Effect of space flight on the behavior of human retinal pigment epithelial ARPE-19 cells and evaluation of coenzyme Q10 treatment

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Abstract
Astronauts on board the International Space Station (ISS) are exposed to the damaging effects of microgravity and cosmic radiation. One of the most critical and sensitive districts of an organism is the eye, particularly the retina, and > 50% of astronauts develop a complex of alterations designated as spaceflight-associated neuro-ocular syndrome. However, the pathogenesis of this condition is not clearly understood. In the current study, we aimed to explore the cellular and molecular effects induced in the human retinal pigment ARPE-19 cell line by their transfer to and 3-day stay on board the ISS in the context of an experiment funded by the Agenzia Spaziale Italiana. Treatment of cells on board the ISS with the well-known bioenergetic, antioxidant, and antiapoptotic coenzyme Q10 was also evaluated. In the ground control experiment, the cells were exposed to the same conditions as on the ISS, with the exception of microgravity and radiation. The transfer of ARPE-19 retinal cells to the ISS and their living on board for 3 days did not affect cell viability or apoptosis but induced cytoskeleton remodeling consisting of vimentin redistribution from the cellular boundaries to the perinuclear area, underlining the collapse of the network of intermediate vimentin filaments under unloading conditions. The morphological changes endured by ARPE-19 cells grown on board the ISS were associated with changes in the transcriptomic profile related to the cellular response to the space environment and were consistent with cell dysfunction adaptations. In addition, the results obtained from ARPE-19 cells treated with coenzyme Q10 indicated its potential to increase cell resistance to damage.

Keywords Retina · Microgravity · Radiation · Retinopathy · Space flight

Introduction
Space radiation and microgravity present in spacecraft and space stations in orbit around the Earth cause several time-dependent health alterations in astronauts both during their missions and after their return to Earth. The most well-known are fluid shifts, loss of bone and muscle mass, cardiovascular imbalances, alterations in immunity, sleep interruption, and circadian misalignment, which have been described for more than two decades in a multitude of scientific reports and reviews [1–6]. At the cellular level, the main pathological effects of microgravity and cosmic radiation...
are structural alterations caused by free radical-mediated molecular damage [7–10].

Space exploration has now entered a new phase in which NASA, ESA, and other national space agencies are working together to plan long-term space missions in order, for example, to create lunar bases and reach other space destinations such as Mars. At this stage, assessing the risk inherent in space missions and challenging the numerous obstacles to astronaut safety are increasingly important in mission planning. In particular, since the microgravity and ionizing radiation inevitably present in the spacecraft environment represent two serious stress factors for astronaut health, the discovery of pharmacological countermeasures is clearly a mandatory prerequisite for mission planning [11, 12]. A basic principle of pharmacological research is that the most valid rational basis for identifying tools capable of blocking or inhibiting a pathological process is knowledge of its pathogenic mechanisms. This principle applies to the range of damage and health impairments known to be endured by astronauts during their lengthy space missions.

The eye, particularly the retina, is severely sensitive to radiation and microgravity damage, which eventually cause apoptotic cell death [9, 10, 13–18]. Approximately 10 years ago, NASA reported that nearly 60% of 300 astronauts returning to Earth after long-term stay aboard the International Space Station (ISS) were affected by several neuro-ophthalmic alterations, including optic disc edema, globe flattening, choroidal and retinal folds, hyperopic refractive error shifts, nerve fiber layer infarcts, and visual acuity reduction [19], comprehensively termed spaceflight associated neuro-ocular syndrome (SANS) [20–23]. The pathogenesis of SANS is not fully understood. The relevance of astronauts’ visual impairment led NASA to consider vision as one of the top health risks for long-term space flight [24].

The ISS, where astronauts are exposed to the space environment for several months [25], offers a great opportunity to directly study astronaut health impairments, but also to execute experiments aimed at unraveling the pathogenesis of damage even at the molecular level and to test the efficacy of potential countermeasures to minimize damage and therefore maximize the feasibility of space missions.

In this paper, we aimed to explore the impact exerted on both the cell structure and on the transcriptomic profile by the transfer of human retinal pigment epithelial ARPE-19 cells (ATCC, Manassas, VA, USA) were cultured in 50% Dulbecco’s modified Eagle’s medium (DMEM, Lonza, Basel, CH) and 50% Ham’s F12 Medium (Lonza) supplemented with 10% fetal bovine serum (FBS; # 35–015-CV; Corning, NY, USA), 100 U/mL penicillin–streptomycin (Lonzas), and 2 mM L-glutamine (Lonza) in a humidified incubator at 37 °C and 5% CO₂. ARPE-19 cells were frozen and shipped to the Kennedy Space Center (KSC) Space Station Processing Facility (SSPF) laboratories (Cape Canaveral, Florida, USA) months before the scheduled launch date. Two weeks before launch, the cells were thawed and cultured in the KSC laboratories. For the experiment on board the ISS, ARPE-19 cells were seeded at a density of 20,000 cells/cm² on cell culture supports, i.e., cyclic olefin copolymer (COC) ibiTreat plastic coverslips (IBIDI, Martinsried, Germany), which allow cell adhesion that is comparable to standard cell culture flasks, flexibility, and good optical performance. Dedicated experimental units (EUs, KEU-ST, Kayser Italia, Livorno, Italy), which are electromechanical devices capable of executing cell culture protocols and are qualified for flights to the ISS, were used for the experiment on board the ISS (Fig. 1A–B). EUs contain five reservoirs: three were loaded with cell culture media (50% DMEM, 50% Ham’s F12, 10% FBS, 100 U/mL Penicillin–Streptomycin, 2 mM L-Glutamine, 25 mM Heps [Lonza]) in the presence or absence of 10 µM CoQ10 (Sigma-Aldrich, St. Louis, USA) dissolved in a commercial vehicle to ensure cellular uptake (0.04% Kolliphor P407 micro, Sigma-Aldrich); one with Dulbecco’s phosphate-buffered saline (DPBS, added with Ca²⁺/Mg²⁺, Lonza) for cell rinse prior to fixation; one with RNAlater (Ambion AM7020, MA, USA) as a fixative solution. At day 1 after seeding, cell cultures on COC plastic coverslips were transferred into the cell culture chambers of the EUs, which were then introduced into Experiment Containers KIC-SL to compose the experimental hardware (EH). EHS were inserted into a passive temperature-controlled transportation.
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for 10 min. The samples were analyzed using a confocal microscope (Nikon TE2000 using EZ-C1 Software; Nikon Corp.) equipped with a 60XA/1.40 oil-immersion objective and digitally captured.

Immunofluorescence

Cells grown on plastic coverslips and treated as previously described were fixed for 5 min with ice-cold acetone. Unspecific binding sites were blocked with PBS containing 3% bovine serum albumin (BSA; Sigma-Aldrich) for 1 h at 22 ± 2 °C. The cells were then incubated overnight at 4 °C with anti-vimentin antibody (#MAB1681, Chemicon, Merck KGaA, Darmstadt, Germany) diluted 1:100 in PBS with 0.5% BSA. After washing three times with PBS-0.5% BSA, samples were incubated for 1 h at 22 ± 2 °C in the dark with fluorescein isothiocyanate-conjugated secondary antibody (anti-mouse IgM [#AP128F, Chemicon] diluted 1:200). The samples were washed three times, mounted on glass slides using Fluoromount™ aqueous mounting medium (Sigma-Aldrich), evaluated using an epifluorescence microscope (Nikon, Firenze, Italy) at 100× magnification, and imaged using a HiRes IV digital CCD camera (DTA, Pisa, Italy).

RNA isolation

RNA extraction was performed using the RNAqueous Total RNA Isolation kit (Ambion AM1912) following the manufacturer’s instructions, and 40 μL RNA was eluted. The samples were vacuum-concentrated using DNA 120 Speedvac® (ThermoSavant, USA) with low vacuum and no heating to avoid RNA fragmentation, and the samples were manually checked every 5 min until the volume reached 20 μL. Residual genomic DNA was removed using a DNA-free DNA Removal kit (Ambion AM1906) following the manufacturer’s instructions. Isolated RNA and library preparation products were quantified using Qubit 3.0 (Invitrogen, USA) and quality was assessed by capillary electrophoresis using an AATI Fragment Analyzer (Advanced Analytical Technologies, Inc., USA).

Gene expression analysis

RNA expression was quantified at Polo d’Innovazione di Genomica, Genetica e Biologia (Polo GGB, Siena, Italy) using RNAseq analysis. Total ribo-depleted cDNA libraries were prepared using SMARTer Stranded Total RNA-Seq Kit Pico Input Mammalian (Takara Bio USA, Inc.) according to the manufacturer’s instructions. Indexed DNA libraries were normalized to a concentration of 4 nM and then pooled in equal volumes to obtain a uniform read distribution. The pool was denatured and diluted to a final loading concentration of 1.4 pM according to the Illumina protocol, with the addition of 1% spike in PhiX Control v3 (Illumina, USA) as a sequencing control. To obtain a minimum of 20 million reads per sample, the pool was sequenced using a NextSeq 550 sequencer (Illumina) using a NextSeq 500/550 Mid Output v2 kit (150 cycles) (Illumina) to perform a paired-end run 2X75 bp read length.

Raw reads for each sample (three treated vs. three untreated replicates) were aligned to the human_g1k_v37 release of the GRch37/hg19 human reference genome using the RNA-seq aligner STAR [30] (version 2.5.2b) with the default parameter settings. Count-based differential expression analysis was performed using featureCounts [31] and DESEQ2 [32]. In detail, we counted the reads mapped to each genomic meta-feature (that is, each gene) with featureCounts (version 1.5.1), using the default parameter settings and the Ensembl Gene Transfer Format (GTF) file for the appropriate reference genome (Ensembl release 82). The count matrix from featureCounts (where each row represents an Ensembl gene, each column is a sequenced RNA library, and the values indicate the raw numbers of fragments that were uniquely assigned to the respective gene in each library) was subsequently converted into a DESeqDataSet object inside the R statistical environment. The downstream differential expression analysis in the DESeq2 R package (release 3.3) uses a generalized linear model of the form K_\_j ~ NB(\_\_i_j, \alpha_j), where K counts (from the count matrix) for gene i and sample j are modeled using a negative binomial (NB) distribution with mean \_\_i_j and gene-specific dispersion \alpha_j, which defines the relationship between the variance of the observed count and its mean value. The estimation of the dispersion values for each gene as well as the fitting of the NB distribution were performed using the DESeq function, and the log fold change (LFC) estimates, p values, and adjusted p values (Wald statistic, treated vs. untreated) were extracted using the results function using the default parameter settings. To generate even more accurate LFC estimates, we eventually calculated shrunken LFC estimates (lfcShrink function, apeglm algorithm): LFC shrinkage uses LFC estimates for all genes to shrink toward the LFC estimates of genes with little information (low counts) or high dispersion, facilitating the downstream assessment of results.

Pathway analysis

The data were analyzed using the iPathwayGuide platform in the context of pathways obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Release 84.0 + /10–26, Oct 17), gene ontologies from the Gene Ontology (GO) Consortium database (2017-Nov6), miRNAs from the miRBase (Release 21), and TARGETSCAN (Targetscan version: Mouse:7.1, Human:7.1) databases. iPathwayGuide scores pathways using the impact analysis method [33], which uses two types of evidence: (i) the
over-representation of differentially expressed (DE) genes in a given pathway and (ii) the perturbation of that pathway computed by propagating the measured expression changes across the pathway topology. These aspects are captured by two independent probability values, pORA and pAcc, which are then combined in a unique pathway-specific p value. pORA expresses the probability of observing the number of DE genes in a given pathway that is equal to or greater than that observed by random chance. pAcc was calculated based on the amount of total gene accumulation measured in each pathway. Once the accumulations of all gene number perturbations are computed, iPathwayGuide computes the total accumulation of the pathway as the sum of all absolute accumulations of the genes in a given pathway. The two types of evidence, pORA and pAcc, are combined into one final pathway score by calculating a p value using Fisher's method, which is then rectified for multiple comparisons using false discovery rate (FDR) or Bonferroni correction. Bonferroni correction, which is simpler and more conservative than FDR, reduces the false discovery rate by imposing a stringent threshold on each comparison adjusted for the total number of comparisons. FDR correction has more power, but only controls the family-wise false positive rate.

**Transferring ARPE-19 cells to the ISS and culturing on board for 3 days did not affect cell proliferation or apoptosis, but severely modified vimentin organization**

Some key cellular parameters that we first evaluated after the flight are shown in Fig. 2. The transfer of ARPE-19 cells (treated or not treated with CoQ10) to the ISS and the 3 days of incubation on board had no impact on their proliferation rate compared to ground controls. In both cases, the cells almost reached confluence and did not show any signs of damage (Fig. 2A) or apoptosis, as assessed by the TUNEL assay (Fig. 2B). However, as indicated in the representative images of immunofluorescence microscopy analysis in Fig. 2C, the organization of the vimentin network underwent profound alterations, including a marked increase in its localization in the cellular perinuclear area and a concomitant redistribution from the cellular borders, which emphasized the collapse of the vimentin intermediate filament network.

**Transferring ARPE-19 cells to the ISS and culturing on board for 3 days dramatically modified the transcriptome profile**

We then explored the possibility that spaceflight alters gene expression by analyzing ARPE-19 transcriptome profiles with respect to ground controls using next-generation sequencing technology, assuming a threshold of 0.05, for statistical significance (p value) and a change in the expression of a log fold with an absolute value of at least 1. Of 23,556 genes analyzed, 5555 were DE after spaceflight with respect to the ground controls (Fig. 3A and Supplementary S1). Among them, 3081 were upregulated and 2474 were downregulated. To predict the impact of the ISS environment on ARPE-19 gene expression pathways, we used the iPathwayGuide to assess the possibility that a specific pathway was perturbed and that the subsequent genes in a pathway were significantly more perturbed than the previous ones. The results predicted that 99 pathways defined in the KEGG database were significantly affected (Fig. 3B and Supplementary S2). The first ten pathways are shown in Table 1, and pathway diagrams containing coherent cascades and DE pathway genes are presented in Supplementary S3A-I. The most significantly impacted pathways were related to cellular responses to space environment adaptation/damage. The analysis of DE genes associated with perturbed pathways revealed down-modulation of metabolic pathways, N-Glycan biosynthesis, protein processing in the endoplasmic reticulum, p53 signaling, cellular senescence, mitophagy, and steroid biosynthesis, and up-modulation of MAPK, TGF-beta, and Rap1 signaling (Fig. 3C). To further explore the functional roles of the DE genes, we performed...
GO analysis (Fig. 4 and Supplementary S4), which demonstrated that the transfer of ARPE-19 cells to the ISS and their culturing for 3 days on board caused an alteration in response to unfolded protein, stress response of the endoplasmic reticulum, and ion binding. The first five GO terms in biological processes, molecular functions, and cellular components are listed in Table 2.

The presence of lncRNAs was screened among the DE genes, based on the lncRNAs noted on the HUGO Gene Nomenclature Committee website, which included a total of 4118 lncRNAs (http://www.genenames.org/cgi-bin/statistics) (Supplementary S5). The expression of 255 lncRNAs was deregulated in ARPE-19 cells cultured on board the ISS; 208 were upregulated and 47 were downregulated (the top ten up- and down-regulated lncRNAs are presented in Table 3). To predict active micro-RNAs (miRNAs) in ARPE-19 cells cultured on board the ISS compared to ground control, the DE genes were also analyzed to determine the presence of enriched DE targets of the miRNAs. For a given miRNA, the analysis computed the ratio between the number of DE targets and all differentially expressed targets and compared it with the ratio of all targets expressed downwards with all targets. This allowed for the calculation of the probability of observing a greater number of differentially downregulated target genes for a given miRNA only by chance. Of a total of 366 screened miRNAs, 19 achieved significant expression values, as described in Fig. 5 and Supplementary S6, with hsa-miR-296-5p, hsa-miR-494-3p, and hsa-miR-128-3p showing the best p values.

We then evaluated the role of CoQ10 treatment on gene expression in ARPE-19 cells cultured on board the ISS. Assuming a threshold of 0.05 for p and of 0.4 for fold change, of the 26,694 genes analyzed, we found 153 DE genes in cells treated with CoQ10 with respect to untreated controls (Fig. 6A and Supplementary S7). Among these DE genes, 81 were upregulated and 72 were downregulated in spaceflight ARPE-19 cells. iPathwayGuide analysis predicted that 22 pathways defined in the KEGG database were significantly affected (Fig. 6B and Supplementary S8), among which type I diabetes mellitus, HIF-1 signaling, ferroptosis, and Notch signaling pathways were the most significantly affected.

Fig. 2 A Representative photomicrographs obtained with phase-contrast microscopy in ARPE-19 cells pre-launch and post-flight. Scale bar: 200 μm. B Evaluation of apoptosis in ARPE-19 cells by TUNEL fluorescent staining. Image size: 250×250 μm. C Evaluation of vimentin cellular localization in ARPE-19 cells by immunofluorescence. Image size: 100×100 μm.
Fig. 3 Gene expression analysis of ARPE-19 cells cultured on board the International Space Station compared to on ground. A The 5555 significantly differentially expressed (DE) genes (shown in red) are represented in the Volcano plot in terms of their measured expression change (x-axis) and the significance of the change (y-axis). The significance is represented in terms of the negative log (base 10) of the p value, such that genes that are more significant are plotted higher on the y-axis. The dotted lines represent the thresholds used to select the DE genes: 1 for expression change and 0.05 for significance. B Pathways perturbation vs over-representation: the pathways are plotted in terms of the two types of evidence computed by iPathwayGuide: over-representation is on the x-axis (pORA) and the total pathway accumulation is on the y-axis (pAcc). Each pathway is represented by a single dot, with significant pathways shown in red, non-significant in black, and the size of each dot is proportional to the size of the pathway it represents. Both p values are shown in terms of their negative log (base 10) values. C DE pathway genes of the most significant impacted pathways. The mean value and standard deviation are indicated in red.

Table 1 Most significantly affected pathways in ARPE-19 cells cultured on board ISS compared to on ground

| Pathway name                                | Pathway ID (KEGG) | p-value         | p-value (FRD)  | p-value (Bonferroni) |
|---------------------------------------------|-------------------|-----------------|----------------|---------------------|
| Metabolic pathways                          | 01,100            | 4.716e−12       | 1.504e−9       | 1.504e−9            |
| N-Glycan biosynthesis                       | 00,510            | 1.374e−7        | 2.192e−5       | 4.385e−5            |
| Protein processing in endoplasmic reticulum | 04,141            | 1.074e−6        | 1.142e−4       | 3.426e−4            |
| p53 signaling pathway                       | 04,115            | 1.160e−5        | 9.248e−4       | 0.004               |
| MAPK signaling pathway                      | 04,010            | 1.649e−5        | 9.861e−4       | 0.005               |
| Cellular senescence                         | 04,218            | 1.855e−5        | 9.861e−4       | 0.006               |
| TGF-beta signaling pathway                  | 04,350            | 4.917e−5        | 0.002          | 0.016               |
| Mitophagy—animal                            | 04,137            | 5.734e−5        | 0.002          | 0.018               |
| Rap1 signaling pathway                      | 04,015            | 8.164e−5        | 0.003          | 0.026               |
| Steroid biosynthesis                        | 00,100            | 9.600e−5        | 0.003          | 0.031               |

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**Fig. 4** Gene Ontology analysis of differentially expressed genes in ARPE-19 cells cultured on board the International Space Station compared to on ground. The top ten Gene Ontology (GO) terms in biological processes (green), molecular functions (red), and cellular components (blue) category are listed.

**Table 2** GO analysis

| Category                  | Identifier | Description                                           | Count DE | Count All | p-value   | p-value (FDR) | P-value (Bonferroni) |
|---------------------------|------------|-------------------------------------------------------|----------|-----------|-----------|---------------|----------------------|
| Biological Processes      | GO:0,034,976 | Response to endoplasmic reticulum stress              | 125      | 261       | 1.10e−11  | 1.29e−02     | 1.29e−02             |
|                           | GO:0,034,620 | Cellular response to unfolded protein                 | 72       | 130       | 7.40e−11  | 4.34e−02     | 8.68e−02             |
|                           | GO:0,006,986 | Response to unfolded protein                          | 86       | 167       | 1.80e−10  | 7.04e−03     | 2.11e−06             |
|                           | GO:0,035,967 | Cellular response to topologically incorrect protein | 77       | 147       | 5.80e−10  | 1.70e−06     | 6.80e−06             |
|                           | GO:0,030,968 | Endoplasmic reticulum unfolded protein response       | 67       | 123       | 8.70e−10  | 2.04e−06     | 1.02e−05             |
| Molecular Functions       | GO:0,043,167 | Ion binding                                           | 1715     | 5615      | 2.50E−06  | 7.34E−03     | 7.34E−03             |
|                           | GO:0,005,509 | Calcium ion binding                                   | 217      | 610       | 4.60E−05  | 1.00E+00     | 1.00E+00             |
|                           | GO:0,004,576 | Oligosaccharyl transferase activity                   | 9        | 10        | 8.80E−05  | 1.00E+00     | 1.00E+00             |
|                           | GO:0,003,777 | Microtubule motor activity                            | 39       | 81        | 1.20E−04  | 1.00E+00     | 1.00E+00             |
|                           | GO:0,043,168 | Anion binding                                         | 784      | 2509      | 2.60E−04  | 1.00E+00     | 1.00E+00             |
| Cellular Components       | GO:0,005,783 | Endoplasmic reticulum                                 | 616      | 1615      | 2.40E−20  | 3.33e−13     | 3.33e−13             |
|                           | GO:0,044,432 | Endoplasmic reticulum part                            | 463      | 1173      | 2.10E−18  | 1.46e−10     | 2.92e−11             |
|                           | GO:0,098,827 | Endoplasmic reticulum Sub-compartment                 | 372      | 931       | 7.60E−16  | 3.16e−07     | 1.06e−07             |
|                           | GO:0,031,984 | Organelle subcompartment                              | 554      | 1489      | 9.10E−16  | 3.16e−07     | 1.26e−07             |
|                           | GO:0,005,789 | Endoplasmic reticulum membrane                        | 370      | 928       | 1.30E−15  | 3.61e−09     | 1.81e−08             |
In the present study, we investigated the impact of the space environment on human retinal pigment epithelial cells. For this purpose, ARPE-19 cells were transferred and cultured for 3 days onboard the ISS; in addition, cells were cultured in the presence or absence of CoQ10. As a control, ARPE-19 cells were cultured on the ground under normal conditions.

Previous ground experiments on ARPE-19 cells using microgravity simulators have led to contrasting results. We previously showed that 3 days of simulated microgravity obtained by the rotating wall vessel bioreactor (RWV) increased the activity of caspase 3/7 and reduced the mitochondrial transmembrane potential, which is evidence of apoptosis induction [26]. In contrast, using a different microgravity simulator (random positioning machinery, RPM), Corydon et al. did not report apoptotic events in ARPE19 cells cultured for 5–10 days under simulated microgravity conditions [34]. In the present study, we found that the transfer of ARPE-19 cells from Cape Canaveral to the ISS and 3-day incubation on board did not affect their proliferation rate nor induce apoptosis with respect to the ground conditions.

### Table 3  Top ten up- and downregulated IncRNAs in ARPE-19 cultured on board ISS

| ID symbol  | IncRNA name    | Chromosome location | Description                                      | Expression (log fc) |
|------------|----------------|---------------------|--------------------------------------------------|---------------------|
| HGNC:44,153| DIO2-AS1       | 14q31.1 NR_038355   | DIO2 antisense RNA 1                              | 6.3                 |
| HGNC:27,121| LINC00906      | 19q12 NR_027318     | Long intergenic non-protein coding RNA 906       | 5.4                 |
| HGNC:45,193| PLCE1-AS1      | 10q23.33 NR_033969  | PLCE1 antisense RNA 1                             | 5.2                 |
| HGNC:27,437| WDR11-AS1      | 10q26.12 NR_033850  | WDR11 antisense RNA 1                             | 5.1                 |
| HGNC:48,727| LINC00968      | 8q12.1 NR_038236    | Long intergenic non-protein coding RNA 968       | 4.7                 |
| HGNC:42,692| LINC00370      | 13q33.3 BF695150    | Long intergenic non-protein coding RNA 370       | 4.6                 |
| HGNC:27,471| TRHDE-AS1      | 12q21.1 NR_026836   | TRHDE antisense RNA 1                             | 4.6                 |
| HGNC:40,587| TM4SF1-AS1     | 3q25.1 NR_046650    | TM4SF1 antisense RNA 1                            | 4.5                 |
| HGNC:49,095| CLSTN2-AS1     | 3q23 NR_108084      | CLSTN2 antisense RNA 1                            | 4.4                 |
| HGNC:44,989| LINC00842      | 10q11.22 NR_033957  | Long intergenic non-protein coding RNA 842       | 4.4                 |
| HGNC:44,064| SLC7A11-AS1    | 4q28.3 NR_038380    | SLC7A11 antisense RNA 1 non-coding RNA           | −3.1                |
| HGNC:40,792| ARHGAP26-AS1   | 5q31.3 NR_046680    | ARHGAP26 antisense RNA 1                          | −3.1                |
| HGNC:48,575| LINC00888      | 3q27.1 NR_038301    | Long intergenic non-protein coding RNA 888       | −3.1                |
| HGNC:41,429| ARHGAP26-IT1   | 5q31.3 NR_046816    | ARHGAP26 intronic transcript 1 non-coding RNA   | −3.3                |
| HGNC:17,263| PART1          | 5q12.1 AF16347      | Prostate te androgen-regulated transcript 1      | −3.4                |
| HGNC:48,498| LUCAT1         | 5q14.3 NR_103548    | Lung ancer associated transcript 1               | −3.5                |
| HGNC:48,574| LINC00887      | 3q29 NR_024480      | Long intergenic non-protein coding RNA 887       | −3.9                |
| HGNC:23,135| RN7SL3         | 14q21.3 NG_011406   | RNA component of signal recognition particle 7SL3| −4.6                |
| HGNC:4713 | H19            | 11p15.5 NR_002196   | H19 imprinted maternally expressed transcript   | −4.6                |
| HGNC:42,812| MRPL23-AS1     | 11p15.5 NR_024471   | MRPL23 antisense RNA 1                           | −5.8                |
controls (Fig. 2A, B). These results could be explained by differences between microgravity simulated by the available simulator devices and between simulated microgravity and real microgravity onboard the ISS, and the use of these devices and the impact of flight hardware on cellular physiology are currently debated [35, 36]. Cells living onboard the ISS are simultaneously subjected to microgravity and space radiation, which typically do not occur in on-ground simulation experiments, where the two treatments are administered separately [37]. Indeed, the constant threat to the integrity of astronauts represented by space radiation, which causes damage to DNA directly or via the production...
of free radicals, and microgravity, which hampers DNA repair, have raised the opportunity to explore the potential synergies between microgravity and radiation, both in space and in Earthly analogs. The results have been conflicting, which indicates the need to perform experiments in a space environment [37]. Furthermore, in our experiment, the 3-day culturing of ARPE-19 cells on board the ISS was preceded by a 5-day permanence into the Biokit at a relatively low temperature (up to approximately 26 °C). It has been demonstrated that hypothermia elicits a strong expression of cold shock proteins in mammalian retinal cells, including retinal pigment epithelial cells [38], where they may act as protective factors against damage and prevent apoptosis [39]. Finally, it should be noted that, in our experiment, ARPE-19 cells were cultured inside the KUBIK on board the ISS for 3 days, however, due to the transfer from the ground to the ISS, the cells underwent microgravity conditions several hours before entering the KUBIK, which could have affected adaptive behavior.

Cytoskeletal disorganization and significant changes in the expression of its main components, actin, tubulin, and vimentin, both at phenotypic and genotypic levels, have been observed in various cell types subjected to altered gravity conditions [40–43] and complete destruction of the microtubule network was observed in Jurkat cells following exposure to real microgravity [44]. Similarly, in endothelial cells, a significant reduction in actin fibers was observed in response to simulated microgravity [45] and a significant increase in stress fibers following hypergravity [46]. ARPE-19 cells cultured for 5 days and 10 days on RPM revealed the appearance of two major phenotypes, i.e., adherent monolayers and 3D multicellular spheroids [34]. In addition, the reduction of F-actin filaments in favor of F-actin structures at the cell boundaries, and the down-regulation of beta-actin, beta-tubulin, keratin 8, vimentin, laminin subunit beta 2, integrin beta 1, integrin beta 3, and collagen 4 gene expression have been revealed, suggesting that simulated microgravity reduces the ability of exterior structures in ARPE-19 cells to retain adhesion and stiffness [34]. Other cell types differ from ARPE-19 cells in their cytoskeleton-related responses to microgravity. For example, RPM induces the upregulation of beta actin gene expression in MCF-7 breast carcinoma [47] and in human primary chondrocytes [48] and of vimentin gene expression in FLG 29.1 leukemic cells [46] and ML-1 follicular thyroid carcinoma cells [49]. Here, we revealed a dramatic change in the cytoskeletal vimentin distribution in ARPE-19 cultured for 3 days onboard the ISS. Vimentin is a cytoskeletal protein responsible for maintaining cellular integrity and is the main constituent of intermediate filaments, which provide structural support for the cytoplasm and play an essential role in the response to mechanical stresses, such as gravity [50]. Recently, it has been demonstrated that the vimentin network maintains the resilience of the cytoplasm and enhances cytoplasmic mechanical strength and toughness under dynamic deformation via poroelastic and viscoelastic relaxation [51]. Thus, vimentin networks may reduce the risk of cell damage during major deformation events [51]. In our study, immunofluorescence analysis revealed deregulated vimentin cellular localization, mostly concentrated in the perinuclear region in cells exposed to real microgravity (Fig. 2C). The different distribution of vimentin in cells exposed to microgravity compared to the control denotes a change in the shape of the cells and suggests an alteration in the capability of cells to interact with others and with extracellular matrix components. Moreover, vimentin analysis revealed that spaceflight ARPE-19 cells contained perinuclear structures resembling aggresomes. Aggresomes are protein inclusions aggregates at the centrosome-nuclear bay, and contain proteins destined to degradation that are located within a cage comprised of intermediate filament, including vimentin [50, 52]. The formation of aggresomes is directly linked to disruption of proper protein homeostasis, in particular when elevated levels of misfolded or damaged proteins accumulate into cells [50]. This observation suggests that the real microgravity determines an alteration of protein processing in ARPE-19 cells, and is consistent with the following transcriptomic data.

The first observation that microgravity altered gene expression occurred > 20 years ago; however, since the most apparent detrimental effect of long-lasting space flights on astronauts was the strong atrophy of their musculoskeletal system, studies mainly focused on gene expression in bone and muscle cells. Subsequently, several studies have highlighted the impact of the spatial environment on gene expression in different tissues, but the real milestone for high-performance studies of the expression of entire panels of genes in response to spatial radiation and microgravity has been the recent application of genomic and proteomic approaches. Understanding the variations in gene expression in their maximum amplitude is key to unraveling the pathogenesis of damage endured by astronauts during spaceflight and, ultimately, to identify effective countermeasures. The present study found a considerable number of upregulated and downregulated DE genes (Fig. 3A), KEGG analysis, impact analysis, and GO annotation revealed that several relevant biological pathways and cellular functions were affected by space flight (Fig. 3B, C, Fig. 4). Among them, protein processing in the endoplasmic reticulum (ER) is one of the most impacted, which is also justified by the vimentin network alterations described above. The ER is a complex endomembrane system in which proteins are folded and transported to distinct cellular districts [53]. Correctly folded proteins are packaged into transport vesicles and shuttled into the Golgi system. Misfolded proteins are retained within the ER, coupled with chaperones, and if terminally misfolded,
subjected to ER-associated degradation. ER accumulation of misfolded proteins determines ER stress and evokes the unfolded protein response (UPR), which aims to inhibit protein translation, degrade misfolded proteins, increase chaperone production, and eventually induce apoptosis [53]. ER stress and UPR have been correlated with microgravity in different cell types [54–56]. We found that the majority of DE genes involved in this pathway were downregulated in ARPE-19 cells cultured onboard the ISS. However, some members of the 70 kDa heat shock protein (HSP70) gene family (HSPA1A, HSPA8, and HSPA2 in particular) and E3 ligase parkin (PARK2) genes, which are involved in chaperone and E3 ubiquitin ligase activity, respectively, were significantly upregulated. Recently, interesting studies have been conducted on human endothelial cells (HUVECs). It has been reported that 4 days of HUVEC culture in RWV simulated microgravity-induced cell stress and promoted HSP70 upregulation, likely sustaining the early phases of the dynamic adaptation of HUVECs to microgravity, while HSP70 silencing impaired cell survival [57]. In addition, reduced mitochondrial function and mitochondrial content have been demonstrated [57], and the contribution of mitophagy to this adaptation, which culminates in a thrifty phenotype, has been demonstrated [41]. In addition to its role in ER metabolism, Parkin is a central regulator of mitophagy [58], another pathway found to be significantly impacted in ARPE-19 cells cultured onboard the ISS. Deteriorous byproducts or oxidative stress lead to mitochondrial dysfunction, which, if the damage is unrepairable, culminates in mitochondria recognition and targeting for degradation via the autophagic process termed mitophagy [58]. Multi-omics analysis recently identified mitochondrial dysregulation as a central biological hub for the pathophysiological effects of spaceflight [59]. In this context, Parkin recognizes altered proteins on the mitochondrial outer membrane and mediates damaged mitochondrial clearance [58]. Microphthalmia-associated transcription factor (MITF) is another gene belonging to the mitophagy pathway [60] that is upregulated in ARPE-19 cells exposed to the space environment. MITF is a transcription factor responsible for retinal pigment epithelial cell development and function [61]. MITF plays a protective role against oxidative stress in ARPE-19 cells, where it upregulates antioxidant gene expression and mitochondrial biogenesis, mainly via peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC1α) and nuclear factor erythroid 2-related factor 2 (NRF2) upregulation [62].

In our experiment, transforming growth factor (TGF)-beta and p53 signaling were deregulated in ARPE-19 cells cultured onboard the ISS. While most of the DE genes involved in TGF-beta signaling were upregulated, the opposite was true for p53 signaling. Using a system biology approach to evaluate rodent transcriptomic data from GeneLab (Genelab.nasa.gov), TGF-beta and p53 were identified as the most prevalent pan-tissue signaling pathways activated in response to microgravity [63]. p53 is a major contributor to cell cycle arrest, DNA repair, and apoptosis induction in response to genotoxic or non-genotoxic damage [64]. p53 signaling is frequently activated in response to microgravity. However, heterogeneous responses have been observed depending on the cell type and culture conditions. For example, p53 pathway activation has been recently described in macrophages undergoing spaceflight or RWV simulated microgravity [65] and in lung cancer cells cultured with RPM [66], while the inhibition of p53 signaling under 3D-clinostat simulated microgravity was found in hepatoblastoma cells [67]. In ARPE-19 cells cultured onboard the ISS, we detected several p53-transactivated pro-apoptotic genes (e.g., BAX, PMAIP1, BBC3), and genes involved in cell cycle arrest (e.g., CDKN1A, GADD45A, GADD45B) and DNA repair and damage prevention (DDB2, GADD45A [67], GADD45B, SESN2) were downregulated. Similarly, we found that cellular senescence pathway genes, and in particular, most of the genes encoding senescence-associated secretory phenotype (SASP) factors [68], such as IL1A, CXCL8, SERPINE1, and IGFBP3, were significantly downregulated. In contrast, the gene coding for the SASP TGF-beta factor was upregulated. TGF-beta is a master regulator that coordinates the systemic response to microgravity at multiple biological scales [63]. TGF-beta is a well-known cytokine that belongs to a family of several protein members, including bone morphogenic proteins, growth differentiation factors, activins, and inhibins, all of which regulate a wide spectrum of cellular functions such as proliferation, apoptosis, differentiation, and immune responses [69]. TGF-beta plays a major role in retinal pigment epithelial cells, particularly in a dynamic transition along a well-differentiated, polarized epithelial to mesenchymal cells, which has been defined as retinal pigment epithelium dysfunction [70]. Functional retinal pigment epithelial cells form a single layer of polarized cells located between the photoreceptors and the choroid. Correct differentiation and polarization of the retinal pigment epithelium are essential for proper functioning. While epithelial features guarantee retinal pigment epithelium physiological homeostasis in the retina, different mechanisms have been found to be involved in retinal pigment epithelium epithelial to mesenchymal transition (EMT) and mesenchymal to epithelial transition (MET), which hinder their functions [70]. Microgravity has been previously demonstrated to trigger EMT in different cell types [65, 71]. TGF-beta signaling is one of the most potent EMT inducers and is, therefore, a major contributor to retinal pigment epithelium dysfunction [70]. High levels of vitreal TGF-beta have been detected in proliferative vitreoretinopathy patients, a pathological condition in which EMT of the retinal pigment epithelium plays an essential pathogenic role [72], which led to the evaluation...
of TGF-beta signaling inhibitors able to counteract EMT for the treatment of proliferative retinal diseases such as proliferative vitreoretinopathy [73]. The Hippo signaling pathway is closely interconnected with that of TGF-beta, and in our experiment, it was significantly affected in ARPE-19 cells cultured on board the ISS. This pathway regulates retinal pigment epithelial cell differentiation and is involved in EMT [74]. Connective tissue growth factor (CTGF) encodes a cysteine-rich extracellular matrix protein that acts downstream of the TGF-beta and Hippo signaling pathways [75, 76]. We found that CTGF is one of the genes overexpressed in ARPE-19 cells cultured on board the ISS. It has been hypothesized that its binding to various cell surface receptors, including integrin receptors, heparan sulfate proteoglycans, and low-density lipoprotein receptor-related proteins, enables CTGF to regulate key cellular functions, such as cell adhesion, proliferation, chemotaxis, differentiation, survival, and extracellular matrix component production [77]. A previous study indicated that CTGF increases the migratory ability of retinal pigment epithelial cells and that it is a major mediator of retinal fibrosis [78]. In addition, it has been recently demonstrated that the Hippo pathway is severely altered in a murine model of retinal degeneration, in which CTGF is markedly upregulated [76].

Analysis of DE genes revealed deregulation of the expression and activity of lncRNAs (Table 3 and Supplementary S5) and microRNAs (Fig. 5 and Supplementary S6), respectively, in ARPE-19 cells. LncRNAs are defined as transcripts longer than 200 nucleotides that generally lack protein-coding potential and can be processed like mRNAs, i.e., spliced and polyadenylated [79]. LncRNAs are involved in several cellular physiological processes, such as adaptation to stress, cell differentiation, maintenance of pluripotency, and apoptosis [79]. The correct balance of lncRNA levels is crucial for the maintenance of cellular equilibrium, and their dysregulation is associated with many disorders [79]. Variations in lncRNA expression have been documented in different cell types exposed to simulated microgravity [80–82]. Here, we demonstrated that real microgravity alters a panel of lncRNAs in ARPE-19 cells, most of which were upregulated. Considering the top ten up- and down-regulated lncRNAs, the only one involved to date in retinal cell physiology is H19, which is involved in retinal cell death and in the inflammatory response of ARPE-19 cells exposed to hyperglycemia [83]. We detected strong downregulation of H19 expression in ARPE-19 cells cultured onboard the ISS, and it has been reported that H19 downregulation correlates with TGF-beta-mediated EMT in retinal endothelial cells [84]. Similarly, another two lncRNAs downregulated in ARPE-19 cells cultured onboard the ISS, namely SLC7A11-AS1 and LUCAT1, are reported to be involved in the EMT process [85, 86]. MicroRNAs are small single-stranded non-coding RNAs, whose functions in RNA silencing and post-transcriptional regulation of gene expression are well-known [87, 88]. A recent milestone study identified a spaceflight-associated microRNA signature in response to simulated short- and long-duration spaceflight and simulated deep-space radiation conditions [89]. In addition, the panel of spaceflight-associated microRNAs has been predicted to interfere with cell signaling and metabolic pathways, partly through interaction with TGF-beta signaling [63]. Some microRNAs that were identified to be activated in ARPE-19 cells cultured on board the ISS are involved in the cell response to spaceflight. Among them, hsa-miR-296 expression was affected in HUVECs cultured in a 3D clinostat to simulate microgravity and was found to influence cell proliferation and vascular function under microgravity conditions [90]. hsa-miR-145-3p, hsa-miR-324-5p, and hsa-miR142-5p act as inhibitors of EMT in different cell types [92–95]; in contrast, high expression and activation of hsa-miR 483-3p, hsa-miR-128-3p are involved in EMT induction.

CoQ10 is an essential cofactor in the electron transport chain. It is endowed with well-known protective actions with respect to different types of damage on various cell types, including those of the retina, which mainly depends on its free radical scavenging ability and the regulation of the mitochondrial permeability transition pore [29]. It is known that the choriocapillaris level of endogenous CoQ10 decrease with aging concomitantly with the progression of apoptosis-related retinal diseases [96]. We previously demonstrated the efficacy of the topical administration (via eye drops) of CoQ10 to protect retinal cell layers commissioned to apoptosis by a variety of noxious stimuli [27, 28]. On these bases, we tested the role of CoQ10 treatment in modulating the transcriptomes of ARPE-19 cells cultured onboard the ISS. We found a limited number of DE genes that were subjected to KEGG and impact analyses (Fig. 6). Among the most affected biological pathways affected by CoQ10 treatment, we focused on HIF-1 signaling and ferroptosis. HIF-1 is a well-known transcription factor discovered by virtue of its role as a master regulator of oxygen homeostasis [97]. HIF-1 signaling is involved in numerous cellular responses to a variety of environmental stresses. Interestingly, it has been demonstrated that, regardless of its function as a transcription factor, HIF-1 has a protective role through its localization into mitochondria, where it reduces reactive oxygen species levels and reverses mitochondrial damage [98]. Previous studies have revealed that microgravity affects HIF-1 pathway activation. Wang et al. demonstrated that 28 days of simulated microgravity induced oxidative stress and HIF-1
activation and a panel of its downstream targets in rat hippocampus, while Vogel et al. demonstrated that HIF-1α- and HIF-1-dependent transcripts were differentially regulated under altered gravity on monocytes and macrophages during parabolic flight and suborbital ballistic rocket campaigns, whereas HIF-1-dependent gene expression adapted after 5 min of microgravity [99]. Ferroptosis is a peculiar type of iron-dependent cell death; it was first characterized in 2012 and results from the accumulation of toxic levels of lipid-reactive oxidative species [100]. Increased intracellular iron accumulation is a classical ferroptosis activator that is directly linked to the failure of glutathione (GSH)-dependent antioxidant defenses. Cellular iron metabolism includes absorption of the ferritin-Fe3+ complex by the transferrin receptor (TFRC), and it has been demonstrated that reducing iron utilization may increase the sensitivity to ferroptosis [101]. GSH production is sustained by the activity of the amino acid antiporter, SLC7A11/xCT/system xc-, which guarantees the exchange of extracellular cystine (which, in turn, generates cysteine, a limiting precursor for GSH synthesis) for intracellular glutamate [100]. It is known that inhibition of system xc- causes GSH depletion to trigger ferroptosis [102]. The direct involvement of CoQ10 in ferroptosis has been recently reported. It is known that the reduced form of CoQ10 is a potent antioxidant that counteracts lethal lipid peroxidation, thus acting as a ferroptosis suppressor. In particular, the NADH-dependent oxidoreductase, FSP1, converts non-mitochondrial oxidized to reduced CoQ10, thus furnishing a GSH-independent protective axis to counteract membrane lipid peroxidation [103]. In addition to this role, we found that CoQ10 treatment of ARPE-19 cells cultured on board the ISS reduced TFRC and induced SLC7A11 gene expression, corroborating the evidence that CoQ10 acts as a direct ferroptosis inhibitor and bona fide increases ferroptosis resistance by reducing iron cellular uptake and enhancing cystine uptake.

**Conclusion**

Our results indicate that the transfer of cultured ARPE-19 human retinal cells to the ISS and their 3-day living on board severely altered cytoskeleton morphology and the transcriptome profile, indicating the emergence of cellular dysfunction (Fig. 7). Although obtained in a single experiment, in which we were able to use only a limited number of ARPE-19 cells in culture to carry out the various analyses, we suggest that our results may, at least partially, reflect the response of the retinal pigment epithelial cells of astronauts’ eyes subjected to space flight. The retinal pigment epithelium has peculiar functions strictly linked to its structure that are essential for normal vision [104]. Alterations in retinal pigment epithelium physiology can lead to retinopathy, which consequently affects vision. Retinal pigment epithelium dysfunction is known to be involved in the pathogenesis of several degenerative retinal diseases including age-related macular degeneration, retinitis pigmentosa, and Stargardt disease [105]. Moreover, retinal pigment epithelium dysfunction accompanies the course of non-degenerative retinal diseases, such as diabetic retinopathy [106], and is of primary importance in proliferative diseases such as proliferative vitreoretinopathy [70]. The results obtained in the present study, highlighting the potential dysfunction of ARPE-19 cells, are consistent with what has been observed in vivo in previous studies in rodents housed in spacecraft for several days [16, 107]. Interestingly, during the same SpaceX CRS-12 mission as our experiment, retinal pigment epithelium thickness reduction was observed in the retinas of mice hosted for 40 days on board the ISS [107]. Therefore, although the evidence we report here does not have an immediate impact on SANS pathogenesis, it poses an alert for the health of the astronauts’ retinas, particularly in view of long-term missions, which may even extend to years. Approximately 60% of astronauts returning from long-term missions exhibit signs of SANS, the etiology of which is currently debated [19]. The causes of SANS are thought to be a mild and chronic increase in intracranial pressure, congestion
of cerebral venous, and alteration of folate-dependent 1-carbon metabolism [23]. Regardless of its causes, reducing the risk of SANS is a priority for long-term spaceflight planning. Among the signs of SANS, two are particularly feared. The first is optic disc edema, which may cause severe vision loss due to the occurrence of increasing blind spots. The second is retinal and choroidal folds, which, if severe and located near the fovea, can cause distortions or visual acuity reduction [108].

The contribution of spaceflight-dependent cellular alterations of individual retinal cell types in the pathogenesis of SANS has not yet been defined. However, results from experiments conducted on cell cultures suggest that the molecular and cellular responses to microgravity and radiation could synergize with macroscopic tissue and organ alterations in the pathogenesis of SANS. A recent example of this hypothesis comes from the observation that choroidal folds in the retina of a crewmember > 5 years post-flight induced alterations in the retinal pigment epithelium [108]. Therefore, the results presented herein suggest a rational basis for future investigations aimed at identifying effective countermeasures against SANS. On this line of evidence, the treatment of ARPE-19 cells with CoQ10 on board reveals its potential to increase cell resilience towards harmful agents in the space environment. Moreover, it can be hypothesized that at least some of the gene deregulations we found in ARPE-19 cells occur in other cell types and tissues of astronauts. Based on this assumption, the evidence that important cell pathways are significantly affected by spaceflight and, if deregulated, play a central role in a variety of severe human disorders, such as cancer, cardiovascular diseases, and diabetes, confirms possible connections to astronauts’ health [109, 110].

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**Data availability** All data and material used are available in the author’s labs.

**Code availability (software application or custom code)** Not applicable.

**Declarations**

**Conflict of interest** The authors declare no conflicts of interest/competing interests.

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