miR302a and 122 are deregulated in small extracellular vesicles from ARPE-19 cells cultured with H$_2$O$_2$

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Age related macular degeneration (AMD) is a common retina-related disease leading to blindness. Little is known on the origin of the disease, but it is well documented that oxidative stress generated in the retinal pigment epithelium and choroid neovascularization are closely involved. The study of circulating miRNAs is opening new possibilities in terms of diagnosis and therapeutics. miRNAs can travel associated to lipoproteins or inside small Extracellular Vesicles (sEVs). A number of reports indicate a significant deregulation of circulating miRNAs in AMD and experimental approaches, but it is unclear whether sEVs present a significant miRNA cargo. The present work studies miRNA expression changes in sEVs released from ARPE-19 cells under oxidative conditions (i.e. hydrogen peroxide, H$_2$O$_2$). H$_2$O$_2$ increased sEVs release from ARPE-19 cells. Moreover, 218 miRNAs could be detected in control and H$_2$O$_2$ induced sEVs. Interestingly, only two of them (hsa-miR-302a and hsa-miR-122) were significantly under-expressed in H$_2$O$_2$-induced sEVs. Results herein suggest that the down regulation of miRNAs 302a and 122 might be related with previous studies showing sEVs-induced neovascularization after oxidative challenge in ARPE-19 cells.
Results

**H$_2$O$_2$-induced oxidative stress in ARPE-19 cells.** ARPE-19 cells exposed for 24 h to H$_2$O$_2$ significantly increased intracellular reactive oxygen species (ROS) levels when using a concentration of 600 μM (106 ± 1.69) and 800 μM (103.1 ± 0.9892), compared to control (100 ± 0). Nevertheless, 400 μM H$_2$O$_2$, or lower, did not increase ROS levels compared to control (Fig. 1A). In order to assess whether H$_2$O$_2$-induced ROS affected ARPE-19 cell viability, a XTT assay was performed. Cell viability was significantly decreased when using 800 μM H$_2$O$_2$ (95.45 ± 0.9999) but not with lower concentrations (Fig. 1B). Moreover, 800 μM H$_2$O$_2$ concentration also increased early apoptosis (3.038 ± 0.1689) with respect to control (1.713 ± 0.4796) (Fig. 1C,D).

**H$_2$O$_2$ increased sEVs release in ARPE-19.** Matching previous results on cell viability (see Fig. 1), 600 μM H$_2$O$_2$ was used to stress ARPE-19 cells without killing them (sub-lethal concentration). After 24 h of 600 μM H$_2$O$_2$ exposure, ARPE-19 cells significantly increased the number of EVs released to the medium (Fig. 2). More precisely, a 40% increase in the release of EVs was observed after 600 μM H$_2$O$_2$ exposure, compared to controls. Number and size of EVs were studied using a nanoparticle tracking system (NanoSight) (Fig. 2A). Besides, EVs were observed using transmission electron microscopy (TEM), which showed that, according to size and morphology, most of the EVs observed can be classified as sEVs (Fig. 2B,C).

**miRNA Expression in ARPE-19 cells and sEVs.** In the present study, 384 miRNAs were analysed, and two different miRNA clusters could be set after hierarchical clustering (Fig. 3). The same proceeding was performed with the extracellular medium to collect sEVs and miRNAs providing also two clusters (Fig. 4).

**miRNA Expression in ARPE-19 cells (Cell miRNA).** As shown in Fig. 3, there is a significant miRNA repression after H$_2$O$_2$ treatment when compared with control ARPE-19 cells. As a result, 306 out of 384 Cell miRNAs were detected by the array. From those, 59 Cell miRNAs were significantly expressed in ARPE-19 cells (p < 0.05) (Table 1). Moreover, seven out of 59 Cell miRNAs were significantly under-expressed in H$_2$O$_2$-treated cells (fold change, FC > 1) than in control conditions. Different detected miRNAs are shown in Fig. 5A,C. It is noteworthy to underline that hsa-miR-205 and hsa-miR-302c presented a dramatic decrease in treated cells when compared to others (FC > 2).

**miRNA Expression in ARPE-19 sEVs (sEV miRNA).** As above-mentioned, ARPE-19 cells released a significantly higher number of sEVs after 600 μM H$_2$O$_2$ exposure than control cells. In contrast, these H$_2$O$_2$-induced sEVs showed a significant lower miRNA expression compared to control. More concretely, 218 out of 384 sEV miRNAs were detected by the array. However, only 2 out of 218 sEV miRNAs were significantly lower in sEVs released from treated cells (p < 0.05) hsa-miR-302a (FC = 1.159) and hsa-miR-122 (FC = 1.576) than in control sEVs (Fig. 5B,D).

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Figure 1. Effect of oxidative stress in ARPE-19 cells. Superoxide anions were measured by DHE after 24 h of H$_2$O$_2$ treatment. (A) XTT cell viability as percentage to control. (B) Early apoptotic cells measured using AnnexinV-PI. (C) Flow cytometry histograms: alive cells (annexin V-, PI-), early apoptosis (annexin V+, PI-) and necrotic cells (annexin V+, PI+). (D) Values are expressed as mean ± SEM (n = 3). Statistically significant differences were set at *p < 0.05 and **p < 0.01.
Microarray validation. Among the miRNAs differently expressed in ARPE-19 cells, seven out of 59 presented a significant fold-change. In order to validate this finding, qRT-PCR was performed by using independent RNA samples. We selected three out of seven significant miRNAs (those with the highest differences observed):
Figure 4. Heatmap of miRNA expression profile from array assay of the sEVs released by ARPE-19 cells. After 24 h of 600μM H₂O₂, the miRNA expression profile of sEVs was analyzed, using an array of microRNA. Hierarchical unsupervised clustering was performed with the expressed miRNAs in ARPE-19-released sEVs. Each column represents an individual cell sample. Orange columns are sEVs released by ARPE-19 cells treated with 600μM H₂O₂ and green columns are sEVs released by ARPE-19 control cells. Control sample (n = 4) and 600μM H₂O₂ sample (n = 4). Overexpressed miRNAs (green) and under-expressed miRNAs (red). The expression values are calculated with respect to the reference genes of the array.

| miRNA      | FC  | P_value | miRNA      | FC  | P_value |
|------------|-----|---------|------------|-----|---------|
| hsa-miR-139-3p | 0.71 | 0.00065 | hsa-miR-371-5p | 0.95 | 0.02665 |
| hsa-miR-192  | 0.74 | 0.00129 | hsa-miR-92a  | 0.70 | 0.02672 |
| hsa-miR-22   | 0.73 | 0.00646 | hsa-miR-361-5p | 0.79 | 0.02674 |
| hsa-miR-15b  | 0.73 | 0.00657 | hsa-miR-521  | 1.35 | 0.02850 |
| hsa-let-7c   | 0.82 | 0.00661 | hsa-miR-30c  | 0.78 | 0.02935 |
| hsa-let-7b   | 0.77 | 0.00670 | hsa-miR-335  | 0.85 | 0.03051 |
| hsa-miR-221  | 0.66 | 0.00723 | hsa-miR-28-5p | 0.94 | 0.03170 |
| hsa-miR-323-5p | 0.79 | 0.01131 | hsa-let-7g   | 0.84 | 0.03204 |
| hsa-miR-15a  | 0.67 | 0.01139 | hsa-miR-29c  | 0.82 | 0.03218 |
| hsa-miR-18b  | 0.70 | 0.01164 | hsa-miR-488  | 0.98 | 0.03271 |
| hsa-miR-151-3p | 0.78 | 0.01193 | hsa-miR-532-3p | 0.86 | 0.03316 |
| hsa-miR-183  | 0.80 | 0.01214 | hsa-miR-338-5p | 1.11 | 0.03332 |
| hsa-miR-518b | 0.76 | 0.01364 | hsa-miR-125a-5p | 0.82 | 0.03352 |
| hsa-miR-10a  | 0.78 | 0.01407 | hsa-miR-499-3p | 0.72 | 0.03376 |
| hsa-miR-23a  | 0.79 | 0.01456 | hsa-miR-29a  | 0.72 | 0.03509 |
| hsa-miR-151-5p | 0.85 | 0.01492 | hsa-miR-20b  | 0.81 | 0.03536 |
| hsa-miR-224  | 0.78 | 0.01537 | hsa-miR-148a | 0.90 | 0.03727 |
| hsa-miR-186  | 0.52 | 0.01561 | hsa-miR-548b-5p | 1.91 | 0.03849 |
| hsa-let-7f   | 0.86 | 0.01619 | hsa-miR-218  | 1.00 | 0.04031 |
| hsa-miR-27a  | 0.80 | 0.01739 | hsa-miR-505  | 0.65 | 0.04090 |
| hsa-miR-148b | 0.66 | 0.01813 | hsa-miR-205  | 2.29 | 0.04350 |
| hsa-miR-25   | 0.83 | 0.01956 | hsa-miR-17   | 0.78 | 0.04392 |
| hsa-miR-98   | 1.00 | 0.01966 | hsa-miR-324-5p | 0.73 | 0.04490 |
| hsa-miR-106a | 0.71 | 0.02016 | hsa-miR-320c | 0.82 | 0.04523 |
| hsa-let-7a   | 1.06 | 0.02102 | hsa-miR-99a  | 0.84 | 0.04595 |
| hsa-miR-300  | 0.89 | 0.02126 | hsa-miR-302c | 2.55 | 0.04598 |
| hsa-miR-18a  | 0.70 | 0.02210 | hsa-miR-30b  | 0.85 | 0.04622 |
| hsa-miR-515-3p | 0.89 | 0.02262 | hsa-miR-28-3p | 0.83 | 0.04717 |
| hsa-miR-27b  | 0.78 | 0.02454 | hsa-miR-99   | 0.77 | 0.04962 |
| hsa-miR-518d-3p | 1.06 | 0.02537 |                  |     |         |

Table 1. miRNAs significantly regulated in ARPE-19 cells under oxidative stress. The miRNAs are ranged according to the P-value.
hsa-miR-205-5p, hsa-miR-521, and hsa-miR-302c; plus a miRNA that was unchanged: hsa-miR-130b, as a control. The outcome confirmed the results obtained, hsa-miR-205-5p, hsa-miR-521 and hsa-miR-302c were down-regulated by 600 \( \mu \text{M} \) H2O2 compared to control (Fig. 5E).

The same procedure, qRT-PCR, was performed using the two sEV miRNAs which expression had changed: hsa-miR-302a and hsa-miR-122. As expected, miRNAs were under-expressed in sEVs released from ARPE-19 cells treated with 600 \( \mu \text{M} \) H2O2 (Fig. 5F).

Pathway analysis and prediction of miRNA targets regulated by oxidative stress. Subsequently, the role of the under-expressed miRNAs in treated ARPE-19 cells and in sEVs from treated cells (seven in cells and two in sEVs), was analysed. For that reason, two independent "in silico" analysis were performed in order to determine potential biological processes related to oxidative stress induction. The analysis of the KEGG pathway, regulated by Let-7a, miR-518d-3p, miR-521, miR-338-5p, miR-548b-5p, miR-205, and miR-302c, shows a large number of pathways involving these miRNAs. Among them, cell cycle, adherent junction, p53 signalling pathway, and HIF-1 signalling pathway are the most relevant (Fig. 6A). Both sEV miRNAs, miR-302a and miR-122, are involved in different pathways, such as TGF-beta signalling pathway, FoxO signalling pathway, and cell cycle (Fig. 7A).

Potential targets of miRNAs. In order to identify biological functions of the validated miRNAs and to select their putative targets, two programs were handled: DIANA TOOLS mirPath and Target Scan Human. The outcome on sEV miRNA and Cell miRNA related pathways are not completely equivalent. Most of the significantly involved KEGG are related to cancer or cell cycle pathways (Figs. 6B and 7B).

Discussion
The study of circulating miRNAs on AMD has been addressed to find biological markers which might help implementing an early diagnosis for the disease, or to find new therapeutic strategies. Although neovascular processes and oxidative stress are well known characters involved on AMD, the origin of the disease is far from being completely understood. Several studies were focused in identifying neovascularization-related miRNAs or oxidative stress-related miRNAs.
stress-related ones\textsuperscript{23–26}. On this line, the use of RPE cell cultures, as ARPE-19 under oxidative challenges (eg. H\textsubscript{2}O\textsubscript{2}, rotenone or EtOH), results useful to study the RPE response to oxidative stimuli\textsuperscript{27–29}.

As mentioned earlier, 600 µM was the highest H\textsubscript{2}O\textsubscript{2} concentration used which increased ROS levels without generating early apoptosis (800 µM H\textsubscript{2}O\textsubscript{2} exposure significantly increased early apoptosis). Other authors have used different H\textsubscript{2}O\textsubscript{2} ranges (200-600 µM) and some differences can be found in terms of cell viability (XTT or MTT), apoptosis and ROS production\textsuperscript{7,27,30}. Plausibly, the differences on time exposure (12–24 h) or cell confluence level could explain these discrepancies.

Fitting with previous data, pro-oxidant challenge resulted on significant high sEVs release from ARPE-19 cells. It is well documented that EtOH or high glucose conditions resulted in the same response increasing sEVs release from ARPE-19 cells\textsuperscript{28,31}.

Extracellular or circulating miRNAs can be included on sEVs, associated to lipoproteins or proteins\textsuperscript{13}. In this respect, we have just focused on miRNAs contained as sEVs cargo (sEV miRNA). In spite of the fact that in sEVs only 218 out of 384 miRNAs could be detected, only two sEV miRNAs were significantly high in control released sEVs than in sEVs from treated cells. The hsa-miR-302a and hsa-miR-122 have already been seen in sEVs confirming our results\textsuperscript{32–35}.

Figure 6. Cell miRNA related pathways after H\textsubscript{2}O\textsubscript{2} exposure in ARPE-19 cells. Graphic representation of cellular pathways (Y) and log\textsubscript{10} P-value (X), circle size represents the number of involved miRNAs up to 7 deregulated miRNAs. (A) KEGG pathways regulated and putative target genes (B).

Figure 7. sEVmiRNA related pathways after H\textsubscript{2}O\textsubscript{2} exposure in ARPE-19 cells (A) Graphic representation of cell pathways (Y) and log\textsubscript{10} P-value (X), circle size represents the number of involved miRNAs up to 2 deregulated miRNAs. (A) KEGG pathways regulated and putative target genes (B).

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When comparing the two sEV miRNAs, hsa-miR-302a and hsa-miR-122, to those miRNAs on ARPE-19 cells (Cell miRNA), no matches could be found. Surprisingly, the sEV miRNA hsa-miR-302a and the Cell miRNA hsa-miR-302c belong to the miR 302/367 La-related protein 7 (LARP7) intragenic cluster. This includes hsa-miR-367, hsa-miR-302d, hsa-miR-302a, hsa-miR-302b, and hsa-miR-302b. This cluster is involved in several processes coordinating proliferation, differentiation, pluripotency maintenance, and cell reprogramming. Moreover, the cluster, regulates TGF pathway, PI3K–AKT and BMP cell signaling. Additionally, hsa-miR-302a acts as a tumor suppressor and repressor of cell division, and more concretely, VEGFA is one of the direct targets for this miRNA. In addition, hsa-miR-122 has been related to VEGFC. Interestingly, low hsa-miR-302a expression levels are inversely related to VEGF levels in hepatocellular carcinoma, promoting vascular changes. In addition, miR-122 seems to have a role against oxidative stress, since the use of pre-miR-122 protects from H2O2-induced oxidative stress, targeting the mitochondrial ribosomal protein S1. VEGF is one of the direct targets for this miRNA. In addition, hsa-miR-122 has been related to VEGFC. Interestingly, low hsa-miR-302a expression levels are inversely related to VEGF levels in hepatocellular carcinoma, promoting vascular changes. In addition, miR-122 seems to have a role against oxidative stress, since the use of pre-miR-122 protects from H2O2-induced oxidative stress, targeting the mitochondrial ribosomal protein S1. VEGF is one of the direct targets for this miRNA. In addition, hsa-miR-122 has been related to VEGFC. Interestingly, low hsa-miR-302a expression levels are inversely related to VEGF levels in hepatocellular carcinoma, promoting vascular changes. In addition, miR-122 seems to have a role against oxidative stress, since the use of pre-miR-122 protects from H2O2-induced oxidative stress, targeting the mitochondrial ribosomal protein S1.

Recent data from other groups indicate how diverse oxidative insults -EtOH or high glucose, and now H2O2 - lead to increased sEVs release from ARPE-19 cells. Furthermore, those oxidative-induced sEVs were capable of promoting neovascular processes in endothelial cell cultures, whereas control-released sEVs inhibited this phenomenon. In view of these findings, we hypothesize that sEVs hsa-miR-122 and hsa-miR-302a decreased levels could play a role on angiogenesis involving oxidative stress related pathways. Several reports on AMD or experimental AMD, have reported significant changes on miRNAs (see Table 2). Among them, hsa-miR-23a is present in five reports. This miRNA seems to be downregulated in both AMD patients and H2O2-treated ARPE-19 cells. In fact, H2O2-induced-apoptotic cell death is significantly observed in ARPE-19 cells after hsa-miR-23a inhibition. Fitting with this, we found significant expression differences on hsa-miR-23a after H2O2 exposure (FC = 0.79). Table 2 summarizes those Cell miRNAs matching with previous reports on AMD or AMD experimental models. Besides, there are reports that locate the sEVs-miRNAs in eye tissue. MiR-302 expression was observed already in RPE cells by Li and collaborators, playing an important role in the RPE differentiation. Other authors observed a miR-122 differential expression on canine retinas, in aqueous humor and are related in fact with diabetic retinopathy. In agreement with others, up/down regulated miRNAs are related to several and different cell signaling pathways. After seeing the results in both sEV miRNA and Cell miRNAs related pathways, cancer related pathways are commonly involved in many of the deregulated miRNAs. More research must be performed on these generic pathways to determine the concrete role of these cell signaling routes. As a conclusion, H2O2 significantly increased sEVs release from ARPE-19 cells compared to control cells. Paradoxically, the miRNA sEVs cargo (hsa-miR-302a and hsa-miR-122) resulted in significantly lower in H2O2-induced sEVs compared to control. Since hsa-miR-302a and hsa-miR-122 regulates vasculoegenic targets, these results support those on ARPE-19 cells indicating that oxidative-induced sEVs promote angiogenesis.

| miRNA     | Reference                      |
|-----------|--------------------------------|
| hsa-miR-139-3p | Szemraj et al. et al.          |
| hsa-miR-192   | Grassmann et al. et al.        |
| hsa-let-7c    | Ren et al. and Szemraj et al.  |
| hsa-miR-18b   | Ren et al. and Wang et al.     |
| hsa-miR-183   | Karali et al. et al. and Xiang et al. |
| hsa-miR-23a   | Li et al., Lin et al., Romano et al., Szemraj et al. and Zhou et al. |
| hsa-miR-27a   | Ren et al., Romano et al., Szemraj et al. |
| hsa-miR-25    | Ren et al. and Zhang et al.    |
| hsa-miR-106a  | Ertekin et al.                |
| hsa-miR-7a    | Ertekin et al. and SanGiovanni et al. |
| hsa-miR-27b   | Ertekin et al. and Howell et al. |
| hsa-miR-518d-3p | Ding et al.                  |
| hsa-miR-92a   | Ding et al., Howell et al., Desjarlais et al. and Walz et al. |
| hsa-miR-361-5p | Grassmann et al. and Szemraj et al. |
| hsa-miR-335   | Ertekin et al. and Grassmann et al. |
| hsa-miR-30c   | Ren et al.                     |
| hsa-miR-28-5p | Ren et al.                     |
| hsa-miR-29a   | Ertekin et al., Howell et al., Walz et al., Zhang et al. and Zhang et al. (2) |
| hsa-miR-205   | Ménard et al.                 |
| hsa-miR-17    | Ertekin et al., Barber et al. and Tian et al. |
| hsa-miR-324-5p | Ertekin et al.                |
| hsa-miR-302c  | Howell et al.                 |
| hsa-miR-302b  | Ding et al., Romano et al., Mazzeo et al. and Haque et al. |
| hsa-miR-28-3p | Howell et al.                 |

Table 2. Relevant miRNAs identified on eye disorders.
Material and Methods

Cell culture. A rising retinal pigment epithelium (ARPE-19) human cell line was obtained from American Type Culture Collection (ATCC, Barcelona, Spain) at passage 19. ARPE-19 cells were cultured in Dulbecco's modified Eagle's DMEM/F12 (Invitrogen, Carlsbad, CA, USA), as previously described. Cells were cultured to 80–90% confluence at a starting density of 1 × 10^6 cells/cm² in different plates depending on the technique. After 2 days, the cells were treated for 24 h with 600 μM H₂O₂ (Scharlau, Senmenat, Spain), using filtered media with 1% of Fetal Bovine Serum, exosome-depleted (FBS; Thermo Fisher Scientific, Gibco, USA). Cells and supernatant were collected and preserved for further experiments.

Determination of intracellular ROS. Intracellular ROS levels were measured using dihydroethidium, (DHE; Thermo Fisher Scientific, Waltham, MA, USA), which is a superoxide indicator. This molecule has a blue fluorescence, but, when oxidized to ethidium, it stains DNA in red. ARPE-19 cells were seeded at 6 × 10^5 cells/ well in a 96 well plate. Cells were rinsed with PBS (phosphate-buffered saline) twice and incubated with 5 μM of DHE during 30 min at 37°C and 5% CO₂. ROS levels were measured by a fluorescence multiplate reader (Victor X5; Perkin Elmer) excited at 518 nm and read at 605 nm.

Apoptosis detection. Number of dead cells by apoptosis or necrosis was measured by flow cytometry using the FITC Annexin V Apoptosis detection kit (Immunostep, Salamanca, Spain) that can discriminate live cells from those in early apoptosis, late apoptosis, or necrosis. A total of 10,000 cells per condition were analysed using the FACS Verse (Beccton Dickinson, New Jersey, USA). Four populations are detected: unmarked Annexin (−)/Propidium Iodide (−) are live cells; double marked Annexin (+)/Propidium Iodide (+) represent apoptotic cells; simple marked Annexin (+)/Propidium Iodide (−) are early apoptotic cells; and simple marked Annexin (−)/Propidium Iodide (+) are necrotic cells.

sEVs isolation and size distribution. sEVs isolation was performed by successive ultracentrifugation as previously reported. The sEVs pellet was stored at 4°C until further processing in PBS solution. For microarray assay, sEVs were isolated using ExoQuick-TC (Systems Biosciences, Mountain View, CA, USA) following the manufacturer's instructions. sEVs identity was confirmed by the nanoparticle tracking system NanoSight NS300 following manufacturer's protocols (Malvern Instruments, Malvern, UK).

Electron microscopy. sEVs pellets were resuspended in PBS and ultracentrifuged at 120,000 × g for 70 min at 4°C. After that, approximately 10 μg of the samples were resuspended in PBS on parafilm. The sample was fixed by depositing a drop of 2% Paraformaldehyde on the parafilm and placing the grid (Mesh with Formvar) on top of the drop. Negative staining was performed with 2% uranyl acetate. Photomicrographs were obtained using the transmission electron microscope FEI Tecnai G2 Spirit (FEI Europe, Eindhoven, Netherlands) using a digital camera Morada (Olympus Soft Image Solutions GmbH, Münster, Germany). EVs were identified under the microscope solely based on size and morphology.

RNA isolation and miRNA expression analysis. To perform microarray analysis, ARPE-19 cells from 4 separate cultures were exposed to control and H₂O₂ 600 μM treatment for 24 h. Total RNA was extracted using SeraMir Kit (System Biosciences, Mountain View, CA, USA) according to the manufacturer's instructions. Therefore, four microarrays were performed for each condition: control, ARPE-19 cells exposed to H₂O₂ 600 μM, sEVs released by control cells, and sEVs released by ARPE-19 cells exposed to H₂O₂ 600 μM.

Total RNA quantity and quality (260/280 absorbance ratio) were assessed using NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA was reversely transcribed (cDNA synthesis) using PeqSTAR 96 Universal Gradient (Peqlab, Erlangen, Germany) under the following conditions: 60°C/5 min, RT/2 min, 42°C/30 min, 95°C/10 min and 15°C/hold. Real-time quantitative PCR (qPCR) was performed using 384 well SeraMir Profiler using RT-PCR QuantStudio™ 3 y 4 system (Thermo Fisher Scientific, Waltham, MA, USA) with the appropriate temperature cycles 50°C/2 min, 95°C/10 min, 40 ciclos; 95°C/15 s, 6°C/1 min. The miRNA expression values were calculated using three endogenous controls: RNU43, RNU1Q and RNU6. The expression was calculated according to the 2−∆∆Ct method.

Array analysis. We analyzed miRNA expression differences between ARPE-19 control cells and ARPE-19 treated with H₂O₂. Moreover, differences in miRNA expression between EVs released by ARPE-19 cells treated and EVs released by ARPE-19 control cells were also studied. Differences were analyzed using a t-test study from genefilter package from R Bioconductor. P-values were adjusted by the Benjamini-Hochberg method. MiRNAs that presented an adjusted p-value < 0.05 were considered statistically significant. Significantly modified miRNAs from different samples were represented in a hierarchical clustering heatmap representation. Heatmaps were performed using heatmap.3 package from R Bioconductor.

Analysis of miRNA target genes. In silico analysis of the pathways in which the miRNAs regulated by H₂O₂ were involved using DIANA TOOLS mirPath v.3 algorithm (http://snf-515788.vm.okeanos.grnet.gr/). Moreover, we carry out an analyse of the putative miRNAs target using TargetScanHuman (http://www.targetscan.org/vert_72/).

Quantitative real-time PCR validation. Quantitative real-time PCR (qRT-PCR) was used to validate the miRNA expression profile of the selected miRNAs in an independent sample set. The RNA was isolated from ARPE-19 cells by miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.
100–300 ng of RNA were retro transcribed using TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA) using specific TaqMan RT primers and the thermocycler P HegSTAR 96 Universal Gradient (PeqLAB, Erlangen, Germany), the cycles used were 16 °C/30 min, 42 °C/30 min, 95 °C/5 min and 4 °C/ infinity. Quantitative real time PCR was performed using TaqMan™ microRNA Assays (Thermo Fisher Scientific, Waltham, MA, USA) with TaqMan Gene Expression master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and RT-PCR Roche 234 LighterCycler 480 with the appropriate temperature cycles (50 °C/2 min, 95 °C/10 min, 40 cycles: 95 °C/15 s and 60 °C/1 min). Normalisation was performed with RNU6B snoRNA and RNU43 snoRNA. Relative expression was calculated as $2^{−\Delta\Delta Ct}$.

**Statistical analysis.** The results of each experiment are presented as mean ± SEM. Statistical significance was determined using t-test and 2-way-ANOVA.

**Data availability**

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Competing interests
The authors declare no competing interests.

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