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What information can be obtained from the tears of a patient with primary open angle glaucoma?

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ABSTRACT

Since tears are a biological fluid, they have a potential diagnostic value for ophthalmic diseases. The aim of this study was to compare tear supernatants and pellets obtained from patients suffering from primary open angle glaucoma (POAG) and healthy persons (HPs) using transmission electron microscopy (TEM) and molecular biological methods.

Tear supernatants and pellets were prepared using ultrafiltration and ultracentrifugation and were examined by negative staining and immunogold labelling TEM. DNA of the pellets was isolated, quantified and sequenced using a MiSeq (Illumina, USA) genomic sequencer with the Reagent Kit v3 (600 cycles, Illumina, USA). MicroRNA was isolated and quantified from the pellets; miR-146b, miR-16 and miR-126 were detected using TaqMan MicroRNA Assays (Applied Biosystems, USA).

TEM of tear supernatants from both POAG patients and HPs revealed identical constituents: spherical or cup-shaped vesicles, “non-vesicles”, cell debris and macromolecular aggregates. Pellets of POAG patients and HPs contained small extracellular vesicles (sEVs) non-labelled vesicles and “non-vesicles”; pellets of sick persons also contained sEVs with “a capsule”. POAG-patient tear pellets showed elevated concentrations of genomic ds-DNA and SINE-repeats, and different expressions of miR-146b, miR-16 and miR-126 and a different set of bacterial DNA in comparison with pellets obtained from the tears of HPs.

The data obtained indicate that the tears of HPs and POAG patients could serve as an object for TEM studies and as a source of sEV-containing preparations (pellets), which, in turn, could be used for the isolation and study of genomic ds-DNA and RNA. Our data provide the basis for using tears for diagnostic applications.

1. Introduction

Studies of tears and their composition are not “a hot topic”, despite the fact that tears are a part of daily life and are necessary for normal eye function. Currently, published information is limited to some data on the tears' proteome [1], mineral composition [2], cytokine profile [3] and lacrimation under various influences [1,4]. Morphologic examination of the tears is missing, apparently because normally tears are a clear fluid, which does not suggest presence of any substantial material for microscopic study. Nevertheless, our electron microscopic study of negatively stained tears collected from healthy persons (HPs) revealed plenty of submicroscopic structures, including extracellular vesicles (EVs) positive for CD63 and CD9 receptors [5]. Study of ultrathin sections in transmission electron microscope (TEM) showed different cells, cellular fragments and debris, mucous granules and lipid droplets in tear sediments obtained from HPs and patients with primary open angle glaucoma (POAG). Components of tear sediments differed between HPs and POAG patients, indicating an influence of disease on tear composition [6].

Differences between morphological parameters of sediments in POAG patients and HPs prompted us to compare submicroscopic structures in a liquid part of the tears collected from POAG patients with those of HPs with a special emphasis at small extracellular vesicles (sEVs), which were previously called “exosomes”. The purpose of our study was the examination, using different methods, of supernatants

Abbreviation: EVs, extracellular vesicles; sEVs, small extracellular vesicles; POAG, primary open angle glaucoma; HPs, healthy persons; EM, electron microscopy; TEM, transmission electron microscope

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and EV-containing preparations (pellets) obtained from the tears of POAG patients and HPs. We chose glaucoma because it is a severe eye disease causing blindness and, in spite of numerous studies, it remains "difficult" for early diagnosis, which is a prerequisite for successful treatment [7]. The special attention paid to sEVs was motivated by data concerning the presence of the sEVs in vitreous [8] and aqueous humours [9] of patients with uveal melanoma and cataracts, as well as the detection of myocilin protein in these vesicles, which is thought to be related to the pathogenesis of POAG [10,11]. The sEVs are a type of EVs containing various RNA, proteins and biologically active lipids that are bounded with a membrane enriched with cholesterol and sphingolipids [12]. The presence of a great variety of biologically important molecules in sEVs makes their samples an attractive object for diagnostic investigations [13–15].

Here, we describe structural composition of supernatants and sEV preparations. The pellets from tears of POAG patients differ from those of HPs in the content of miR-146b, miR-126 and miR-16 in addition to the main types of DNA repeats and coding regions and differences in species-specific bacterial DNA fragments.

2. Materials and methods

2.1. Samples of the tears

Collection of all tear samples adhered to the tenets of the Declaration of Helsinki and was performed as mandated by the Bioethics Committee of "The Acad. S.N. Fyodorov Eye Microsurgery Complex". All persons were approached using approved ethical guidelines, and those who agreed to participate in this study gave written informed consent.

The tears were sampled only once from patients with POAG (n = 33, age 52–81 years, median 65). Diagnosis of POAG was based on standard ophthalmic investigation (Table 1). The patients with any secondary forms of glaucoma (like traumatic, uveitic, steroid glaucoma, neovascular), previous surgery, dry eye, history of contact lens wear, history of inflammatory or allergic disorders, were excluded from the study. Healthy persons (n = 29, age 44–79 years, median 63) did not wear contact lenses and did not have any medical history within the previous six months of systemic disease, ocular surface disease, uveitis, glaucoma, retinal disease or ocular trauma.

Tears were individually collected from each person (80–150 μl) using a modified method described earlier [16]: instead of nasal stimulation, the lacrimation was stimulated by sulphacetamide sodium monohydrate (DOSFARM, Kazakhstan), about 10 μg of which were introduced into the conjunctival sac.

Volume (μl) of all samples was measured after collection of the tears, and then the tears were centrifuged at 20,000 ×g for 15 min to pellet cells and cell debris. Then 20 μl of each supernatant were used for TEM, and the remaining supernatant was used for isolation of sEVs (Fig. 1).

Table 1
General clinical characteristics of POAG-patients.

Parameters		Median (Min-Max)	N (%)
Sex	Male		11 (33.3)
	Female		22 (66.7)
Glaucoma stages	Early (< 6 dB)		16 (49)
	Moderate (6–12 dB)		10 (30)
	Severe (> 12 dB)		7 (21)
Glaucoma complication	No complication		4 (12)
	Cataract		14 (42)
	Missing information		15 (46)
Visual acuity	Early stage	1 (0.5–1)	
	Moderate stage	0.33 (0.20–0.65)	
	Severe stage	Missing	
IOP (eyes merged, mmHg)	Early stage	17.5 (13–29)	
	Moderate stage	17 (16–28)	
	Severe stage	Missing	
Biometry (IOLMaster)	Early stage	23.81 (22.37–25.29)	
	Moderate stage	23.67 (21.54–25.7)	
	Severe stage	Missing	
Pachymetry	Early stage	553 (507–583)	
	Moderate stage	560 (488–588)	
	Severe stage	Missing	
Total no. of patients			33 (100%)

2.2. Isolation of sEVs-containing preparation from the tears

Individual supernatants (70–120 μl) were diluted in 4 ml of PBS and passed through 100-nm pore-size syringe filters (Minisart high flow, 16,553-K, Sartorius) providing low pressure and thereby preserving the morphology of structures. Then, the filtrates were centrifuged for 90 min at 100,000 ×g (4 °C). The obtained pellets were suspended in 4 ml of PBS, and were centrifuged again for 90 min at 100,000 ×g (4 °C). Then, supernatants were removed, and each pellet was suspended in 150 μl of PBS (Fig. 1). Thus, we prepared fractions that were supposed to contain sEVs [17]. These preparations will be called "pellets" to differentiate them from the "supernatants" of the tears. The term "pellet" is often used for definition of sEVs-containing preparations and generally for most solid results of centrifugation [17].

2.3. Electron microscopy

For negative staining, 10 μl of supernatant or pellet were adsorbed for 1 min on copper grids covered with carbonised formvar film. Then, the grids were exposed for 5–10 s on a drop of 0.5% uranyl acetate or 2% phosphotungstic acid.

Specific markers for sEVs, CD63 or CD9 were detected as described earlier [5] using 10 μl of pellets and corresponding monoclonal antibodies (Abcam, UK).

All grids were examined in JEM 1400 (Jeol, Japan) TEM supplied with digital camera Veleta (EM SIS, Germany). The measurements were made directly on the camera screen using iTEM (EM SIS, Germany) software.

2.4. Nanoparticle tracking analysis

The analysis of EV quantities was performed using the nanoparticle tracking analysis (NTA) system of the NanoSight NS300 (Malvern, UK). Depending on the concentration of the particles, samples were diluted 2- to 4-fold with 0.1 μm filtered PBS to obtain an optimum concentration for NTA. Each sample was measured in triplicate at camera setting "15" with an acquisition time of 30 s and a detection threshold setting of "5". At least 200 completed tracks were analysed per video. NTA version 2.3 analytical software was used for data analysis and capture.

2.5. DNA isolation and quantification

DNA was isolated from the pellets using the "DNA Isolation Kit" (BioSilica Ltd., Russia) according to the manufacturer protocols and concentrated by precipitation in acetone as triethylammonium salts [18]. In brief, 100 μl of pellet were mixed with 5 μl of glycogen (20 mg/ml, Fermentas, Republic of Lithuania), then 10 μl of 50 μM triethylamine and 500 μl of acetone were added. The precipitation was carried out at −20 °C for 15 h. Then, the samples were centrifuged for 20 min at 4 °C and 15,000 ×g, and nucleotide material was dissolved in 12 μl of water. The samples were mixed with 1 μl of DNase I (1 e.u./ml, without RNase activity, Fermentas, Republic of Lithuania) and 1.4 μl 10× DNase buffer; or 1 μl of RNase A (10 e.u./ml, without DNase activity, Fermentas, Republic of Lithuania) and 1.4 μl 10× TE buffer. After incubation for 30 min at 37 °C, DNase was inhibited by EDTA and heating (65 °C, 10 min), and then nucleic acids were precipitated by triethylamine and acetone as described above.

Concentration of DNA in pellets was measured by the TaqMan multiplex real-time PCR of human LINE1 fragments and α-satellite

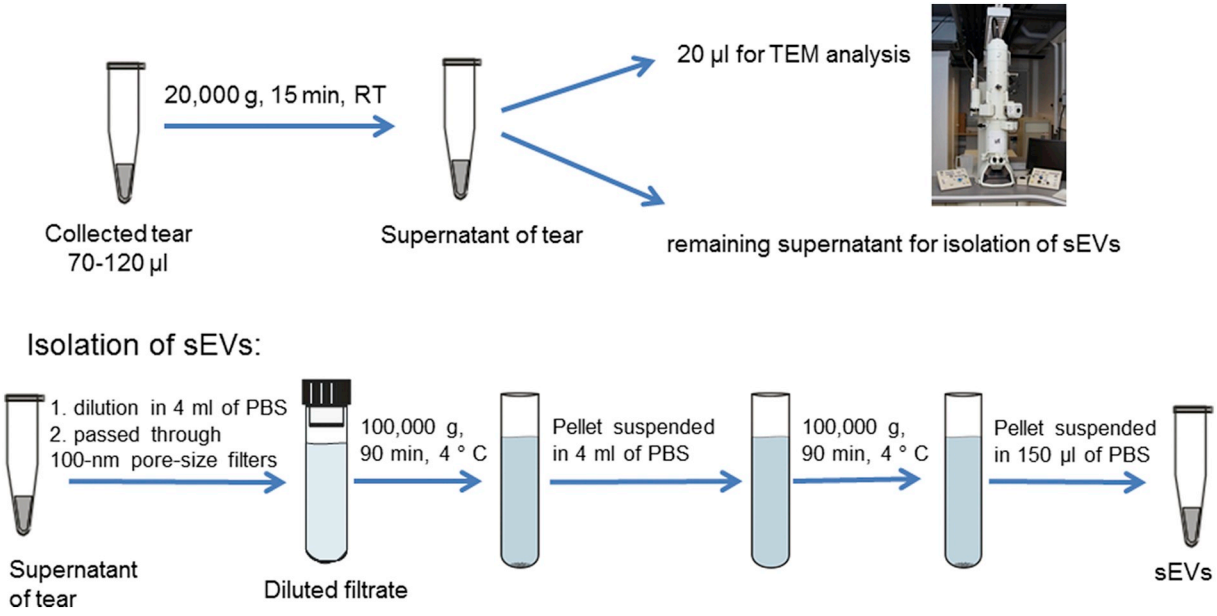

Fig. 1. Isolation of sEVs from the tears.

repeats [18]. Genomic DNA from human leukocytes served as a standard for obtaining the calibration curves. The Q-PCR was performed with an ICycler iQ5 (Bio-Rad, USA). The DNA concentration was estimated according to the initial volume of each tear sample. The sizes of DNA in pellets were evaluated using an Agilent High Sensitivity DNA Kit and Bioanalyzer (Agilent Technologies, Germany).

The pellet DNA (total pellets from six HPs and ten individual samples from POAG patients) was eluted by the method used to remove the DNA from the cell surface [19], or digested by DNase I treatment. In brief, 8 µl of DNase I (1 e.u./ml) and 52 µl of 10× DNase buffer were added to 460 µl of pellets and incubated for 30 min at 37 °C. Then the enzyme was inhibited as described above. After DNase I treatment, the samples were ultracentrifuged (100,000 ×g, 4 °C, 90 min), and supernatants and pellets were collected for DNA isolation and subsequent evaluation of sizes and concentrations.

2.6. DNA sequencing

DNA isolated from pellets was treated with RNase A, concentrated by precipitation and then fragmented using Covaris S2 shearing (Covaris, USA). A fragment library was prepared using the NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs, USA). The sequencing was performed in a SB RAS Genomics Core Facility (ICBFM SB RAS) on a MiSeq (Illumina, USA) genomic sequencer with the Reagent Kit v3 (600 cycles, Illumina, USA). The obtained data were analysed using CLC GW 8.5 (Qiagen, Germany). Paired reads were filtered for quality (ambiguous limit = 2, quality limit = 0.031). The sequence mapping was performed with default parameters using human reference genomes (hg19) with Ensemble annotation GRCh37.75. RepeatMasker tracks were downloaded from www.genome.ucsc.edu. Then, 10 K sets of unmapped reads were analysed by BLAST against the NCBI nucleotide database (15.03.2016). Unmapped reads were analysed by using the Blastx command of Diamond [20] against the NCBI nr database (08.2017). Blastx results were extracted by the metagenomic analysis tool MEGAN v.6.8 [21]. Principal component analysis was performed on the data using Python's scikit-learn package [22]. For principal component analysis, count table was normalised by function LogLin with the MCMC.OTU package in R.

2.7. MicroRNA isolation and quantification

MicroRNA was isolated from the pellets using the "*mir*Vana™ miRNA Isolation Kit" (Ambion, USA) according to the manufacturer instructions and was concentrated up to 12 µl by precipitation with glycogen and isopropanol [23]. The purity and quality of isolated microRNA were examined by OD260/280 using a Nanodrop ND-1000 (Thermo Scientific, USA) and by a RNA 6000 Pico Kit and an Agilent 2010 Bioanalyzer (Agilent Technologies, USA).

Each RNA sample was reversely transcribed to cDNA using the TaqMan® MicroRNA Reverse Transcriptions Kit (Applied Biosystems, USA). Single stranded cDNA was synthesised from 10 µl of isolated microRNA using specific primers (TaqMan MicroRNA Assay, PN 4427975, Applied Biosystems, USA). Samples without RNA templates and preparations of genomic DNA were used as negative controls.

In a 30-µl PCR reaction, 5 µl of cDNA served as a template. PCR products were amplified using specific primers (TaqMan MicroRNA Assay) and TaqMan Universal PCR Master Mix (PN 4324018, Applied Biosystems, USA), and products were detected using ICycler iQ5 (Bio-Rad, USA). PCR reactions for each sample were run in triplicate, including blank controls without cDNA. The TaqMan MicroRNA Assays used in this study were obtained from Applied Biosystems, and the following were used for microRNA detection: miR-146b (001097), miR-16 (000391), miR-126 (000508). The expression levels of microRNAs were normalised to 100 µl of tears.

2.8. Statistical analysis

The significance of miRNA and DNA concentrations were determined by the Mann-Whitney test and using STATISTICA 6.0 software. All *P*-values that were two-sided and < 0.05 were considered statistically significant. The sensitivity and specificity were calculated from ROC curves established for discriminating POAG patients and healthy donors by a commonly used method [24]. All statistical calculations were performed by GraphPad PRISM 5 software (GraphPad Software, USA). The significance of changes in tear volume was determined by *t*-test; all *P*-values that were < 0.05 were considered statistically significant.

3. Results

3.1. Characteristics of tear supernatants

Basic characteristics of the tears, such as the volume of basal and reflex tears, stability of the lipid layer in the tear film (tear break up

time test), osmolarity and others are widely used in clinics for diagnosis and monitoring of ophthalmic diseases [25]. We measured reflex tear volume and found it to be reduced in 20 POAG patients (126 ± 7 μl) in comparison with those of 20 HPs (162 ± 11 μl), $p < .05$, Student test. A change in reflex tear volume could be related with the development of "dry eye" syndrome, which, in turn, often is associated with POAG progression [26].

The description of electron microscopic images requires some clarification of definitions. We used the term "vesicles" to define all vesicles that are observed in TEM, and the term "non-vesicles" means compact spherical structures without a membrane. All together, these structures are called "submicroscopic structures".

Tear supernatants obtained from HPs showed a collection of structures in TEM that were identical to those reported earlier [5]: spherical or cup-shaped vesicles (40–250 nm in size), non-vesicles of different sizes, cell debris and macromolecular aggregates (Fig. 2A, B). The same structures were observed in supernatants obtained from the tears of POAG patients (Fig. 2C–F). All samples, both from healthy and sick persons, contained vesicles 40–100 nm in diameter with spherical or cup-shaped morphologies (Fig. 2A–C). Some vesicles (70–130 nm) in the tears of patients with advanced POAG had "a capsule" of amorphous substance (30–50 nm in thickness) with low electron density (Fig. 2F). Such vesicles were not found in the supernatants from HPs and patients with early and moderate POAG.

Amount of vesicles, which were observed in TEM, varied in a large extent in the supernatants of POAG patients; however, it was about the same in all samples obtained from HPs. The amount of vesicles at early and moderate stages of POAG exceeded those in tear samples from advanced POAG and HPs.

"Non-vesicles" (previously called "microparticles" [5]) are another essential component of all studied tear supernatants obtained from POAG patients and HPs. "Non-vesicles" are 20–40 nm in size and represent rounded particles of low electron density, without limiting membranes, observed as individual units or loose clusters (Fig. 2 B, D). In the samples from POAG patients, they were less frequent and were mostly located in loose macromolecular aggregates. "Non-vesicles" of larger sizes (40–100 nm) also were present in all tear supernatants, and it was sometimes difficult to differentiate them from the vesicles.

The tears wash anterior eye surfaces and collect the life-waste of cells in the cornea and conjunctiva, and so supernatants obtained from tears of HPs contain some cellular debris, which appears as shapeless structures (40–250 nm) of middle electron density, without limiting membranes (Fig. 2E). Supernatants of the tears collected from advanced POAG patients contained much more cell debris than those of early and moderate POAG patients and HPs. Particles of cell debris in the tears of patients with advanced POAG differed from other samples by polymorphism, dentate borders and irregular electron density.

Thus, negatively stained supernatants obtained from the tears of POAG patients are suitable for examination in TEM and contain components, identical to those in supernatants of HPs. Supernatants from advanced POAG patients contain distinctive vesicles covered with a "capsule" and show increased amounts of cell debris.

3.2. Characteristics of EVs in the tears of POAG patients and healthy persons

To isolate sEVs from the tears of HPs and POAG patients, we applied ultracentrifugation and ultrafiltration, which removed loose macromolecular aggregations and large vesicles.

To characterise sEVs isolated from tears, NTA and TEM combined with immunogold detection of CD63, CD9 and CD24 in EV membranes were used. NTA was executed in triplicate for each sample; the reproducibility of counting between runs was around 15%. The concentration of sEVs in tears from POAG patients was higher than in HPs (mean 3.78 ± 0.31 × 10^9/ml of tear and 3.63 ± 0.40 × 10^8/ml of tear, correspondingly). At the same time, hydrodynamic sizes of sEVs in tears from POAG patients and HPs were similar: 269.6 ± 7.3 nm and 192.2 ± 19.7 nm, correspondingly. It should be noted that NTA is capable of enumerating individual nano-sized particles independently from their nature and structure; thus, this method cannot distinguish membrane-coated EVs from protein/proteolipid aggregates and aggregates of membrane-coated vesicles.

The TEM of resulting pellets revealed many rounded or cup-shaped vesicles with diameters < 100 nm and "non-vesicles" (Fig. 3A–C).

Although both NTA and TEM showed similar characteristics for sEV size distributions, in general, the value of results measured by TEM could be considered lower than NTA as recently described [27–29]. Moreover, TEM demonstrated that all studied sEV preparations contained contaminating low electron density particles without limiting membranes–"non-vesicles". Two main types of the "non-vesicles" found in the sEV preparations were particles of 10–30 nm in size, and particles of 40–100 nm in size. The proportion of "non-vesicles" in the samples was about 17%. Morphology of the "non-vesicles" corresponded with intermediate and low density lipoproteins (10–30 nm) as well as very low density lipoproteins (40–100 nm), and their sizes were analogous with sEVs [30].

The detection of the tetraspanins CD63 and CD9, which are a constituent part of EV membranes [31,32], using gold-labelled monoclonal antibodies, is "the gold standard" for EV identification [33]. Incubation of the pellet samples with these antibodies showed the presence of positively labelled vesicles (including those covered with a "capsule") (Fig. 3D–F). Each antibody was applied to a separate grid, and about a half of all sEVs were labelled both in POAG patients and HPs. Taking into account that cells produce sEVs bearing different types of tetraspanins [34,35], the total amount of CD63- and CD9-positive sEVs exceeded 50% in tear pellets of sick and HPs.

3.3. Examination of DNA, isolated from the CD63- and CD9-positive sEVs of tears

Previously, we showed the presence of genomic double-stranded DNA (size 3–9292 kbp) in pellets obtained from the tears of HPs [5]. Examination of pellets isolated from HPs and POAG patients in this study also found genomic double-stranded DNA (Fig. 4B).

The DNA concentration in the pellets was evaluated by TaqMan Q-PCR, specific for fragments of L1 repeating sequences, which are uniformly distributed across the human genome. DNA was present in pellets of 83% of samples derived from healthy and sick persons. The concentration of pellet DNA had a normal distribution (chi-square test, $p < .05$) in an average concentration of 15 ± 3 ng/ml of the tears of HPs. In tear samples from POAG patients, the average L1 repeating sequence concentration was 233 ng/ml of tears (range of 0–1315 ng/ml, median 78 ng/ml, Fig. 4A), and was not correlated with the stage of disease. The difference between the POAG patients and HPs was statistically significant (Mann-Whitney U test, $P < .05$).

The detection of genomic DNA raised the question about where the DNA was located. It could be inside sEVs or on their outer surface, and the DNA could be bound with the "non-vesicles". We treated the pellets with DNase I and found that DNA was accessible for cleavage with the enzyme. Elution of the DNA from the surface of vesicles (or "non-vesicles") also showed the location of DNA on the outer surface of the structures. These results indicated that DNA was located on the outer surface of pellet sEVs or "non-vesicles".

DNA in the pellets was sequenced with average coverage 200 and 400 Mb for HPs and POAG patients, respectively. In all cases, > 97% of reads mapped to human genome. Bioinformatic analysis showed that in average 24.4% and 31.9% of reads were mapped to repeat regions for healthy and POAG groups respectively (Table 2). In both groups, the main repeat classes were presented by LINE, LTR, Satellite and SINE with the total contribution of not < 90% of reads. The most notable differences were observed for SINE repeats, the value increased by 1.6 times for POAG (16%) patients in comparison with HPs (10%). In the

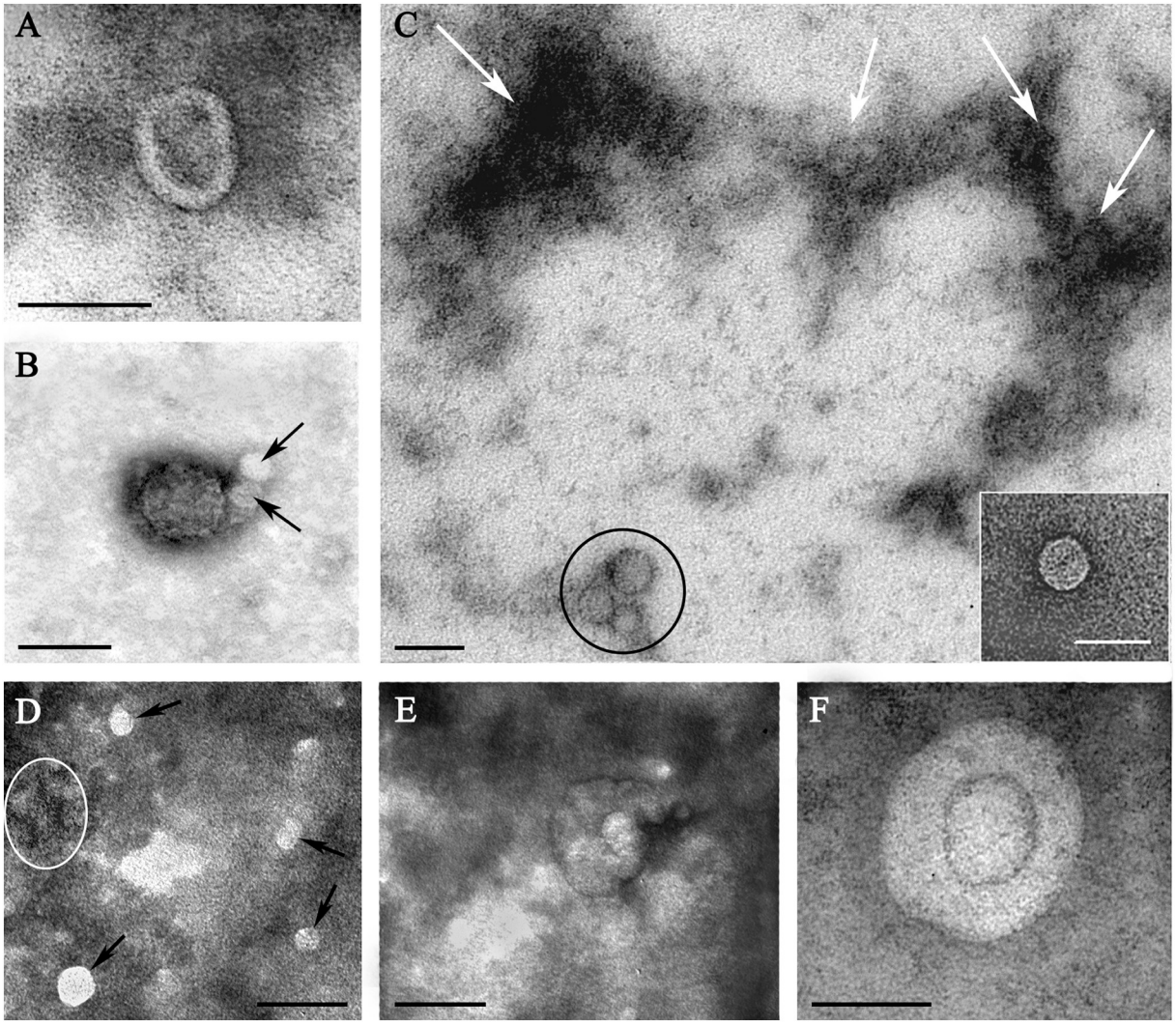

Fig. 2. Submicroscopic structures in supernatants of the tears. A, B–vesicles (40–100 nm), arrows show "non-vesicles". C–general view of a sample from POAG patient; arrows show macromolecular aggregates; a circle encloses the "non-vesicles"; a vesicle is shown in the insert. D–arrows show "non-vesicles"; a circle encloses macromolecular aggregates. E–debris. G–vesicle with a capsule. A, B–samples from HPs, D–F–samples from POAG patients. Scale bars correspond to 100 nm. TEM, negative staining.

case of another variant annotation (coding regions) which could intersect the previous variant, we showed that representation level of reads related to genes was about 52%, and a difference was not found for the groups under consideration. However, differences were present for mRNA and CDS. Value for CDS were about 1.5 times more for tear samples from POAG patients compared to HPs. The differences between HPs and POAG patients were statistically significant with $p < .05$ for all types of repeats and for CDS and mRNA.

< 3% of total reads were not mapped to human genome and were analysed by the Blastx command of Diamond for the identification of species, with further analysis in MEGAN. < 1% of reads were not assigned to any species. The most represented category was bacteria, with > 95% reads in both HPs and POAG patients. Analysis of metagenomic distribution (Fig. 5 A) showed that the main bacterial genera were *Lactobacillus*, *Pseudomonas*, *Cutibacterium*, *Micrococcus* and others. The greatest differences were the prevalence of *Lactobacillus delbrueckii* in POAG patients and *Pseudomonas* sp. in HPs. Principal component analysis made the clustering of samples possible (Fig. 5B).

Thus, analysis of CD63- and CD9-positive sEV-related double-stranded DNA revealed increases in total pellet DNA concentrations and SINE repeats in POAG patients, and detected different presentations of bacterial repeats in comparison to HPs.

3.4. Characterisation of microRNA isolated from the CD63- and CD9-positive sEVs

We examined tear pellets containing CD63- and CD9-positive sEVs using quantitative PCR after reverse transcription. The quality of isolated RNA was checked by capillary electrophoresis. The Agilent small RNA kit enabled the detection of small RNAs in the interval of 6–150 nucleotides, including the microRNA region (18–25 nucleotides). Electrophoregrams showed the presence of primarily short RNA molecules (10–50 nt) in the pellets derived from the tears of POAG patients (Fig. 6A).

Involvement of miR-146b and miR-126 in development in some ophthalmic diseases has been shown previously [8,36], and we chose these two species for our study. We measured the contents of miR-146b, miR-126 and miRNA-16 by quantitative PCR as standard normalisers [23]; data are presented as Ct levels (Fig. 6B). It was found that levels of miR-146b, miR-16 and miR-126 expression were significantly different in HPs and POAG patients, suggesting a contribution of these microRNAs to glaucoma progression (Table 3). We found low a Ct level of miR-16, and significant difference between the pellets obtained from tears of healthy and sick persons, which indicated that the search of optimal microRNA for data normalisation remains actual.

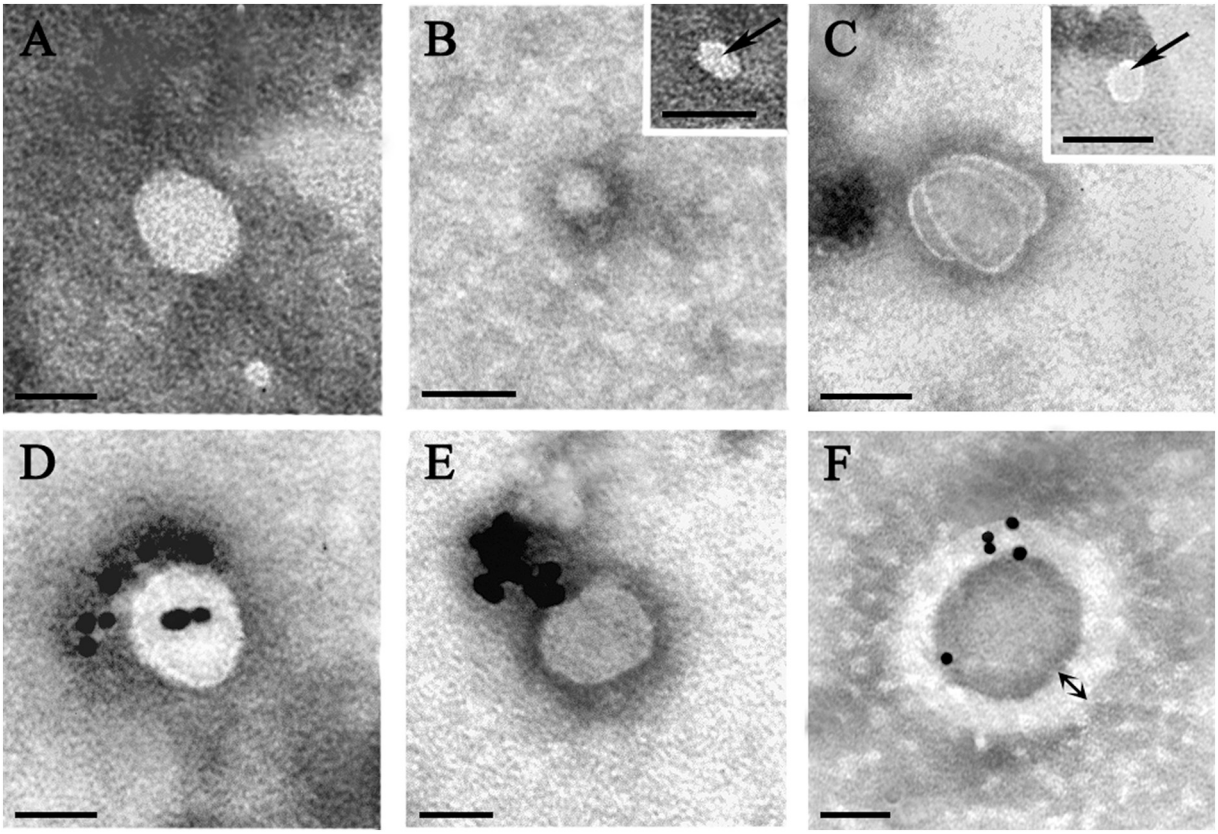

Fig. 3. Submicroscopic structures in the pellets obtained from the tears for sEVs studies. A–sEVs, B–"non-vesicle". C–vesicles, insert shows "non-vesicle". sEVs in pellets obtained from POAG patient samples labelled with gold-conjugated monoclonal antibodies to CD63 (D) and CD9 (E, F). F–vesicle with a capsule. A, B–samples from HPs, D–F–samples from POAG patients. Scale bars correspond to 100 nm. TEM, negative staining.

The diagnostic efficacy of microRNAs from tear pellets was confirmed by ROC curve analysis. The discriminatory accuracy for differentiating between POAG patients and HPs was AUC = 0.849, 0.824 & 0.935 for miR-146b, miR-126 and miR-16, respectively (Fig. 6C–E). The discriminatory accuracy for differentiating between POAG patients at early and moderate stages of disease and HPs was the highest with a combination of miR-126 and miR-16 (sensitivity 89%, specificity 90%), showing the diagnostic potential of these two microRNAs from CD63- and CD9-positive sEVs.

In summary, the data indicate that changes in the levels of the studied microRNAs in preparations of tear pellets is detectable at POAG early stage and, apparently, could play a role in disease development.

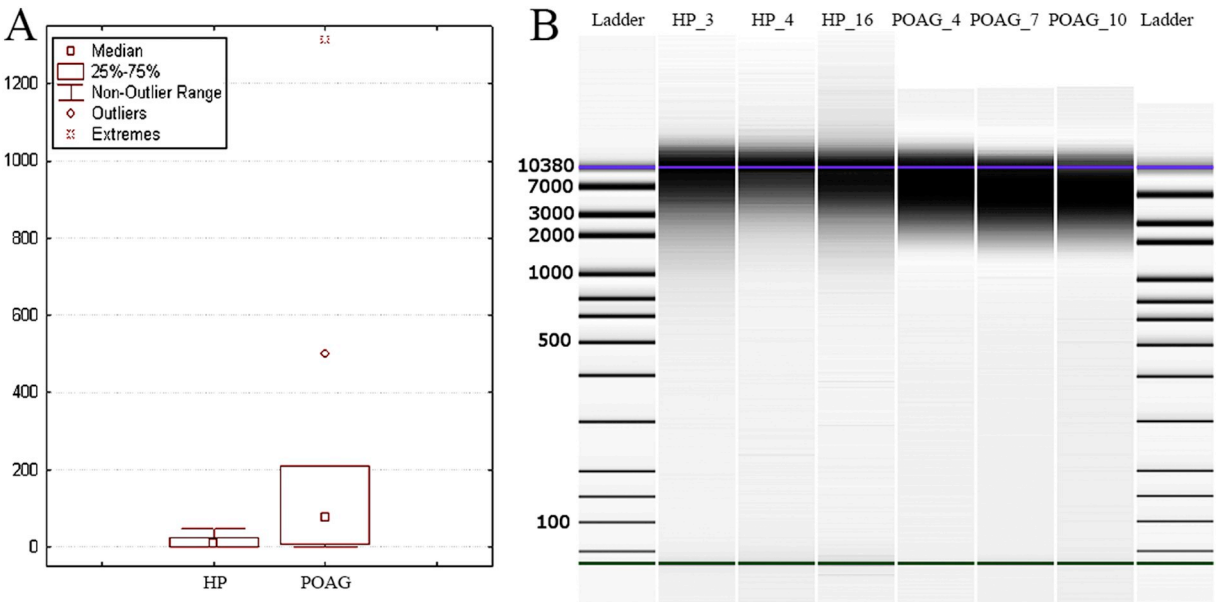

Fig. 4. A–DNA in the pellets obtained from the tears of HPs and POAG patients. B–Genomic double-stranded DNA (size 3–9292 kb) in pellets obtained from the tears from HPs and glaucoma patients.

Table 2
Results of sequencing and mapping reads.

	HPs				POAG-patients			
	H-04	H-05	H-06	Mean	GS-04	GS-07	GS-10	Mean
Raw reads, Mb	409	466	442	439	203	240	232	225
Reads mapped on human genome, %	98.7	97.5	98.3	98.2	99.0	98.5	98.6	98.7
Reads on repeats, %	22.8	25.0	25.3	24.4	33.0	30.9	31.8	31.9
Reads on genes, %	53.2	50.5	51.5	51.7	52.2	52.9	53.0	52.7

4. Discussion

All biological fluids have their own unique features that need to be taken in consideration in studies of sEVs [37,38]. For example, isolation of sEVs from viscous human saliva became possible only after application of ultracentrifugation with a sucrose density gradient [37], while centrifugation without a gradient provides for the isolation of sEVs from blood, urine and cell culture fluids [39,40]. We isolated CD63- and CD9-positive sEVs from the tears of HPs and found high contents of these structures and a relative purity of preparations [5]. The tears of POAG patients also were shown to contain high amounts of CD63- and CD9-positive sEVs. Additionally, in the case of advanced POAG patients, the tears contained sEVs covered with a "capsule". Evaluation of amount of CD63- and CD9-labelled sEVs showed that about 50% of the vesicles were labelled with each antibody in individual grids prepared from pellets obtained from the tears of POAG patients and HPs. It is known that sEVs derived from one cell can differ in the types of tetraspanins in their membrane, due to different ways of proteins sorting to sEVs [35]. TEM data about tetraspanin content in sEVs or pellets were absent, so it was impossible to compare our data with other publications.

TEM study of the pellets showed high purity of these preparations: vesicles larger than 100 nm and macromolecular admixtures were absent. However, the combination of ultrafiltration and double ultracentrifugation did not remove "non-vesicles", which are another component of all pellets. Previously we showed the presence of "non-vesicles" in sEV preparations isolated from blood and urine of HPs and cancer patients, culture fluids collected from various cell lines, breast milk and ascetic fluid of cancer patients using TEM [6]. We proposed that "non-vesicles" correspond to particles of lipoproteins, and this is in line with data of Sodar and colleagues [41], who reported presence of different density lipoproteins in sEV preparations and the inability to remove these admixtures from sEV samples using various methods. Lipoproteins of high density were mentioned as a part of sEV preparations in "Methodological Guidelines to Study Extracellular Vesicles" [42]. Usage of the "non-vesicle" term to describe the particles without membrane envelope that are observed in sEV preparations by TEM is correct, because exact identification of lipoproteins requires the application of corresponding antibodies. At the same time, "non-vesicles" should be mentioned in TEM results of sEV preparations studies, because they could carry various functionally active molecules, and thereby influence the properties of sEV preparations.

The presence of DNA in sEV preparations had been in doubt for a long time; however, with the accumulation of corresponding evidence, DNA is now considered as a part of these preparations [42]. We found genomic ds-DNA in pellets obtained from the tears of HPs and showed that it was localised on the external surfaces of CD63- and CD9-positive sEVs or "non-vesicles" [5]. The examination of pellets obtained from the tears of POAG patients revealed the presence of genomic ds-DNA with the same localisation, and we found an increase in total pellet DNA concentration and SINE repeats in POAG patients. Another interesting feature of pellet DNA in POAG patients was the different presentation of bacterial repeats in comparison with HPs. Numerous studies of eye microbiomes have been published [43–45]; however, it is impossible to apply that data for analysis of bacteria DNA found in tear pellets, because no studies examining DNA in CD63- and CD9-positive sEV preparations have been reported. Obviously, the signs of presence of different bacteria species in tear pellets obtained from sick and HPs are preliminary and need further study; however, we think that these data should be available for researchers, firstly, since there is a bacterial theory of glaucoma development [46].

CD63- and CD9-positive sEVs produced by the cells in pathological status contain disease-specific microRNA, and thus sEVs circulating in biological fluids of a patient could be a valuable source of diagnostic material [8,47–49]. The existence of such sEV microRNAs indicates that sEV microRNAs in tears could serve as markers for the detection of ophthalmic diseases. Indeed, members of miR-24 and miR-204/211 families are expressed in cells of trabecular meshwork and influence the expressions of some genes related with glaucoma development [50]. It has been proposed that decrease in the expression of miR-126 is related to an increase of fibronectin secretion in patients with diabetic retinopathy [51] because the miR-126 is expressing in endotheliocytes and

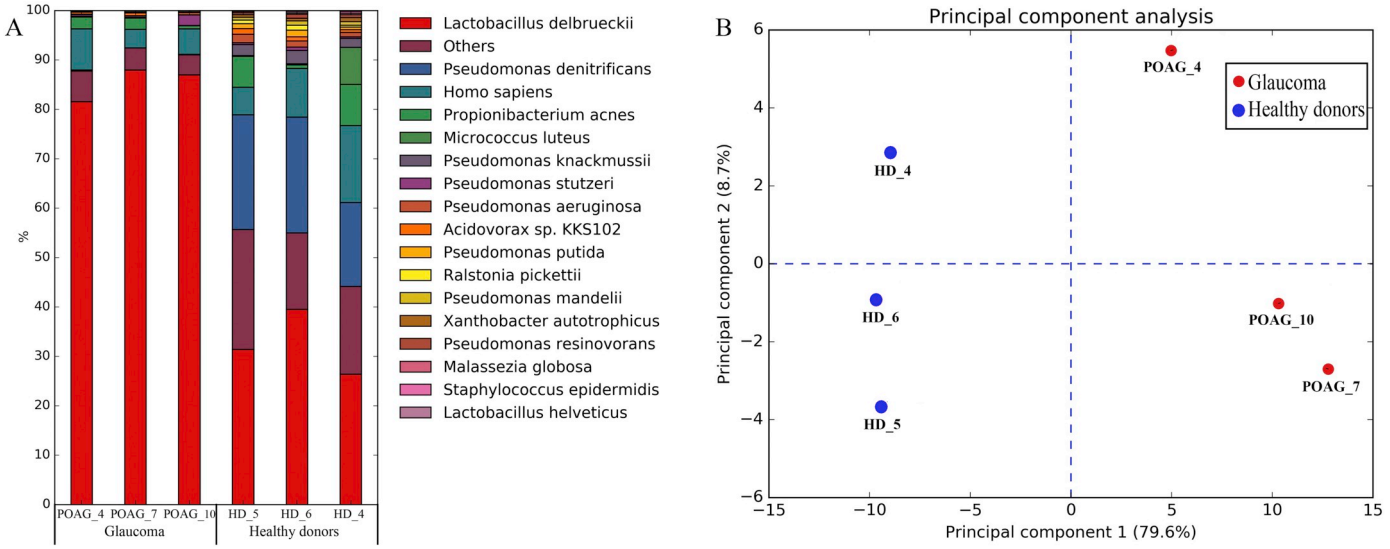

Fig. 5. A–Taxonomic distribution in DNA from sEVs of HPs and POAG patients. B–Principal component analysis for HPs (blue) and POAG patients (orange) EVs microbiota. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

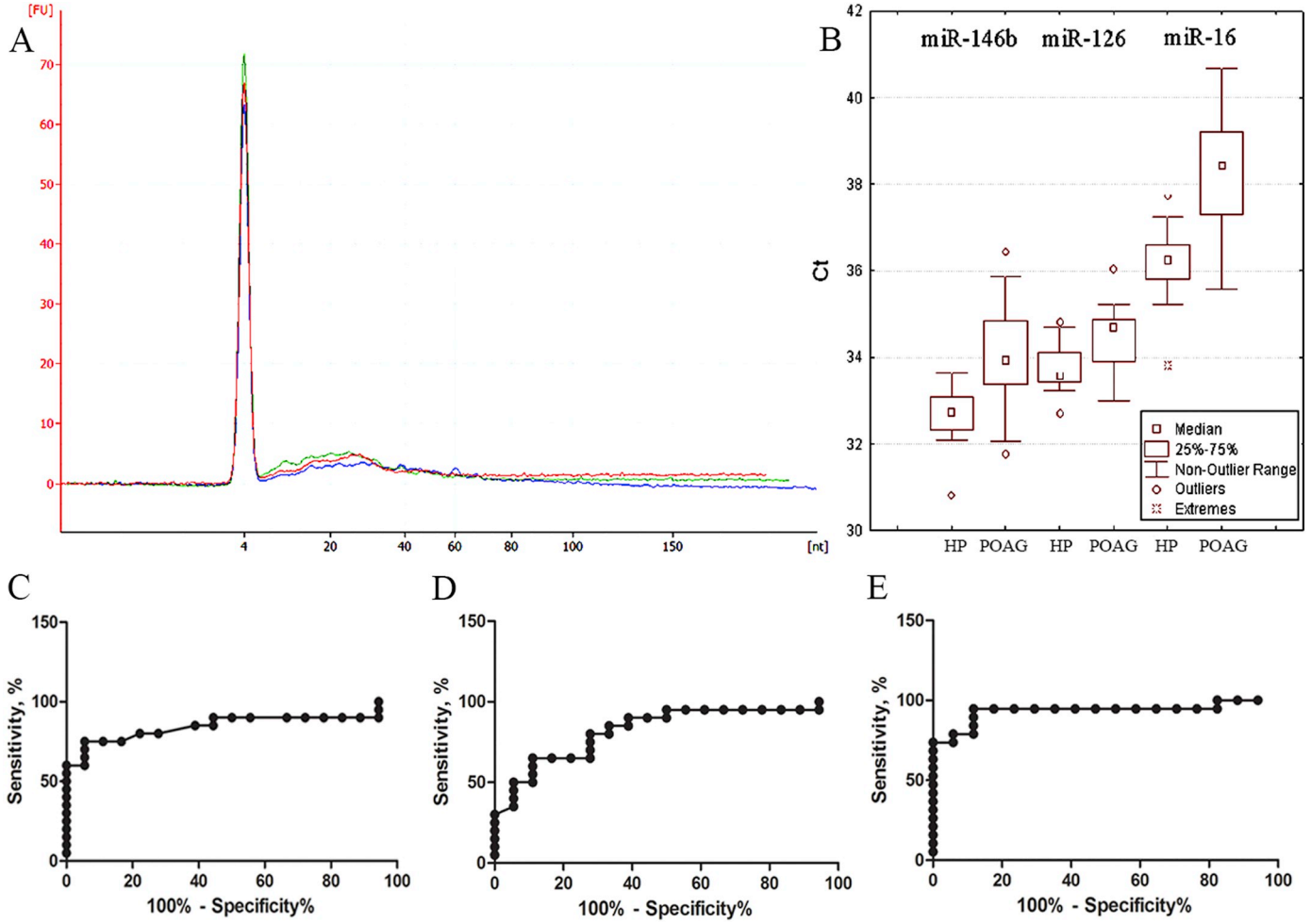

Fig. 6. A–Bioanalyzer electrophoregrams showed the presence of primarily short RNA molecules (10–50 nt) in samples derived from tears of POAG patients. B–MicroRNA in the pellets obtained from the tears of HPs and POAG patients. C–E–Diagnostic potential of sEVs microRNA for POAG. In ROC-curve analysis individual sEVs miR-146b (C), miR-126 (D) and miR-16 (E) were found to have a discriminatory accuracy of 0.824–0.935.

Table 3
Quantification of miRNA levels in sEVs of tears of HPs and POAG patients.

microRNA	Mean Ct ± SD		Significant difference by Mann-Whitney U test
	HPs (n = 18)	POAG-patients (n = 20)	
miR-146b	32.70 ± 0.67	34.03 ± 1.17	P = .000242
miR-126	33.71 ± 0.54	34.48 ± 0.70	P = .000660
miR-16	36.15 ± 0.88	38.36 ± 1.24	P = .000045

involved in vasculogenesis [52]. The miR-146 was found to be associated with inflammatory and tumour diseases of vision organ [8,36,53]. Finally, > 300 microRNAs were identified in the tears [54]; however, microRNAs of the sEVs of the tears remained outside of the studies. Our study found different expression of miR-146b, miR-16 and miR-126 in tear pellets obtained from HPs and POAG patients, and the data obtained suggest the possibility to use these microRNAs as POAG diagnostic markers. The most important finding is the detection of the changes at an early stage of POAG, which is still difficult to diagnose.

5. Conclusions

Our study showed that the tears of HPs and POAG patients could serve as an object for electron microscopic studies and as a source of CD63- and CD9-positive sEVs-containing preparations (pellets). The pellets contain EVs bearing specific CD63 and CD9 markers and “non-vesicles” devoid of membrane envelope. The tears of sick persons contained sEVs with “a capsule”. CD63- and CD9-positive sEVs comprise not less than a half of total mass of structures visualised using TEM in the pellets. Pellets obtained from the tears of POAG patients showed elevated concentrations of genomic ds-DNA and SINE repeats in addition to different expressions of miR-146b, miR-16 and miR-126 in comparison to those in pellets obtained from the tears of HPs. All changes in molecular biological parameters were detected at the initial stage of POAG, and so could be used for the development of diagnostic means.

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