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Review article

Extracellular vesicles in degenerative retinal diseases: A new therapeutic paradigm

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ABSTRACT

Nanoscale extracellular vesicles (EVs), consisting of exomers, exosomes and microvesicles/ectosomes, have been extensively investigated in the last 20 years, although their biological role is still something of a mystery. EVs are involved in the transfer of lipids, nucleic acids and proteins from donor to recipient cells or distant organs as well as regulating cell-cell communication and signaling. Thus, EVs are important in intercellular communication and this is not limited to sister cells, but may also mediate the crosstalk between different cell types even over long distances. EVs play crucial functions in both cellular homeostasis and the pathogenesis of diseases, and since their contents reflect the status of the donor cell, they represent an additional valuable source of information for characterizing complex biological processes. Recent advances in isolation and analytical methods have led to substantial improvements in both characterizing and engineering EVs, leading to their use either as novel biomarkers for disease diagnosis/prognosis or even as novel therapies. Due to their capacity to carry biomolecules, various EV-based therapeutic applications have been devised for several pathological conditions, including eye diseases. In the eye, EVs have been detected in the retina, aqueous humor, vitreous body and also in tears. Experiences with other forms of intraocular drug applications have opened new ways to use EVs in the treatment of retinal diseases. We here provide a comprehensive summary of the main *in vitro*, *in vivo*, and *ex vivo* literature-based studies on EVs' role in ocular physiological and pathological conditions. We have focused on age-related macular degeneration, diabetic retinopathy, glaucoma, which are common eye diseases leading to permanent blindness, if not treated properly. In addition, the putative use of EVs in retinitis pigmentosa and other retinopathies is discussed. Finally, we have reviewed the potential of EVs as therapeutic tools and/or biomarkers in the above-mentioned retinal disorders. Evidence emerging from experimental disease models and human material strongly suggests future diagnostic and/or therapeutic exploitation of these biological agents in various ocular disorders with a good possibility to improve the patient's quality of life.

1. Extracellular vesicles and cellular communication

The role of extracellular vesicles (EVs) has been extensively investigated during the last 20 years. It is now evident that EVs are key determinants in intercellular communication in both physiological and pathological contexts [1]. As reported by the International Society for Extracellular Vesicles (ISEV), these biological entities are defined by a precise range size, the presence of a phospholipid bilayer containing specific markers (*e.g.*, tetraspanins, CD63), and the ability to exert biological functions (*e.g.*, to convey anti- or pro-inflammatory stimuli) [2]. The term "extracellular vesicle" is generic and refers to a variety of vesicles that can be classified according to the following characteristics: (1) exomers; non-membranous nanoparticles with a < 50 nm diameter; (2) exosomes; whose size is between 30 and 150 nm; (3) microvesicles/

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ectosomes; that can have diameters in a range of 100 to 1000 nm; (4) apoptotic bodies; which have a characteristic size similar or higher to that of microvesicles [3–6]. However, since the nomenclature is still non-standardized, the advice from ISEV is that one should always specify the nature of EVs when referring to certain characteristics, such as: (a) size (*e.g.*, small, or medium/large EVs); (b) biochemical composition (*e.g.*, the surface markers); (c) conditions of the EVs-releasing cells, what they have overcome (*e.g.*, hypoxic EVs if released from cells under hypoxic conditions) [2]; in this case, it is possible to define the vesicular bodies involved in specific biological processes, such as oncosomes [7] or migrasomes [8]. Despite the differences in their classifications, all the EVs are characterized by a phospholipid bilayer in which bioactive lipids, proteins, and carbohydrates are immersed, and they enclose a cargo composed of proteins, DNA, RNA, miRNAs, and lncRNA [2]. While exosomes and ectosomes may overlap in terms of their size, it is possible to distinguish each type when one takes into account their biogenesis and also their surface markers. In particular, it seems that ectosomes derive mainly from the budding of the plasma membrane, whereas exosomes are generated inside the cytoplasm within the endocytic pathway, where they are assembled into multivesicular bodies (MVBs) and released after fusion with the plasma membrane [9]. Exosomes are also characterized by the presence of specific surface markers that derive mostly from their biogenesis route. The membranes of exosomes contain annexin, integrins, cofilin and they carry a cargo consisting of many proteins *e.g.*, tubulin, actin, myosin, heat shock proteins (HSPs), ALG-2-interacting protein X (ALIX), vacuolar protein sorting-associated protein 4 (VPS4), intercellular adhesion molecule 1 (ICAM-1), tetraspanin-8 (TSPAN8), and several CD molecules, such as CD9, CD37, CD53, CD63, CD81, CD82, and CD106 [10] (Fig. 1). Interestingly, EVs have been isolated from almost all biological fluids, including saliva [11], urine [12], breast milk [13], mucus [14], and tears [15]. This advantage led to the consideration that EVs could be collected from liquid biopsies and function as novel potential biomarkers for several disorders. Furthermore, it has been demonstrated that proteins within exosomes are very stable and can be stored for long periods [16,17]. Studies performed on human and other model organisms are collated in the EVpedia website (https://evpedia.info/evpedia2_xe/, last access May 2023, 22nd; latest update September 2021, 27th), which is linked to ISEV and the Journal of Extracellular Vesicles. As reported in this database, with respect only to *Homo sapiens,* currently there are 25,119 findings for proteins, 15,386 for mRNAs, and 1833 for miRNAs. Lipids (1139) and metabolites (669) are collected together with those of other species.

2. Physiological and pathological role of EVs in the cellular communication within the eye

During the last decade, the role of EVs in ocular tissues has become clarified, such as their importance in cell-cell communication in both the healthy and pathological conditions of this complex organ. The first article on EVs in this context dates from 1996; subsequently, interest on ocular EVs has grown exponentially in the new Millennium. Indeed, searching in PubMed for either "extracellular vesicles AND eye" or "extracellular vesicles AND retina", yields about 600 and 260 articles, respectively, all of which have been published in the last 15 years. However, many of the articles are not pertinent and are not really

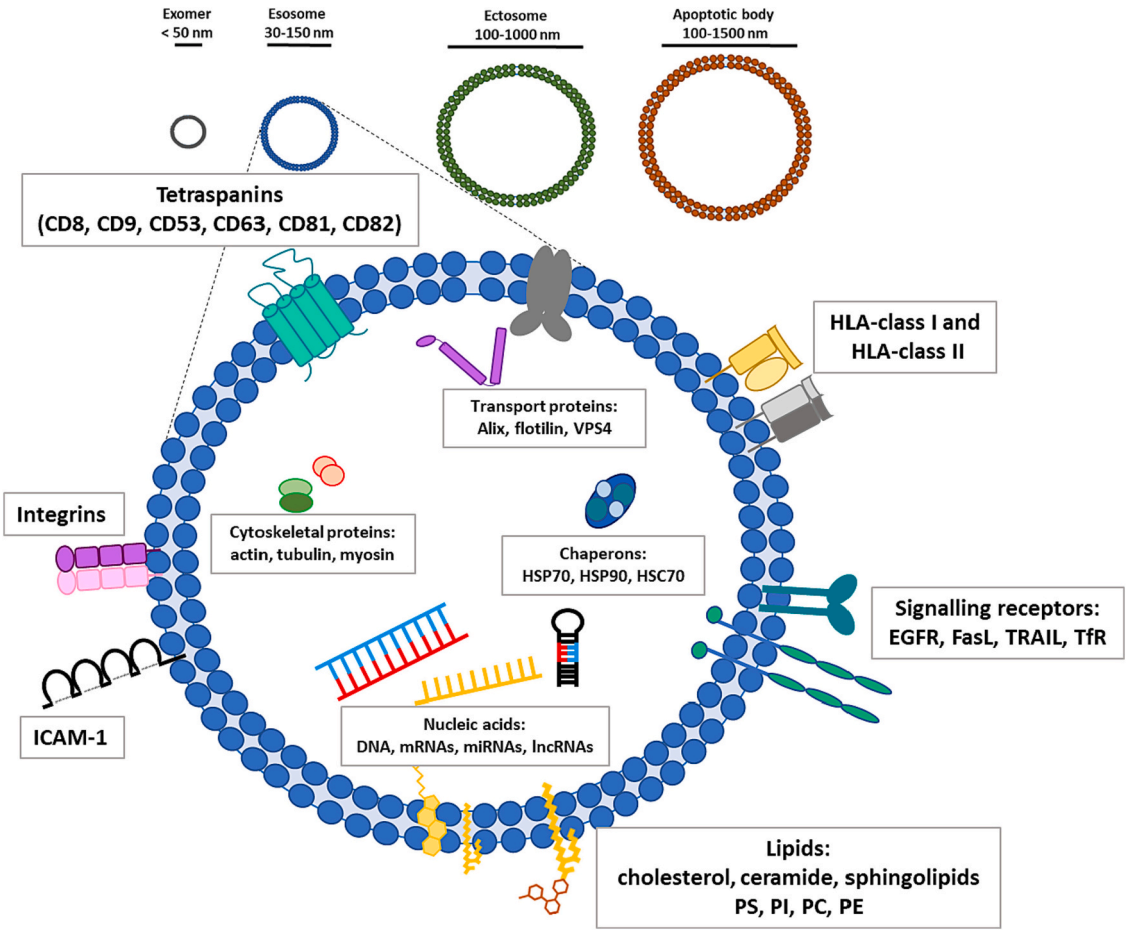

Fig. 1. A schematic representation of cellular vesicles with their relative dimensions. The structure of an exosome with its membrane- and cargo-associated proteins and nucleic acids is also shown.

related to the properties of EVs in the eye/retina. In fact, the same search using the corresponding Medical Subject Headings (MeSH) terms, led to 176 results for "extracellular vesicles AND eye" and 111 results for "extracellular vesicles AND retina". A subsequent analysis of the collected articles aimed at excluding both reviews and research papers reported twice in the two bibliographic searches resulted in a total of 221 articles related to extracellular vesicles/exosomes, retina, and the eye (last search dated March 30th, 2023).

When examined in depth, only a limited number of reports (40/221) had investigated the constitutive release of EVs from cells/tissues at the ocular level, in particular from the retina [18], highlighting the need to better understand EVs' physiological functions in this tissue. Instead, the majority of the literature has focused on the therapeutic potential of EVs – mostly exosomes - derived from mesenchymal stem cells (MSCs) in the treatment of eye diseases [19,20].

The following sections provide a comprehensive and critical summary of the main *in vitro*, *in vivo*, and *ex vivo* literature-based evidence supporting a role for EVs in ocular pathophysiology, followed by a review on how EVs may be potential therapeutic agents in retinal diseases.

Notably, if one considers all of the EVs, then it does seem that exosomes have been the most extensively investigated, since they are associated with all the cellular components of the eye and are present in all the ocular fluids, including tears [21]. However, other types of EVs have been also detected and represent objects of intriguing research aimed at clarifying the function of released vesicles in either physiological or pathological eye contexts, or even both states. We will use the abbreviation "EVs" to refer to vesicles that were not characterized in greater detail in the cited study.

2.1. Trabecular meshwork

A few investigators have examined the composition of EVs and their role in cell-cell communication in the context of glaucoma, with much of the literature focusing on the therapeutic potential of MSC-derived EVs in the treatment of this irreversible degenerative retinal disease [22]. A unique study performed on a 3D human retinal organoid revealed that the constitutive release of EVs was fundamental in the regulation of many retinal homeostasis and developmental processes, including ganglion and photoreceptor cell differentiation [23].

Many researchers working with EVs have focused on the trabecular meshwork cells (TMCs); these cells constitute the main system for regulating the intraocular pressure (IOP) and aqueous humor outflow, a cornerstone in glaucoma-related research, since elevated IOP is the main risk factor associated with this disease of retinal ganglion cells. One of the first pieces of evidence regarding the role of exosomes in the drainage system, and more generally, in the cellular communication at the ocular level, came from the analysis of a protein, myocilin (MYOC), which is found in the extracellular space of TMCs. While is still unclear how alterations in the *MYOC* gene, and the protein for which it codes exert their fundamental effects, they have been related to >100 diseases, including juvenile glaucoma [24]. An *in vitro* study on various human TMC-derived cell lines showed that the MYOC protein is present in the culture medium and associated with exosome membranes, suggesting that it is being released into the extracellular space inside vesicles instead of the canonical secretory pathway [25]. This evidence has been corroborated in both *in vitro* and *ex vivo* studies which demonstrated that in human TMCs and ocular specimens, respectively, MYOC was exosome-associated, with its release being stringently regulated (*via* corticosteroids and aqueous humor, [AH], release) [26,27]. Proteomic techniques were then used to characterize in greater detail the primary TMC-derived exosomes. Interestingly, the presence in exosomes of 108 out of 143 typical exosomal proteins as well as other proteins specific to the investigated cell lines, has been demonstrated, *i.e.*, elastin microfibril interface-located protein 1 (EMILIN-1), neuropilin-1 (NRP-1), and MYOC [28]. As mentioned, exosomes are also key mediators of cell-matrix interactions; an *ex vivo* study conducted in human TM explants revealed that TMC-derived exosomes were enriched in annexins A2 and A6, suggesting that the exosomal binding to fibronectin was mediated by heparan sulphate [29]. Subsequent analyses revealed that exosomes are important in cell-cell communication between TMCs and other cell types, such as non-pigmented ciliary epithelium (NPCE, the AH producing cells) and Schlemm's canal endothelial (SCE) cells, and that this occurred in both physiological and pathological conditions. In particular, *in vitro* studies with human cell lines have revealed that NPCE-derived exosomes carry miRNAs (~ 600) and proteins (~ 200) which are involved in TMC homeostasis [30], whereas exosomes isolated from TMCs treated with transforming growth factor-β2 (TGF-β2) and collagen type-1 (Col-1) exhibited a dysregulation in the amounts of miR-23a-5p, miR-3942–5p, and miR-7515 [30,31]. It is known that miR-7515 is able to increase the expression of vascular-endothelial growth factor A (VEGFA) and its VEGFR2 receptor, the platelet and endothelial cell adhesion molecule 1 (PECAM1), and the tyrosine-protein kinase receptor (Tie2) in SCE cells, suggesting a role for exosomal miRNAs (exomiRs) in inflammation and cell reprogramming [31]. Another piece of evidence of the impact of EVs on the crosstalk between tissues belonging to the ocular drainage system was the finding that the Wnt signaling pathway and MMPs activity in normal TMCs was modulated by NPCE-derived exosomes [30,32,33]. Table 1 summarizes the literature related to EVs and TMCs.

2.2. Retinal pigment epithelium

The physiological and pathological roles of EVs in the retinal pigment epithelium (RPE) are an area of growing scientific interest. The RPE is organized as a monolayer and, in conjunction with the endothelial cells, it constitutes the retinal blood barrier; RPE cells are located close to the choroid on their basal side and, on their apical side, in front of the photoreceptors. The RPE exerts numerous essential functions to guarantee the homeostasis of the outer retina, for example, it transports nutrients and oxygen from the choriocapillaris and regulates the visual cycle according to the circadian rhythm [34] as well as the production

Table 1
List of papers (in chronological order) describing the physiological role of EVs in TMCs. The type of study (*in vitro*, *ex vivo*, or *in vivo*), the model used, and the principal findings are also reported. According to the list, it does seem that with time, the main topic has shifted from MYOC to exomiRs.

Author and Year	Type of study	Model	Findings
Hardy et al., 2005 [25]	*In vitro*	HTM cell strains	MYOC is secreted and associated with exosomes
Perkumas et al., 2007 [26]	*Ex vivo*	Effluent from human anterior segments or AH	MYOC is associated with exosome membranes in fresh samples
Hoffman et al., 2009 [27]	*In vitro/ Ex vivo*	HTM cell strains and porcine AH	Environmental factors regulate exosome-associated MYOC
Stamer et al., 2011 [28]	*In vitro/ex vivo*	Primary HTM, urine, and AH	TMC-derived exosomes contain MYOC, EMILIN-1, and NRP-1
Dismuke et al., 2016 [29]	*Ex vivo*	HTM explants	Environmental factors regulate the interaction of HTM-derived exosome and extracellular matrix
Lernet et al., 2017 [32]	*In vitro*	HTM, NPCE, and human ciliary muscle (HCM) cells	NPCE cells regulate the Wnt pathway in TMCs through the release of exosomes
Tabak et al., 2018 [33]	*In vitro*	HTM, NPCE, and RPE cells	Exosomes affect TMCs in a dose-dependent manner
Lerner et al., 2020 [30]	*In vitro*	Primary NPCE and HTM cells	ExomiRs in NPCE-derived exosomes regulate TMCs homeostasis
Takahashi et al., 2021 [31]	*Ex vivo*	Primary monkey TM and SCE cells	ExomiRs from TMC-derived exosomes play a role in SCE cells reprogramming

and secretion of several growth factors including VEGF and PEDF and taking care of ionic balance in the outer retina [35]. A dysfunction of RPE is associated with many degenerative and dystrophic retina diseases, of which AMD has been the best recognized. One of the first pieces of evidence regarding the physiological role of EVs in the adult retina emerged from studies conducted on RPE cells, in particular with respect to αB-crystallin. This molecular chaperone, which itself plays a key role in the biogenesis of exosomes [36], is secreted into the extracellular space through $CD63^{+}$ vesicles, providing neuroprotection against oxidative stress and inflammation [37–39]. The sorting and release of exosomes from the RPE are also regulated by other proteins, such as the G-protein coupled receptor 143 (GPR143), semaphorin 4 A (Sema4A), and possibly MYOC [40,41]. Interestingly, mass spectrometry profiling revealed that the content of RPE-derived exosomes is different according to whether release occurs from the basolateral or apical side of these vesicles. Specifically, exosomes released from the basolateral membrane are enriched in ~300 unique proteins; conversely, those released from the apical membrane display ~100 unique proteins, thus further corroborating the specificity of these vesicles in cell-cell communication [42]. The cargo of RPE exosomes is influenced not only by genomic DNA, but also by mitochondrial DNA (mtDNA). Particularly, one paper described the association between EVs and haplogroups, which are mtDNA haplotypes correlated with the geographic origins of populations (*i.e.*, haplogroup H is the most common in Europe whereas haplogroup J in Near East). Specifically, ARPE-19 cells carrying mitochondria of the haplogroup J (J-cybrids) released EVs enriched in fibronectin and annexin A2, which were able to reduce transepithelial resistance once they had been internalized by naïve RPE cells through the involvement of HDAC6. No effects were observed instead for EVs derived by H-cybrids [43]. Subsequently, it was reported that the RPE communicated with endothelial cells and immune cells through exosomes; for example, RPE actively exchanged miR-21 with retinal microglia and utilized EVs to regulate the homeostasis of these immune cells in a p53-mediated manner [44]. Furthermore, these EVs promoted angiogenesis in stressful conditions by conveying several pro-angiogenic factors, such as VEGFR2 to the endothelial cells [45,46]. The immunoregulatory effects of RPE-derived EVs have been examined in T-cells, monocytes, and macrophages. EVs originating from an RPE stimulated with cytokines- (*i.e.*, interleukin-1β, IL-1β, interferon-γ, IFN-γ, and tumor necrosis factor-α, TNF-α) were demonstrated to inhibit the proliferation of T-cells and induce the $CD14^{++}CD16^{+}$ phenotype of monocytes [47]. Furthermore, EVs derived from the RPE are believed to regulate the activity of macrophages and, consequently of innate immunity through different mediators (*e.g.*, IL-6, IL-8, VEGF, and monocyte chemoattractant protein-1, MCP-1) as well as evoking a suppression of apoptosis [48,49]. Table 2 summarizes the literature related to EVs and RPEs.

Table 2
List of papers (in chronological order) describing the physiological role of EVs in RPE cells. The type of study (*in vitro*, *ex vivo*, or *in vivo*), the model used, and the principal findings are also reported. From this list, it is evident that one of the main topics examined has involved the association of αB-crystallin with EVs.

Author and Year	Type of study	Model	Findings
Sreekumar et al., 2010 [37]	*In vitro/ex vivo*	Human RPE cells, retinal cryosections from 129 svE mice	αB-crystallin is released through exosomes by RPE cells, whose uptake by PRs in times of oxidative stress promotes their protection
Gangalum et al., 2011 [38]	*In vitro*	ARPE-19	Exosome-mediated αB-crystallin transport is mediated by lipid rafts
Bhat et al., 2011 [39]	*In vitro*	ARPE-19	Speculations regarding the role of exosome-mediated αB-crystallin release in pathological contexts
Toyofuku et al., 2012 [41]	*In vitro/in vivo*	ARPE-19 and C57BL/6 J mice (wt, Sema4A−/−. prosaponin −/−)	Sema4A is critical for PRs' survival and phototransduction
Gangalum et al., 2016 [36]	*In vitro*	ARPE-19	Inhibition of αB-crystallin reduces exosome release
Knickelbein et al., 2016 [47]	*In vitro*	ARPE-19, human PBMCs	EVs from RPE cells modulate the immune response
Klingeborn et al., 2017 [42]	*In vitro*	RPE cells	The content of RPE-derived exosomes is different according to whether there is a basolateral or apical release of these vesicles
Atienzar-Aroca et al., 2018 [45]	*In vitro*	ARPE-19, HUVEC	Exosomes from RPE promote new vessels formation
Nicholson et al., 2020 [43]	*In vitro*	ARPE-19 cybrids	mtDNA haplogroup affects EVs' cargo and release
Morris et al., 2020 [44]	*In vitro/ex vivo*	Human RPE cells, mice primary retinal microglia, RPE-choroid-sclera mice explants	miR-21 is exchanged between RPE cells and microglia through exosomes
Fukushima et al., 2020 [46]	*In vitro*	ARPE-19, HUVEC	EMT-associated factors induce the release of pro-angiogenic exosomes from RPE cells
Otsuki et al., 2021 [48]	*In vitro*	pmRPE cells, RAW 264.7	Exosomes from RPE cells promote a crosstalk with macrophages
Sanjiv et al., 2021 [49]	*In vitro/in vivo/ex vivo*	ARPE-19, RAW 264.7, immunized C57BL/6 mice	Extracellular soluble membranes from RPE cells act as a vehicle to transmit pro-apoptotic signals to macrophages

2.3. Retinal neuronal cells

In mammalian species, the release of EVs is an evolutionarily conserved process, and it plays an important role in the maintenance of homeostasis in the photoreceptors (PRs) as well as in communication with other cell types (*e.g.*, Müller glial cells and RPE) [50]. Notably, EVs seem also to play a crucial function during the development and differentiation of the adult retina [51]. Retinal progenitor cells release EVs containing a variety of mRNAs coding for transcription factors such as paired box protein 6 (Pax6), hairy and enhancer of split-1 (Hes1), the sex determining region Y-box 2 (Sox2), Nestin which have been implicated in the maintenance of retinal morphology as well as in its growth [52].

While Müller glial cells are not only the target of RPE-derived EVs, these cells can actively secrete exosomes positive for a vesicle-associated membrane protein 5 (VAMP5), which led to the identification of a glial-specific secretome in the retina [53]. It does seem that exosomes exert a physiological protective role in PRs; *in vivo* experiments have demonstrated that thioredoxin (Trx) can regulate the secretion of exosomes in PRs in an autophagy-dependent manner, conferring protection on these cells. Moreover, RPE homeostasis was reported to rely on the internalization of exosomes from PRs [54]. The crosstalk between RPE and PRs has also been highlighted in other experiments demonstrating the rescue of damaged PRs by RPE-derived exosomes [55]. Exosomes communicate the presence of cellular damage in the eye; specifically, RPE cells can accumulate EVs from degenerating PRs and *vice versa* and this process seems to be accentuated in particular pathological contexts [56,57].

The role of exosomes in ocular physiology is also highlighted by their importance in the regulation of the visual response *i.e.*, a response that also involves the central nervous system (CNS). Particularly, proteomic and subsequent GO functional analyses revealed that most of the EV proteins implicated in the visual response are associated with endocytosis and transport of vesicles as well as morphology of neurons, neurotransmission and axonogenesis [58,59]. An analysis of the content of EVs derived from adult retina revealed the presence of proteins which

have been implicated in the function of cells from the originating tissue. In particular, cadherin related family member 1 (Cdhr1), castor zinc-finger 1 (Casz1), syndecan-binding protein (Sdcbp), retinol dehydrogenase 5 (Rdh5), and neuronal-specific nuclear protein (NeuN) have been detected as cargo in retinal EVs [60]. Table 3 summarizes the literature related to EVs and various retinal neuronal cells.

3. EVs' roles in important retinal pathological processes and diseases

3.1. Inflammation and vascularization

Considering the plethora of physiological effects exerted by exosomes in ocular tissues, it is not surprising that their involvement in pathological processes has led to the speculation that this extends to several ocular diseases, such as Age-related Macular Degeneration (AMD), Diabetic Retinopathy (DR), Glaucoma and Retinitis Pigmentosa (RP). In particular, two important biological processes seem to be stringently regulated through the release of EVs by ocular cells: inflammation and vascularization. Under conditions of oxidative stress, RPE cells release exosomes enriched in many phosphoproteins (*e.g.*, Bcl-2, Akt, Src, Erk1/2, AMPK) [61] as well as two regulators of the complement system *i.e.*, CD46 and CD59, which were detected from RPE cells in presence of oxidized low-density lipoproteins (oxLDLs) [62]. The apoptotic protease activating factor-1 (Apaf-1) is another protein upregulated in stress RPE-derived exosomes. It is known that Apaf-1 can trigger caspase-9-mediated apoptosis in naïve RPE cells [63]. Other proteins playing a key role in this inflammatory pathway (*i.e.*, IL-1β, IL-18, and caspase-1) are over-expressed in the exosomes secreted by RPE cells under blue light-induced oxidative stress [64]. Notably, oxidative stress was reported to lead to an increase in the release of EVs which can propagate inflammatory stimuli to neighboring cells [65]. The spreading of detrimental messages through EVs in RPE cells is also modulated by the up-regulation of integrins, proteoglycans, and annexin A2 on the membrane of stressed RPE and the relative ligands in the membrane of EVs [66]. The number of vesicles released under stressful conditions can be also influenced by the effects of lncRNAs, as in the case of *CYLD-AS1*, which regulates the expression of the anti-oxidant regulator nuclear factor erythroid 2-related factor 2 (Nrf2) and the immune response *via* the presence of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in the secreted EVs [67]. Not surprisingly, reactive oxygen species (ROS) can induce inflammation in the RPE also through damage to mtDNA, which promotes the *Z*-DNA-binding protein 1 (ZBP-1)-mediated expression of pro-inflammatory pathways, an elevated release of EVs, and the propagation of inflammatory stimuli to microglia [68].

The response of RPE cells to oxidative stress is strictly associated with the release of pro-angiogenic factors through EVs directed to the vascular endothelial cells. In particular, it has been demonstrated that ROS or other oxidative stress can stimulate RPE cells to release EVs enriched in VEGF and VEGFR and impoverished in miR-122 and miR-302a, and these alterations can directly or indirectly influence the vascularization process [69–72]. Another vascularization inducer, miR-155, once internalized, was able to trigger a pro-angiogenic phenotype in the RPE cells; its inhibition conferred retinal protection and reduced the severity of the inflammation [73,74]. Retinal glial cells also play an important role in the maintenance of vascular integrity in this tissue. Specifically, retinal astroglial cells (RACs) can suppress angiogenesis through the release of EVs enriched in endostatin, TIMP metallopeptidase inhibitor 1 (TIMP1), pigment epithelium-derived factor (PEDF), MMP3, and MMP9 [75]. Certain pro-survival pathways, such as autophagy, which is induced as a defense mechanism by ROS, regulate this process. Notably, the release of EVs can be suppressed not only by exosome inhibitors (*e.g.*, GW4869) but also by autophagy inhibitors (*e.g.*, 3-methyladenine, 3-MA) [76], stressing the link between EVs and the autophagic pathway. The beneficial effects exerted by EVs derived from retinal glial cells depended on the healthy status of the parental cells; analogously, exosomes from stressed microglia conveyed pathological signals to RPE cells, thus contributing to the propagation of the inflammatory stimuli [77]. Table 4 summarizes the literature related to the involvement of EVs in inflammation and oxidative stress with a special focus on the retina.

Table 3
List of papers (in chronological order) describing the physiological role of EVs in retinal neuronal cells, particularly Müller cells and PRs. The type of study (*in vitro*, *ex vivo*, or *in vivo*), the model used, and the principal findings are also reported.

Author and Year	Type of study	Model	Findings
Ho et al., 2015 [58]	*In vivo*	C576BL/J mice and Dark Agouti rats	The visual response is mediated by the release of ATP through vesicles released from dopaminergic neurons
Zhou et al., 2018 [52]	*In vitro*	Mouse retinal progenitor cells	EVs from mRPCs contain transcription factors, miRNAs and proteins involved in retinal development
Ropelewski and Imanishi, 2020 [56]	*In vivo*	Frog (*X. laevis*)	RPE cells internalize microvesicles originating from degenerating PRs
Mighty et al., 2020 [60]	*Ex vivo*	Retina from C57BL/J mice	EVs are constitutively released in adult neural retina and convey factors involved in retinal physiology
Ashok et al., 2021 [57]	*In vitro/in vivo*	ARPE-19, C59BL/J mice	EVs mediate iron overload in PRs
Zhou et al., 2021 [51]	*In vitro*	iPSC-derived 3D retinal organoids	EVs promote retinal development as well as the differentiation of RGCs and PRs
Wang et al., 2021 [55]	*In vitro/in vivo*	ARPE-19, C57BL/J mice	Exosomes from RPE cells could rescue PRs in a model of retinal degeneration
Demais et al., 2022 [53]	*In vivo*	C57BL/J mice	Müller cells release VAMP5-positive EVs *in vivo*
Ren et al., 2022 [54]	*In vitro/in vivo*	Transgenic C57BL/J mice, 661w cells	Thioredoxin up-regulation protects PRs through exosome secretion and autophagy
Schiapparelli et al., 2022 [59]	*In vivo*	Wistar rats	Exosomes mediate the diversification of protein transport in the neurons involved in the visual cycle

3.2. AMD

EVs, in particular exosomes, play an important role in the pathological processes underlying AMD. This disease is usually classified into either its dry (85–90%) or wet (10–15%) forms [35]. The early clinical hallmarks in both forms include an accumulation of lysosomal lipofuscin and extracellular drusen deposits and the degeneration of the RPE. In wet AMD, new blood vessels (neovascularization) sprout from the choriocapillaris into the retina and trigger a detrimental edema in response to VEGF upregulation. Nowadays, anti-VEGF agents are used to suppress this retinal edema. The clinical hallmarks of dry and wet AMD are illustrated in Fig. 2.

Oxidative stress-induced damage to the RPE is considered to be a key factor in the pathology underpinning AMD [78]. RPE cells are constantly exposed to oxidative stress and this may lead to an accumulation of damaged cellular proteins, lipids, nucleic acids, and cellular organelles, including mitochondria. The ubiquitin-proteasome and the lysosomal/autophagy pathways are the two major proteolytic systems involved in the removal of damaged proteins and organelles. It has been demonstrated that increased oxidative stress, protein aggregates and disturbed clearance are triggers for inflammation [35]. Different studies regarding the RPE's response to the AMD-related inflammatory stimuli demonstrated that EVs can propagate detrimental messages, particularly

Table 4
List of papers (in chronological order) describing the role of EVs in oxidative stress and vascularization, two hallmarks of ocular inflammation. The type of study (*in vitro*, *ex vivo*, or *in vivo*), the model used, and the principal findings are described. As reported, most of the papers utilized *in vitro* models.

Author and Year	Type of study	Model	Findings
Biasutto et al., 2013 [61]	*In vitro*	ARPE-19	Oxidative stress induces the secretion of certain phosphoproteins from RPE cells through exosomes
Hajrasouliha et al., 2013 [75]	*In vitro/ in vivo*	B6 mRMVECs, C57BL/J mice and bone marrow DCs	Exosomes derived from retinal astrocytes act as a vehicle for delivery of antiangiogenic factors
Ebrahimi et al., 2014 [62]	*Ex vivo*	RPE of early AMD eyes	Altered expression of CD46 and CD59 in RPE in the presence of oxLDLs
Azientar-Aroca et al., 2016 [69]	*In vitro*	ARPE-19, HUVEC	ROS increase the release of exosomes from RPE cells and promote angiogenesis in endothelial cells
Yoon et al., 2016 [73]	*In vitro*	ARPE-19, Raji, HUVEC	miR-155 alters RPE cells' functionality
Zhang et al., 2019 [64]	*In vitro*	ARPE-19	Blue-light stimulation promotes the secretion of exosomes from RPE cells and the activation of the inflammasome
Maisto et al., 2019 [70]	*In vitro*	ARPE-19, HUVEC	VEGF-containing exosomes from ARPE-19 promote neovascularization
Oltra et al., 2019 [71]	*In vitro*	ARPE-19	miR-302a and miR-122 are downregulated in EVs derived from ARPE-19 under oxidative stress
Ke et al., 2020 [63]	*In vitro*	ARPE-19	Exosomes derived from inflamed RPE cells can propagate inflammatory stimuli to normal RPE cells
Yang et al., 2020 [65]	*In vitro*	ARPE-19	Oxidative stress in RPE cells is exacerbated by EVs
Nicholson et al., 2020 [66]	*In vitro/ ex vivo*	ARPE-19, primary porcine RPE cells	On the membranes of EVs secreted under stressful stimuli there are ligands that promote the rapid uptake of the EVs
Du et al., 2020 [67]	*In vitro*	RPE cells	LncRNA *CYLD-AS1* promotes inflammation in RPE cells
Zhu et al., 2020 [76]	*In vitro*	Human retinal astrocytes, HUVEC	Oxidative stress-induced autophagy in retinal astrocytes mediates exosomes release
Aires et al., 2020 [77]	*In vitro/ in vivo*	BV-2 cells, retinal microglia, C57BL/J mice	Exosomes derived from stressed microglial cells transmit detrimental signals to retinal cells
Aggio-Bruce et al., 2021 [74]	*In vivo*	C57BL/J mice	miR-155 inhibition attenuates inflammation in the retina
Saada et al., 2022 [68]	*In vitro/ ex vivo*	ARPE-19, HMC3, normal human RPE cells, MEFs from WT and ZBP1 KO C57BL/J mice	Oxidative stress promotes the release of mtDNA (mainly with EVs) from RPE cells with a consequent activation of microglial cells through ZBP1
Oltra et al., 2022 [72]	*In vitro*	ARPE-19, HUVEC	miR-302a-3p in small EVs released from RPE cells induce angiogenesis in conditions of oxidative stress

through the carriage of pro-inflammatory and pro-angiogenic factors [61,62,64,65,67,75,79]. *In vitro* experiments demonstrating the involvement of exosomes-delivered miRNAs in AMD-related pathological contexts [71] were corroborated by analyses performed on exosomes obtained from AMD patients. Sera-isolated exosomes from AMD individuals were enriched in miRNAs believed to be involved in the VEGF signaling pathway (*i.e.*, miR-19a, miR-126, and miR-410) [80]; furthermore, the content of the miRNAs in the exosomes differed between dry and wet AMD patients, showing a differential up-regulation of let-7a-5p, miR-17-5p, miR-195-5p, miR-26b-5p, and miR-30c-5p in dry AMD, which are related to apoptotic events, while wet AMD, as expected, was characterized by exomiRs promoting angiogenesis [80]. A content analysis of the EVs derived from the AH of AMD patients made it possible to identify some proteins that were differentially expressed between AMD patients and control subjects, and whose secretion was impaired by anti-VEGF treatments. It has also been demonstrated that AH from AMD patients is impoverished of both microvesicles generated by neural cells and in the general protein content. Conversely, RPE-derived exosomes from these AMD patients have elevated amounts of Heat Shock Protein 70 (Hsp70), which is produced by cells under stressful conditions in an attempt to repair damaged proteins. Thus, these represent differences in the expression of secreted proteins implicated in the autophagy-lysosomal pathway and in the epithelial-mesenchymal transition (EMT). Among them, the levels of actin, myosin-9, galectin 3-binding protein, lysozyme, metalloproteinase inhibitor, PEDF, vitamin D-binding protein, complement factor C3, annexin A1, cytokeratin 14, and cathepsin D, have been found to be higher in AMD subjects than in controls [81]. A differential expression (*i.e.*, up-regulation in AMD) in the secretion of the complement factor C3 was also identified in a subsequent study, together with that of apolipoprotein A1 (APOA1) and clusterin (CLU). Furthermore, anti-VEGF treatments reduced the exosome-mediated secretion of SERPINA1 and the zinc-alpha2-glycoprotein (AZGP1) [82]. Several investigators have shown that the secretion of exosomes plays a role in drusen formation in AMD. Particularly, *in vitro* and *ex vivo* experiments demonstrated that the amounts of two exosome markers, CD63 and LAMP2 (Lysosome-associated membrane protein 2), were increased in ARPE-19 cells and accumulated between RPE and choroid of old mice [83]. Similarly, immunostaining experiments detected LAMP2, CD81, and CD63 in drusen of AMD patients, with CD63 co-localizing with some drusen-associated proteins, such as amyloid-β and αB-crystallin [83]. Moreover, exosomes released from RPE cells seemed to be the cause of the down-regulation of two complement regulator proteins, CD46 and CD59, in the plasma membrane of RPE cells in the retinal areas affected by AMD [84]. Notably, *post-mortem* studies identified Alix and Fibulin-3 (Fib3) positive puncta in the drusen of AMD patients, suggesting the existence of different EV subpopulations in the pathological area of the retina [85]. Further evidence regarding the role of EVs in the formation of drusen emerged from the work of Flores-Bellver and colleagues [86]; they demonstrated the active secretion of drusen-associated proteins through EVs release by RPE cells in the presence of stress conditions mimicking AMD. This evidence, together with the results obtained through multi-omics approaches, highlights the key role of EVs in the induction of the pathological phenotype associated with AMD. Furthermore, EVs derived from the RPE taken from AMD patients have been characterized by an enrichment in mRNAs, proteins, and lipids involved in specific AMD-pathways, such as oxidative stress, drusen accumulation, and angiogenesis. Importantly, these EVs can propagate their detrimental effects into naïve RPE cells [87]. The molecular mechanisms that contribute to the release of EVs in the AMD pathological condition have been partially elucidated. There is evidence in the literature for the involvement of the $P2X_7$ receptor, an ATP-gated ion channel, when EVs are secreted in times of oxidative stress. Particularly, the knock-out (KO) of this receptor was able to protect mice from the appearance of an AMD-like phenotype and the accumulation of EVs [88]. A similar effect on the release of EVs was obtained by treating

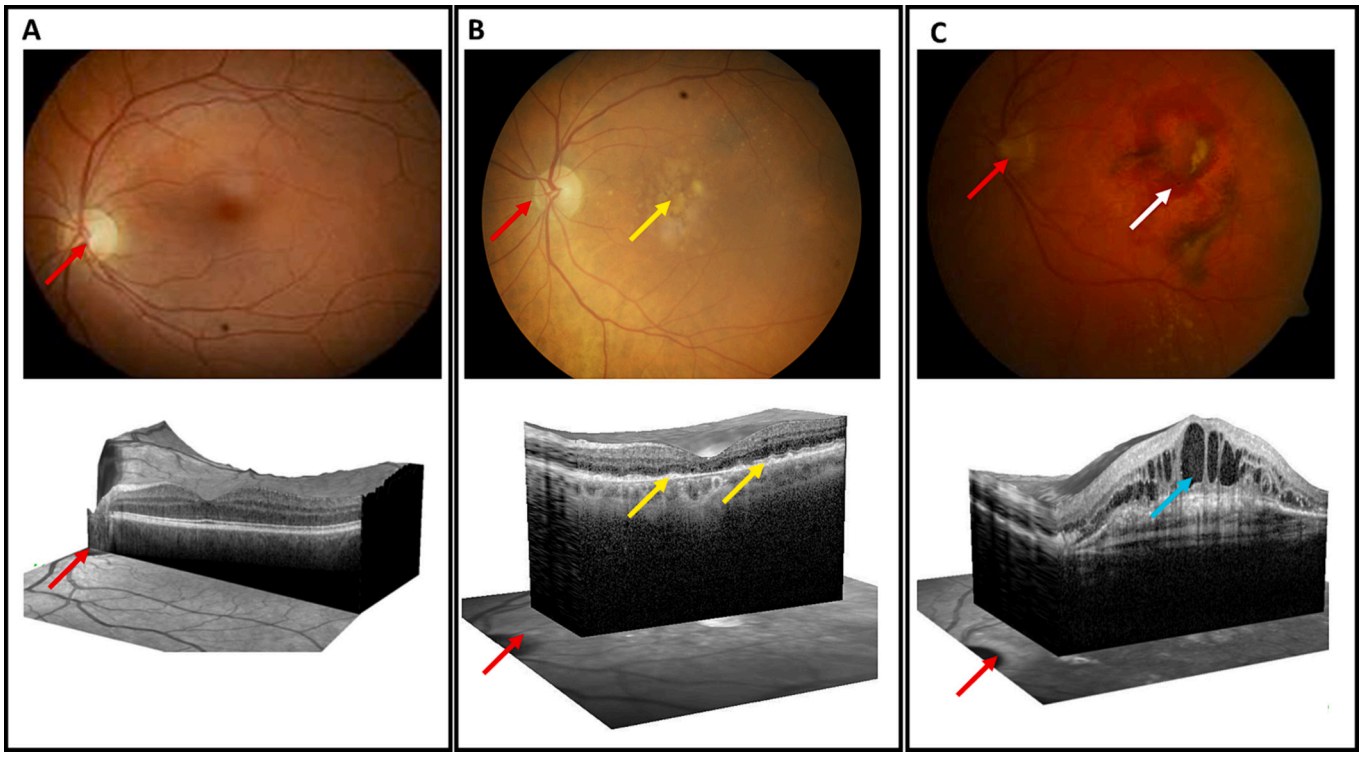

Fig. 2. Representative fundus photographs and optical coherent tomography (OCT) images from healthy (**A**), dry AMD (**B**), and wet AMD (**C**) cases. Optic disc (red arrows), drusens (yellow arrows), neovascularization hemorrhage (white arrow), and macular edema (cyan arrow) are indicated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

primary RPE cells with *N*-acetylcysteine amide [89]. ExomiRs are also involved in the induction of epigenetic modifications: the presence of hyperhomocysteinemia, a condition associated with AMD and diabetic retinopathy (DR), was shown to induce the expression of miRNAs capable of activating histone deacetylase (HDAC) and DNA methyltransferase (DNMT), as well as an involvement in oxidative stress, inflammation, hypoxia, and angiogenesis. Interestingly, the inhibition of HDAC and DNMT activity reduced many dysfunctional events in the blood retinal barrier [90]. Recently, it has been demonstrated *in vivo* that the release of exosomes represents an important endogenous neuroprotective factor in the retina. In particular, a decrease in exosome secretion from PRs has been found to be associated with retinal damage in mice exposed to photo-oxidative damage [91]. This result was confirmed by administration of GW4869, an inducer of exosome depletion, which exacerbated retinal dysfunction and damage, at least partially through the death of PRs and the appearance of inflammation. Furthermore, mice with a depressed exosome release displayed an impaired ability to exchange of PR-derived miRNAs regulating the immune response [91]. Table 5 summarizes the literature related to EVs and AMD.

3.3. Diabetic retinopathy (DR)

EVs play an important role in the pathogenesis of DR. This disease is characterized by progressive microvascular complications in the retina with the presence of microaneurysms, hemorrhages, exudates, changes in the veins, neovascularization and retinal thickening. It can affect both the peripheral retina and the macular area. High glucose concentrations, tissue hypoxia and increased inflammation are present in advanced diabetic retinopathy [92]. The clinical characteristics of DR are illustrated in Fig. 3.

As already mentioned, exosomes from RACs contain anti-angiogenic factors that dampen neovascularization and that, once altered, not only contribute to the development of the typical hallmarks of DR [75] but they can also directly trigger the release of EVs capable of influencing vessel neogenesis, as demonstrated for EVs obtained from MSCs [93]. As reported by Mazzeo and colleagues [94], one possible molecular mechanism is based on the downregulation of the expression of miR-126 in human retinal pericytes (RPs) induced by MSC-EVs obtained in DR-like conditions. On the other hand, some exomiRs (*i.e.*, miR-150-5p, miR-21-3p, miR-30b-5p) are differentially expressed in diabetic patients with ocular complications in comparison with healthy subjects, and subsequently they have been considered as potential biomarkers. In this regard, EVs from DR patients have been able to induce DR-like modifications in *in vitro* models, possibly by transferring the forms of miRNAs associated with angiogenic dysfunctions [95,96]. A good example is represented by miR-30b, which is present at a high concentration in plasma-derived EVs of DR mice models; this miR has pro-angiogenic effects mediated through the inhibition of sirtuin-1 (SIRT1) in cells in the microvasculature [97]. Recently, it has been demonstrated that exosomes can trigger microvascular dysfunctions if circRNAs are present as their cargo. In particular, hypoxia-stressed pericytes transfer *circEhm1* through exosomes to endothelial cells, thus promoting their protection *in vitro* under hyperglycemic conditions [98]. Similarly, there are *in vitro* studies demonstrating that RPE cells under hyperglycemic conditions released EVs containing protective factors (such as miR-202–5p) which were able to inhibit the TGF-βR2 signaling pathway in endothelial cells cultured in hyperglycemic conditions, thus preventing the endothelial-to-mesenchymal transition [99]. Several proteins which are known to be involved in the maintenance of cellular homeostasis regulate the secretion of EVs carrying protective factors *e.g.* thioredoxin (Trx), whose upregulation in PRs enhanced the release of protective EVs in both *in vitro* and *in vivo* DR models [54], thus preventing cell degeneration. Other experiments, performed on the circulating EVs derived from the platelet-poor plasma of DR patients, demonstrated an enrichment of cytokines and pro-angiogenic factors, such as basic fibroblast growth factor (bFGF), VEGF-2, angiostatin, TNF-α, TIMP1, TIMP2, and RANTES, in the EVs' content [100]. Notably, RANTES may

Table 5
A list of papers (in chronological order) describing the role of EVs in AMD. The type of study (*in vitro*, *ex vivo*, or *in vivo*), the model used, and the principal findings are also described. From the reported list, it is evident that most papers involved *ex vivo* experiments. Moreover, several studies have focused on the involvement of EVs in the formation of drusen.

Author and Year	Type of study	Model	Findings
Wang et al., 2009 [83]	*Ex vivo*	Eyes from AMD donors and controls, tissue from C57BL/J mice	Autophagy and exosomes contribute to the release of intracellular proteins and the formation of drusen in AMD
Ebrahimi et al., 2013 [84]	*Ex vivo*	RPE from early AMD eyes	Altered expression of CD46 and CD59 in RPE in the presence of oxLDLs
Kang et al., 2014 [81]	*Ex vivo*	Exosomes from AH	Identification of exosomal proteins as novel candidate biomarkers in AMD
Carver and Yang, 2016 [89]	*Ex vivo*	Primary RPE cells	*N*-acetylcysteine prevents the oxidative stress induced by the secretion of microparticles from RPE cells
Carver et al., 2017 [88]	*In vivo*	KO mice for P2X7 and Sod1	Defects associated with AMD and the accumulation of microvesicles can be prevented by P2X7 KO
Elmasry et al., 2018 [90]	*In vitro/in vivo*	HREC, ARPE-19, mice models	Role of epigenetic modifications in AMD
Wooff et al., 2020 [91]	*In vivo*	C57BL/J mice w/wo specific mutations	Exosomes from PRs are associated with retinal damage
ElShelmani et al., 2021 [80]	*Ex vivo*	Exosomes from sera of AMD patients and controls	Identification of exomiRs associated with AMD
Grillo et al., 2021 [85]	*Ex vivo*	Post-mortem eyes	Presence of Fibulin-3 and Alix in drusen of AMD patients
Flores-Bellver et al., 2021 [86]	*In vivo*	iPSC-derived RPE, IN2–5 and ic4–4 cells	Drusen proteins are released by RPE cells in response to AMD stressors
Tsai et al., 2022 [82]	*Ex vivo*	Exosomes from AH	Identification of the exosomal protein profile in AMD patients under therapy or not. Identification of novel candidate targets for AMD treatment
Kurzawa-Akambi et al., 2022 [87]	*In vitro*	iPSC-RPE from AMD patients and control, WT1 and WT3 cells	EVs from RPE cells can induce an AMD-like phenotype

represent a novel biomarker for DR since its levels were elevated in diabetic patients and they increased with DR progression [101]. Additionally, exosomes derived from platelet-rich plasma from diabetic rats induced the production of ROS and malonyldialdehyde (MDA) as well as increasing the expression of the immunological agent, TLR4 in human retinal endothelial cells (HREC), leading to cell death; in an *in vivo* experiment, these vesicles directly induced retinal damage though the delivery of CXCL10 [102]. Other effects of these plasma-derived vesicles from diabetic rats were described on Müller cells; in these cells, they activated the proliferative and migratory pathways linked with Yes-associated protein (YAP) and the PI3K/Akt complex as well as the expression of fibrogenic proteins (*e.g.*, connective tissue growth factor and fibronectin) [103]. Notably, the shedding of EVs plays a role in DR, contributing to the process of thrombosis, an event commonly associated with this disease [104]. Moreover, the activation of the complement pathway, which contributes to the vascular damage in DR, was reported to be mediated by exosomes with a cargo composed of C1q, C3, and C4. One consequence was the formation of the membrane attack complex (MAC) and cellular damage [105,106]. Interestingly, ocular complications in diabetes can also be affected by the EVs released from the pancreas: increased miR-15a levels were detected in the plasma of diabetic patients and these were linked principally to pancreas-derived exosomes that, once in contact with retinal Müller cells, triggered an oxidative stress and an activation of apoptosis pathways [107], highlighting the importance of this kind of remote cell-cell communication. Due to the substantial amount of *in vivo* and human studies in DR, the potential use of EVs and their cargos as biomarkers has been suggested and evaluated. In particular, an analysis of the EVs' protein content sampled from the plasma of diabetic patients and healthy controls led to the identification of tumor necrosis factor-α-induced protein 8 (TNFAIP8) as a biomarker [108]. There are also other proteins that have been isolated from the EVs derived from retinal tissue and in urine of DR patients that may serve as novel biomarkers *e.g.*, junction plakoglobin, a member of the catenin protein family which has been implicated in the development of the BRB [109]. Additional studies led to the identification in EVs of miR-431-5p and miR-26b-5p; these were speculated to be useful as additional potential biomarkers for DR [110,111]. Other interesting candidates, such as IL-5, IL-18, MMP3, and MMP9, have been identified also through the analysis of tears-derived exosomes from proliferative and non-proliferative DR patients [112]. The characterization of exosomes from tears was further improved using a system based on incorporated tear-exosome analysis with rapid isolation systems (iTEARS) which led to the identification of dysregulated miRNAs in DR patients (*i.e.*, miR-145-5p, miR-214-3p, miR-218-5p, miR-9-5p) [113]. Not surprisingly, also lncRNAs were found to be associated with DR; for example, an analysis of plasma exosomes led to the isolation of *DLX6-AS1* as a risk factor for DR, whereas *PRINS* and *FAM190A-3* functioned as protective actors [114]. The damage induced by DR in endothelial cells was also recently investigated through an analysis of the exosomes derived from DR and diabetic nephropathy (DN) patients, using the sophisticated approaches of metabolomics and proteomics. As a result, up-regulation of the alpha subunit of the coagulation factor fibrinogen (FIBA), which has been implicated in exacerbations of cellular injuries, was observed in human glomerular endothelial cells treated with serum-derived exosomes from DR/DN patients; conversely, in the same samples, a down-regulation was reported of 1-methylhistidine (1-MH), a molecule involved in cytoprotection against the hyperglycemic condition [115]. Nowadays, there are novel scalable and flexible systems to allow the isolation of EVs from biological fluids [116]; these can help to identify metabolomic variations in the EVs and acquire specific signatures for DR and other ocular pathologies. Table 6 summarizes the literature related to EVs and DR.

3.4. Glaucoma

Glaucoma is a multifactorial disease and while the exact pathogenic mechanism is still unknown, the disease development involves the apoptotic death of retinal ganglion cells (RGCs), a process that may be related to increased intraocular pressure [117]. The glaucoma-oriented research has focused on two major aspects, the dynamics of the aqueous humor (production, secretion, and outflow) and the degeneration of RGCs themselves [118]. The clinical characteristics of glaucoma are illustrated in Fig. 4.

Some of the initial evidence regarding the role of EVs in the pathogenesis of glaucoma emerged from the analyses of exosomes in the human AH and TMCs, which revealed that they contained the presence of the juvenile glaucoma-causing MYOC, and pointed to a role for EVs in the control of intraocular pressure [26,28] Alterations in the miRNAs inside the EVs were detected in TMCs as a response to TGF-β2 treatment, a factor which is responsible for extracellular matrix remodeling and inflammation [119]. In particular, as mentioned, the combined use of TGF-β2 and collagen type I led to changes in the levels of the pro-inflammatory exomiRNAs in TMCs [31]. Some miRNAs such as miR-182, and specifically its variant rs76481776 seem to be associated with the onset glaucoma, this latter form has been identified in TMC-derived exosomes, suggesting that it has a role in primary open-angle

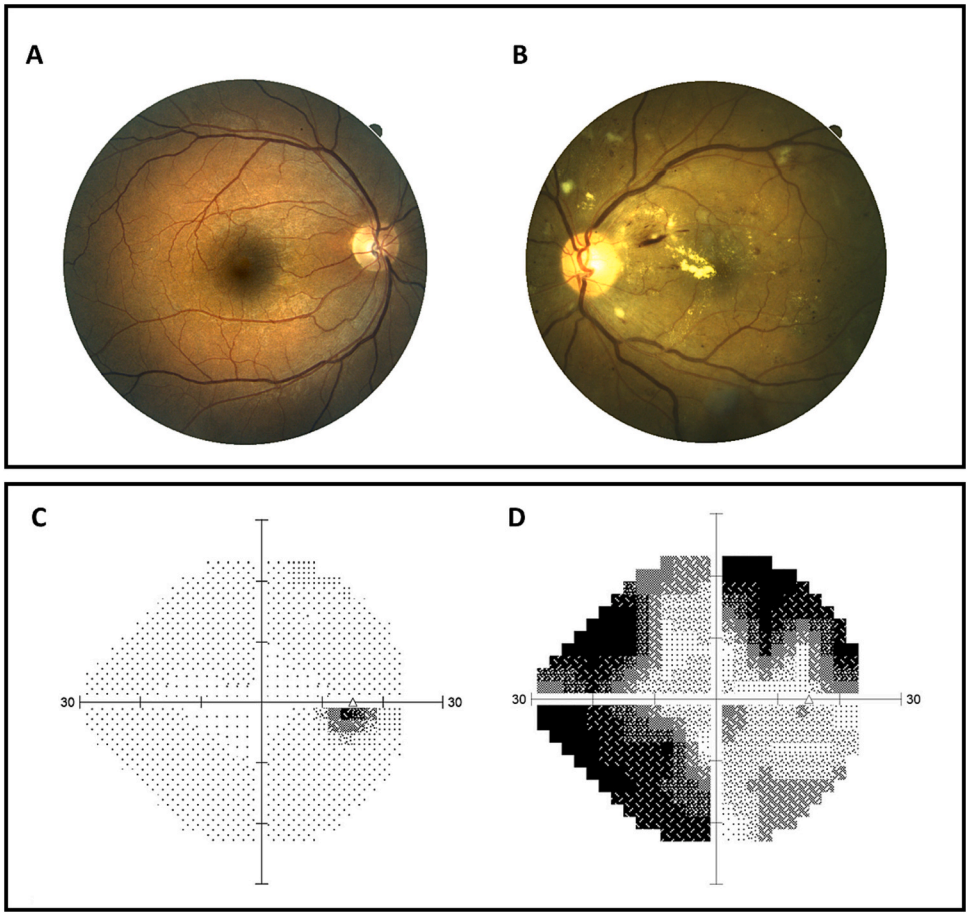

Fig. 3. In the upper panels, representative fundus photographs from healthy (**A**) and DR (**B**) patients. In the lower panels examples of visual field tests from a healthy subject (**C**) and a DR patient (**D**).

glaucoma (POAG) [120]. The exchange of EVs is significant in the cross talk between TMCs and NPCE cells; in particular, the mechanism underlying the uptake of NPCE EVs by TMCs has been described by Tabak et al. [121], who stated that this communication represented a paradigm for a form of signaling common to many tissues, and therefore it would be relevant beyond the pathophysiology of glaucoma disease. In this regard, EVs derived from NPCE cells exposed to a non-lethal oxidative stress contained factors which could trigger an endogenous detoxifying response in TMCs, such as Nrf2 activation and Wnt inhibition, and therefore would be able to protect these cells from a subsequent direct oxidative insult [30,122]. Interestingly, some anti-glaucoma drugs, *i.e.*, β-blockers and carbonic anhydrase inhibitors, affect the physicochemical characteristics of EVs and thus interfere with TMCs' uptake properties, and this has implications for the communication between TMCs and the NPCE [123].

Other cellular components are participants in the biological processes involved in the onset of glaucoma. Proteomic studies on human sclera found evidence of a differential protein expression between patients with and without POAG, with six proteins enriched in POAG patients (*i.e.*, vimentin, annexin A2, serum albumin, serum amyloid P component, angiopoietin-related protein 7, and thrombospondin-4) and accordingly when bioinformatic approaches were applied, these were possibly associated with EVs [124]. As already mentioned, exosomes derived from microglial exposed to high pressure promote inflammation in retinal cells in an *in vivo* glaucoma model, further evidence that EVs are a relevant system contributing to propagating the inflammatory state and promoting neurodegeneration in glaucomatous conditions [77]. Metabolomic and proteomic approaches on EVs derived from POAG patients' tears revealed that the numbers of these vesicles were extremely high in comparison with healthy subjects and according to previous evidence, their cargo was enriched in certain proteins involved in the recruitment of neutrophils, such as lysozyme (LYZ) [125]. A better understanding of the exosome signaling pathways in the context of glaucoma would also be potentially useful when designing novel therapeutic approaches [29,32]. One example is represented by fibrillogenesis, a pathway dysregulated in POAG, and physiologically regulated through the exchange of EVs between NPCE cells and TMCs. Since NPCE-derived EVs regulate the deposition of collagen type I, the delivery of EVs could represent a valuable approach in counteracting POAG dysfunction in the deposition of the extracellular matrix [126]. Based on this evidence, other approaches have been used *in vitro* to counteract fibrotic processes; for example, the use of siRNA molecules targeted against *decapentaplegic homolog 7* (*SMAD7*) on TMCs through NPCE-derived EVs produced encouraging preliminary results [127].

Very little is still known about the neuronal pathomechanisms underpinning glaucoma. Some recent studies have shed new light into the ways that RGCs function as a part of a syncytial formation in various retinal cells. It is known that both the direct and paracrine cross-talk between neighboring cells may participate in either the death or the survival of RGCs in glaucoma [128]. The direct communication is represented by gap junctions (GJs), but it has very recently been discovered that RGCs axons are directly coupled by sophisticated synapses allowing for passage of ions and small signaling molecules between cells and consequently an involvement in the regulation of homeostasis [129]. However, it has been speculated that EVs have a physiological role in homeostasis in RGCs; in the development of glaucoma there are disturbances in signaling systems involving EVs. In this situation, the paracrine mechanism of cellular cross-talk can be considered as an

Table 6
List of papers (in chronological order) describing the role of EVs in DR. The type of study (*in vitro*, *ex vivo*, or *in vivo*), the model used, and the principal findings are also reported. From the list, it is evident that the majority of papers involved *in vitro* and *ex vivo* experiments. In comparison with AMD and glaucoma, DR shows the highest number of studies. Moreover, several research groups have focused on the possible use of EVs as biomarkers and on exomiRs.

Author and Year	Type of study	Model	Findings
Beltramo et al., 2014 [93]	*In vitro*	Human retinal pericytes, MSCs, and human microvascular EC	EVs derived from MSCs cultured in diabetic-like conditions induce a DR-like phenotype
Tokarz et al., 2015 [100]	*Ex vivo*	EVs from diabetic patients w or wo ocular complications and controls	EVs in diabetic patients with ocular complications carry angiogenic factors and cytokines
Mazzeo et al., 2015 [94]	*In vitro*	Human retinal pericytes and MSCs	EVs derived from MSCs cultured in diabetic-like conditions lead to a down-regulation of miR-126 and the release of pro-angiogenic factors from pericytes
Kamalden et al., 2017 [107]	*In vitro/ ex vivo*	Plasma from adult volunteers, MIO-M1, rMC-1, HRECs, HRPEs, and INS-1 cells	miR-15a released from the pancreas through exosomes contributes to the ocular complications associated with diabetes
Mazzeo et al., 2018 [95]	*Ex vivo*	EVs from serum/plasma of diabetic patients (w or wo DR) and controls	Identification of exomiRs that may represent novel biomarkers for DR
Su et al., 2018 [104]	*Ex vivo*	Microparticles from DR patients and controls	Microparticles in DR patients promote pro-coagulant activities
Huang et al., 2018 [105]	*In vivo*	STZ-mouse model of DR	Microvascular damage is triggered by classical complement activation mediated by exosomes
Mazzeo et al., 2019 [96]	*In vitro/ ex vivo*	EVs from DR patients and controls, human retinal pericytes and human microvascular ECs	Characterization of the exomiRs identified in the previous work (Mazzeo et al., 2018), which may represent a prognostic biomarker for DR
Tokarz et al., 2019 [101]	*Ex vivo*	EVs from diabetic patients and control	The RANTES concentration in CCR5-positive EVs correlates with the severity of the retinopathy
Zhang et al., 2019 [102]	*In vitro/ in vivo*	Diabetic rats, HRECs	Exosomes from platelet-rich plasma induce endothelial damage
Zhang et al., 2020 [103]	*In vitro/ ex vivo*	Diabetic rats, human Muller cells	Exosomes from plasma of diabetic rats promote fibrogenic activity in human Muller cells
Gu et al., 2020 [99]	*In vitro*	ARPE-19, HUVEC	miR-202–5p secreted through exosomes by RPE cells protects against DR
Huang et al., 2020 [106]	*In vitro/ in vivo*	STZ-mouse model of DR, HRECs	Retinal endothelial damage is promoted by MAC deposition after the complement activation mediated by exosomes
Ye et al., 2021 [98]	*In vitro*	Murine retinal microvascular pericytes and endotheliocytes	Exosomal *circEmth1* plays a role in microvascular dysfunction
Xiao et al., 2021 [108]	*In vitro/ Ex vivo*	Small EVs from diabetic patients w or wo DR, HRMECs	TNFAIP8 is a novel biomarker for DR
Yu et al., 2021 [110]	*Ex vivo*	Small EVs from DR patients plasma	miR-431-5p is a possible biomarker for proliferative DR
Wang et al., 2022 [97]	*In vitro/ in vivo*	STZ-mouse model of PDR, retinal microvascular ECs	miR-30b is delivered by plasma-EVs to the PDR and contributes to angiogenesis
Ren et al., 2022 [54]	*In vitro/ in vivo*	Transgenic C57BL/J mice, 661w cells	Thioredoxin up-regulation protects PRs through exosome secretion and autophagy
Mighty et al., 2022 [109]	*Ex vivo*	EVs from human DR retinal tissues and urine	Plakoglo bin protein is over-expressed in EVs derived from retinal tissue and urine of DR patients
Zhang et al., 2022 [111]	*Ex vivo*	EVs from serum of DR patients	miR-26b-5p is a possible biomarker for DR
Amorim et al., 2022 [112]	*Ex vivo*	Small EVs in tears of T2D patients and controls	Diabetic patients with retinopathy show alterations in the profile of tear fluid proteins
Hu et al., 2022 [113]	*Ex vivo*	Exosomes from tears	iTEARS technology identified dysregulated miRNAs associated with DR development
Ye et al., 2022 [114]	*Ex vivo*	Plasma exosomes from diabetic patients w or wo DR	lncRNAs are associated with DR progression (*e.g.*, *DLX6-AS1*) or protection (*e.g.*, *PRINS* and *FAM190A-3*)
Yang et al., 2022 [115]	*In vitro/ ex vivo*	HGECs, serum exosomes	Metabolomic and proteomic analyses confirm that serum exosomes in DR patients contribute to endothelial damage
Pan et al., 2022 [116]	*Ex vivo*	Plasma EVs	TiO2 microbeads achieve a high purification of EVs and this can be used to obtain a metabolic signature in DR

extracellular extension of GJ-based cell-cell direct contact [130]. Table 7 summarizes the literature related to EVs and glaucoma.

3.5. Other retinopathies

According to the evidence reported in the previous paragraphs, it is possible to hypothesize that EVs would play a significant role also in other types of retinal pathologies, such as retinitis pigmentosa (RP). This disorder is an inherited ocular condition that leads to a progressive retinal degeneration. Its symptoms include night blindness, onset of tunnel vision and progressive decrease of central vision. Moreover, RP patients suffer from decreased visual acuity, dyschromatopsia and dark pigmentary clumps in the fundus ("bone spicules") [131]. The clinical characteristics of RP are illustrated in Fig. 5.

There are still rather few published studies clarifying the role of EVs in RP, with most of them focused on RPE cells. *In vivo* studies have demonstrated that RPE cells can engulf the EVs originating from rods. Furthermore, it has been demonstrated that in *Xenopus laevis* expressing the class I mutant rhodopsin, the EVs containing the mutated protein, are released from degenerating rods, and phagocytized by RPE cells [56]. The alterations in the contents of the EVs and their increased uptake agree with the RPE-derived EV-mediated transport of detrimental proteins, such as α-synuclein. This is known to occur in RP as in other ocular disorders like AMD [132]. Moreover, it has been described that the total amount of EVs in *rd10* mice did not differ from healthy controls, despite the fact that there was an increase in the numbers of $CD9^{+}$ vesicles in the outer nuclear layer in the mutated mice. The secretion of these vesicles seemed to be dependent on the action of poly (ADP-ribose) polymerase (PARP) [133]. The role of PARP in the release of EVs had been previously examined; it was demonstrated that the impaired activity of this enzyme was mediated by αB-crystallin [37]. Again, retinal

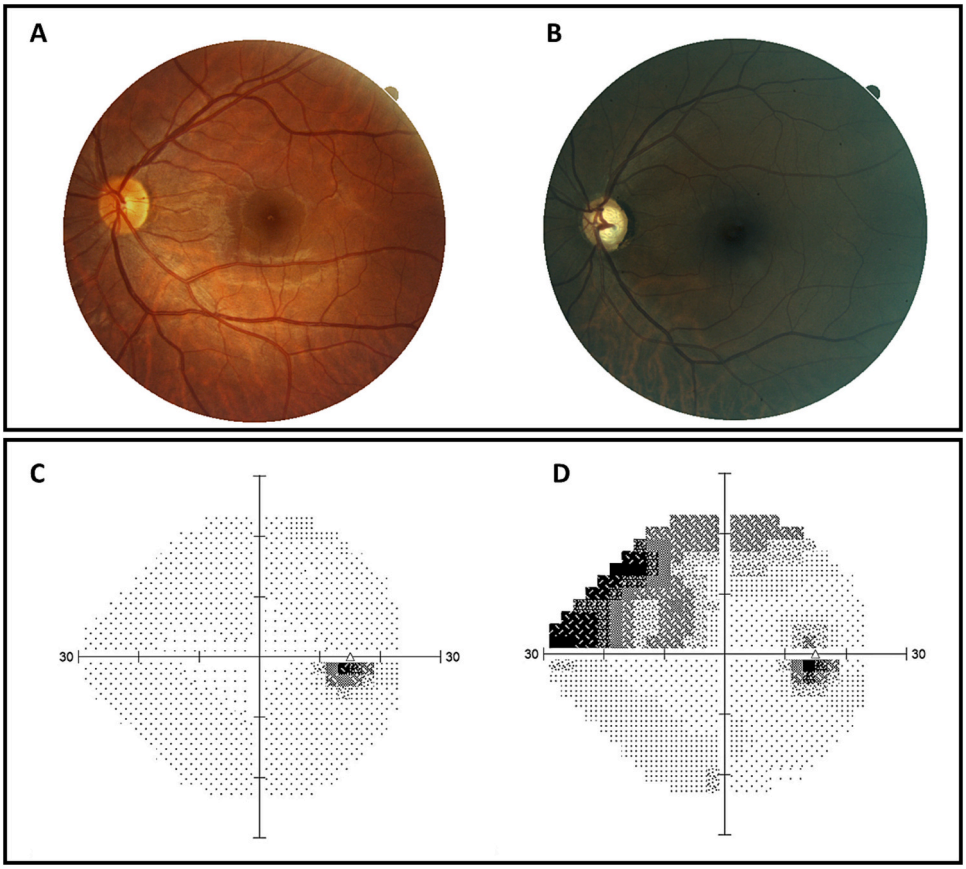

Fig. 4. In the upper panels, representative fundus photographs from healthy (**A**) and glaucoma (**B**) patients. In the lower panels, examples of visual field tests from a healthy subject (**C**) and a glaucoma patient (**D**).

EVs from *rd10* mice were enriched in rhodopsin whereas no differences were observed with respect to RPE65 protein [133]. Alterations in rhodopsin vesicular transport had been described in transgenic mice carrying the P347S mutation in the same gene, suggesting that retinal degeneration is also due to dysregulation in the EV-mediated trafficking of rhodopsin [134]. Other experiments performed in *rd1* mice models demonstrated that also the secretion of EVs was impaired by ageing as well as by the presence of the mutation [135]. Retinal degeneration due to a mutation in the *phosphodiesterase 6D* (*PDE6D*) gene has been characterized by PR dysfunctions induced by EVs and alterations in protein trafficking, possibly due to derangements in the interaction with cargo proteins, such as serine/threonine protein kinase NIM1 (NIM1K) and ubiquitin-like protein 3 (ULB3) [133,136]. Other mutations, such as those occurring in the *TUB like protein 1* (*TULP1*) gene, can affect the homeostasis of EVs, leading to an accumulation of vesicles in the interphotoreceptor matrix [137]. Recently, experiments performed on both human lymphoblasts derived from two families affected by the distinctive syndrome SHRF (Short stature, Hearing loss, Retinitis pigmentosa, and distinctive Facies) and *in vitro/in vivo* models of this disease, led to the identification of a mutation in the *RNA exosome component exosome component 2* (EXOSC2). Notably, in *Drosophila,* this mutation plays a critical role in altering the development of the insect's eye [138]. EVs from other cell types might play a protective role against genetically induced retinal degeneration. Specifically, the injection of exosomes derived from either microglial or RPE cells from healthy animals led to an attenuation of the degenerative process at the retinal level in an *in vivo* model of retinopathy [55,139], confirming that vesicles from other cell types can carry physiologically protective factors to the PRs. The use of exosomes to deliver protective factors to the retina has been further confirmed in additional experiments, in which exosomes were used to carry anti-angiogenic factors to inhibit neovascularization in a model of oxygen-induced retinopathy [140]. The role of EVs in other rare ocular disorders is highlighted by their involvement in proliferative vitreoretinopathy (PVR). Exosomes play an important role in the epithelial-mesenchymal transition (EMT); for example, it was demonstrated in an *in vitro* PVR model that exomiRs contribute to inducing EMT in RPE cells [141]. Notably, also the migrasome, a particular class of extracellular vesicles related to RPE migration and proliferation, has been implicated in the development of PVR [142]. A recent proteomic analysis performed on exosomes derived from patients with severe PVR revealed that proteins associated with proliferation, inflammation, EMT, and gliosis, were differentially expressed. Furthermore, some proteins already mentioned, such as CLU, COL2A1, and SEMA7A, were found to be up-regulated in PVR patients [143]. This evidence in the scientific literature underlines the belief that EVs play a role also in rare monogenic and polygenic retinal disorders. Table 8 summarizes the literature related to EVs and other various retinal pathologies.

4. EVs as potential therapeutic tools in retinal diseases

4.1. State of art

Since the discovery of the biological properties of these vesicles, it has been postulated that EVs may have potential beneficial properties as therapeutic agents. In the ophthalmological field, some of the first evidence came from the use of an αB-crystallin-derived peptide engineered into a polymer nanoparticle to rescue the health of the RPE in stressful conditions [144]. A similar approach was used to enhance the delivery of lipophilic compounds with engineered liposomes being created to improve retinal penetration, thus overcoming the issues regarding the delivery of ocular drugs [145,146]. Notably, *in vitro* experiments have

Table 7
List of papers (in chronological order) describing the role of EVs in DR. The type of study (*in vitro*, *ex vivo*, or *in vivo*), the model used, and the principal findings are also described. As is evident from the list, the majority of published papers involved *in vitro,* and *ex vivo* experiments. Moreover, several studies were focused on the crosstalk between TMCs and NPCE.

Author and Year	Type of study	Model	Findings
Perkumas et al., 2007 [26]	*Ex vivo*	Effluent from human anterior segments or AH	MYOC is associated with exosome membranes in fresh samples
Stamer et al., 2011 [28]	*In vitro/ ex vivo*	Primary HTM, urine, and AH	TMC-derived exosomes contain MYOC, EMILIN-1, and NRP-1
Liu et al., 2016 [120]	*Ex vivo*	Post-mortem ocular tissues	A variant in miR-182 is associated with PAOG
Dismuke et al., 2016 [29]	*In vitro/ ex vivo*	HumanTM explants and cell strains	TMC-derived exosomes are enriched in the heparin/ heparan sulfate binding of annexins A2 and A6
Lerner et al., 2017 [32]	*In vitro*	NPCE cells	NPCE-derived exosomes can affect the Wnt pathway in TMCs
Rossi et al., 2019 [125]	*Ex vivo*	Tears from glaucoma patients	Multi-Omics approaches identified the enrichment of pro-inflammatory proteins in EVs from glaucoma patients' tears
Lerner et al., 2020 [122]	*In vitro*	NPCE and TMCs	EVs derived from NPCE cells exposed to non-lethal oxidative stress contain factors capable of triggering an endogenous detoxifying response in TMCs
Lerner et al., 2020 [30]	*In vitro*	NPCE and TMCs	The crosstalk between NPCE and TMCs result in a modulation of the Wnt pathway and EMC remodeling
Iomdina et al., 2020 [124]	*Ex vivo*	Sclera samples from POAG patients and controls	Proteins involved in EMC remodeling are altered in the sclera of POAG patients
Aires et al., 2020 [77]	*In vitro/in vivo*	BV-2 cells, retinal microglia, C57BL/ J mice	Exosomes derived from stressed microglial cells amplify detrimental signals to retinal cells
Zhang and Wang, 2021 [119]	*In vitro*	Primary TMCs	TGF-β2 lead to alterations in the miRNAs in TMC-derived EVs
Takahashi et al., 2021 [31]	*Ex vivo*	Primary monkey TM and SCE cells	ExomiRs from TMC-derived exosomes play a role in the reprogramming of SCE cells
Tabak et al., 2021 [121]	*In vitro*	NPCE cells, TMCs, and RPE cells	TMCs and NPCE crosstalk is mediated by EVs
Tabak et al., 2021 [123]	*In vitro*	NPCE and TMCs	Drugs for glaucoma treatment influence the uptake of EVs in TMCs
Tabak et al., 2021 [126]	*In vitro*	NPCE and TMCs	EVs from NPCE can inhibit the collagen formation from TMCs
Tabak et al., 2021 [127]	*In vitro*	NPCE and TMCs	*SMAD7* siRNA in NPCE-derived EVs modulate the Wnt pathway in TMCs

demonstrated that exosomes can affect the pharmacokinetics of therapeutic agents, as in the case of bevacizumab, which is internalized and secreted by RPE cells through CD63+ vesicles [147]. There is an abundance of data in the scientific literature demonstrating the beneficial role of EVs derived from mesenchymal stem cells (MSCs) in different pathological contexts. MSC-derived exosomes were able to confer protective effects on RGCs in a model of laser-induced retinal injury *via* a decline in MCP-1 levels, thus preventing apoptosis and inflammation [148]; notably, TNF-α can enhance the release of exosomes from the kinds of MSCs which are particularly enriched in PEDF and VEGF-AA, two growth factors exerting neuroprotective effects on RGCs [149]. The protection of RGCs, at least in an *in vivo* model of some disorders, was also traced to the actions attributable to the miRNA content, as demonstrated by an abrogation of the biological effect of MSC-exosomes after Argonaute-2 knockdown [150]. Most of the evidence collected in the literature has focused on the beneficial effects of MSC-EVs in diabetes/DR or hyperglycemic damage; for example, this has been described in experiments utilizing a live imaging technique [151]. Moreover, the uptake and distribution of MSC-EVs in the retina have been described recently in *in vitro*, *ex vivo*, and *in vivo* models, increasing our understanding of this promising approach [152]. MSC-exosomes obtained from the bone marrow were shown to modulate the Wnt/ β-catenin pathway in streptozotocin-induced diabetic rats, also reducing the levels of iNOS, VEGF, NF-κB, and GFAP [153]. There is other evidence demonstrating that MSC-exosomes were capable of inhibiting VEGF-A expression in the retinal vascular endothelium [154]. The injection of MSC-exosomes in streptozotocin-induced diabetic rabbits led to a reduction in retinal degeneration, probably through an induction of miR-222 [155]. The use of a similar model led to the identification of miR-192 as the cargo inside MSC-EVs, and this miR was speculated to be able to ameliorate the progression of DR *via* a negative regulation of integrin induced subunit α1 (ITGA1) [156]. There are other miRNAs playing protective roles in diabetic rats with retinal damage induced by hyperglycemia; in particular, miR-126, once it had been transferred into MSC-derived exosomes, was able to reduce the action of high-motility group box 1 (HMGB1) as well as suppressing the properties of NLRP3 and NF-κB. These results were also corroborated in an *in vitro* model based on human endothelial retinal cells (HRECs) [157]. Furthermore, an inhibition of apoptosis and inflammation in mouse- and rat-based DR models was observed after the delivery of miR-17-3p and miR-18b through exosomes isolated from MSCs of the human umbilical cord [158,159]. Exosomes derived from the abovementioned source (*i.e.*, umbilical cord MSCs) have also been claimed to exert a neuroprotective role in neurons of hyperglycemic rats [160]. Other factors in addition to miRNAs are delivered by EVs/exosomes and help to promote the protection of the retina. One example is the lncRNA *SNHG7*, which was able to inhibit the epithelial-to-mesenchymal transition in an *in vitro* model of human retinal microvascular endothelial cells treated with a high glucose concentration; the effect was mediated through the suppression of the activity of miR-34a-5p [161]. Furthermore, in the context of glaucoma and retinal degeneration, MSC-derived EVs delivered beneficial effects, as demonstrated in *in vitro* and *in vivo* models of glaucoma or AMD [162–164]. With regard to retinal degeneration, miR-21 has been identified as a key player behind the observed neuroprotective effects [165]. EVs derived from embryonic stem cells also displayed the ability to slow retinal degeneration *via* an up-regulation of Oct4 [166]. Other miRNAs exhibited similar characteristics in an *in vivo* model of retinitis pigmentosa; in particular, MSC-EVs induced anti-inflammatory responses in *rd10* mice this being mainly mediated through the miR-146a-Nr4a3 axis [167]. Despite the above-mentioned beneficial effects of MSC-derived EVs in different retinal pathologies, it has become clear that *in vitro* experiments are subject to their inherent environmental context as this can influence the response evoked by these vesicles; for example, if they originate from cells cultured in high-glucose/hypoxia conditions, they have been shown to induce angiogenesis and a DR-like phenotype [93]. Similar results were obtained using exosomes derived from adipocytes [168]. Furthermore, it has been demonstrated that exosomes derived from MSCs under hypoxic condition, administered either subretinally or intravitreally, exerted no beneficial effects in a model of inherited retinal degeneration, also when administered in combination with CD34+ cells [169], suggesting again that the culture condition of the progenitor cells was a critical issue. In contrast to the MSCs, fewer studies have been performed on the possible administration of EVs derived from other cell types. Despite the amount of evidence highlighting the positive role exerted by RPE-derived exosomes on other cell types, there is only one published paper that has described *in vivo* the possible therapeutic applications of intraocular administration of these exosomes in rescuing PRs homeostasis. This experiment was conducted in an MNU-mouse model of retinal degeneration [55]. Notably, similarly

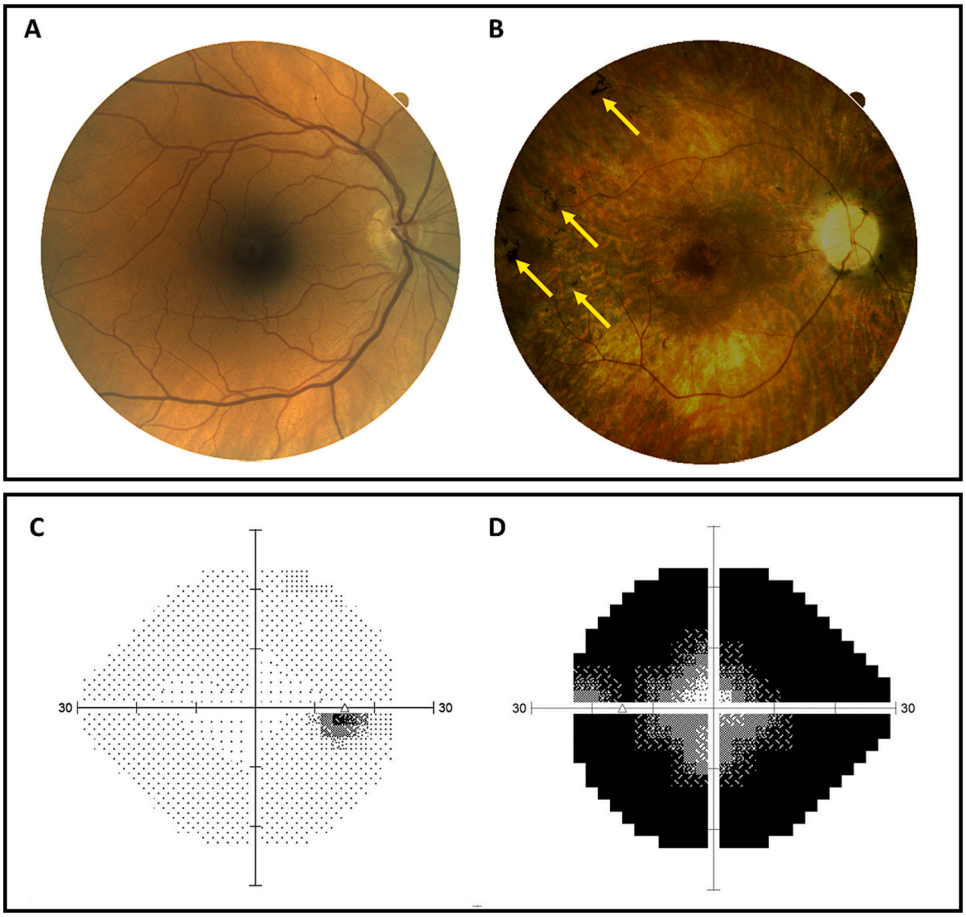

Fig. 5. In the upper panels, representative fundus photographs from a healthy (**A**) and an RP (**B**) patient. Yellow arrows indicate "bone spicules". In the lower panels, examples of visual field tests from a healthy subject (**C**) and an RP patient (**D**). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

as is the case for MSCs, *in vitro* experiments have demonstrated that if the progenitor cells had been exposed to an environmental stress this could affect the cargo carried by these exosomes and, as a consequence, they could convey detrimental messages [170]. When one considers the neural and retinal progenitor cells, there are only a few entries in the literature; these tend to support the beneficial role of exosomes derived from these cells in counteracting the retinal degeneration occurring in animal models [171,172].

5. Advantages, limitations, and future perspective of EV-based therapies

The evidence in the literature summarized here suggests that also in the ophthalmological field, EVs might be valuable tools for both the diagnosis (*e.g.*, EVs isolated from AH) and treatment of many ocular diseases. Clearly, improvements in the currently available technology will be required before novel therapeutical strategies are approved. Currently, cell-based therapy as well as AAV2-based gene therapy for monogenic disorders have been the two best-studied approaches for treating several diseases. Despite the encouraging results, both of these strategies belong to the so-called Advanced Therapy Medicinal Products (ATMPs), which are regulated in different ways by FDA and EMA, thus leading to distinctive final recommendations that may limit the marketing of these therapies in different countries. For example, currently, both the number and the type of ATMPs differ between the two regulatory agencies [173]. Compared to cells, EVs possess several advantages that could bypass some of limitations encountered in cell-based therapy, thus leading to the development of alternative EV-based treatments. In particular, (1) EVs are easier to store than cells, and their protein cargo is more stable [16,17]; (2) the intravenous administration and consequent biodistribution of EVs are less complicated than the corresponding processes with cells, which makes them more suitable for a variety of applications [174]; (3) their components can be modularized and are not influenced by environmental factors [175]; (4) there is no risk for malignant transformation or a harmful immune response [176]. The following statement was issued by the ISEV, EVs are biological medicinal products that should be regulated according to their functional moiety. Indeed, it should be possible to categorize the medicinal product type for each EV-based therapy, and thus to follow relatively specific regulations [177,178]. For the subgroups of EVs whose cellular sources are "substantially manipulated", ATMP guidelines should be considered [179]. Nowadays, the use of EVs as a biological therapy is characterized by several challenges and pitfalls that still need to be addressed. The isolation of EVs is the first critical step of particular relevance, since there are a multitude of isolation methods (>190) and no standardized protocols [180]. The isolation procedure as well as the storage can affect the overall quality of the collected sample, and thus they introduce variables and biases possibly interfering with the comparability of studies conducted in different laboratories. Specifically, the EVs' phenotype and stability can be altered by storage conditions, even leading to a loss of material; furthermore, as far as we are aware, no exhaustive comparison of the quality of "fresh" and "stored" EVs has been ever performed. In addition, the source from which the EVs have been isolated should be accurately detailed, in order to standardize the use of these vesicles and make comparable the results emerging from different laboratories. *In vitro* experiments should indicate the exact

Table 8
The list of papers (in chronological order) describing the role of EVs in RP, retinopathy, and PVR. The type of study (*in vitro*, *ex vivo*, or *in vivo*), the model used, and the principal findings are also described. As is evident from the list, most papers involved *in vivo* experiments.

Disorder	Author and Year	Type of study	Model	Findings
Retinitis pigmentosa	Li et al., 1996 [134]	*In vivo*	RP mouse model	The dominant P347S mutation in the rhodopsin gene alters the transport of the related protein
	Hagstrom et al., 1999 [137]	*In vivo*	RP mouse model	*TULP1* mutations affects EVs homeostasis
	Sreekumar et al., 2010 [37]	*In vitro/ex vivo*	Human RPE cells, retinal cryosection from 129 svE mice	αB-crystallin is released through exosomes by RPE cells, whose uptake by PRs under oxidative stress promote their protection
	Vidal-Gil et al., 2019 [133]	*In vivo*	RP mouse model	The activity of EVs contributes to the degeneration of PRs in the presence of the PDE6 mutation
	Sahaboglu et al., 2019 [135]	*In vivo*	RP mouse model	CD9- and CD81-positive vesicles contribute to retinal degeneration
	Ropelewski and Imanishi, 2020 [56]	*In vivo*	Frog (*X. laevis*)	RPE cells internalize microvesicles originating from degenerating PRs
	Yang et al., 2020 [138]	*In vitro/ex vivo/in vivo*	HEK293T, *EXOSC2*-mutated B cells, primary human keratinocytes, *D. melanogaster*	*EXOSC2* mutations affect the autophagy pathway
	Pinelli et al., 2021 [132]	*Ex vivo*	Human retina	α-synuclein and exosomes co-localize in pathological retina
	Faber et al., 2023 [136]	*In vitro*	HEK293T, mIMCD3 cells	*PDE6D* mutations affects protein trafficking in EVs
Retinopathy	Xu et al., 2019 [139]	*In vitro/in vivo*	BV-2 cells, mouse model of oxygen-induced retinopathy	Microglia-derived exosomes protect PRs from injury
	Wang et al., 2021 [55]	*In vivo*	MNU-mice model of RD	Exosomes from RPE cells can restore homeostasis in PRs
	Dong et al., 2021 [140]	*In vitro/in vivo*	HUVEC, HRMECs, C57BL/J and CD-1 mice, SD rats	The delivery of anti-angiogenic factors through exosomes inhibits angiogenesis in pathological contexts
	Zhang et al., 2020 [141]	*In vitro*	ARPE-19	EMC is triggered by exomiRs
Proliferative vitreoretinopathy	Wu et al., 2022 [142]	*In vitro/ex vivo/in vivo*	RPE from human eyes, animal model of PVR	TSPAN4-positive vesicles from RPE contribute to PVR development
	Nair et al., 2022 [143]	*Ex vivo*	Exosomes from VH of PVR patients and controls	Exosomes implicated in PVR are enriched in proteins involved in inflammation, EMT, cellular proliferation, and growth of connective tissue

composition of the medium as well as the type of cells, their passage and seeding density and, if present, their polarization. The same concerns extend to *in vivo* studies, where the details of the animals or human subjects should be well characterized (*e.g.*, sex, age, diet, body mass index, time of collection) [2]. Overall, these aspects have undoubtedly contributed to the paucity of information regarding the overall efficacy and safety of EVs, thus delaying the possible approval of EV-based therapies. Accordingly, nowadays there are no FDA-approved EV-based products approved for clinical use. However, according to ClinicalTrials.gov website, 84 clinical trials based on exosomes have been completed, and 110 studies are in the recruiting phase, demonstrating the evident interest in EV-based therapies (last access on 30th October 2023). However, of these, only nine trials are focused on eye disorders, and six involve exosomes. In more detail, these trials are recruiting participants with the aim of verifying the efficacy of exosomes derived from Wharton jelly MSC in patients with RP (NCT05413148) and from umbilical MSCs in patients affected by dry eye associated with GVHD (NCT04213248). Notably, another trial is focused on the safety and effectiveness of eye drops containing PSC-MSC-derived exosomes to treat dry eye syndrome post-refractory surgery in presence of blepharospasm (NCT05738629, not yet recruiting). Exosomes derived from MSCs are also under investigation to evaluate their role in promoting healing of large and refractory macular holes (NCT03437759, unknown status). The use of exosome-associated miRNAs as biomarkers for ocular disorders is also the topic of two additional trials, respectively focused on ocular muscle myasthenia gravis (NCT05888558, enrolling by invitation) and DR (NCT03264976, unknown status).

The delivery of active compounds is one of the most problematic aspects in ophthalmology. Since the eyeball represents an enclosed and immune privileged organ with multiple hydrophilic/lipophilic layers, the topical delivery on the eye surface is likely to be associated with poor penetration for example, as little as 1–4% of the administered dose reaching the vitreous humor [181]. Nonetheless, the penetration is higher for lipophilic agents, making exosomes, with their structure of phospholipid membranes, and exosome-based vesicles suitable candidates for topical ocular delivery [182] (Fig. 6). With respect to topical ocular delivery, there are several extremely lipophilic agents, like cyclosporin A, prostaglandin analogues, amphotericin B that could benefit from delivery inside exosomal carriers [183]. On the other hand, intravitreal injection of exosomes, the most popular and most effective route of intraocular delivery of active compounds, would potentially limit the triggering of immune reactions to the pharmaceutics, since they would be encapsulated in the EVs' phospholipid bilayer. Extracellular vesicles used as a drug carrier would potentially enhance the delivery efficacy of gene vectors, like AAVs [184] and would make it possible to achieve benefits of cell therapies without the need to deliver actual cells into the eye. It is likely that this approach would overcome many of the risks associated with cell therapies and bypass some of ethical problems of this approach. Lastly, by engineering of an individual's own retinal cells, it may be possible to induce them to secrete EVs with a certain content to achieve a protective effect in the case of neurodegenerative diseases, *i.e.*, glaucoma, RP or antiangiogenic in AMD or DR. In the last years, cell-penetrating peptides (CPPs) have also attracted the scientific community due to their biochemical properties, which make them suitable as drug-delivery systems in the eye [185]. However, despite the encouraging results, they seem to generate off-targets effects due to their free diffusion and they are still characterized by instability at room temperature and *in vivo*, because of their susceptibility to ubiquitous proteases [186]. The partial degradation of CPPs as well as their aggregation or post-translational modifications are factors leading to their rapid clearance and thus likely determining short-term activities. Currently, numerous efforts are devoted to optimizing this therapeutic strategy; however, since their appearance in the scientific panorama 30 years ago, none CPP-based therapies has been

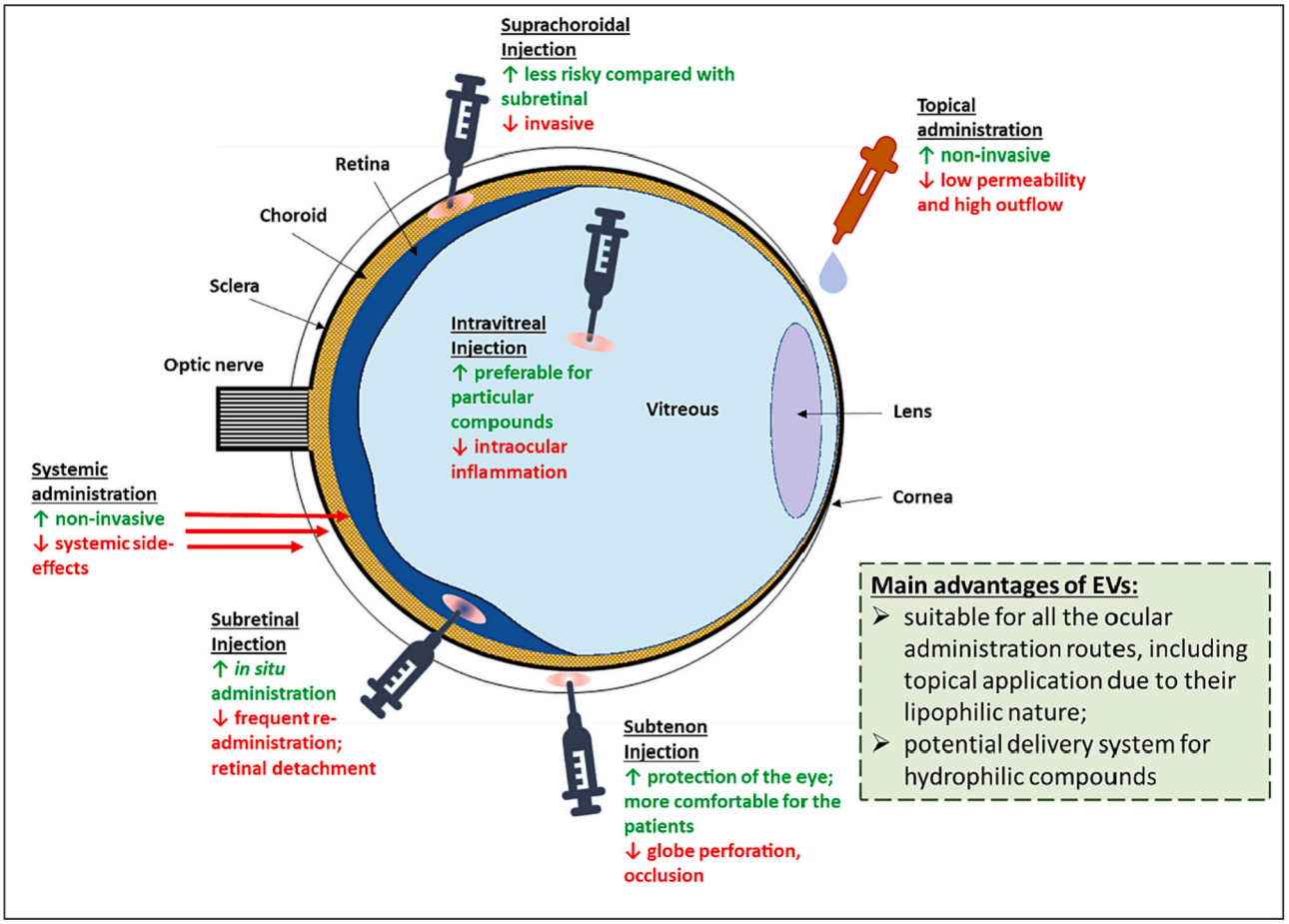

Fig. 6. Schematic representation of administration routes targeting the eye and suitable for EVs use. Green ↑ and red ↓ indicate advantages and disadvantages, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

approved by FDA due to *in vivo* instability, low selectivity and limited efficacy [187]. Compared to CPPs, EVs represent a valuable alternative in virtue of their stability in all biological fluids.

Considering these aspects, some specific observations regarding EV-based therapies in ocular disorders are here reported. As already mentioned, EVs transfer different molecules (*e.g.*, small RNAs and proteins) from a donor to a recipient to vehicle information and communicate specific signals to neighboring cells. Moreover, EVs might be used as biomarkers of disease severity and progression, and of response to treatment in retinal disorders. Accordingly, Hernandez and colleagues [188] demonstrated that chronic low-level oxidative stress is able to induce exosome release in polarized primary RPE cells: notably, the protein signature of these vesicles changed when compared to normal RPE cells and was detected before the onset of morphological changes and cell dysfunction. This evidence suggests that EVs might be used as potential pre-symptomatic biomarkers in ocular disorders. Interestingly, the same protein signature was observed in iPSC-RPE cell lines derived from patients with a high-risk for AMD in absence of oxidative stress, thus further underlining the diagnostic and prognostic potential of EVs.

The isolation and characterization of EVs associated with specific cytoprotective, anti-inflammatory, anti-angiogenic, and anti-fibrotic small RNAs and/or proteins might be used in cell-targeted therapy in ophthalmology. For example, miRNAs implicated in autophagy modulation in AMD can be delivered through EVs [189]. This approach, as well as others based on different therapeutical molecules (*e.g.*, antagomiRs, agomiRs, oligonucleotides, and proteins), may rely on two main administration strategies. One approach implies the EVs extraction from donor cells and incorporation of the therapeutic material in a cell-free context; another strategy is based on the *in vitro* treatment of donor cells with the molecules of interests, their internalization, and the subsequent isolation of the enriched EVs [190]. This latter approach presents some obvious limitations and may require substantial implementation depending on the molecules used. Vesicles produced with both these strategies may be administered through either intravitreal or subconjunctival injection, taking advantage of the direct route constituted by the vitreous humor and the phagocytic activity of RPE cells [190]. Evidently, topical administration would represent a more valuable and safer alternative strategy than the mentioned approaches. The use of cells from ocular tissues to derive EVs may be desirable; however, several pre-clinical studies demonstrated that vesicles isolated from other more accessible cells (*e.g.*, MSCs) exert beneficial and therapeutic effects in different ophthalmological disorders, as summarized in Table 9. This approach may represent a good alternative to overcome issues associated with the isolation of EVs from the eye or its fluids. Indeed, the collection of vesicles from tears is poorly known, despite the disease-associated biomarkers which can be identified through this source [191]. Other limitations to use tears as well as aqueous humor and vitreous body in therapy are represented by the repetitive sample collection required to obtain enough volume for EVs isolation, the presence of free proteins in these biologic specimens and the need of improved separation methods [192]. According to some evidence reported in literature [103,108,111,116], blood is a putative easy-to-collect specimen to isolate EVs for molecular characterization and therapeutic applications. High-risk patients or patients already affected by a disease might benefit from EVs derived from low-risk healthy individuals.

In our opinion, considering the encouraging evidence summarized in this review, EV-based therapies may constitute a useful tool for the

Table 9
A list of papers (in chronological order) describing the role of EV-based treatments against different ocular disorders. The type of study (*in vitro*, *ex vivo*, or *in vivo*), the model used, and the principal findings are also reported. From the list, it is evident that most of the publications involved experiments based on corneal tissue.

Disorder	Author and Year	Type of study	Model	Findings
Retinal ischemia	Moisseiev et al., 2017 [193]	*In vivo*	C57BL/J mice model of oxygen-induced retinopathy	Exosomes from MSCs (intravitreal administration) decrease the severity of retinal ischemia
	Mathew et al., 2019 [194]	*In vitro/ in vivo*	Oxygen-glucose deprivation in R28 cells, Wistar rats as a model of retinal ischemia	MSC-derived exosomes promote retinal recovery and decrease the severity of neuroinflammation when administered once in the vitreous
	Ma et al., 2020 [195]	*In vivo*	Rat retinal detachment model (Sprague-Dawley)	Exosomes isolated from MSCs suppress PRs apoptosis and maintain a normal retinal structure
	Mathew et al., 2023 [196]	*In vivo*	Rat model of retinal ischemia	Engineered EVs over-expressing miR-424 reduce inflammation in retinal microglia as well as reducing the effects of ROS in Müller cells and microvascular endothelial cells
Optic nerve crush/injury	Pan et al., 2019 [197]	*In vivo*	Rat model of ONC	Administration of umbilical MSC-derived exosomes promotes the survival of RGCs and the activation of glial cells
	Mead et al., 2020 [198]	*In vitro/ in vivo*	RGCs, rat model of ONC	AAV2 expressing the six miRNAs identified in exosomes from bone marrow MSCs confer neuroprotection in RGCs
	Seyedrazizadeh et al., 2020 [199]	*In vivo*	Rat model of optic nerve injury	EVs from human MSCs help in the regeneration of RGCs
	Cui et al., 2021 [200]	*In vivo*	Rat model of optic nerve injury	Intravitreal administration of exosomes from rat MSCs leads to the regeneration of RGCs
Corneal disorders	Samaeekia et al., 2018 [201]	*In vitro/ in vivo*	Human corneal epithelial cells, mice with corneal debridement	Exosomes from corneal human MSCs accelerate the repair of a corneal wound
	Shojaati et al., 2019 [202]	*In vivo*	Murine model of a corneal wound	EVs originating from corneal stromal stem cells exert a regenerative function possibly through the delivery of miRNAs
	Wang et al., 2020 [203]	*In vivo*	Corneal epithelial defect model based on SD rats	Exosomes from iPSs evoke higher therapeutic effects compared with those isolated from MSCs
	Buono et al., 2021 [204]	*In vitro*	Human corneal endothelial cells cultured with serum deprivation and tunicamycin	EVs isolated from MSCs protect human corneal endothelial cells from the apoptosis induced by ER stress
	Tang et al., 2022 [205]	*In vivo*	*In vivo* model of a corneal scar	A thermosensitive hydrogel loaded with exosomes derived from both iPSs and MSCs can promote a corneal regeneration
	Escandon et al., 2022 [206]	*In vitro*	Primary corneal stromal cells	Salivary exosomes promote the migration and regeneration of human corneal stromal cells
	Liu et al., 2022 [207]	*In vitro/ in vivo*	Human corneal epithelial cells, SD rats	EVs isolated from human umbilical MSCs help in the healing of a corneal wound through the delivery of miR-21
	Widyaningrum et al., 2022 [208]	*In vitro*	Corneal endothelial cells	EVs derived from platelets lead to a recovery of the corneal endothelial
	Zhao et al., 2023 [209]	*In vivo*	Mice with corneal injury	Exosomes derived from MSCs and loaded with siRNAs against c-Rel accelerate the repair of a corneal wound
Other ocular disorders	Zhang et al., 2018 [210]	*Case report*	Patients with refractory macular holes	MSC-derived exosomes promote the recovery after surgery for macular holes
	He et al., 2018 [211]	*In vitro/ in vivo*	RPE cells, laser retinal injury mouse model	Exosomes from MSCs exert beneficial effects on blue light stimulated RPE cells as well as on a retinal injury
	Hong et al., 2020 [212]	*In vitro*	Human lens epithelial cells	Adipose stem cell-derived exosomes protect HLECs from UVB-induced damage
	Wang et al., 2021 [213]	*In vitro/ in vivo*	HEK-293 T, C57BL/J mice	Exosome-associated AAV administration promotes an enhanced RS1 gene delivery. Possible implication for the treatment of X-linked retinoschisis.
	Li et al., 2021 [214]	*In vitro/ in vivo*	Human skin fibroblasts, ARPE-19, C57BL/J mice model of retinal fibrosis	miR-27b in exosomes derived from human umbilical cord MSCs rescue retinal fibrosis through EMT suppression
	Jiang et al., 2021 [215]	*In vitro/ in vivo*	T-cells, Lewis rats as a model of experimental autoimmune uveitis	Exosomes derived from immunized uveitis rats inhibit the immune response in T-cells
	Hadady et al., 2022 [216]	*In vitro/ in vivo*	PC12 cells, rat model of retinal degeneration	EVs from dental stem cells enriched with two different methods promote a reduction in retinal degeneration
	Zhou et al., 2022 [217]	*In vivo*	C57BL/J and NCG mice, patients with chronic GVHD-associated dry eye	Exosomes from MSCs administered as eye drops reduce the symptoms associated with GVHD-associated dry eye in both humans and mice through the action of miR-204
	Polallis et al., 2022 [218]	*In vivo*	Laser induced choroidal neovascularization mouse model	Engineered exosomes derived from mouse retina exert beneficial effects on choroidal neovascularization
	Ma et al., 2023 [219]	*In vivo*	Mouse model of dry eye disease	MSC-derived exosomes associated with ascorbic acid lead to corneal epithelium recovery, reduce the severity of inflammation and restore the secretion of tears
	He et al., 2023 [168]	*In vitro*	3 T3-L1 preadipocytes and mouse retina microvascular endothelial cells	LINC00968 in the exosomes derived from 3 T3-L1 cells cultured under high glucose conditions triggers dysfunctions in microvascular endothelial cells
Other applications	Zhao et al., 2018 [220]	*Ex vivo/ in vivo*	Vitreous from post-mortem donors or C57BL/J mice	Vitreous is enriched in the exosomes carrying retinal proteins suggesting their possible use as liquid biopsies

treatment of several ocular disorders. As clearly shown in Table 9, the potential usefulness of EV-based treatments is not limited to the retina and related disorders, which have been thoroughly discussed in this review, but it also relates to other ocular tissues, *e.g.*, cornea, vitreous, displaying indeed encouraging results in a variety of ocular diseases.

EVs carrying beneficial molecules (*e.g.*, nucleic acids, proteins, lipids, or synthetic therapeutic compounds) might be used either in prevention for high-risk patients, or as protective agents for subjects in non-advanced stages of the disorders. Compared with other approaches, such as those based on nanoparticles (NPs) (*e.g.*, nanoemulsions,

nanomicelles, quantum dots, liposomes, or inorganic NPs), EVs seems to be more suitable for ophthalmological application. Indeed, although pre-clinical results on NPs seem to be encouraging, in some cases short-term toxicity was detected (*e.g.*, subacute toxicity of subconjunctival and vitreous cavity in rabbits) and long-term toxicological assessments still lack [221]. Another advantage of EVs-based therapies is their possible application and usefulness in multifactorial ocular disorders (*e.g.*, AMD, glaucoma, and DR), caused by a complex association between genetic and environmental factors.

Interesting gene-based approaches have been developed for monogenic disorders, such as RP, although with some limits. Specifically, Luxturna® (voretigene neparvovec) is a gene therapy approved for RP that gave encouraging results in a phase III clinical trial on 31 patients. However, this therapy can benefit only subjects carrying mutations in the *RPE65* gene, detectable in a small sub-population accounting for 0.3–1% of all RP patients [222]. As known, inherited retinal degeneration (IRD) syndromes are associated with mutations in multiple genes: as reported in the RetinoGenetics database (https://www.retinogenetics.org/) mutations in 186 genes were found to be associated to IRDs [223]. On these premises, to cover the wide-range spectrum of IRD-causing mutations, an undetermined number of gene therapies should be developed; besides its arguable feasibility, this approach implies obvious consequences in terms of timing and affordability. Effective EV-based therapies tailored on a given pathology, or targeting features shared by different IRD syndromes, have the potential to bypass these limitations and might be used, alone or in combination with other therapies - if available - to prevent or slow down retinal degeneration in early stages of various disorders, including inherited diseases.

Other strategies have been hypothesized for ocular disorders, all associated with more or less impactful limitations. Therapies based on AAV vectors are invasive, CRISPR-Cas gene editing has a low performance rate, siRNAs are susceptible to degradation, the use of iPSCs is held back by ethical problems as well as concerns regarding the epigenetic memory of these cells or their teratogenic potential [222]. Optogenetics, which may represent an alternative approach to gene therapy, recently gave encouraging results leading to the partial recovery of visual function in a blind patient [224]. An important aspect that should be taken into consideration is the cost of all these therapies: indeed, gene therapies are expensive to develop and produce (some therapies can reach costs up to 3 million dollars), thus dampening the possibility to introduce them into clinical practice [225]. EV-based approaches have also the advantage to be less costly, thus making them more attractive for large-scale clinical use.

6. Conclusions

The characterization of EVs and their molecular cargos has contributed to elucidating the roles of these structures in the ethiopathogenesis and progression of several diseases. In many respects, it can be stated that for what concern EVs, we are witnessing the opening of a new era of potential biological therapies. Recent observations have revealed that the use of EVs as biomarkers and as EV-based treatments holds the potential to change the current management of ocular pathologies. In particular, EV-mediated therapies may provide breakthroughs in the treatment of retinal and optic nerve diseases, such as AMD, DR, RP or glaucoma. When one considers the many advantageous features of EVs, it can be argued that it may be possible to administer them by all the presently used routes to exert ocular effects, even topical application may not be ruled out. Thus, in the future, EVs-based therapies may have moved from being an intriguing paradigm to a common treatment mode in eye clinics.

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Consent for publication

All the authors agree with the publication.

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F.M. and M.A. wrote the main manuscript text. F.M. and S.C. prepared Fig. 1; F.M., A.S., and K.K. prepared Figs. 2–5; F.M. and M.A. prepared Fig. 6. All authors reviewed the manuscript.

Authors' information

Not applicable.

CRediT authorship contribution statement

Federico Manai: Writing – review & editing, Writing – original draft, Conceptualization. **Adrian Smedowski:** Writing – review & editing. **Kai Kaarniranta:** Writing – review & editing. **Sergio Comincini:** Writing – review & editing. **Marialaura Amadio:** Writing – review & editing, Writing – original draft, Conceptualization.

Declaration of Competing Interest

None of the authors declares any kind of competing interests.

Data availability

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