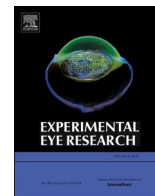




Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.



Research article

Tracing the SARS-CoV-2 infection on the ocular surface: Overview and preliminary corneoscleral transcriptome sequencing

Umberto Rosani^a, Claudia del Vecchio^b, Elisa Franchin^b, Paola Brun^b, Stefano Ferrari^c, Diego Ponzin^c, Andrea Leonardi^{d,*}

^a Department of Biology, University of Padova, Italy

^b Department of Molecular Medicine, University of Padova, Italy

^c Fondazione Banca degli Occhi del Veneto, Venice, Italy

^d Department of Neuroscience, Ophthalmology Unit, University of Padova, Italy



ARTICLE INFO

Keywords:

SARS-CoV-2

Ocular surface

Cornea

Corneoscleral ring

RNA-Sequencing

ABSTRACT

COVID-19's impact on the ocular surface has already been recognized, however the molecular mechanisms induced by the infection on the ocular surface are still unclear. The aim of this paper is to provide a first overview of the transcriptional perturbations caused by SARS-CoV-2 on the ocular surface by analyzing gene expression profile of corneoscleral ring samples from post-mortem SARS-CoV-2 positive donors (PD). The presence of SARS-CoV-2 on the ocular surface, in tears and corneal tissues has rarely been detected in infected individuals in both the presence and the absence of ocular manifestations. In this preliminary study, 6 human corneoscleral tissues of 3 PD and two tissues from a negative donor (CTRL) were obtained at the local eye bank. The presence of genomic and sub-genomic SARS-CoV-2 RNAs was assessed by qRT-PCR, while transcriptome analysis (RNA-sequencing) was performed by Illumina. Principal Component Analysis (PCA), search for differentially expressed genes (DEGs) and Gene Ontology (GO)-enrichment analysis were performed. Three samples from PD were found positive for SARS-CoV-2 genomic RNA, although the absence of sub-genomic RNAs indicated an inactive virus. PCA analysis grouped 3 different clusters, one including CTRL, and the other two including, respectively, PD with undetected SARS-CoV-2 (PD-SARS-neg) and PD with detected SARS-CoV-2 (PD-SARS-pos). The DEGs in common with the 2 PD clusters included several genes associable to the interferon pathway, such as ADAMTS4, RSAD2, MMP1, IL6, ISG15 and proinflammatory cytokines. Among the down-regulated genes we found AQP5. GO analysis revealed 77 GO terms over-represented in PD-SARS-neg vs. CTRL, and 17 GO terms in PD-SARS-pos vs. CTRL.

The presence of SARS-CoV-2 RNA and RNA-sequencing reads in ocular surface tissues supports the possibility that the eye acts as an entry route. The modulation of early responsive genes, together with several ISGs suggests a potential protective responsiveness of the ocular tissues to SARS-CoV-2.

1. Introduction

The random combination of morbidity, high infectivity and the ability of Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) to target several human organs and subvert antiviral defenses contributed to the diffusion of COVID-19 worldwide (Lam et al., 2020). The 12 Open Reading Frames (ORFs) encoded along the positive-sense RNA genome of SARS-CoV-2 codified for structural and non-structural proteins (Fernandes et al., 2020), the latter responsible for the interactions with host receptors and antiviral mediators, to promote viral

entry and the overall infection process (Blanco-Melo et al., 2020). *In-vivo* studies have revealed a hyper-activation of Interferon Stimulated Genes (ISGs) as the molecular footprint of SARS-CoV-2 infection in lungs, known as “cytokine storm” (Song et al., 2020). Mechanistically, how SARS-CoV-2 may interfere with immune responses is not completely known, although the reported modulation of long non-coding RNAs (lncRNAs) can dysregulate the fine-tuning mechanisms controlling the immune system (Turjya et al., 2020).

The presence of SARS-CoV-2 on the ocular surface and in tears has rarely been detected in infected individuals in both the presence and the

* Corresponding author. Department of Neuroscience, Ophthalmology Unit, University of Padua via Giustiniani 2, 35128, Padua, Italy.

E-mail address: andrea.leonardi@unipd.it (A. Leonardi).

<https://doi.org/10.1016/j.exer.2022.108975>

Received 30 August 2021; Received in revised form 23 January 2022; Accepted 1 February 2022

Available online 5 February 2022

0014-4835/© 2022 Elsevier Ltd. All rights reserved.

absence of ocular manifestations (Zhang et al., 2020; Wu et al., 2020). In fact, the reported prevalence of viral RNA detection in tears varies from 0% to 24%, with higher positivity rates in patients with severe COVID-19, suggesting that a significantly higher possibility of viral transmission exists through tears in patients with moderate to severe COVID-19 (Arora et al., 2021).

In addition, low RNA loads and sub-genomic RNA of SARS-CoV-2 were detected post-mortem in the cornea of patients with COVID-19 viremia in the absence of SARS-CoV-2 infectious particles in any of the samples analyzed, suggesting that the risk of COVID-19 infection via corneal transplant is low even in donors with SARS-CoV-2 viremia (Casagrande et al., 2021). However, COVID-19 infection should remain a contraindication for donation for both penetrating keratoplasty and endothelial keratoplasty (Wan et al., 2021).

Conjunctivitis or keratoconjunctivitis may be a sign of COVID-19 prior the onset of respiratory symptoms (Inomata et al., 2020; Cheema et al., 2020) or be the only sign of the infection (Scalinci and Trovato Battagliola, 2020, 19), suggesting that the ocular surface can be both a potential gateway for the SARS-CoV-2 to enter the body and a potential source of infection. On the contrary, the risk for an isolated conjunctival viral activity in patients with a negative nasopharyngeal swab-based RT-PCR seems to be absent or extremely low, suggesting no need to perform additional conjunctival swabs in patients with negative nasopharyngeal swabs (Rokohl et al., 2020, 2021).

Although lungs represent the main SARS-CoV-2 targets, other mucosal tissues can mediate the SARS-CoV-2 entry (Douglas et al., 2020; Collin et al., 2021). We and others reported the expression of SARS-CoV-2 entry receptor ACE2 and associated host protease TMPRSS2 in human cornea and conjunctiva by immunohistochemistry, RNA-sequencing, single-cell RNA-sequencing, Western blot, and assay for transposase-accessible chromatin using sequencing (ATAC-seq) (Leonardi et al., 2020; Mencucci et al., 2021; Zhou et al., 2020; Ma et al., 2021; Grajewski et al., 2021) supporting the possibility of an eye-mediated entry of SARS-CoV-2 (Douglas et al., 2020). Interestingly, the limbal region seems to be particularly prone to infection and may serve as a potential entry route for the virus (Eriksen et al., 2021). However, eye antiviral immunity has been rarely investigated and a recent study indicated that SARS-CoV-2 infection is restricted in human corneas, differently from Zika and HSV-1 (Miner et al., 2020).

Animal studies confirmed the ability to detect virus in the nasolacrimal and pulmonary system upon SARS-CoV-2 infection via the conjunctiva in monkeys (Deng et al., 2020) and in golden hamsters (Hoagland et al., 2021). In this last model, the eye transmission route showed evidence of a host respiratory response with elevated levels of interferon-stimulated protein ISG15 and the pro-inflammatory chemokine C-X-C motif chemokine ligand 11 (CXCL11) in the lungs. In the same study, it was suggested that dissemination of virus-derived PAMPs may be responsible for the systemic inflammation observed across tissues, reflecting the inconsistency of the tropism and inflammation in COVID-19 affected patients and the different clinical manifestations.

A recent report showed that SARS-CoV-2 RNA has been isolated from corneal specimens without inducing cytopathic effects and plaque formation in VeroE6 cell cultures (Ferrari et al., 2020). Therefore, the fate of SARS-CoV-2 in the eye is unclear. Based on the availability of ocular samples of SARS-CoV-2-positive donors, we report here preliminary data on the gene expression profile by RNA-sequencing of corneoscleral ring samples.

2. Materials and methods

2.1. Sample collection and description

According to the Italian guidelines set by the National Transplant Center (Rome, Italy), for every tissue donor post-mortem nasopharyngeal swabs are analyzed by means of quantitative Real-Time PCR (qRT-PCR) for identification of the presence of SARS-CoV-2 RNA before the

tissue is used for transplantation. Only corneal tissues from donors with a negative post-mortem swab test can be transplanted (Ferrari et al., 2021).

The ocular tissues of 3 donors with positive post-mortem nasopharyngeal swab test for SARS-CoV-2 RNA (positive donor, PD), plus 2 of a negative post-mortem nasopharyngeal swab donor (control donor, CTRL) were obtained at the Fondazione Banca degli Occhi del Veneto (Venice, Italy) (Ferrari et al., 2020). By Italian law, written informed consent from a donor's next of kin was obtained for the use of tissues for transplantation or, alternatively, for research purposes in agreement with the Declaration of Helsinki. The tissues were used in accordance with the laws of the National Transplant Center (Rome, Italy). Corneas were maintained in storage medium at 31 °C until sample processing (Ferrari et al., 2020).

2.2. SARS-CoV-2 diagnosis

Total nucleic acids (DNA and RNA) were purified from corneal specimens and storage medium by using a MagNA Pure 96 System (Roche Applied Sciences, Penzberg, Germany). Detection of SARS-CoV-2 RNA and sub-genomic RNA were performed by an in-house qRT-PCR method, which were developed, respectively, according to the protocol designed by CDC that targeted the genes N2 and sequence analysis of E gene sub-genomic mRNA (Wölfel et al., 2019, 2020). RT-qPCR assays were performed in a final volume of 25 µl, containing 5 µl of purified nucleic acids, using One Step Real Time kit (Thermo Fisher) on an ABI 7900HT Fast Sequence Detection System (Thermo Fisher).

2.3. RNA extraction and sequencing

Tissues were homogenized with a manual pestle in liquid nitrogen. Subsequently, 1 ml of Trizol (Life Technologies, Carlsbad, CA, USA) was added to each sample and they were finely homogenized using a T-10 Ultra-Turrax (IKA, Staufen, Germany). Samples were centrifuged at 800×g to remove the cellular pellets, and further mixed with 200 µl of Chloroform (Sigma-Aldrich, St. Louis, US) and centrifuged at 12,000×g for 15 min at 4 °C. The water phase was collected and used for RNA extraction with the RNAeasy micro kit (Qiagen, Hilden, Germany) following manufacturer instructions. A DNA removal step was applied using 500 units of RNase-free DNase (Qiagen) at room temperature for 15 min. Total RNA was resuspended in RNase-free water (Thermo Fisher, Carlsbad, US) and the RNA/DNA concentrations in each sample were quantified using the Qubit RNA and DNA high-sensitivity Assay Kit (Thermo Fisher). RNA qualities were assessed with an Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). Due to the low RIN values (below 5), a 3'-end sequencing procedure was adopted using the QuantSeq 3' mRNA-Sequencing Library Prep Kit FWD for Illumina (Lexogen, Vienna, Austria) for 7 suitable RNA samples out of 8. Libraries were sequenced using an Illumina NextSeq500 (Cribi, UniPD, Padova, Italy) with a 75 single end read layout.

2.4. RNA sequencing data analysis

The raw Illumina reads were trimmed for quality using *trimmomatic* (Bolger et al., 2014), setting a minimal Phred quality of 25 and removing the sequencing adaptors. FASTQ files were imported in the CLC Genomic Workbench v.21 (Qiagen, Hilden, Denmark) and analyzed as follows. To identify differentially expressed genes (DEGs) the trimmed reads were mapped on the human reference genome (hg19, Ensembl v.99) applying the following parameters: Mismatch cost = 2; Insertion cost = 3; Deletion cost = 3; Length fraction = 0.8; Similarity fraction = 0.8 and adopting a "forward" strand specificity and expression values were counted as Read Per Kilobase of Mapped reads (RPKM). A Baggerley test with false discovery rate (FDR) p-value correction was applied to identify differentially expressed genes (DEGs), setting a cutoff of 2-Fold changes (FC) and a 0.01 of FDR p-value. A GO-enrichment analysis

based on Uniprot gene ontology (goa_human_20200423_hg19) was performed, removing hits with mean RPKM below 5.0, absolute fold change below 2 and with an FDR p-value higher than 0.01. Raw RNA-sequencing data were deposited at the NCBI SRA archive with accession ID PRJNA732263.

3. Results

Three post-mortem nasopharyngeal swabs of corneal donors were detected positive for genomic SARS-CoV-2 RNA (Ferrari et al., 2020). The presence of SARS-CoV-2 RNA was tested in the explanted corneas and two tissues were found positive (Donor 1 and 3, Table 1), whereas SARS-CoV-2 RNA was found in the conservative media of one cornea only (Donor 3, right cornea). We exploited the remaining parts of the explanted corneas, mostly referring to the corneoscleral ring, to perform RNA extraction and transcriptome analysis (RNA-sequencing), to identify differentially expressed genes in comparison to two control samples of a non-infected individual processed similarly (CTRL) (Table 1). The hardness of the corneoscleral tissue required a strong homogenization step and this probably contributed to the overall low RNA quality. We obtained a total of 0.26–2.1 µg of RNA per sample with RNA integrity values (RIN) below 5, whereas for one sample (D3_L) the RNA quality was not sufficient to proceed with library preparation. The absence of contaminating DNA was achieved through a DNase step and verified using DNA- and RNA-specific quantifications (Table 1). qRT-PCR revealed the presence of SARS-CoV-2 RNA only in two of the extracted RNAs (D3_R and D1_L) (Table 1), whereas sub-genomic SARS-CoV-2 RNAs was undetectable in all the samples. Illumina sequencing yielded 29.7 million (M) of high-quality reads (3.4–5.5 M reads per sample). As much as 1,850 reads of sample D3_R mapped to the 3'-end of the SARS-CoV-2 genome, whereas SARS-CoV-2 reads were not present in the other samples.

3.1. Corneoscleral gene expression profile by RNA-sequencing

Principal Component Analysis (PCA) based on the whole gene expression profiles grouped the 7 samples in 3 clusters according to the presence of SARS-CoV-2 RNAs in cornea or corneoscleral tissues, with the positive donors Donor 1, 2 and 3 clustered together on the PC1 axis (explaining 26.5% of the variation) and the control samples being more distributed on the PC2 axis (18.9% of the variation, Fig. 1). The first cluster included the samples of Donor 2 (positive donor with undetected SARS-CoV-2, PD-SARS-neg), the second cluster included samples of positive donors with detected SARS-CoV-2 in the eye (PD-SARS-pos), whereas the third cluster included CTRL samples. The comparison of gene expression profiles of these 3 clusters revealed 816 Differentially Expressed Genes (DEGs) (Fig. 2 and Supplementary Tables 1) and 462 DEGs comparing PD-SARS-neg 1 vs. CTRL and 235 DEGs comparing PD-SARS-pos vs. CTRL. The 114 DEGs common to these two comparisons

Table 1

Sample description and sequencing results. The donor code, cause of death, post-mortem sampling time and the results of the nasopharyngeal swab, detection of SARS-CoV-2 RNA in the cornea, conservative medium and corneoscleral ring were reported. The presence of SARS-CoV-2 was evaluated in the cornea and in the corneoscleral RNAs. Total obtained RNA in micrograms, the sequencing library ID and number of high-quality reads were also indicated per sample.

CODE	DONOR	CAUSE OF DEATH	POST-MORTEM TIME	RESULT OF NS	SARS-CoV-2 in cornea	SARS-CoV-2 in conservative medium	SARS-CoV-2 in CR	RNA (µg)	HQ reads
D1_L	Donor 1	Lung cancer	4h 25m	positive	Detected	n.d.	Detected (30)	2,17	6,641,526
D1_R					n.d.	n.d.	n.d.	1,27	7,016,032
D2_L	Donor 2	Prostate cancer	15h 55m	positive	n.d.	n.d.	n.d.	0,52	6,158,830
D2_R					n.d.	n.d.	n.d.	0,67	6,336,917
D3_L	Donor 3	Glioblastoma	8h 40m	positive	n.d.	n.d.	n.d. (33)	\	\
D3_R					Detected	Detected	Detected (29)	0,26	9,731,146
C1_L	Control	Lung cancer	3h 30m	negative	n.d.	n.d.	n.d.	0,80	7,531,443
C1_R	-Donor				n.d.	n.d.	n.d.	0,53	7,171,372

D = donor; C = control; CR = corneoscleral ring; NS = nasopharyngeal swab; n.d. = not detected.

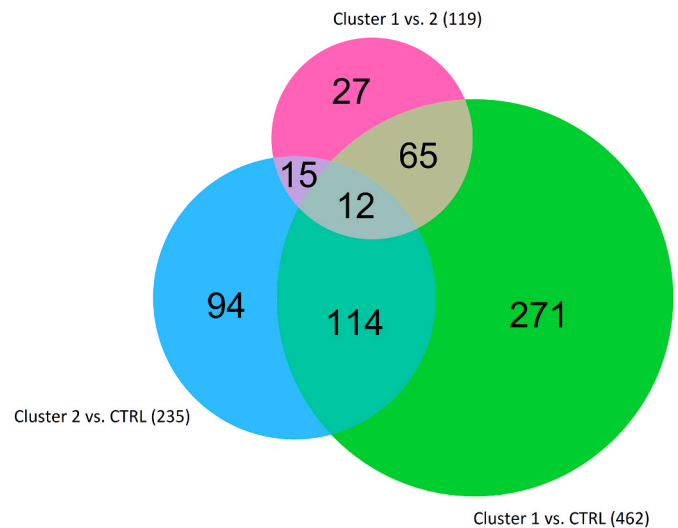


Fig. 1. Principal Component Analysis based on whole transcriptome expression data. The samples labeled in blue are negative controls, whereas the ones labeled in red refer to positive donors. Triangles indicate samples negative or not-detected for SARS-CoV-2, whereas dots are samples with SARS-CoV-2 detected in the cornea and in the corneoscleral tissue (D1-D3). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(Fig. 2 and Supplementary Table 1) included several genes associate to the Interferon pathway, like ADAMTS4 (5.9x and 8.9x of log2 Fold Change, FC in the first and second comparison, respectively), RSAD2 (4.4x and 6.2x), MMP1 (7.5x and 63x), IL6 (5.9x and 7.6x) and ISG15 (5x and 5.5x). Proinflammatory cytokines like CXCL1, 2, 3, 5 and 6, and IL23a were also upregulated (Supplementary Table 1). A total of 12 DEGs were common to all the three comparisons (ADAMTS4, CSRN1, NFKBIZ, TM4SF1, CXCL2, IRF1, CCDC71L, CTSL, NINJ1, TNFAIP2, SOCS3 and PLAUR) (Figs. 2 and 3 and Supplementary Table 1) and are characterized by incremental expression values, starting from CTRL, PD-SARS-pos and PD-SARS-neg samples. Among the down-regulated genes we found AQP5 (−4.8x), ANK3 (−2.2x) and KRT19 (−3.6x). Only in the comparison between PD-SARS-neg samples vs. CTRL, intracellular dsRNA receptors appeared upregulated, such as MDA5 (3x), RIG-I (3x) and ZNFX1 (2.9x).

Using a hypergeometric test performed on the Gene Ontology (GO) Biological processes annotations, we reported 77 GO terms over-represented comparing PD-SARS-neg vs. CTRL, and 17 GO terms comparing PD-SARS-pos vs. CTRL, whereas 5 GO terms differentiated PD-SARS-pos vs. PD-SARS-neg samples (Supplementary Table 2). A total of 8 common GO terms, including 159 non-redundant genes, are detectable when comparing PD vs. CTRL samples, and included *response*

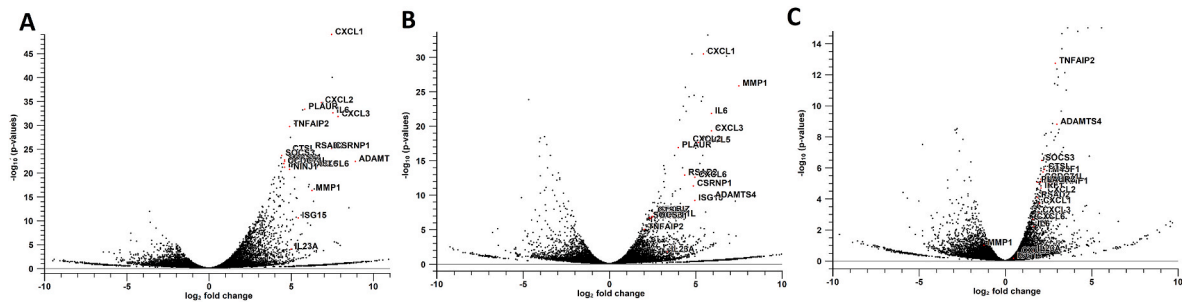


Fig. 2. Volcano plot depicting gene expression and FDR-corrected p-values computed comparing PD-SARS-neg vs. CTRL samples (A), PD-SARS-pos vs. CTRL sample (B) and PD-SARS-neg vs. PD-SARS-pos samples (C). A selection of genes discussed in the present paper is highlighted by red dots their names are reported on the graphs.

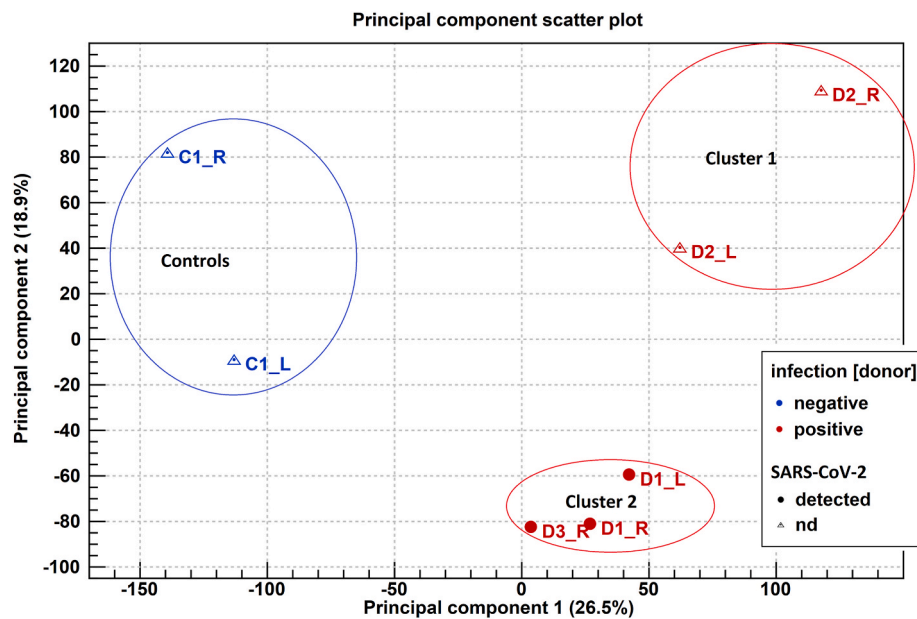


Fig. 3. Venn diagram depicting exclusive and common DEGs in the comparisons between cluster1 (positive donors with undetected SARS-CoV-2), cluster 2 (positive donors with detected SARS-CoV-2) and controls (CTRL).

to chemical, response to organic substance, response to stress, cytokine-mediated signaling pathway, cellular response to chemical stimulus, response to stimulus, response to biotic stimulus (Supplementary Table 2). If considered alone, these 159 genes clustered the seven RNA-sequencing datasets according to the SARS-CoV-2 infection status and not according to the donor origin, indicating that they displayed incremental expression levels from control to PD-SARS-pos and with the highest expression levels in PD-SARS-neg (Supplementary Fig. 1).

3.2. RNA editing enzymes are poorly regulated by SARS-CoV-2 infection

Since in our previous study we found high basal expression levels of editing enzymes in healthy corneal and conjunctival tissues (Leonardi

et al., 2020), here we investigated if the expression levels of the double-stranded RNA-specific adenosine deaminase (ADAR-1) and apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC) genes in PD vs. CTRL samples are modified. We retrieved detectable expression levels (>3 TPMs) for ADAR1, ADARB2 and ADARB1 and for APOBEC3A, C and G. ADAR1, ADARB2 and APOBEC3A reached considerable expression levels in some samples, often exceeding 100 TPMs, although only ADAR1 and APOBEC3A appeared somewhat upregulated in SARS-CoV-2 infected samples (Table 2).

4. Discussion

The host response to SARS-CoV-2 extends beyond the typical

Table 2

RNA editing enzymes. For ADAR and APOBEC3 genes, the fold change (FC) and corresponding p-value are reported for each comparison, plus the Mean expression levels per individual.

Name	Description	D1_L	D1_R	D3_R	D2_L	D2_R	C1_R	C1_L
ADAR	dsRNA-specific adenosine deaminase	90,7	66,8	141,6	77,6	98,5	67,9	54,4
ADARB2	dsRNA-specific editase B2	47,8	93,0	84,2	108,6	102,4	397,4	301,4
ADARB1	dsRNA-specific editase 1	38,1	20,3	67,9	43,0	32,6	46,5	58,6
APOBEC3A	DNA dC- > dU-editing enzyme APOBEC-3A	57,9	50,1	39,8	144,3	260,1	41,5	17,3
APOBEC3C	DNA dC- > dU-editing enzyme APOBEC-3C	25,8	21,5	35,6	16,3	6,0	42,2	42,5
APOBEC3G	DNA dC- > dU-editing enzyme APOBEC-3G	3,2	30,4	25,5	9,4	0,8	8,6	32,9

respiratory tract in several organs distal to the site of infection. As observed in cell culture and animal models, it is clear that the host response to SARS-CoV-2 results in an early aberrant IFN response juxtaposed with an overproduction of chemokines (Blanco-Melo et al., 2020). This response is defined by low levels of type I and III interferons with a moderate IFN-stimulated genes (ISGs), contrasted by elevated expression levels of chemokines and IL-6.

It is unclear if non-respiratory organs, such as the ocular surface, can contrast SARS-CoV-2 infection because of a potential heightened immunity of these tissues. This tissue, which expressed SARS-CoV-2 receptors such as ACE-2 and associated protease TMPRSS2, often showed undetectable levels of SARS-CoV-2 and mild possible associable symptoms, even in severe COVID-19 cases (Rokohl et al., 2020).

To investigate the ocular surface involvement in COVID-19 and to further investigate potential implications in corneal transplantation, we produced RNA sequencing data of the corneoscleral ring tissue, based on five SARS-CoV-2 positive and two negative samples. RNA-sequencing expression profiles clustered these seven samples firstly according to the SARS-CoV-2 infection of the donors and subsequently by the presence of SARS-CoV-2 RNAs either in the cornea or in the corneoscleral ring. We can suppose that these corneoscleral ring expression profiles are stimulated by punctual intakes of SARS-CoV-2 mediated by hand touching or viral particles in aerosols (Dawood, 2021). An alternative hypothesis is that the secretion or spillover of sub genomic material, forming a protected ribonucleoprotein complex, may be a prevalent PAMP that is disseminated from the primary site of replication (Hoagland et al., 2021). This will indicate that the systemic response promoted by SARS-CoV-2 infection is strong enough to consistently modulate gene expression profiles in distal tissues, such as the eye, making it a responsive organ more than a secret place to hide the virus (Rokohl et al., 2020, 2021).

The presence of different expression profiles between SARS positive donors with RNA-positive cornea and SARS positive donors with RNA-negative cornea may also suggest different infection stages. The higher number of DEGs in PD-SARS-neg and the upregulation of dsRNA sensors exclusively in these samples suggested that an antiviral response is mounting, possibly after a recent viral uptake. Notably, the zinc-finger helicase ZNFX1, which was recently reported as an early dsRNA sensor able to boost the antiviral response by stimulating ISGs (Wang et al., 2019), resulted highly expressed and induced in PD-SARS-neg samples. ZNFX1 was reported as negatively correlated with SARS-CoV-2 loads, and to be possibly involved in the early detection of this virus promoting an immediate antiviral response (Qin et al., 2021). Arguably, ZNFX1 and other helicases have been shown to share short peptides with coronavirus genomes, with possible outcomes in virus-induced autoinflammatory conditions (Venkatakrishnan et al., 2020). The role of ZNFX1 in the eye is unknown and will require further dedicated studies.

Our preliminary data suggests that a cytokine response is mounted by SARS-CoV-2 in the eye, as previously reported in other tissues *in-vivo* and also in *in-vitro* infection models (Song et al., 2020; Desai et al., 2020).

Besides the activation of well-studied ISGs, several proinflammatory cytokines, such as CXCL1, 2, 3, 5 and 6, IL-6 and IL-23a were also upregulated together with the over-representation of the GO term *cytokine-mediated signaling pathway* in both SARS positive donors with RNA-positive or RNA-negative cornea, is in line with the hypothesis that a substantial host response can be mounted also in an organ at the distal site of infection or just as a consequence of a spillover of SARS sub-genomic material. If this response may be responsible of the low prevalence of ocular surface clinical manifestation in COVID-19 patients remains to be elucidated.

Many of the genes related to normal limbus functions, including cytokeratins, mucins, aquaporins, and tight junctions, have shown to be suppressed in the limbus of infected donors (Eriksen et al., 2021). In our study, the under-regulation of aquaporin 5 (ACQ5) in infected

tissues seems particularly interesting for the ocular surface. In a recent systematic review and meta-analysis including a total of 8,219 COVID-19 patients, dry eye or foreign body sensation were the most common reported ocular symptoms (16%) (Nasiri et al., 2021). The reason for the high frequency of dry eye symptoms has been explained by the use face masks and the increased tear evaporation due to the expiratory air current toward eyes. Since AQP5 is strongly expressed in the normal cornea playing a role in corneal hydration (Verkman et al., 2008) and a AQP5 defect has been associated to dry eye in Sjögren's syndrome (Tsubota et al., 2001), the decreased expression of AQP5 in infected corneas could be a molecular explanation of the dry eye symptoms in COVID-19 patients.

Arguably, negative-feedback mechanisms are required to avoid cellular damages because of excessive production of antiviral proteins (Lamers et al., 2019). Hyper-editing of endogenous dsRNAs performed by the enzymes of the ADAR family can reduce dsRNA detection by cytoplasmic receptors such as RIG-I, MDA5 and ZNFX1 and the consequent activation of the immune system (Lamers et al., 2019; George et al., 2016; Liddicoat et al., 2016). We previously reported a high basal expression levels of RNA editing enzymes on the ocular surface (Leonardi et al., 2020, 2021). Abundant C-to-U variations traced on SARS-CoV-2 RNAs and along SARS-CoV-2 evolution (Wang et al., 2020) are indicative of the activity of *apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like* (APOBEC), whereas less frequent A-to-G variations marked the activity of *double-stranded RNA-specific adenosine deaminase* (ADAR-1) (Di Giorgio et al., 2020). Although these RNA editing enzymes appeared poorly modulated in our samples, the determination of presence and possible modulation of post-transcriptional modifications during infection will require dedicated studies, since our 3'-RNA-sequencing are not suitable to detect RNA editing events.

5. Conclusions

We reported gene expression profiles of corneoscleral samples from SARS-CoV-2-positive individuals. The modulation of Interferon responsive genes suggested a good responsiveness of the ocular tissues to SARS-CoV-2, either mediated by a continuous input of viral particles from the micro-environment of infected individuals or by a systemic response. The detection of early response genes, such as ZNFX1 and other dsRNA receptors in PD-SARS-neg samples suggested a temporal diversification of the analyzed samples with possible outcomes in the restriction of infection. The presence of SARS-CoV-2 reads in one corneoscleral sample supported the possibility that the eye acts as an entry route, although additional studies will be necessary to understand the resilience of ocular tissues to the viral infection. The use of Nanopore direct RNA-sequencing aimed to detect gene expression levels together with RNA modifications at single base resolution (Harel et al., 2019) might disentangle the role of RNA editing enzymes during SARS-CoV-2 infection.

Commercial relationship

None.

Source of funding

Supported by MIUR DOR1952345/19 and DOR2092417/20 from Italian Institute of Health.

Declaration of competing interest

Authors have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.exer.2022.108975>.

[org/10.1016/j.exer.2022.108975](https://doi.org/10.1016/j.exer.2022.108975).

References

- Arora, R., et al., 2021. Evaluation of SARS-CoV-2 in tears of patients with moderate to severe COVID-19. *Ophthalmology* 128, 494–503. <https://doi.org/10.1016/j.ophtha.2020.08.029>.
- Blanco-Melo, D., et al., 2020. Imbalanced host response to SARS-CoV-2 drives development of COVID-19. *Cell* 181, 1036–1045. <https://doi.org/10.1016/j.cell.2020.04.026> e9.
- Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*. *Oxf. Engl. J.* 30, 2114–2120. <https://doi.org/10.1093/bioinformatics/btu170>.
- Casagrande, M., et al., 2021. Presence of SARS-CoV-2 RNA in the cornea of viremic patients with COVID-19. *JAMA Ophthalmol* 139, 383–388. <https://doi.org/10.1001/jamaophthalmol.2020.6339>.
- Cheema, M., et al., 2020. Keratoconjunctivitis as the initial medical presentation of the novel coronavirus disease 2019 (COVID-19). *Can. J. Ophthalmol. J. Can. Ophthalmol.* 55, e125–e129. <https://doi.org/10.1016/j.cjco.2020.03.003>.
- Collin, J., et al., 2021. Co-expression of SARS-CoV-2 entry genes in the superficial adult human conjunctival, limbal and corneal epithelium suggests an additional route of entry via the ocular surface. *Ocul. Surf.* 19, 190–200. <https://doi.org/10.1016/j.jtos.2020.05.013>.
- Dawood, A.A., 2021. Transmission of SARS CoV-2 virus through the ocular mucosa worth taking precautions. *Vacunas Engl. Ed.* 22, 56–57. <https://doi.org/10.1016/j.vacune.2021.01.007>.
- Deng, W., et al., 2020. Ocular conjunctival inoculation of SARS-CoV-2 can cause mild COVID-19 in rhesus macaques. *Nat. Commun.* 11, 4400. <https://doi.org/10.1038/s41467-020-18149-6>.
- Desai, N., et al., 2020. Temporal and spatial heterogeneity of host response to SARS-CoV-2 pulmonary infection. *Nat. Commun.* 11, 6319. <https://doi.org/10.1038/s41467-020-20139-7>.
- Di Giorgio, S., Martignano, F., Torcia, M.G., Mattiuz, G., Conticello, S.G., 2020. Evidence for host-dependent RNA editing in the transcriptome of SARS-CoV-2. *Sci. Adv.* 6, eabb5813 <https://doi.org/10.1126/sciadv.abb5813>.
- Douglas, K.A.A., Douglas, V.P., Moschos, M.M., 2020. Ocular manifestations of COVID-19 (SARS-CoV-2): a critical review of current literature. *Vivo Athens Greece* 34, 1619–1628. <https://doi.org/10.21873/invivo.11952>.
- Eriksen, A.Z., et al., 2021. SARS-CoV-2 infects human adult donor eyes and hESC-derived ocular epithelium. *Cell Stem Cell* 28, 1205–1220. <https://doi.org/10.1016/j.stem.2021.04.028> e7.
- Fernandes, J.D., et al., 2020. The UCSC SARS-CoV-2 genome browser. *Nat. Genet.* 52, 991–998. <https://doi.org/10.1038/s41588-020-0700-8>.
- Ferrari, S., et al., 2021. Absence of severe acute respiratory syndrome coronavirus 2 RNA in human corneal donor tissues: implications for transplantation. *Cornea* 40, e3. <https://doi.org/10.1097/ICO.0000000000002594>.
- Ferrari, S., et al., 2020. Detection of severe acute respiratory syndrome coronavirus 2 in corneas from asymptomatic donors. *Acta Ophthalmol.* <https://doi.org/10.1111/aos.14692>.
- George, C.X., Ramaswami, G., Li, J.B., Samuel, C.E., 2016. Editing of cellular self-RNAs by adenosine deaminase ADAR1 suppresses innate immune stress responses. *J. Biol. Chem.* 291, 6158–6168. <https://doi.org/10.1074/jbc.M115.709014>.
- Grajewski, R.S., et al., 2021. A missing link between SARS-CoV-2 and the eye?: ACE2 expression on the ocular surface. *J. Med. Virol.* 93, 78–79. <https://doi.org/10.1002/jmv.26136>.
- Harel, N., Meir, M., Gophna, U., Stern, A., 2019. Direct sequencing of RNA with MinION Nanopore: detecting mutations based on associations. *Nucleic Acids Res.* <https://doi.org/10.1093/nar/gkz907>.
- Hoagland, D.A., et al., 2021. Leveraging the antiviral type I interferon system as a first line of defense against SARS-CoV-2 pathogenicity. *Immunity* 54, 557–570. <https://doi.org/10.1016/j.immuni.2021.01.017> e5.
- Inomata, T., et al., 2020. Clinical and prodromal ocular symptoms in coronavirus disease: a systematic review and meta-analysis. *Invest. Ophthalmol. Vis. Sci.* 61 <https://doi.org/10.1167/iovs.61.10.29>, 29–29.
- Lam, T.T.-Y., et al., 2020. Identifying SARS-CoV-2-related coronaviruses in Malayan pangolins. *Nature* 583, 282–285. <https://doi.org/10.1038/s41586-020-2169-0>.
- Lamers, M.M., van den Hoogen, B.G., Haagmans, B.L., 2019. ADAR1: ‘editor-in-chief’ of cytoplasmic innate immunity. *Front. Immunol.* 10, 1763. <https://doi.org/10.3389/fimmu.2019.01763>.
- Leonardi, A., et al., 2021. Antiviral response in vernal keratoconjunctivitis may be protective against COVID-19. *Allergy*. <https://doi.org/10.1111/all.15048>.
- Leonardi, A., Rosani, U., Brun, P., 2020. Ocular surface expression of SARS-CoV-2 receptors. *Ocul. Immunol. Inflamm.* 28, 735–738. <https://doi.org/10.1080/09273948.2020.1772314>.
- Liddicoat, B.J., Chalk, A.M., Walkley, C.R., 2016. ADAR1, inosine and the immune sensing system: distinguishing self from non-self. *Wiley Interdiscip. Rev. RNA*. 7, 157–172. <https://doi.org/10.1002/wrna.1322>.
- Ma, R., et al., 2021. High expression of SARS-CoV-2 entry factors in human conjunctival goblet cells. *Exp. Eye Res.* 205, 108501. <https://doi.org/10.1016/j.exer.2021.108501>.
- Mencucci, R., et al., 2021. Co-expression of the SARS-CoV-2 entry receptors ACE2 and TMPRSS2 in healthy human conjunctiva. *Exp. Eye Res.* 205, 108527. <https://doi.org/10.1016/j.exer.2021.108527>.
- Miner, J.J., et al., 2020. HSV-1 and Zika virus but not SARS-CoV-2 replicate in the human cornea and are restricted by corneal type III interferon. *Cell Rep.* 33, 108339. <https://doi.org/10.1016/j.celrep.2020.108339>.
- Nasiri, N., et al., 2021. Ocular manifestations of COVID-19: a systematic review and meta-analysis. *J. Ophthalmic Vis. Res.* 16, 103–112. <https://doi.org/10.18502/jovr.v16i1.8256>.
- Qin, S., et al., 2021. Analyzing master regulators and scRNA-seq of COVID-19 patients reveals an underlying anti-SARS-CoV-2 mechanism of ZNF proteins. *Briefings Bioinform.* <https://doi.org/10.1093/bib/bbab118>.
- Rokohl, A.C., et al., 2020. More than loss of taste and smell: burning watering eyes in coronavirus disease 2019. *Clin. Microbiol. Infect. Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis.* 26 <https://doi.org/10.1016/j.cmi.2020.08.018>, 1560.e5–1560.e8.
- Rokohl, A.C., et al., 2021. No secret hiding place? Absence of SARS-CoV-2 on the ocular surface of 1145 hospitalized patients in a pandemic area. *Graefes Arch. Clin. Exp. Ophthalmol. Albrecht Von Graefes Arch. Klin. Exp. Ophthalmol.* 259, 1605–1608. <https://doi.org/10.1007/s00417-021-05086-3>.
- Scalinci, S.Z., Trovato Battagliola, E., 2020. Conjunctivitis can be the only presenting sign and symptom of COVID-19. *IDCases* 20, e00774. <https://doi.org/10.1016/j.idcr.2020.e00774>.
- Song, P., Li, W., Xie, J., Hou, Y., You, C., 2020. Cytokine storm induced by SARS-CoV-2. *Clin. Chim. Acta Int. J. Clin. Chem.* 509, 280–287. <https://doi.org/10.1016/j.cca.2020.06.017>.
- Tsubota, K., Hirai, S., King, L.S., Agre, P., Ishida, N., 2001. Defective cellular trafficking of lacrimal gland aquaporin-5 in Sjögren’s syndrome. *Lancet Lond. Engl.* 357, 688–689. [https://doi.org/10.1016/S0140-6736\(00\)04140-4](https://doi.org/10.1016/S0140-6736(00)04140-4).
- Turjya, R.R., Khan, MdA-A-K, Mir, Md, Khademul Islam, A.B., 2020. Perversely expressed long noncoding RNAs can alter host response and viral proliferation in SARS-CoV-2 infection. *Future Virol.* 15, 577–593. <https://doi.org/10.2217/fvl-2020-0188>.
- Venkatakrishnan, A.J., et al., 2020. Benchmarking evolutionary tinkering underlying human-viral molecular mimicry shows multiple host pulmonary-arterial peptides mimicked by SARS-CoV-2. *Cell Death Dis.* 6, 1–14. <https://doi.org/10.1038/s41420-020-00321-y>.
- Verkman, A.S., Ruiz-Ederra, J., Levin, M.H., 2008. Functions of aquaporins in the eye. *Prog. Retin. Eye Res.* 27, 420–433. <https://doi.org/10.1016/j.preteyeres.2008.04.001>.
- Wan, K.H., Chow, V.W.S., Lam, D.S.C., 2021. Risk of SARS-CoV-2 transmission via corneal transplant from donors with COVID-19. *JAMA Ophthalmol.* <https://doi.org/10.1001/jamaophthalmol.2021.2148>.
- Wang, R., Hozumi, Y., Zheng, Y.-H., Yin, C., Wei, G.-W., 2020. Host immune response driving SARS-CoV-2 evolution. *Viruses* 12. <https://doi.org/10.3390/v12101095>.
- Wang, Y., et al., 2019. Mitochondria-localised ZNF1 functions as a dsRNA sensor to initiate antiviral responses through MAVS. *Nat. Cell Biol.* 21, 1346–1356. <https://doi.org/10.1038/s41556-019-0416-0>.
- Wölfel, R., et al., 2020. Virological assessment of hospitalized patients with COVID-2019. *Nature* 581, 465–469. <https://doi.org/10.1038/s41586-020-2196-x>.
- Wu, P., et al., 2020. Characteristics of ocular findings of patients with coronavirus disease 2019 (COVID-19) in Hubei province, China. *JAMA Ophthalmol* 138, 575–578. <https://doi.org/10.1001/jamaophthalmol.2020.1291>.
- Zhang, X., et al., 2020. The evidence of SARS-CoV-2 infection on ocular surface. *Ocul. Surf.* 18, 360–362. <https://doi.org/10.1016/j.jtos.2020.03.010>.
- Zhou, L., et al., 2020. ACE2 and TMPRSS2 are expressed on the human ocular surface, suggesting susceptibility to SARS-CoV-2 infection. *Ocul. Surf.* 18, 537–544. <https://doi.org/10.1016/j.jtos.2020.06.007>.