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Vitreous composition modification after transpalpebral electrical stimulation of the eye: Biochemical analysis

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ABSTRACT

Electrical stimulation (ES) of the eye represents a therapeutic approach in various clinical applications ranging from retinal dystrophies, age-related macular degeneration, retinal artery occlusion and nonarteritic ischemic optic neuropathy. In clinical practice, ES of the eye is mainly performed with a transcorneal or transpalpebral approach. These procedures are non-invasive and well-tolerated by the patients, reporting only minimal and transient adverse events, while serious adverse effects were not observed. Despite the growing literature on animal models, only clinical parameters have been investigated in humans and few data are available about biochemical changes induced by ES of the eye. The purpose of this study is to investigate the possible mechanism that regulates the beneficial effects of ES on retinal cells function and survival in humans. 28 patients undergoing pars plana vitrectomy (PPV) for idiopathic epiretinal membrane (iERM) were randomly divided in two groups: 13 patients were treated with transpalpebral ES before surgery and 15 underwent surgery with no prior treatment. Vitreous samples were collected for biochemical analysis during PPV. ES treatment leads to a reduction in the vitreous expression of both proinflammatory cytokines, namely IL-6 and IL-8, and proinflammatory lipid mediators, such as lysophosphatidylcholine. Indeed, we observed a 70% decrease of lysophosphatidylcholine 18:0, which has been proven to exert the greatest proinflammatory activities among the lysophosphatidylcholine class. The content of triglycerides is also affected and significantly decreased following ES application. The vitreous composition of patients undergoing PPV for iERM displays significant changes following ES treatment. Proinflammatory cytokines and bioactive lipid mediators expression decreases, suggesting an overall anti-inflammatory potential of ES. The investigation of the mechanism by which this treatment alters the retinal neurons leading to good outcomes is essential for supporting ES therapeutic application in various types of retinal diseases.

1. Introduction

The first traces of the application of electrical stimulation (ES) to treat eye diseases was dated back to 1873 when a Swiss ophthalmology named Henri Dor used this approach in various pathologies such as glaucoma, amblyopia, amaurosis, retinochoroiditis and white optic atrophy (Dor, 1873). However, only in the last two decades, ES of the retina aroused renewed interest, since Chow et al. reported amelioration of residual vision in patients carrying an inactive sub-retinal prosthesis which produced only sub-threshold currents even in the area of the retina far from the implant (Chow et al., 2004). It was hypothesised that the release of neurotrophic factors could explain such evidence, and,

Abbreviations: ES, electrical stimulation; (IL)-1β, interleukin; (TNF)-α, tumor necrosis factor; BDNF, brain-derived neurotrophic factor; CNTF, ciliary neurotrophic factor; RGCs, retinal ganglion cells; iERM, idiopathic epiretinal membrane; NT, non-treated group; T, treated group; OCT, Optical Coherence Tomography; PPV, Pars Plana core Vitrectomy; PLS-DA, partial least square-discriminant analysis; LPC, lysophosphatidylcholines; Cer, ceramides; PC, phosphatidylcholines; SM, sphingomyelins; TAG, triacylglycerols; CE, cholesterol esters; VEGF, Vascular endothelial growth factor; FFA, free fatty acids.

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since that, many groups investigated the effects of ES on ocular diseases *in vitro*, in animal models and clinical studies. To date, we understood that mechanism of action of ES is multifactorial. In an *in vitro* model, Wen-ting Zhou et al. reported that ES had a prominent inhibitive effect on the secretion of interleukin (IL)-1β and tumor necrosis factor (TNF)-α in microglia.

Furthermore, they described a positive regulative effect on the production of brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF) in Müller cells, both preventing photoreceptor degeneration and ameliorating photoreceptor damage in a light-damaged photoreceptor model (Zhou et al., 2012). Consistently, ES was shown to induce the upregulation of IGF-1 produced by Müller cells, to promote the survival of retinal ganglion cells (RGCs) after transection of the optic nerve in rats (Henrich-Noack et al., 2013; Morimoto et al., 2005), and to sustain the survival of photoreceptors in both animal models of autosomal recessive and autosomal dominant retinitis pigmentosa (Morimoto et al, 2007, 2012). To further explore the mechanism of action of ES, Kanamoto et al. performed a proteomic analysis of the expression patterns of proteins induced by transcorneal ES. They found twenty-five differently expressed proteins among physiological factors, cellular signalling molecules, metabolic proteins, immunological and structural proteins, thus indicating that ES has various effects on retinal cells (Kanamoto et al., 2015). Clinical applications of ES range from retinal dystrophies, age-related macular degeneration, retinal artery occlusion and nonarteritic ischemic optic neuropathy (Chaikin et al., 2015; Fujikado et al., 2006; Inomata et al., 2007; Schatz et al, 2011, 2017, 2011; Wagner et al., 2017). For practical reasons, ES of the eye was performed in clinical studies using transcorneal or transpalpebral approaches: these procedures are non-invasive and well tolerated by the patients. Severe adverse effects have not been reported in any peer-reviewed publication on the use of ES on patients. Across these studies, adverse effects appear minimal and transient.

Nevertheless, clinical evidence is likely weaker than those described in animals and *in vitro* studies. In retinitis pigmentosa, Schatz et al. reported only a significant improvement of light-adapted single flash b-wave after one year of treatment while Wagner et al. did not find any significant change when comparing the control with the treated eye. In two small case series of three patients, functional parameters improved after ES treatment in patients affected by longstanding retinal artery occlusion (Inomata et al., 2007) and nonarteritic ischemic optic neuropathy (Fujikado et al., 2006). Only clinical parameters have been investigated in humans, while limited data is available on biochemical changes induced by ES in the eye. Such changes can be viably characterised in the vitreous through vitreous biopsy, a method of diagnostic analysis increasingly used in ocular diseases such as uveitis or endophthalmitis due to the improvements in posterior segment surgery and the development of specific and sensitive tools for the analysis. The vitreous fluid is collected through a pars plana vitrectomy and represents a useful tool to gain information on the pathophysiology of many posterior segment disease (Mastropasqua et al., 2019; Patnaik et al., 2020). The aim of the study was to investigate the possible mechanism that regulates the effects of ES on retinal cells function and survival in humans, in order to fill the gap between pre-clinical evidence and clinical applications of ES. Since ES treatment could elicit a multifactorial response in the vitreous composition, we focused on the biochemical analysis of proteins and bioactive lipids known to be involved in inflammatory response. In fact, among other beneficial effects, ES treatment seems to correlate with attenuation of the inflammatory phenotype. Hence, we treated a group of patients undergoing pars plana vitrectomy for idiopathic epiretinal membrane (iERM) with transpalpebral ES before surgery, and we collected vitreous samples. In the vitreous fluid of patients either treated or not with transpalpebral ES before surgery, we determined the concentration of IL-6, IL-8 and IL-10 as the main cytokines correlated with ES treatment. Furthermore, since VEGF plays a relevant role in the pathogenesis of some retinal disorders which provide for clinical applications of ES, we evaluated its expression too. Finally, we performed a lipidomic analysis focusing on the amount of ceramides and lysophosphatidylcholines as the relevant (sphingo)lipid classes potentially involved in inflammation.

2. Materials and methods

2.1. Reagents

The reagents were all analytical grade. Methanol, propanol, acetonitrile, ammonium formate, acetic acid, potassium hydroxide and formic acid (all analytical grade) were supplied from Merck (Darmstadt, Germany). Water was MilliQ grade. Lipids standards were purchased by Avanti Polar Lipids (Alabaster, USA).

2.2. Study population

Patients affected by iERM with a clinical indication for surgery were screened at vitreoretinal service of ASST Santi Paolo e Carlo Hospital, University of Milan, in one year. Exclusion criteria were considered: eye surgery or other eye procedures in the last 12 months, history of retinal laser photocoagulation, glaucoma or any other eye disease that requires chronic therapy, except for artificial tears and other lubricating eye drops, history of uveitis or other eye disease characterised by inflammatory reactions, i.e. diabetic retinopathy or other retinal vascular diseases. Patients undertaking systemic anti-inflammatory drugs, or patients affected by diabetes mellitus, systemic inflammatory diseases or any other disease for which eye involvement cannot be excluded were considered not eligible for the study as well as patients without psychophysical requirements to understand the aim of the study adequately, to complete the required ES cycles and to provide the informed consent. A total of 28 patients were randomised: 15 were included in non-treated (NT) group and 13 in treated (T) group. At the baseline visit, complete medical and ocular history was obtained to verify if all eligibility criteria were checked. Visual acuity test was performed along with anterior segment and dilated fundus examinations, IOP evaluation and Optical Coherence Tomography (OCT).

2.3. Ethical statements

The vitreous fluid was obtained by patients undergoing pars plana vitrectomy (PPV) for idiopathic epiretinal membrane (iERM) under appropriate approval by the Ethical Committee of the ASST Santi Paolo e Carlo, Milano, Area A (n°409, May 03, 2019). All participants signed informed consent forms approved by the Ethical Committee before specimen collection. The privacy rights of human subjects have always been observed. All the procedures have been performed in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). The manuscript is in line with the Recommendations for the Conduct, Reporting, Editing and Publication of Scholarly Work in Medical Journals and aims for the inclusion of representative human populations.

2.4. Transpalpebral electrical stimulation

ES of the eye was administered transpalpebral using Subeye, research use only (RUO) device (SubVision, Milan - Italy), generating biphasic square-wave pulses of 500 ms/phase with frequency ranging among 300 to 1 Hz during the 20 min of treatment and intensity incrementable from 0 to 30 V. We chose the transpalpebral approach, with electrodes placed on the eyelids, for practical and safety reasons since patients performed the treatment at home on their own. Each patient was trained during the baseline visit: after receiving detailed instructions on the procedure, the first treatment was completed in the Eye Clinic with the supervision of the clinician. Patients were asked to increase the level of intensity until they perceived phosphenes. Surgery was then planned after six weeks, and patients had to perform

transpalpebral ES five days a week (from Monday to Friday) until the surgery. The last treatment was administered the morning of the surgery in the clinic. At day 7 and 28, a visit was scheduled to verify the absence of side effects of ES; on these occasions, a proper smartphone application connected to the device through the bluetooth allowed to check if patients correctly completed the scheduled sessions. Surgery consisted of a standard three-port 25 G Pars Plana core Vitrectomy (PPV) using Constellation Vision System (Alcon Laboratories, Fort Worth, Texas) and ERM peeling. The vitreous sample was collected as the first step of the surgery, after inserting three valved trocars and before opening the infusion line: 0.2–0.5 ml of vitreous fluid was collected in a 1 ml syringe connected to the vitreous cutter. At the same time, the surgeon digitally checked the intraocular pressure during the procedure. Patients were dismissed after a few hours, and a next day visit was scheduled. Postoperative day 1 visit only included a slit-lamp examination with anterior and posterior segment evaluation, along with IOP measurement, to check the absence of any adverse event. Next visits were scheduled at day 7, 30, 60 and 180 postoperatively: the presence of any adverse event potentially related to ES treatment was checked.

2.5. ELISA test

The protein content of the cytokines IL-6, IL-8 and IL-10 together with VEGF were determined in the vitreous from control untreated (NT), and transpalpebral ES treated (T) patients by biomarker multiplex immunoassays on Luminex® Platform. Every determination has been performed in duplicate.

2.6. Lipidomic analysis

Vitreous (50 μl, 50–100 μg protein content) was diluted (1:1, v/v) with pure water and lipids extraction was achieved using a monophasic extraction with chloroform:methanol (850 μl, 1:2, v/v). After concentration, purified extracts (5 μl) were analyzed by a Shimadzu UPLC coupled with a Triple Tof 6600 Sciex (Concord, ON, CA), running top-20 data-dependent acquisition with positive electrospray ionization (Dei Cas et al., 2020b). All samples were analyzed in duplicate for the identification and semi-quantification of main lipids. The separation was achieved by an Acquity CSH C18 column 1.7 μm 2.1 × 100 mm (Waters, MA, USA) using mobile phase (A) water/acetonitrile (60:40) and mobile phase (B) 2-propanol/acetonitrile (90:10) both containing 10 mM ammonium acetate and 0.1% of formic acid. The lipidomics data analysis was processed using MS-DIAL (ver. 3.4). Data analysis and plotting were achieved with MetaboAnalyst (ver. 4.0), after being normalized by the sum, log-transformed and auto-scaled. Statistical analysis included volcano plots (significant alterations were considered for $p < 0.05$ and $0.8 < FC < 1.25$) and partial least square-discriminant analysis (PLS-DA) for biomarker analysis.

2.7. Statistical analysis

Data were analyzed with R (Version 3.6.) and R Studio (RStudio Team, Boston, MA. Version 1.2), and plotted with GraphPad Prism (GraphPad Software, San Diego, California USA. Version 8.1). Statistics for categorical variables were investigated by Fisher's exact test. Descriptive statistics were calculated for the control group (NT, n = 15) and treatment group (T, n = 13). Due to their skewed distribution, the concentration of cytokines and lipids was summarised by their median and interquartile range. Differences between treatment and control group were tested by means of two-tailed Mann-Whitney tests with an alpha level of 0.05, and effect sizes were calculated as Wilcoxon's R. Spearman's rank correlation coefficient was calculated on continuous variables and reported as a correlation matrix.

3. Results

3.1. Population

Twenty-eight patients were recruited for the study: n = 15 patients were randomly assigned to NT group, n = 13 patients to T group. Mean age in NT group was 75.53 ± 9.07 years and in T group was 73.15 ± 6.76 years (p = 0.435). NT group was composed of 9 females and 6 males, whereas T group of 9 females and 4 males (Table 1).

3.2. Pro- and anti-inflammatory cytokines expression

The vitreous levels of different interleukins were determined in both NT and T group (Fig. 1 and Table 2). The proinflammatory markers IL-6 and IL-8 and the anti-inflammatory marker IL-10 were determined, and a decreasing trend in proinflammatory cytokines was established after ES treatment. As opposite, the level of IL-10 was unaffected by the treatment. Vascular endothelial growth factor (VEGF) was studied as well. It was recognised to be not significantly different after ES treatment (median [interquartile range]; 3.86 [1.57–9.66] vs 7.09 [3.95–10.760] pg/ml), although a slight increase was apparent in the T group.

3.3. Lipidomic profile

Lipids are involved in cell membrane structural partitioning, energy storage, and cell signalling that affects cellular functions such as apoptosis, proliferation, stress response, and inflammation (Casares et al., 2019; Dei Cas et al., 2020a; Van Meer et al., 2008). In this study, we focused on the amount of the main lipid classes for each functional category: ceramides (Cer) and lysophosphatidylcholines (LPC) for cell signalling lipids (Biemmi et al., 2020), phosphatidylcholines (PC) and sphingomyelins (SM) for structural lipids and triacylglycerols (TAG) and cholesterol esters (CE) for energy storage lipids. The amount of structural and storage lipids is slightly inferior in T group as compared to NT (Fig. 2 and Table 2), though not reaching a statistical significance except for TAG which showed a decrease of about 26% (median [1st quartile – 3rd quartile]; 3.808×10^{-4} [3.47×10^{-4} – 4.35×10^{-4}] vs 2.809×10^{-4} [2.38×10^{-4} – 3.53×10^{-4}]); intensities expressed as arbitrary unit, p = 0.036). However, it's worth noting the relevant reduction of SM expression following ES treatment (median [1st quartile – 3rd quartile]; 6.12 × 10–4 [5.01 × 10 -4 – 2.37 × 10–3] vs 3.855 × 10–4 [1.54 × 10 -4 – 2.43 × 10–3]). As it concerns signalling lipids, while Cer content was unaltered, proinflammatory lipids (LPC) follow the same decreasing trend in T versus NT group, as indicated by Wilcoxon's R values: 0.28. Vitreous displays a slight content of lipids as compared to other biological fluids, such as plasma (Bowden et al., 2017). A total of n.4313 features were detected in positive ionization modes, but the putative identification procedure by MS/MS spectral match resulted in 215 lipids (about 5%). Vitreous displayed a slight content of lipids as compared to other biological fluids, such as plasma (Bowden et al., 2017). The supervised multivariate discriminant analysis displays the entire lipidome in vitreous of patients without (NT) or with (T) transpalpebral electrostimulation (Fig. 3A). However, the NT and T samples were partially separated by PLSDA, showing a consistent overlapped area that comprises the 65% of the samples. At first glance, ES treatment

Table 1
Demographic data of the study population. NT: untreated; T: treated. p-values were calculated with unpaired *t*-test with an alpha level of 0.05 for continuous variables and Fisher's exact test for categorical ones.

	NT (n = 15)	T (n = 13)	p-value
Age (mean ± SD)	75.53 ± 9.07	73.15 ± 6.76	0.435
Male	6 (40%)	4 (30.1%)	0.705
Female	9 (60%)	9 (69.9%)	

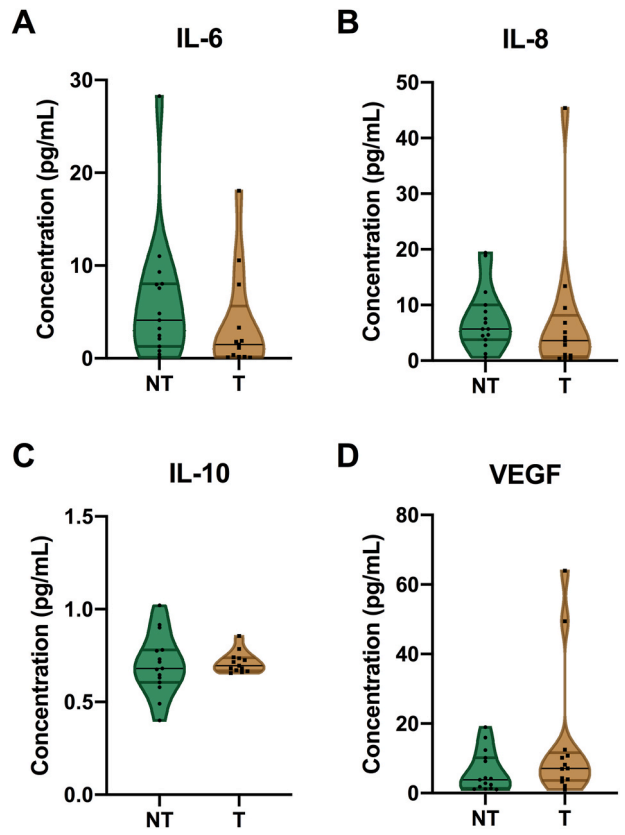

Fig. 1. Pro- and anti-inflammatory cytokines expression in vitreous liquid from TES-treated and untreated patients. Concentration of interleukins, expressed as pg/ml, in patients untreated (NT) and treated (T) with TES. Panel **A**: Interleukin 6 (IL-6); panel **B**: Interleukin 8 (IL-8); panel **C**: Interleukin (IL-10) and panel **D**: Vascular Endothelial Growth Factor (VEGF).

Table 2
Comparative expression of different analytes in untreated (NT) and treated (T) patient groups. Effect sizes were calculated as the Wilcoxon's R. p-values were calculated with two-tailed Mann-Whitney test with an alpha level of 0.05. 1st quartile and 3rd quartile were reported in square brackets. Interleukines concentrations are expressed as pg/mL whereas lipids as intensities (arbitrary unit).

Analyte	NT group (n = 15)	T group (n = 13)	Effect size	p-value
IL-6	4.11 [1.70–8.00]	1.58 [0.16–3.32]	- 0.30	0.117
IL-8	5.70 [4.12–9.42]	3.59 [0.95–6.81]	- 0.28	0.142
IL-10	0.68 [0.62–0.78]	0.70 [0.67–0.74]	+0.10	0.628
VEGF	3.86 [1.57–9.66]	7.09 [3.95–10.760]	+0.25	0.189
Cer	1.75×10^{-5} [1.34×10^{-5} - 2.23×10^{-5}]	1.98×10^{-5} [1.04×10^{-5} - 2.12×10^{-5}]	+0.08	0.723
LPC	4.87×10^{-5} [3.56×10^{-5} - 1.62×10^{-4}]	3.74×10^{-5} [2.64×10^{-5} – 7.93×10^{-5}]	- 0.28	0.178
PC	3.93×10^{-3} [2.42×10^{-3} – 1.34×10^{-2}]	1.56×10^{-3} [4.03×10^{-4} - 1.46×10^{-2}]	- 0.26	0.216
SM	6.12×10^{-4} [5.01×10^{-4} – 2.37×10^{-3}]	3.855×10^{-4} [1.54×10^{-4} – 2.43×10^{-3}]	- 0.27	0.196
CE	4.251×10^{-6} [2.59×10^{-6} – 1.41×10^{-5}]	3.093×10^{-6} [6.74×10^{-7} – 1.08×10^{-5}]	- 0.17	0.428
TAG	3808×10^{-4} [3.47×10^{-4} – 4.35×10^{-4}]	2.809×10^{-4} [2.38×10^{-4} – 3.53×10^{-4}]	- 0.42	0.036

seems to modify the lipid content of the vitreous slightly. The single subclasses of lipids were then investigated in both T and NT group by a heatmap showing the mean distribution of each lipid classes (Fig. 3B) and by fold-change comparison, as well (Fig. 3C). Both the representations evidenced that the lipid classes are quite similarly expressed except for the lysophosphatidylcholines (LPC) in the T group that are about 60% less expressed than in the NT group (T/NT = 0.41). The univariate analysis by Volcano plot selected eight lipids (Fig. 4A, green dots (in the web version)) which decrease significantly after ES treatment. In particular, it is worth noting the 70% decrease of proinflammatory LPC 18:0 (*m/z* 524.37067 [M + H^+], $p < 0.05$) (Fig. 4B). This observation was indeed in line with the reduction of IL-8 and IL-6 as reported by the positive correlation between these proinflammatory mediators in the scatter plots of Fig. 5.

4. Discussion

Our study investigates the possible mechanism regulating the effects of ES on retinal cells function and survival in humans, by analyzing the composition of vitreous fluid of patients who underwent PPV for idiopathic epiretinal membrane (iERM) with transapalpebral ES before surgery. iERM was chosen as it is the most frequent cause of PPV that does not recognize an inflammatory or infectious aetiology, thus avoiding this possible source of bias. The patients enrolled in our study underwent six weeks of ES of the eye as per protocol: for practical reasons, the treatment was performed transpalpebral, and the only reported adverse effect was a transient eyelid redness. None of the patients discontinued the 6-weeks treatment regimen. Accordingly, in a prospective single-arm observational safety study, repeated delivery of transcorneal ES in 105 retinitis pigmentosa patients induced dry eye sensation as the most common adverse event recorded while serious adverse effects were not observed (Jolly et al., 2020). A single application of ES in ten healthy human subjects promotes a gradual and sustained increase of chorioretinal blood flow with minimal variations of the systemic blood circulation and the intraocular pressure (Kurimoto et al., 2010). In our study, microcurrent stimulation was administered with biphasic square-wave pulses. Even if some authors suggest that sinusoidal stimulation requires lower thresholds for the activation of the retinal network (Kelbsch et al., 2018), most of the literature on animal studies and clinical evaluations used square or rectangular biphasic current pulses (Sehic et al., 2016). Despite the growing literature on animal models of ocular pathologies, as recently reviewed by Sehic A. and colleagues (Sehic et al., 2016), ES eye application in human subjects is still poorly investigated. Therefore, we analyzed the protein and lipid content of vitreous fluid from iERM patients in an attempt to identify molecular mediators potentially involved in ES mediated beneficial effects. The vitreous is a gel-like connective tissue that accumulates various biological compounds which come from the retina and exert paracrine functions. It comprises of 98% water, and the remaining 2% consists mainly of proteins (87%) and, to a lesser extent, of lipids (9%) and carbohydrates (4%) (Reddy et al., 1986). As it concerns the proteins, we focused on mediators of inflammation such as interleukins IL-6, IL-8 and IL-10 since previous *in vitro* studies have demonstrated that electrostimulation of the retina reduces the secretion of proinflammatory and toxic cytokines by microglia. As compared to the untreated vitreous, we observed a decreasing trend in proinflammatory IL-6 and IL-8 expression after ES treatment, while the level of IL-10 was unaffected. In accordance, light-damaged cone-derived cells (661 W) co-cultured with microglia exhibit increased survival following electrostimulation because of inhibitive effect on the secretion of proinflammatory mediators in microglia (Zhou et al., 2012). Willman G. et al. (Willmann et al., 2011) report that ES applied to wild-type rat's retina attenuates the expression of some genes of the tumor necrosis factor family, a group of proinflammatory cytokines, *versus* sham-stimulated retina, thus identifying a set of transcriptional changes with potential neuroprotective effects. In a DBA2/J mouse model of glaucoma, ES application reduces the neurodegenerative process by decreasing the expression of inflammatory microglial (Iba1+ cells) and T cells ($CD3^+$ cells) (Jassim et al., 2020).

Lipids serve as cell membrane components, energy storage compounds, and play a pivotal role as cell signalling mediators (Casares et al., 2019; Dei Cas et al., 2020a; Van Meer et al., 2008). In our study, lipidomic analysis reveals that overall lipids are quite similarly

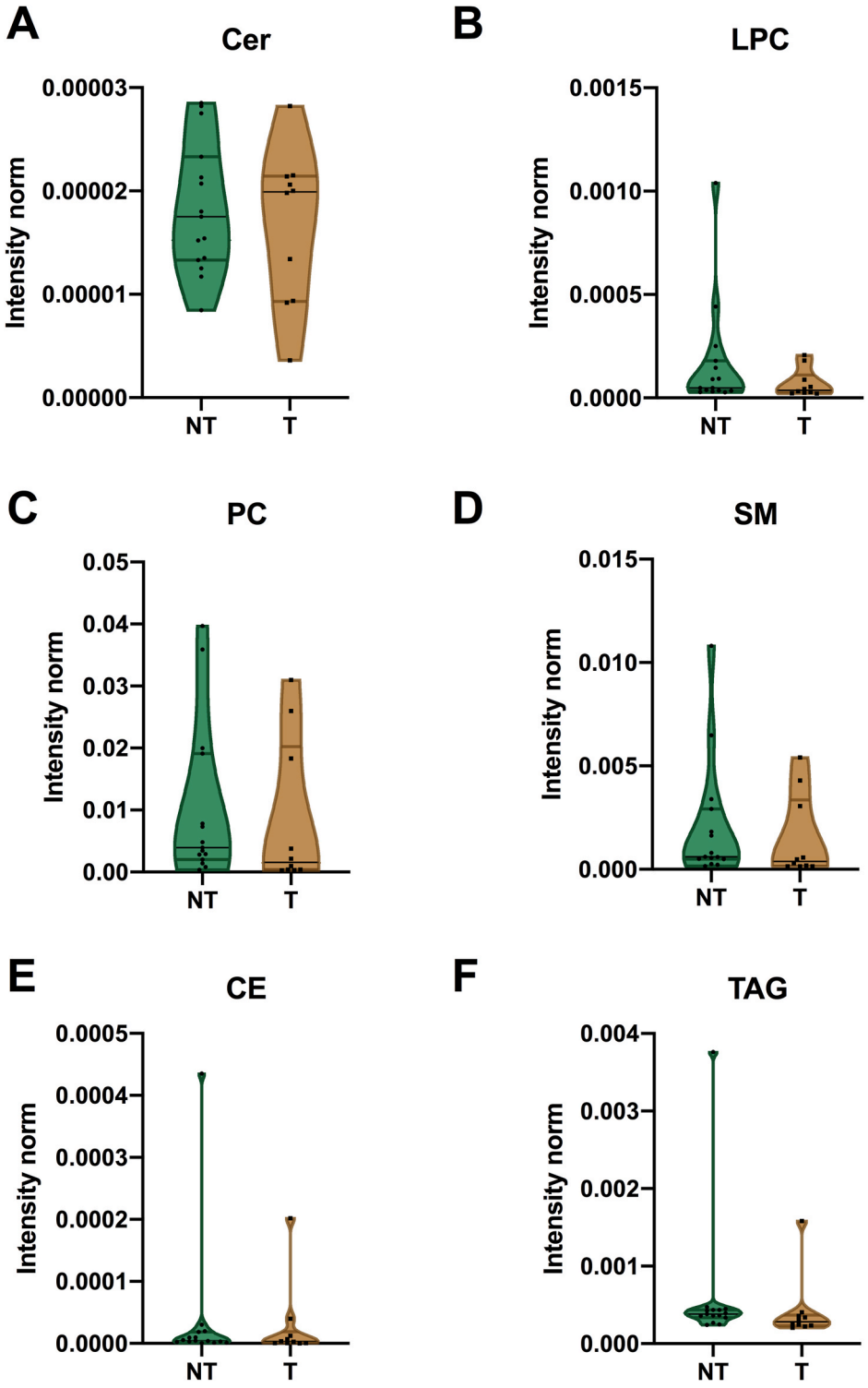

Fig. 2. Concentration of relevant lipid classes in vitreous liquid from TES-treated and untreated patients. Concentration of relevant lipid classes, expressed as intensity after normalization (intensity norm) in control untreated (NT) and treated (T) with TES patients. Panel **A**: Ceramide (Cer); panel **B**: Lysophosphatidylcholine (LPC); panel **C**: Phosphatidylcholine (PC); panel **D**: Sphingomyelin (SM); panel **E**: Cholesterol Esters (CE) and panel **F**: Triacylglicerols (TAG). Intensity after normalization (Intensity norm) is the ratio of the sum of peak intensities of each lipid in a class over the total ion count (TIC) of each samples.

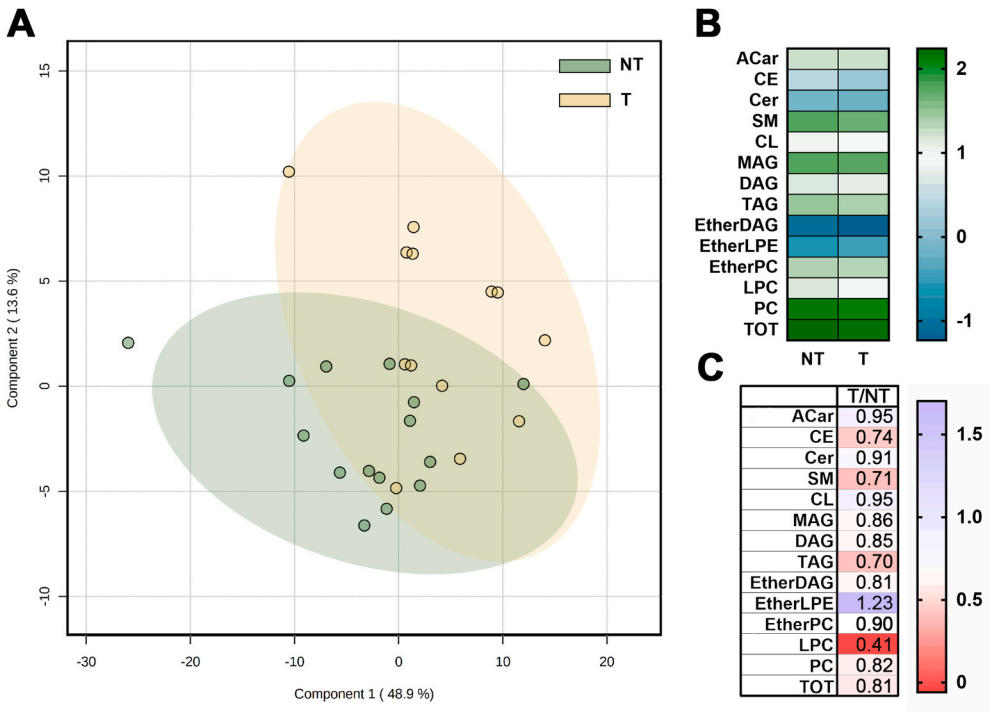

Fig. 3. Lipid profile of vitreous liquid from TES-treated and untreated patients. (**A**) Comparison of vitreous lipidome in patients without (NT) or with (T) TES by multivariate discriminant analysis (PLSDA). The axes are ranked according to their importance in the group discrimination. In the abscissa axis, component 1 (PC1, 49%) represents the maximum of the separation that can be reached within these clusters and variables, which is the direction in the original data that contains the most variance between the groups. In the ordinate axis, component 2 (PC2, 13.6%) represents the direction that contains the most remaining variance. The coloured area indicates the 95% confidence interval of each cluster. (**B**) Heatmap reports the mean distribution of the main lipid classes in the vitreous of NT and T patient group. The entity of the fold change has been visualized by colour gradient from the smallest (cerulean) to the highest value (green), passing through the baseline (white). (**C**) Fold-change between lipid levels in the vitreous of T *versus* NT patient group have been visualized by colour gradient from the smallest (red) to the highest value (lilac). In both the colour scale of panels **B** and **C**, the white was used to indicate baseline values. Acar: acylcarnitine; CE: cholesterol ester; Cer: ceramide; SM: sphingomyelin; CL: cardiolipine; MAG: monoacylglycerol; DAG: diacylglycerol; TAG: triacylglycerol; LPC: lysophopshatidylcholine; PC: phosphatidylcholine; TOT: total lipids; ether- : indicate fatty acid complexed with lipids by an ether linkage instead of the usual ester bond.

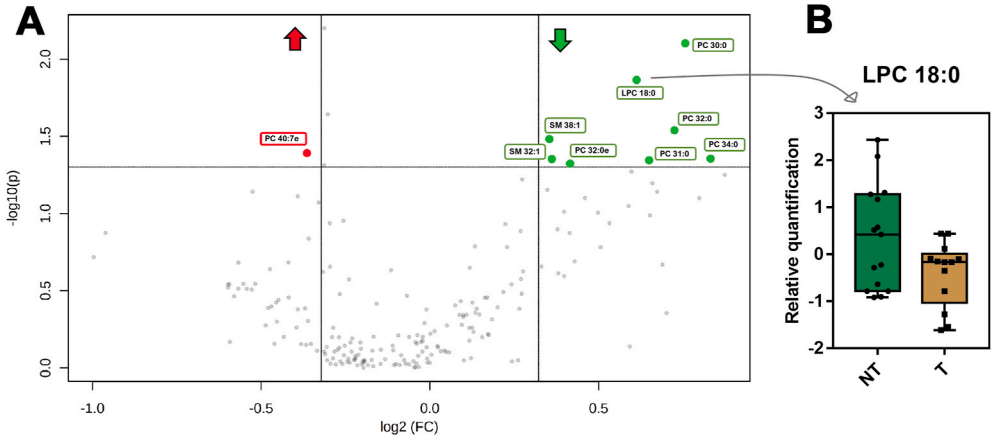

Fig. 4. Volcano plot analysis of selected lipids in vitreous liquid from TES-treated and untreated patients. Discriminant analysis by Volcano plot: (**A**), significant alterations were considered for $p < 0.05$ and fold-change FC<0.8 or FC>1.25, depicted in red and green, respectively. (**B**) The relative concentration of proinflammatory lysophospholipid LPC 18:0 was selected as a significant variable for the discrimination between control untreated (NT) and treated (T) groups. LPC content was reported as relative quantification that is the log transformed and mean centered peak intensity of *m/z* 524.37067 [MH+] among the samples.

distributed in the vitreous from ES-treated and untreated patients except for lysophosphatidylcholines (LPC) and triacylglycerols (TAG), both highly reduced in the ES group. LPC is a bioactive proinflammatory lipid produced by the metabolism of phosphatidylcholines (PC). LPC accumulation correlates with a set of pathologies, including neurodegeneration, inflammatory diseases (Liu et al., 2020), and diabetic retinopathy (Iwase et al., 2008). LPC can increase the expression of chemokines to attract inflammatory cells and induce the release of inflammatory factors, such as IL-1β, IL-8, IFN-γ, IL-6 and IL-5 (Bach et al., 2010). Reports indicate that among acyl-lysophospholipid species, the saturated LPC16:0 and LPC18:0 exert the greatest proinflammatory activities (Liu-Wu et al., 1998; Spangelo and Jarvis, 1996). In accordance, we have observed the 70% decrease of LPC 18:0 in vitreous liquid after ES treatment together with the reduction of the proinflammatory cytokines, IL-8 and IL-6. The presence of lipids in the human vitreous has been described as far as 1976 (Kim and Cotlier, 1976) but only recently, the expression of inflammatory lipid mediators has been reported. Schwartzman ML and colleagues demonstrate an increase in vitreous proinflammatory eicosanoids (Schwartzman et al., 2010). Furthermore, both bioactive sphingolipids and lysophospholipids potentially implicated in inflammation signalling, have been found altered in pathological versus normal vitreous (Abu El-Asrar et al., 2014; Wilmott et al., 2019). We observed a significant reduction ($p < 0.05$) in TAG expression level in ES-treated versus untreated vitreous, To the best of our knowledge, data related to TAG expression in the vitreous fluid are still lacking. However, in biological samples other than the vitreous, some evidence supports the efficacy of electrical stimulation in reducing free fatty acids (FFA) and triglycerides content, together with inflammatory

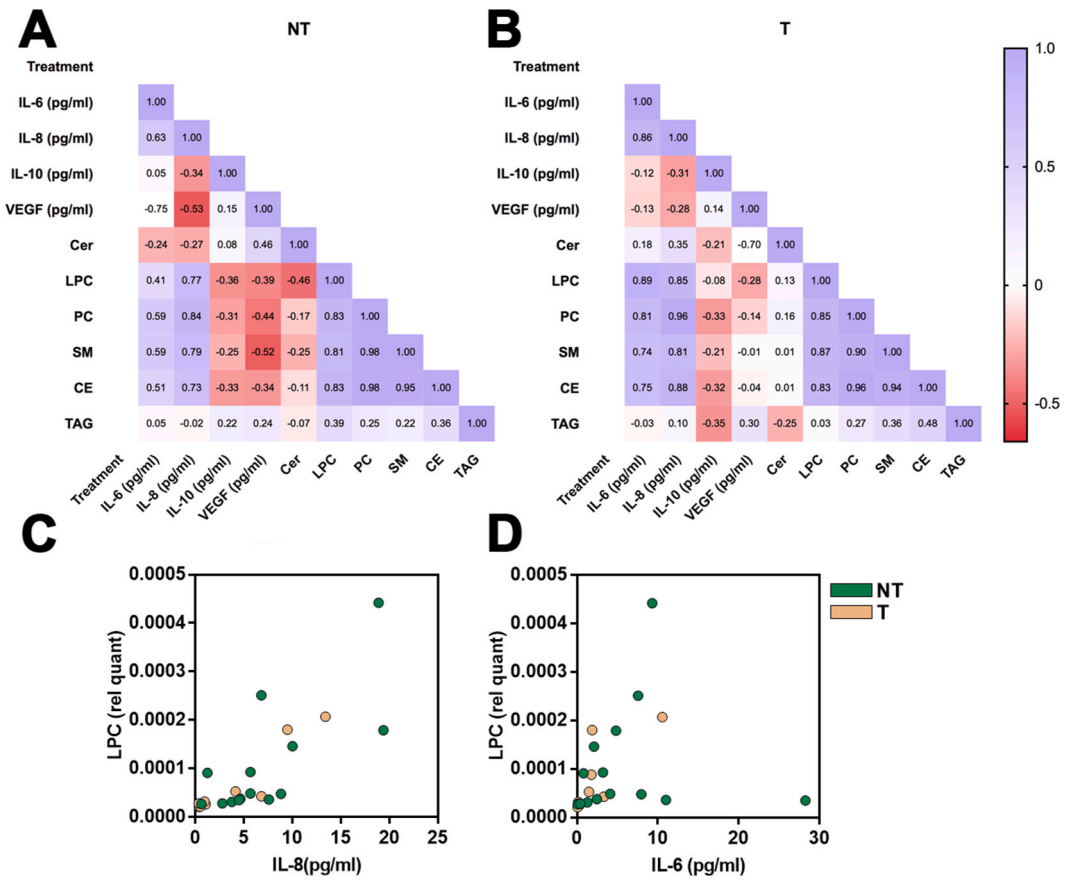

Fig. 5. Relationship between biochemical variables. Correlation matrix for control untreated (NT, panel **A**) and treated (T, panel **B**) groups, using Spearman's rank correlation coefficient. The colour scale summarizes the values of Spearman's rank correlation coefficient (red: negative values; blue: positive values). Scatter plot (panel **C**; **D**) for the correlation of proinflammatory cytokines and lipids: IL-8 and IL-6 *versus* LPC expression are reported. Cytokines concentration was expressed as pg/ml while LPC content was reported as relative quantification that is the log transformed and mean centered peak intensity of *m/z* 524.37067 [MH+] among the samples. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

cells infiltration. Electrical pulse stimulation (EPS) significantly reduces mouse C2C12 myotubes content of FFA, TAG and total cholesterol (Li et al., 2019).

Moreover, in a rat model of insulin resistance, electrical stimulation combined with diet treatment greatly diminished FFA, TAG and cholesterol content likely through PI3K/Akt/mTOR signalling pathway, thus ameliorating inflammatory processes in the liver and the pancreatic islet (Huang et al., 2019).

As it concerns the structural lipids, it is worth noting the significant reduction of SM in the treated group. Sphingomyelin is a structural lipid involved in cell membrane partitioning and it serves as a precursor of ceramide by hydrolysis. It has been reported that sphingomyelin accumulates into the vitreous humor of diabetes patients as compared to healthy subjects. In fact, diabetic patients have a weakening of the retinal barrier which becomes more permeable thus allowing the distribution of phospholipids and glucose in the vitreous humor (Schnepf et al., 2020). Accordingly, Wilmott LA et al. (Wilmott et al., 2019) found significantly elevated content of sphingomyelin (together with ceramides and lactosyl-ceramides) in diabetic vitreous as compared to normal samples. The relevant reduction in sphingomyelin expression that we observed after ES treatment could be part of the possible mechanisms that orchestrate the beneficial effects on retinal cells function and survival.

The overall expression changes induced by ES in the vitreous from iERM patients were relatively small in our study, but comparable with previous results of electrically stimulated wild-type (non-pathological) rat's retina tissue (Willmann et al., 2011) and Müller cells (Sato et al., 2008). Though the data hardly reach statistical significance, we can observe a homogeneous trend towards an anti-inflammatory potential of ES treatment as suggested by both proinflammatory proteins and lipids down-regulation.

5. Conclusions

ES affects the vitreous fluid composition of patients who underwent PPV for idiopathic epiretinal membrane (iERM), which is the most frequent cause of PPV without an inflammatory or infectious aetiology. The expression of proinflammatory cytokines and bioactive lipid mediators, such as LPC and TAG, shows the same decreasing trend suggesting an overall anti-inflammatory potential of ES. Our findings in iERM patients, which can be considered as a physiological model, need to be further investigated in pathological conditions. We can hypothesize that the anti-inflammatory effect could be even more significant in diseases with a demonstrated neuroinflammatory substrate, such as retinitis pigmentosa, age-related macular degeneration or diabetic retinopathy (Massengill et al., 2018), in which ES could play a role in disease progression. The investigation of the mechanism by which this treatment alters the retinal neurons leading to good outcomes is essential for supporting ES therapeutic application in various types of retinal diseases.

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Declaration of competing interest

The authors declare no conflict of interest.

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