three doses of vac-
cine (1 mg: Needle or NFIS; 2 mg: Needle or NFIS) four weeks apart via syringe and needle or NFIS on days 0, 28 and 56. Follow-up visits were scheduled after each vaccination until day 84 (End-of-study). In this study, Pharmajet Tropis® device as NFIS was used for intradermal administration of the vaccine. Participants were monitored in the intensive observation unit for 24 h post the first dose of vaccine and 4 h post the second and third dose of vaccine for solicited adverse reactions (injection site pain, redness, swelling and itching). Close monitoring in terms of frequent vital signs and electrocardiogram (ECG) assessments were done before and after each vaccine dose. Subjects were also provided a diary card to record any solicited sys-
temic symptoms (fever, headache, fatigue, vomiting, diarrhea, nau-
sea, arthralgia, and muscle pain) and local adverse events (AEs) for
7 days post each vaccine dose and any other unsolicited AEs within 28 days post each dose. Serious AEs self-reported by participants were documented throughout the study.

Adverse events were self-reported by the participants, but veri-
fied by investigators throughout the study after vaccination. Adverse events were graded according to a standard toxicity grading scale [16]. Laboratory safety tests including hematolgy, biochemistry, uri-
nalysis and serology were conducted as per the protocol (Appendix 1) to assess any toxic effects post-vaccination. Blood samples were taken from participants as per the protocol for the immunogenicity assessment. The follow-ups were scheduled at days 70 and 84 (end-of-study) post vaccination for safety and immunogenicity assess-
ment.

2.4. Assessment of binding antibody (IgG and neutralizing antibody) and cellular response

For Phase 1 part of the study, immunogenicity assessment for serum IgG by ELISA, was done at baseline, day 28, day 42, day 56, day 70 and day 84. Neutralizing antibody titres and cellular response were also assessed at baseline, day 28, day 56 and day 84.

An indirect ELISA was used to measure anti-S1 SARS CoV-2 IgG antibodies present in the human sera samples post vaccination with ZyCoV-D vaccine. We used antigen from Acro Biosystems. The anti-
gen from the same manufactuer was also used by Innovio in their ELISA assay [17]. We used reference standard from National Institute for Biological Standards and Control (NIBSC) which is a WHO refer-
ence laboratory. We obtained research reagent for anti-SARS-CoV-2 Ab NIBSC code 20/130 and against this we defined unitage as ELISA Unit (EU). Plaque Reduction Neutralization Test (PRNT) was used for estimation of NAB titer in human serum samples against anti SARS-
CoV-2 virus. The SARS-CoV-2 virus (8004/IND/2020/PUNE), Accession number – MT416726 was used for PRNT assay.

Cell-mediated responses were assessed using IFN-γ ELISPOT assay in separated peripheral blood mononuclear cells (PBMCs). Serum samples from vaccinated subjects were also analyzed for the cyto-
kines levels (IFN-γ, IL-2, IL-6, IL-4, IL-10, TNF alpha, Th-17A) using MILLIPLEX® MAP multiplex magnetic bead-based antibody detection kits. Details regarding the methodology of these tests are provided in supplementary material.

2.5. Outcomes

The primary endpoint was the overall incidence and severity of adverse reactions within 7 days after each of the vaccination and AEs within 28 days across the treatment groups were also analyzed as safety endpoints.

The secondary endpoints included seroconversion rate based on IgG antibodies against S1 antigen (by ELISA), NAB titers and IFN-γ cellular immune responses after 3 doses of vaccine. Seroconversion was defined as antibody-negative subjects at baseline who become antibody-positive after vaccination, and subjects having antibody titre at baseline who have four fold rise in antibody titre after vacci-
nation.

2.6. Statistical analysis

The sample size was not determined on the basis of statistical power calculations. Sample size was based on non-probability sam-
pling method. However, a minimum sample size of 48 participants for this vaccine trial has been selected. We assessed the incidence and severity of participants’ adverse reactions post vaccination and compared safety profiles across the dose groups. The antibodies against SARS-CoV-2 were presented as geometric mean titers with 95% confidence intervals (CIs) and the cellular responses were shown as a proportion of positive responders. We used the pearson chi-
square test to analyze categorical data, ANOVA to analyze the log transformed antibody titers. Hypothesis testing was two-sided with an α value of 0.05. Statistical analyses were done by a statistician using SAS (version 9.4).

Geometric mean titres (GMTs) was calculated as: anti-Ln(mean [Ln Xi]) where Xi was the assay result for subject i. 95% CIs of GMTs were calculated assuming log normal distribution.

Geometric Mean Fold Rise (GMFR) were calculated as: GMFR = anti-Ln (mean [Ln Yi/ Bi]) where Yi was the post dose assay result for subject i; and Bi was the baseline assay result for subject i. Baselines were taken as Day 0 assay results.

2.7. Role of the funding source

The study sponsor, Cadila Healthcare Limited, designed the study and oversaw its conduct and data analysis. The sponsor collected, managed, and analyzed data according to a pre-specified statistical analysis plan. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Development of ZyCoV-D was supported by a grant-in-aid from COVID-19 Consortium under National Biopharma Mission, Depart-
ment of Biotechnology, Government of India, to Cadila Healthcare Ltd. (Grant no. BT/COVID0003/01/20).
3. Results

3.1. Study population demographics

Between July 2020 and October 2020, 126 participants were screened, 51 subjects failed in screening, 27 subjects passed but not enrolled in the study and a total of 48 subjects were enrolled into and vaccinated in the Phase 1 study. A total of 43 (89.0%) participants completed the study and 5 subjects were discontinued from the study (Fig. 1). All of the 48 participants (100%) were Asian male healthy subjects. Baseline demographics (age, height, weight and BMI) were well-balanced among the 4 treatment arms. Overall, the mean (SD) age was 34.9 ± 7.18 years and the mean BMI (SD) was 24.28 ± 3.0 kg/m². The patient disposition, baseline and demographic characteristics are provided in Table 1.

3.2. Vaccine safety and tolerability

A total of 48 subjects were vaccinated with the first dose of vaccine, 45 subjects were vaccinated with the second dose of vaccine (except 1 subject in arm 1 and 2 subjects in arm 4 who received only one dose) and 43 subjects were vaccinated with the third dose of vaccine (except 2 subjects in arm 3 who received only two doses). A total of 43 subjects completed the study.

Of the five subjects who discontinued, two subjects were discontinued because of withdrawal of consent (1 subject in each arm 1 and 3); one subject in arm 3 did not receive the third dose of vaccine due to an ongoing unsolicited adverse event (typhoid fever) that occurred 14 days after the second vaccination which was considered not related with vaccination; one subject in arm 4 was discontinued due to an ongoing anti-rabies vaccination for dog bite reported 7 days after the first vaccination; and one subject in arm 4 was withdrawn due to asymptomatic positive COVID-19 rapid antigen detection test, 27 days after the first vaccination.

There were no deaths or SAEs reported in Phase 1 of the study. Overall, 12/48 (25%) subjects reported at least one AE (i.e. combined solicited and unsolicited) during the study. The number of subjects with at least one solicited adverse event and at least one unsolicited adverse event was 7 (14.58%) subjects and 6 (12.5%) subjects, respectively (Figs. 2 and 3).

The number of subjects with solicited AEs across all four treatment arms were similar [i.e. 2 subjects in each Arm 1 (1 mg: Needle), Arm 2 (1 mg: NFIS) and Arm 4 (2 mg: NFIS); 1 subject in Arm 3 (2 mg: Needle)]. Overall, all solicited AEs reported were mild to moderate in severity, related with study vaccination and resolved with or without medication. The majority of solicited AEs were reported after the first dose of vaccine (i.e. 6 subjects, 12.5%) compared to the second dose (0%) and the third dose of vaccination (1 subject, 2.08%). Most solicited AEs were mild in severity (6 subjects, 12.5%) except 1 subject (2.08%) who reported an adverse event of moderate severity. No subject was discontinued from the study because of a solicited adverse event. The reported solicited AEs were injection site pain (3 subjects), injection site pruritus (1 subject), pyrexia (1 subject), arthralgia (1 subject) and diarrhea (1 subject).

There were no abnormal laboratory values that were deemed clinically significant except proteinuria (1 subject on day 14), considered possibly related to study drug and low WBC count (one subject on day 56), considered not related to the study drug by the investigator throughout the study period. There were no clinically significant changes reported in vital signs and 12-lead ECG evaluated during the monitoring period after vaccination of each dose as well as follow-up visits till day 84. For all the physical examinations performed, no major abnormal findings were reported till day 84.

3.3. Immune responses

Seroconversion was defined as antibody negative subjects at baseline who become antibody positive after vaccination and subjects...
having antibody titre at baseline who have four-fold rise in antibody titre after vaccination.

As mentioned in the method section, we used NIBSC reference standard sera in our ELISA assay. Using this standard we established the standard curve range from 1.41 EU as below limit of (BLQ) to 45.23 EU as upper limit of quantification (ULQ). We also tested panel of negative pre-COVID-19 sera sample during assay validation and samples were below the BLQ value of 1.41EU. NIBSC standard was also used in ELISA assay performed by Oxford group for their immunogenicity evaluation of ChADOx-1 SARS-CoV-2 vaccine candidate [18]. The proportion of subjects who seroconverted based on IgG titers on day 84 (i.e. 28 days after third vaccine doses) was 4 (36%) 4 (33%), 10 (100%) and 8 (80%) in the treatment Arm 1 (1 mg: Needle), Arm 2 (1 mg: NFIS), Arm 3 (2 mg: Needle) and Arm 4 (2 mg: NFIS), respectively. This suggests a higher seroconversion rate with the 2 mg vaccine dose, irrespective of method of delivery, compared to the 1 mg vaccine dose. When Arm 1 (1 mg: Needle), and Arm 4 (2 mg: NFIS), seroconversion rates were compared for day

Table 1
Disposition, Baseline and Demographics Characteristics – Safety Population.

| Parameters/Statistic | ZyCoV-D 1 mg (Needle) (N = 12) | ZyCoV-D 1 mg (NFIS) (N = 12) | ZyCoV-D 2 mg (Needle) (N = 12) | ZyCoV-D 2 mg (NFIS) (N = 12) | Overall (N = 48) |
|---------------------|---------------------------------|-------------------------------|---------------------------------|-------------------------------|-----------------|
| Disposition         | All subjects, n (%)             | 12 (100)                      | 12 (100)                        | 12 (100)                      | 12 (100)        |
|                     | Subjects who completed the study, n (%) | 11 (91.67)                   | 12 (100)                        | 10 (83.33)                    | 10 (83.33)      |
|                     | Subjects discontinued from the study, n (%) | 1 (8.33)                     | 0 (0)                           | 2 (16.67)                     | 2 (16.67)       |
| Demographics Age (Years) | Mean ± SD | 35.4 ± 6.56                   | 31.8 ± 7.44                     | 35.1 ± 6.95                   | 37.2 ± 7.53     |
|                     | Median (Range)                   | 36.5 (27, 45)                 | 31.5 (22, 48)                   | 36.0 (20, 45)                 | 36.5 (26, 48)   |
| Sex, n (%)          | Male                            | 12 (25%)                      | 12 (25%)                        | 12 (25%)                      | 12 (25%)        |
|                     | Height (cm)                      | Mean ± SD                     | 168.5 ± 4.08                    | 166.9 ± 4.01                  | 165.8 ± 4.53    |
|                     | Median (Range)                   | 170.0 (161, 173)              | 167.0 (159, 173)                | 165.5 (159, 173)              | 170.0 (159, 181) |
| Weight (kg)         | Mean ± SD                       | 68.52 ± 7.878                 | 65.32 ± 7.751                   | 70.32 ± 8.562                 | 67.97 ± 11.052  |
|                     | Median (Range)                   | 67.80 (57.6, 82.8)            | 66.65 (54.2, 76.2)              | 70.30 (56.8, 85.7)            | 66.60 (54.7, 87.5) |
| BMI (kg/m²)         | Mean ± SD                       | 24.178 ± 3.0745               | 23.416 ± 2.3624                 | 25.593 ± 3.0485               | 23.720 ± 3.5076 |
|                     | Median (Range)                   | 24.355 (19.98, 29.71)         | 23.860 (18.98, 26.40)           | 26.690 (18.98, 29.31)         | 23.805 (18.71, 29.31) |

Abbreviation(s): N = number of subjects in respective treatment arm; n = number of subjects in specified category; NFIS = Needle Free Injection System; BMI = body mass index; SD = standard deviation. Note: Percentages are based on the number of subjects in the specified treatment arm.

Fig. 2. Solicited (local and systemic) adverse events reported within seven days after administration of each dose of vaccine. Adverse events were graded according to the common terminology criteria for adverse events (CTCAE) scale.
28, day 42, day 56, day 70 and day 84, a statistical significant (p value = 0.0019) difference was found for day 70. Similarly for Arm 2 (1 mg: NFIS) vs Arm 4 (2 mg: NFIS), statistical significant (p value = 0.0427) difference was found for day 70 and day 84 (Supplementary Table S2).

The proportion of subjects who achieved seroconversion based on IgG on day 28, day 42, day 56, day 70 and day 84 is mentioned in Table 2. We have also included NAB titers of convalescent sera sample from individuals recovered after SARS-CoV-2 infection (Fig. 4).

The proportion of subjects getting seroconverted based on NAB titers on day 84 was 0.2 (18.18%), 0.2 (16.67%), 0.5 (50.00%) and 0.8 (80.00%) in the treatment Arm 1 (1 mg: Needle), Arm 2 (1 mg: NFIS), Arm 3 (2 mg: Needle) and Arm 4 (2 mg: NFIS) respectively. The proportion of subjects getting seroconverted based on NAB titers on day 56 was lower i.e. 0.0 (00.00%), 0.2 (16.67%), 0.2 (20.00%) and 0.1 (10.00%) in the treatment Arm 1 (1 mg: Needle), Arm 2 (1 mg: NFIS), Arm 3 (2 mg: Needle) and Arm 4 (2 mg: NFIS) respectively. When Arm 1 (1 mg: Needle), and Arm 4 (2 mg: NFIS), seroconversion rates based on NAB titers were compared for day 56 and day 84, a statistical significant (p value = 0.0089) difference was found for day 84. Similarly for Arm 2 (1 mg: NFIS) vs Arm 4 (2 mg: NFIS), statistical significant (p value = 0.0083) difference was found for day 84 (Supplementary Table S3).

Geometric mean titer of IgG (EU) on day 56 (28 days after two doses of vaccine) was 34.75 and 17.46 in the treatment Arm 3 (2 mg: Needle) and Arm 4 (2 mg: NFIS), respectively, which increased to 1019.61 and 720.25 in the treatment Arm 3 (2 mg: Needle) and Arm 4 (2 mg: NFIS) respectively on day 70 (14 days after three doses of vaccine) and remained stable at 748.46 and 884.04 in the treatment Arm 3 (2 mg: Needle) and Arm 4 (2 mg: NFIS) respectively on day 84 (28 days after three doses of vaccine) See, Table 3 and Fig. 5. When Arm 1 (1 mg: Needle), and Arm 4 (2 mg: NFIS), Geometric mean titer of IgG (EU) were compared for day 28, day 42, day 56, day 70, and day 84 a statistical significant (p value = 0.0006) difference was found for day 70 and day 84 (p value = 0.0027). Similarly for Arm 2 (1 mg: NFIS) vs Arm 4 (2 mg: NFIS), statistical significant (p value = 0.0376) difference was found for day 70 and p-values= 0.0259 for day 84 (Supplementary Table S4).

Table 2

| Time point | Seroconversion | ZyCoV-D 1 mg (Needle) (N = 11) | ZyCoV-D 1 mg (NFIS) (N = 12) | ZyCoV-D 2 mg (Needle) (N = 10) | ZyCoV-D 2 mg (NFIS) (N = 10) |
|------------|----------------|-------------------------------|-----------------------------|-------------------------------|-------------------------------|
| Day-28, n (%) | No | 9 (81.82) | 9 (75.00) | 6 (60.00) | 7 (70.00) |
| | Yes | 2 (18.18) | 3 (25.00) | 4 (40.00) | 3 (30.00) |
| Day-42, n (%) | No | 10 (90.91) | 9 (75.00) | 7 (70.00) | 7 (70.00) |
| | Yes | 1 (9.09) | 3 (25.00) | 3 (30.00) | 3 (30.00) |
| Day-56, n (%) | No | 9 (81.82) | 7 (58.31) | 4 (40.00) | 6 (60.00) |
| | Yes | 2 (18.18) | 5 (41.67) | 6 (60.00) | 4 (40.00) |
| Day-70, n (%) | No | 10 (90.91) | 8 (66.67) | 0 (0.00) | 2 (20.00) |
| | Yes | 1 (9.09) | 4 (33.33) | 10 (100.0) | 8 (80.00) |
| Day-84, n (%) | No | 7 (63.64) | 8 (66.67) | 0 (0.00) | 2 (20.00) |
| | Yes | 4 (36.36) | 4 (33.33) | 10 (100.0) | 8 (80.00) |

Abbreviation(s): N = number of subjects in respective treatment arm; n = number of subjects in specified category; NFIS = Needle Free Injection System.
Seroconversion defined as a positive antibody response as at least a four-fold increase in post-vaccination titer from baseline.
Geometric mean titer of NAB on day 84 (28 days after three doses of vaccine) was 39.17 in the treatment Arm 4 (2 mg: NFIS). Geometric mean titer and fold rise of NAB are presented in Table 4.

When Arm 1 (1 mg: Needle), and Arm 4 (2 mg: NFIS), Geometric mean titer of NAB were compared for day 56, and day 84 a statistical significant ($p$ value = 0.0055) difference was found for day 84. Similarly for Arm 2 (1 mg: NFIS) vs Arm 4 (2 mg: NFIS), statistical significant ($p$ value = 0.0251) difference was found for day 84 (Supplementary Table S5).

Seroconversion rates of IgG with 2 mg needle (Arm 3) and 2 mg NFIS (Arm 4) on day 56 and day 84 were observed higher than 1 mg needle and 1 mg NFIS (arm 1 and 2). Seroconversion rates of NAB with 2 mg NFIS (Arm 4) on day 84 were observed higher than 1 mg needle, 1 mg NFIS and 2 mg needle (Table 5). The seroconversion rate (defined as subjects sero-negative at baseline becoming sero-positive post vaccination and four fold rise in antibody titres post vaccination in subjects sero-positive at baseline)) based on humoral responses measured by ELISA were observed in 100% and 80% of the participants who received three doses of 2 mg vaccine either via needle and syringe or NFIS device respectively. The seroconversion rate based on NAB, measured by live virus neutralization assay, was seen in 50% (05/10) and 80% (08/10) of participants who received three doses of 2 mg vaccine either via needle or syringe or NFIS device respectively.

### 3.4. Cell-mediated responses

In our study, ZyCoV-D vaccine, when administered intradermally via NFIS at 2 mg dose, showed peak cellular response in terms of IFN-γ ELISPOT assay at Day 56 with 41.5 spot forming cells (SFC) per million PBMCs and was maintained till Day 84 with median 45.5 SFC per million PBMC. A similar trend was observed with 1 mg NFIS arm with Day 84 median 73 SFC per million PBMCs. ZyCoV-D vaccine when administered by conventional syringe and needle showed some response in IFN-γ ELISPOT assay at Day 56 which declined on Day 84 after reaching the peak on day 56 (Fig. 6).

In our study, there were no significant changes observed in cytokine levels like IFN-γ, IL-2, IL-6, IL-4, IL-10, TNF alpha, Th-17A analysed by Luminex in all four-treatment arms throughout the study compared to baseline.

### 4. Discussion

We report the findings from Phase 1 part of clinical trial on the safety, tolerability and immunogenicity of ZyCoV-D, a SARS-CoV-2 DNA vaccine encoding the spike protein. This first-in-human Phase 1 study of ZyCoV-D DNA vaccine was carried out in an intensive observational unit with frequent monitoring of vital signs and ECGs for at least 24 h post administration of the first

### Table 3

| Time Point | Statistics | ZyCoV-D 1 mg (Needle) (N = 11) | ZyCoV-D 1 mg (NFIS) (N = 12) | ZyCoV-D 2 mg (Needle) (N = 10) | ZyCoV-D 2 mg (NFIS) (N = 10) |
|------------|------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| Day-0      | GMT(95% CI)| 15.82 (4.61, 54.25)          | 26.73 (7.45, 95.89)           | 7.00 (7.00, 7.00)             | 7.00 (7.00, 7.00)             |
| Day-28     | GMT(95% CI)| 26.92 (6.46, 112.12)         | 66.39 (14.11, 312.27)         | 11.42 (6.80, 19.17)           | 14.15 (4.79, 41.79)           |
| Day-42     | GMT(95% CI)| 1.70 (0.93, 3.07)            | 2.48 (0.93, 6.67)             | 1.63 (0.97, 2.74)             | 2.02 (0.98, 5.97)             |
| Day-56     | GMT(95% CI)| 55.67 (12.48, 248.37)        | 1.01 (0.62, 19.26)            | 1.57 (0.90, 2.75)             | 2.23 (0.65, 7.30)             |
| Day-70     | GMT(95% CI)| 115.91 (23.41, 573.98)       | 34.75 (8.11, 148.89)          | 17.46 (4.81, 63.32)           | 4.89 (0.69, 9.05)             |
| Day-84     | GMT(95% CI)| 4.34 (1.14, 16.44)           | 0.16 (0.16, 22.7)             | 0.29 (0.09, 1.49)             | 0.49 (0.09, 1.49)             |

Abbreviation(s): N = number of subjects in respective treatment arm; n = number of subjects in specified category; NFIS = Needle Free Injection System. Seroconversion defined as a positive antibody response as at least a four-fold increase in post-vaccination titer from baseline.

![Fig. 4.](neutralising antibody titres with error bars, representing geometric mean (95%CI) at baseline (Day 0) and Day 84 with comparative NAB of convalescent serum.)
dose of vaccine and for at least 4 h post administration of the second and the third dose of vaccine. Each vaccination was followed by frequent safety follow-up with subjects till 28 days of the last dose of vaccine. ZyCoV-D vaccine was well-tolerated in 48 healthy adults in all four dose groups with no vaccine-related severe or SAEs. The safety profile of ZyCoV-D vaccine supports further development of ZyCoV-D in at-risk populations who are at more serious risk of complications from SARS-CoV-2 infection, including the elderly and subjects with comorbidities. Our findings also correlate with previous clinical evaluation of other DNA vaccine candidates which were reported to be safe and well-tolerated in healthy subjects [17,19–21].

The majority of solicited AEs reported in this trial were after the first dose of vaccine, while the second and third dose of vaccination were found to be well-tolerated. The ZyCoV-D Phase 1 safety data further suggest that the vaccine could be a safe booster as there was no increase in frequency of side effects after the third dose compared to the first dose, an important aspect for the safety profile of SARS-CoV-2 vaccines. One attractive feature of DNA vaccines, like ZyCoV-D, is that the immunizations could be boosted without significant

Fig. 5. (a) - 5 (f) IgG Antibody Titres with error bars, representing geometric mean titre (95%CI) at baseline (Day 0), Day 28, Day 42, Day 56, Day 70 and Day 84.
limitations such as dosing-incremented toxicities or anti-vector responses and additional boosting with other DNA vaccines have resulted in higher levels of cellular and humoral immune responses without increased toxicity [22].

ZyCoV-D also generated balanced humoral and cellular immune responses in participants displaying either or both antibody or T cell responses following three doses of vaccine. Humoral responses were lower in subjects who received 1 mg vaccine irrespective of method of administration. The exact reason for this is not known but it is likely that when the vaccine is administered at the low dose of 1 mg at single intradermal site, it may lead to inefficient transfection, in the host cells and thus lower the expression of antigen. Our data corroborates well with the Rhesus Macaques challenge study, where the vaccination of 2 mg dose with Pharmajet NFIS elicited significant SARS CoV-2 specific IgG, NAB titers and lower viral loads in animals post challenge (Data on file). Further; a Phase II study in 1000 subjects is currently ongoing which will provide better understanding of immunogenicity of ZyCoV-D vaccine in a larger sample size.

In our study, three doses of 2 mg ZyCoV-D DNA vaccine administered intradermally at two different sites via NFIS device 28 days apart have shown good humoral and cellular immune response at Day 70 onwards. Presently, correlation of protection for vaccine against SARS-CoV-2 is unknown, and the roles of the specific antibodies or T cells in building effective protection are not yet well-defined. Therefore, we are only able to demonstrate immune response induction following vaccination and not protection to SARS-CoV-2 following DNA vaccination on the basis of the vaccine-elicited immune responses in this study. A double-blind, placebo controlled Phase III
study in 28,216 subjects aged 12 years and above is also currently ongoing which will help evaluate efficacy of the ZyCoV-D 2 mg dose administered via NFIS device in protection against COVID-19 infection. The study is registered with CTRI/2021/01/030416.

Previous studies investigating SARS and Middle East Respiratory Syndrome (MERS) found that there is a temporary rise in specific antibodies which dropped rapidly in subjects after recovery, and the CD4+ and CD8+ T-cell responses played a vital role in memory response and protection against future exposure to virus [23]. A similar rapid decline of the specific antibody amounts in subjects with COVID-19 after recovery was also noted [23] suggesting that both specific cellular and humoral immunity are potentially important for a successful COVID-19 vaccine. Here, we report immune response till 28 days after the last dose of vaccine.

ZyCoV-D vaccine also induced cellular response as measured by IFN-γ ELISPOT which was maintained till Day 84 in subjects who received vaccination 1 mg or 2 mg via NFIS device. This clearly indicates that vaccination with ZyCoV-D induces cellular response with fold rise. However, the sample size per arm is too small to reach a definitive conclusion on the levels of IFN-γ in different arms and the results should be interpreted in the context of variability of the immunological responses among individuals enrolled in the trial.
Phase II data with a higher sample size will help to understand cellular response obtained with ZyCoV-D vaccine. This first-in-human study of ZyCoV-D DNA vaccine has some limitations. First, this open-label, non-randomized Phase 1 trial report is based on a modest sample size (48) in all vaccine arms and, therefore lacks a comparator group. Larger sample-sized randomized placebo controlled blinded trials may be needed to show the true immunogenicity difference between the dose groups. Second, this report only involves healthy Indian male subjects aged between 18 and 55 years. This is due to societal limitation, COVID-19 related lockdown and completion of recruitment with male subjects at study center. The results of this study are not generalizable to other ethnic groups and female subjects. In this regard, female subjects were part of Phase 2 and 3 studies. SARS-CoV-2 infection has more severe and fatal outcomes in older individuals. In this regard, the Phase 3 trial will evaluate individuals of higher age group. Third, only data within the first 84 days of vaccination is being reported, and this report does not include data about the durability of the vaccine-induced immunity. In previous clinical trials with similar DNA vaccines, durable immune responses up to 1 year following vaccination were reported [14,22]. Fourth, the study showed good humoral and cellular immune response at Day 70 onwards after administration of the third dose, while most other approved vaccines showed immune response after administration of the second dose.

In this study, two different vaccination strategies were used. One is injection and needle, and the second is needle-free injection. i.e. NFIS device. This technology has evolved significantly over the last 50 years and is now accepted in many routine immunization settings as a safe and effective vaccine delivery method. Disposable syringe jet injectors are now being used for the delivery of vaccines to eradicate polio, measles, mumps, rubella and influenza, and are showing promising results in vaccine clinical trials for the Zika virus and human papillomavirus. Vaccine administration using NFIS device offers some distinct advantages compared to the conventional method of vaccination using needle and syringe, like improved compliance and better coverage; no needle trash and needle stick injuries; higher immunogenic response; calibrated for specific volume with minimal vaccine wastage; auto disabling and eliminating possibility of re-use; efficient vaccine delivery; and the workflow is 25% faster than a conventional needle-syringe and is less painful [24]. Tebas et al. reported better immune response after administration of two doses with intradermal DNA vaccine followed by electroporation (EP) technique [17]. However, Pharmajet Tropis device has been used in DNA vaccine clinical trials and has been reported as a better administration technique in terms of ease of administration, reliability, and precision. Use of Tropis is also reported to be cost-effective and have better local tolerance compared with EP [25].

Our data suggests that ZyCoV-D demonstrates a good safety profile and that vaccination induces both cellular and humoral responses, supporting its further development to prevent infection and death related to COVID-19 in the global population. The safety and immunogenic profile are important parameters for vaccination for high-risk populations, such as the elderly and those living with co-morbid conditions.

Over the past decade, the vaccine industry and clinical research centers have been asked to provide urgent responses to epidemics of emerging infectious diseases, such as H1N1 influenza, Ebola virus, Zika, MERS, and now SARS-CoV-2 [26]. The risk of COVID-19 caused by SARS-CoV-2 is ongoing, making the need for effective vaccines even more urgent [27]. Previous findings suggested that those vaccines expressing full-length spike glycoprotein can induce good immune responses and protective efficacy. The full-length spike was chosen in most of the viral vectored, mRNA, or DNA COVID-19 vaccines in development [23].

There have been recent reports of emergence of new SARS-CoV-2 viral strains like B.1.1.7 in UK, B.1.351 in South Africa, P.1 in Brazil [28]. The emergence of new strains of virus has raised the doubts about efficacy of vaccines which were already approved for emergency use authorization. Currently ongoing clinical trials with ZyCoV-D vaccine will provide important insights into efficacy and safety of DNA vaccine platform. DNA vaccines are based on plug and play platform, which allows rapid development of new constructs in case mutant strains develop, and possibility of generating a new vaccine candidate in very short time, thus providing protection against mutated viral strains.

### Table 4
Summary results of neutralization titers at day 28, 56 and 84.

| Time Point | Statistics | ZyCoV-D 1 mg (Needle) (N = 11) | ZyCoV-D 1 mg (NFIS) (N = 12) | ZyCoV-D 2 mg (Needle) (N = 10) | ZyCoV-D 2 mg (NFIS) (N = 10) |
|------------|------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| Day-0      | GMT(95% CI) | 5.98 (4.01, 8.92)             | 8.88 (4.52, 17.48)            | 5.00 (5.00, 5.00)             | 5.00 (5.00, 5.00)             |
| Day-28     | GMT(95% CI) | 6.79 (4.29, 10.73)            | 11.12 (4.91, 25.19)          | 5.00 (5.00, 5.00)            | 6.06 (3.53, 9.34)            |
| Day-56     | GMT(95% CI) | 1.13 (0.82, 1.57)             | 1.25 (0.62, 2.52)            | 1.00 (1.00, 1.00)           | 1.21 (0.79, 1.87)            |
| Day-84     | GMT(95% CI) | 1.08 (0.91, 1.29)             | 1.46 (0.63, 3.42)            | 1.74 (0.62, 4.94)           | 1.27 (0.74, 2.17)           |

### Table 5
Summary of Seroconversion for Neutralization Titers.

| Time point | Seroconversion | ZyCoV-D 1 mg (Needle) (N = 11) | ZyCoV-D 1 mg (NFIS) (N = 12) | ZyCoV-D 2 mg (Needle) (N = 10) | ZyCoV-D 2 mg (NFIS) (N = 10) |
|------------|---------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| Day-28 n (%) | No | 10 (90.91) | 10 (83.33) | 10 (100.0) | 9 (90.00) |
|             | Yes | 1 (9.09)  | 2 (16.67) | 0 (0.00)  | 1 (10.00) |
| Day-56 n (%) | No | 11 (100)  | 10 (83.33) | 8 (80.00) | 9 (90.00) |
|             | Yes | 0 (0.00)  | 2 (16.67) | 2 (20.00) | 1 (10.00) |
| Day-84 n (%) | No | 9 (81.82) | 10 (83.33) | 5 (50.00) | 2 (20.00) |
|             | Yes | 2 (18.18) | 2 (16.67) | 5 (50.00) | 8 (80.00) |

Abbreviation(s): CI = confidence interval; GMT = geometric mean titer; GMFR = geometric mean fold rise; N = number of subjects in respective treatment arm; n = number of subjects in speciﬁed category; NFIS = Needle Free Injection System.

Seroconversion deﬁned as a positive antibody response as at least a four-fold increase in post-vaccination titer from baseline.
Declaration of Competing Interest

All authors declared no competing interests. TM, KK, HP, SS, BS, JP, RM, JS, KM, AD, HC, CR, HPR, PK and AN are employee of Cadila Healthcare Limited, Ahmedabad, India. DP is an employee of Zydus Discovery DMCC, Dubai, United Arab Emirates.

Contributors

KK, JS, RM and DP were involved in conceptualization of the study. TM and HP were the study investigators. TM and JP were involved in data interpretation, manuscript writing, and manuscript review. SS was involved in statistical analysis, designing, programming and generation of Tables, Listing, Figures and aided in interpretation of results. BS was a pharmacist for this study. KM was involved in conceptualizing, designing, developing the vaccine candidate and guiding on data analysis, AD was involved in designing, developing vaccine candidate, perform data analysis for ELISPOT and Luminex assay. HC and CRTM were involved in development of analytical procedures for testing of the vaccine and data analysis for ELISA, neutralization. HPRP was involved in developed process for vaccine production and manufacture Phase-1 vaccine batches, AN was the responsible for quality assurance and regulatory support. Each author contributed important intellectual content during manuscript drafting or revision and accepts accountability for the overall work by ensuring that questions pertaining to the accuracy or integrity of any portion of the work are appropriately investigated and resolved. All authors approved the final version of the manuscript for submission.

Data sharing statement

Deidentified data are in the process of being deposited on the Data Repository for the Cadila Healthcare Limited, and the corresponding author can be contacted for data access.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.eclinm.2021.101020.

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Antiviral drugs prioritization for COVID-19 management based on rational selection

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The SARS-CoV-2 infection has resulted in COVID-19 pandemic worldwide. It has infected around 0.1 billion individuals and caused 2 million fatalities across the globe till mid-January 2021. Drug repurposing has been utilized as the most preferred therapeutic intervention for COVID-19 mitigation due to its necessity and feasibility. To prioritize therapeutic regime against COVID-19, we used 61 antiviral drugs and their combinations. Selected molecules were subjected to virtual screening against: (i) human angiotensin-converting enzyme 2 receptor binding domain (hACE-2) which serves as an anchor for virus attachment and entry, (ii) SARS-CoV-2 RNA dependent RNA polymerase (RdRp) responsible for viral RNA replication, and (iii) SARS-CoV-2 main protease (MPro) needed for viral polyprotein slab proteolytic processing. Based on docking score, pharmacodynamic and pharmacokinetic parameters, combinations of Daclatasvir, Elbasvir, Indinavir, Ledipasvir, Paritaprevir and Rilpivirine were analysed further. Our analysis suggested Sofosbuvir in combination with Ledipasvir and Daclatasvir as potential therapeutic agents for SARS-CoV-2. The combined score suggests that these combinations have superior anti-SARS-CoV-2 potential than Remdesivir and other investigational drugs. The present work provides a rationale-based approach to select drugs with possible anti-SARS-CoV-2 activity for further clinical evaluation.

Keywords: Drug repurposing, hACE-2, main protease, RNA dependent RNA polymerase, SARS-CoV-2.

GLOBAL spread and infection of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) resulted in COVID-19 pandemic. More than 0.1 billion have been infected with a mortality rate up to 5% till the end of January 2021 (ref. 1). Due to the unavailability of a specific therapeutic regime, COVID-19 management is challenging. Drug repurposing offers a speedy solution for COVID-19 mitigation. Ongoing application of repurposed drugs aims to control symptoms of the disease or attain antiviral effect (viral replication cycle). Danoprevir, Darunavir, Favipiravir, Lopinavir/Ritonavir, Oseltamivir, Remdesivir and Umifenovir have been tested clinically against SARS-CoV-2 (ref. 2).

Furthermore, non-antiviral drugs like Camostat or Nafamostat, Chloroquine, Hydroxychloroquine, and Ivermectin have shown anti-SARS-CoV-2 potential. Literature suggests that targeting multiple closely interacting pathogenesis-related proteins can provide effective intervention. Hence, several treatments and clinical trials used combinations of antiviral drugs for viral infection management. We selected three targets from the interaction network of pathogenesis. These targets are the virus entry point, Human angiotensin-converting enzyme 2 receptor binding domain (hACE-2), SARS-CoV-2 RNA dependent RNA polymerase (RdRp) for viral RNA replication and SARS-CoV-2 main protease (MPro) for virus maturation.

SARS-CoV-2 attaches and infects human cells through hACE-2 receptor.6,7 The extracellular domain of hACE-2 serves as SARS-CoV-2 spike (S) receptor. Cleavage product of S protein, S1, interacts with hACE-2 and anchors to the viral membrane by S2 protein.8-8 SARS-CoV-2 shows strong binding (~10 fold) with hACE-2 compared to other coronaviruses, hence, serving as a vital target for intervention.7 Multiprotein complex facilitates the replication of SARS-CoV-2 RNA genome and serves as a therapeutic target. RNA-dependent RNA polymerase (RdRp or NSP12), a cleavage product of viral polyproteins (ORF1a and ORF1b), catalyses the replication and transcription cycle of the virus. Due to its essentiality, RdRp has been explored as one of the primary targets for nucleoside analogues antivirals, e.g. Remdesivir.9,10 Self-maturation and processing of viral replicase enzymes can be targeted by inhibition of papain-like main protease (MPro).9,12,13 Due to low similarity with human proteases, inhibitors of MPro show minimal cross-reactivity.11,13 Molecules inhibiting viral entry, replication and maturation can have the potential anti-SARS-CoV-2 activity. Sixty one approved antiviral drugs were screened in silico for binding against selected targets. A combined activity

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score was calculated based on differential binding energy to targets. Further, pharmacokinetic and pharmacodynamic parameters were calculated to prioritize drug candidates for repurposing for COVID-19 management.

Methods

Library and target molecules preparation

Approved antivirals library was generated using three-dimensional structures from PubChem14. They were checked for stereochemical properties, followed by conversion to *.pdbqt format by Autodock Tools15. The library was used for further docking studies. The necessary methodology was as described by an earlier study4.

Experimental structures of hACE-2 complexed with viral spike protein (PDB ID: 6VW1) and MPro (PDB ID: 6Y2F) were downloaded from PDB7,11. After the receptor preparation process on MPro, the grid for docking simulation was set using AutoGrid program around active site residues H41 and C145 with 36 × 56 × 40 Å dimension15 and structure converted to *.pdbqt format. Similarly, the structure of SARS-CoV-2 RdRp (PDB: 7BTF) was prepared by generating a grid of 34 × 34 × 36 Å dimension around RNA binding pocket9. Furthermore, the grid for hACE2 was 20 × 38 × 24 Å spanning the viral spike protein recognition residues (K31, E35, D38, M82, K353)7. These target molecules were then further used for virtual screening.

Virtual screening using combined score analysis

The prepared receptor molecules from custom-made libraries were set for virtual screening by AutoDock Vina using default parameters16. Top hits of ligands were selected based on their docking scores. Comparative analysis of binding score and the combined score was performed using heatmap analysis, followed with hierarchical clustering and rank product analysis. Relative weightage was assigned as follows: MPro = 20%; RdRp = 20% and hACE-2 = 60%. The combined activity score was calculated as ((binding energy MPro *0.2) + (binding energy RdRp *0.2) + (binding energy hACE-2*0.6)). Ligands with high solubility and bioavailability were further taken for interaction analysis. A detailed methodology of interaction analysis was as described by an earlier study4.

Results and discussion

Outcomes of in silico screening of antiviral drugs demonstrate several molecules’ potential to intervene SARS-CoV-2 infection cycle. They can potentially offer avenues to manage COVID-19. Remdesivir, Lopinavir/Ritonavir (Kaletra)17,18, Favipiravir19 and Umifenovir are among top antivirals being focused on for COVID-19 management. Nucleotide analogue, Remdesivir, serve as broad-spectrum antiviral against RNA viruses like Coronavirus. Initial preclinical and clinical studies indicated Remdesivir causing a reduction in viral load through RdRp inhibition10. Remdesivir exhibited binding energy –7.8, –8.2 and –7.2 kcal/mol against hACE-2, MPro and RdRp respectively (Figure 1). Remdesivir has a higher
Binding energy and combined activity scores for approved antiviral drugs exhibiting hACE-2 binding better than or comparable to Remdesivir.

Table 1. Drug candidates exhibiting strong binding to MPro/RdRp/hACE-2

| Target | Binding energy range* (kcal/mol) | Drug        | Binding energy (kcal/mol) | Combined activity score |
|--------|----------------------------------|-------------|---------------------------|-------------------------|
| MPro   | -3.2 to -10.2                    | Remdesivir  | -8.2                      | 7.8                     |
|        |                                  | Ledipasvir  | -9.4                      | 8.8                     |
|        |                                  | Saquinavir  | -9.0                      | 7.5                     |
|        |                                  | Danoprevir  | -8.7                      | 7.7                     |
|        |                                  |Raltegravir  | -8.7                      | 7.3                     |
| RdRp   | -3.2 to -9.4                     | Remdesivir  | -7.2                      | 7.8                     |
|        |                                  | Ledipasvir  | -10.2                     | 8.8                     |
|        |                                  | Elbasvir    | -10.0                     | 7.8                     |
|        |                                  |Danoprevir   | -9.8                      | 7.7                     |
|        |                                  | Paritaprevir| -9.4                      | 8.0                     |
| hACE-2 | -2.8 to -8.9                     | Remdesivir  | -7.8                      | 7.8                     |
|        |                                  | Daclatasvir | -8.9                      | 8.4                     |
|        |                                  | Rilpivirine | -8.7                      | 8.2                     |
|        |                                  | Ledipasvir  | -8.2                      | 8.8                     |

Drugs exhibiting low binding energy to either MPro/RdRp/hACE-2 are shortlisted. *Range of 61 antiviral drugs screened.

Binding energy and combined activity scores for approved antiviral drugs exhibiting hACE-2 binding better than or comparable to Remdesivir.

Table 2. Binding energy and combined activity scores for approved antiviral drugs against SARS-CoV-2.

| Drug       | Binding energy (kcal/mol) | Combined activity score |
|------------|---------------------------|-------------------------|
|            | MPro                      | RdRp                    | hACE-2                   |
|            | Combined activity score significantly better than Remdesivir |
| Ledipasvir | -9.4                      | -10.2                   | -8.2                     | 8.8                     |
| Daclatasvir| -7.8                      | -7.6                    | -8.9                     | 8.4                     |
| Rilpivirine| -7.3                      | -7.7                    | -8.7                     | 8.2                     |
| Delviridine| -8.4                      | -7.9                    | -8.1                     | 8.1                     |
| Paritaprevir| -8.6                    | -9.4                    | -7.4                     | 8.0                     |
| Letermovir | -8.3                      | -7.6                    | -8.0                     | 8.0                     |
| Dolutegravir| -7.6                    | -8.2                    | -7.7                     | 7.8                     |
| Remdesivir*| -8.2                      | -7.2                    | -7.8                     | 7.8*                    |
| Indinavir  | -7.4                      | -8.1                    | -7.7                     | 7.7                     |
| Sofosbuvir*| -7.6                      | -7.3                    | -7.6                     | 7.5                     |
| Darunavir  | -8.2                      | -6.7                    | -7.3                     | 7.4*                    |
| Abacavir*  | -6.5                      | -6.9                    | -7.3                     | 7.4*                    |
| Tenofovir* | -6.8                      | -7.1                    | -7.4                     | 7.1*                    |

*These drugs are converted intracellularly to active metabolites. The binding energy of known key active metabolite present intracellularly was considered for MPro and RdRp for combined activity score determination.

Combined activity score compared to selected repurposed antiviral and non-antiviral drugs against SARS-CoV-2. Selected drugs and their metabolites binding energy and combined activity are provided in Supplementary Table 1.

Binding energy and combined activity score for selected antiviral drugs are shown in Supplementary Table 2. The binding energy of approved antiviral drugs for respective target is outlined in Table 1.

Ledipasvir, Elbasvir, Danoprevir, Saquinavir and Paritaprevir showed strong binding with (< -9 kcal/mol) either target. Candidates having lower binding energy against hACE-2 than Remdesivir were shortlisted for further evaluation (Table 2).

Daclatasvir and Ledipasvir depicted better combined activity score than Remdesivir (> 8.2) and strong hACE-2 binding (≤ -8.2 kcal/mol). Daclatasvir and Ledipasvir are used as combination therapy against Hepatitis C Virus (HCV) infection. They inhibit HCV RNA replication and assembly of virions by blocking non-structural Protein 5A (NS5A). They are FDA approved against HCV infections as the fixed-dose combination (FDC) with Sofosbuvir.

From in silico analysis, Ledipasvir was found to be a top hit for drug repurposing. For MPro and RdRp, it has a binding energy of < -9 kcal/mol and against hACE-2 binding ≤ -8.2 kcal/mol. Amongst the screened antivirals, Daclatasvir binds strongly with hACE-2 and interfere with its binding to spike protein of SARS-CoV-2. Daclatasvir interaction with the binding pocket of targets is shown in Figure 2, and pharmacokinetic overview of these drugs is summarized in Table 3.

Remdesivir (GS-5734) is used as a reference molecule in the current study. It is a prodrug form of adenosine analogue (GS-441524) and can be intracellularly metabolized to an active nucleoside triphosphate (NTP). Pro- and metabolite forms of Remdesivir exhibit strong binding against RdRp and MPro (Supplementary Table 1). The prodrug can bind to hACE-2 weakly for a prolonged time due to its extended half-life, while, the parent molecule, Remdesivir, has effective hACE-2 inhibition for a shorter duration due to short half-life (Table 3).

The half-life for Ledipasvir and Daclatasvir is 47 h and 12 to 15 h respectively. Due to longer half-life, they can have a long-term binding with hACE-2. Additionally, due to significant intracellular concentrations, they show noteworthy binding against RdRp and MPro. Hence,
Daclatasvir and Ledipasvir are considered for repurposing against COVID-19.

Route of administration for Remdesivir is intravenous, whereas Daclatasvir and Ledipasvir, are orally administered. Combinations of Ledipasvir and Daclatasvir with Sofosbuvir are clinically approved. In docking analysis, Sofosbuvir also exhibited high combined activity score of 7.5. Thus, the additive putative synergistic effect could be expected in these approved antiviral drug combinations and they provide merit over other drugs’ combinations for COVID-19 management. Due to moderate to better protein binding capacity of these molecules, high drug concentration becomes available to bind against the extracellular hACE-2 target. Furthermore, the circulating half-life of Sofosbuvir is 0.4 h. It is metabolized as triphosphate form GS-46103 (2’-deoxy-2’-α-fluoro-β-C-methyluridine-5’-triphosphate) and dephosphorylated metabolite GS 331007 subsequently, which has an elimination half-life of 27 h. Thus, Sofosbuvir can bind for a longer time to intracellular targets such as RdRp and M<sub>pro</sub> (Supplementary Table 3).

In the case of approved Daclatasvir and Sofosbuvir combination with Ribavirin for HCV treatment, Ribavirin did not exhibit good combined activity score (6.2) against SARS-CoV-2 targets. Hence, Ribavirin was not considered for prioritization (Supplementary Table 2).<sup>23</sup> Similarly, another fixed-dose combination (FDC) of Daclatasvir with Asunaprevir against HCV showed low combined activity score (5.7) for Asunaprevir. Hence Asunaprevir was dropped from further analysis.

Combined scores for Rilpivirine, Viridine, Paritaprevir, Letermovir and Dolutegravir are better than Remdesivir (7.8 to 8.3).<sup>24</sup> Rilpivirine binds strongly to hACE-2 and Paritaprevir showed higher binding to RdRp and M<sub>pro</sub>. All these drugs can bind to hACE-2 in pro-form and have high protein binding (Supplementary Table 4). Non-nucleoside reverse transcriptase inhibitors (NNRTI) like Rilpivirine and Delviridine are approved against HIV-1 infections.<sup>25–27</sup> As a second-line therapy drug Delviridine is inconvenient due to its dosing schedule, it is therefore dropped from further evaluation.

Paritaprevir in combination with Ombitasvir, Dasabuvir, Ritonavir, and Ribavirin is used to treat HCV<sup>28</sup>. Most of these antivirals exhibited low binding score against all targets and thus have negligible anti-SARS-CoV-2 potential.

Abacavir, Darunavir, Indinavir, Sofosbuvir and Tenofovir showed high binding to SARS-CoV-2 target. Short half-life, moderate protein binding (60%), acceptable safety profile and strong hACE-2 binding (7.7 kcal/mol) makes Indinavir a potential agent. Indinavir and Ritonavir FDC is used for HIV treatment. Ritonavir has a low combined activity score, but it blocks the intracellular conversion of Indinavir and thus prolongs its half-life. Hence, the FDC of Indinavir and Ritonavir can be anti-SARS-CoV-2 (ref. 29). The development of new FDC of these drugs for intravenous use can be considered in critically ill patients (Supplementary Table 1).

Short half-life (1.54 ± 0.63 h) and moderate protein binding (50%), with a binding energy of −7.3 kcal/mol against hACE-2 suggest the potential of Abacavir for effective intervention. Abacavir and its metabolite Carbovir triphosphate depicted high binding energy against M<sub>pro</sub> and RdRp (Supplementary Table 3).<sup>30</sup> In HIV infection, Abacavir combination with Lamivudine, Zidovudine and Dolutegravir is used for the treatment. The Lamivudine and Zidovudine have combined activity scores 5.3 and 6.3 respectively. Due to good combined activity score, high protein binding and half-life of 14 h, Dolutegravir and Abacavir combination are prefered over a combination with Lamivudine or Zidovudine for COVID-19. Elbasvir has the potential against SARS-CoV-2 replication and maturation due to high binding to RdRp (< −10 kcal/mol) and combined activity score of 7.8. This

| Status | Investigational | Approved | Approved |
|--------|-----------------|----------|----------|
| Indication (*in clinics) | SARS-COV-2 | Chronic HCV genotype 1a, 1b, 4, 5 and 6 infection in combination with Sofosbuvir (Harvoni) | Chronic HCV genotype 1, 3 and 4 infection in combination with Sofosbuvir, Ribavirin or interferon |
| The key known target for the approved indication | RdRp inhibition by triphosphate metabolite (NTP) | Prevent hyperphosphorylation of NS5A | Prevent hyperphosphorylation of NS5A |
| Bio-availability | Not available | 76% | 67% |
| Protein binding | Not available | >99.8% | 99% |
| Elimination half-life | 0.4 h parent (non-human primate (NHP)) 20 h for NTP metabolite in humans, 14 h in NHP | 47 h (median terminal) | 12–15 h |
| Metabolism | Not available | No detectable metabolism excretion–unchanged in faeces | Faecal (53% as unchanged drug), kidney |

**Table 3.** Pharmacokinetic properties of lead candidates and reference drug (Remdesivir*)
drug has a half-life (geometric mean) of 24 h and can be evaluated as a candidate drug for COVID-19. Elbasvir is a direct-acting antiviral which inhibits HCV NS5A protein and approved as the FDC with Grazoprevir or Ribavirin and is a part of combination therapy to treat HCV. Grazoprevir exhibits a combined activity score of 7.2 and shows low binding energy for RdRp (−8.7 kcal/mol), superior to Ribavirin. Hence, FDC of Elbasvir with Grazoprevir can be checked for COVID-19 management.

Danoprevir, NS3/4A protease inhibitor approved for HCV, exhibits distinctly better binding for MPro (−8.7 kcal/mol) and RdRp (−9.8 kcal/mol). Danoprevir half-life and protein binding information are not available in the public domain; also, it showed poor binding to hACE-2. It does not offer an advantage over other lead candidates. Another protease inhibitor, Saquinavir, exhibits high binding to MPro (−9.0 kcal/mol). Due to its low combined activity score (7.5) and poor bioavailability, it is not considered for prioritization. Earlier in silico studies have identified two non-antiviral drugs Ergotamine and Ubrogepant as potential anti-SARS-CoV-2 agents (Supplementary Table 5). These molecules exhibited the potential to bind to all three targets. Binding energy at the crucial target of interest hACE-2 for Ubrogepant was −7.0 kcal/mol. Ergotamine showed strong binding against all three targets as compared to Ledipasvir (Supplementary Table 5), while Daclatasvir showed stronger hACE-2 binding compared to Ergotamine. It has been observed that cardiovascular drugs like ACE inhibitors and angiotensin receptor antagonists do not show affinity toward hACE-2 (ref. 34). Hence, this further strengthens the case for Daclatasvir and Ledipasvir’s prioritization as lead candidates for COVID-19 over Ergotamine and other similar drugs.

Cleavage of S2 protein is catalysed by a human cell surface serine protease, TMPRSS2. Therefore, along with hACE-2, TMPRSS2 can be considered for effective targeting of viral replication and maturation. Camostat and Nafamostat have demonstrated inhibition of TMPRSS2. Hence, Camostat and Nafamostat and their combinations with antiviral drugs with high binding for hACE-2 such as Daclatasvir and Rilpivirine can offer synergistic effects against SARS-CoV-2.

We suggest that molecules with potential for strong hACE-2 binding with RdRp and MPro interaction can be repurposed against SARS-CoV-2. Additionally, due to better protein binding and long half-life, suggested drug/drug combinations can exhibit activity better than other drugs under investigation. Further, shortlisted drugs are orally administered and thus offer an advantage over others. There is a need for systematic preclinical and clinical assessment for these drugs and their FDC for anti-SARS-CoV-2 activity. These repurposed drugs might provide potential antiviral effect against SARS-CoV-2 better than other drugs under trials and tested investigational drugs.
Conclusion

We virtually identified drugs with potential to bind to multiple targets like SARS-CoV-2 M\textsuperscript{Pro} and RdRp; and hACE-2 (Figure 3). These repurposed drugs are likely to have anti-SARS-CoV-2 activity by impacting virus entry, replication and maturation.

Daclatasvir, Elbasvir, Indinavir, Ledipasvir, Paritaprevir and Rilpivirine were predicted as potential anti-SARS-CoV-2 based on combined activity score, pharmacokinetic and pharmacodynamic parameters. Ledipasvir and Daclatasvir emerged as lead candidates with high combined activity scores and prolonged half-life, ensuring significant extracellular hACE-2 engagement along with RdRp and M\textsuperscript{Pro}. With good safety profile and oral administration of Ledipasvir, Daclatasvir and other drugs selected through this screening, can provide an advantage over others. These drugs and their FDCs can be considered for systematic fast track preclinical and clinical evaluation for COVID-19 management. Our findings provide a scientific rationale for applying Ledipasvir and Daclatasvir in combination with Sofosbuvir for COVID-19 management. Recent initial clinical trials data from Iran with Ledipasvir and Daclatasvir in combination with Sofosbuvir against COVID-19 are encouraging. Based on our analysis and available preclinical and clinical data, we recommend prioritization and aggressive perusal of clinical evaluation of these drug combinations.

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