Research article

Comparative study of the lipid profile of tears and plasma enriched in growth factors

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A B S T R A C T

The Tear Film Lipid Layer (TFLL) acts primarily as an interface between the aqueous layer and air. Tear film lipid is composed of a thin layer of polar lipids that interact with the secretory layer of the underlying mucosa and a thicker layer of non-polar lipids at the air interface. The tear film has a complex structure and composition that protects the cornea, promotes wound healing, and maintains high-quality vision. Plasma Rich in Growth Factor (PRGF) eye drops emerged as an exciting new treatment for corneal epitheliopathies, including aqueous deficient dry eye. The purpose of this study was to compare the lipidomic profile of eye drops obtained from PRGF with tear lipidome to determine whether PRGF drops could be an adequate complement to tears in patients with impaired TFLL. To address this study, tears and blood was collected and processed from healthy donors to obtain PRGF eye drops. Samples were aliquoted and stored at −80 °C until use. The lipid profiles of these samples were analysed by Ultrahigh Performance Liquid Chromatography (UHPLC) using a Vanquish UHPLC system to obtain untargeted lipidome profiles on a Q-Exactive HF-X hybrid quadrupole-Orbitrap mass spectrometer. In PRGF eye drops, 408 lipids were identified in ESI+ mode and 183 in ESI- mode, and they were grouped into 15 different lipid classes from four distinct categories. By contrast, 112 lipid species were identified from tear samples in ESI+ mode and 36 in ESI- mode, belonging to 12 lipid classes from six different categories. The relative abundance of most lipid species was much greater in the PRGF eye drops than in the tear, although there were some lipids present in tears that were not found in the PRGF, such as wax esters and (O-acyl)-ω-hydroxy fatty acids. In summary, these results suggest that the lipids present in PRGF eye drops could serve as a tear supplement in individuals in whom tear lipid composition is altered, although there are differences in the lipid profile of these two fluids.

1. Introduction

The tear film (TF) keeps the surface of the eye moist and lubricated, protecting it from pathogens while providing optimal visual transparency (Tiffany, 1987). The TF lipid layer (TFLL) is a thin layer at the surface of the cornea (Bron and Tiffany, 1998; Bron et al., 2004; Butovich, 2009a, 2009b) that may help prevent the evaporation and break-up of the TF. The TFLL is 80 times thinner than the muco-aqueous

layer and approximately 80% of the lipids found in it come from the Meibomian glands (MGs) (Cory et al., 1973; Mathers and Lane, 1998; McCulley and Shine, 1997; Mudgil et al., 2016). The MG secretes a complex mixture of polar and non-polar lipids, containing cholesterol esters (ChEs) and wax esters (WEs), diesters, triacylglycerol (TG), free cholesterol and free fatty acids (FAs) (Chen et al., 2010), as well as peaks that include (O-acyl)-ω-hydroxy fatty acids (OAHFAs) (Butovich et al., 2009) that may help maintain the stability of the TF. This complex mixture of lipids spreads across the surface of the eye, limiting evaporation while establishing a barrier that protects the eye from microbial agents and organic matter, such as dust and pollen (Butovich et al., 2009). The average thickness of the TFLL in healthy subjects is 90–100 nm (Yokoi et al., 2014), orders of magnitude greater than the size of typical lipid molecules or even proteins, hinting at the formation of complex multi-layered structures in the TFLL (Craig and Tomlinson, 1997). The model proposed at The International Workshop on Meibomian Gland incorporates proteins (e.g., lipocalin, lysozyme, and the surfactant proteins B and C) in and/or adsorbed to the outer lipid layer, which influence the physical properties and surface tension of the TFLL. The proposed model also features very long chain OAHFAs, which may form an intermediate surfactant lipid sublayer between the outermost non-polar lipids and the aqueous layer of the tear film (Nichols et al., 2011).

One characteristic of the TFLL is that it is continuously renewed with each blink. This occurs approximately every 10 s and the new layer of lipids introduced moves up the film due to the differences in surface tension (Marangoni effect) until the film breaks and the process starts again with the next blink (Yokoi et al., 2014). The principal role of the TFLL is to stabilize the tear film, and the distribution and thickness of this film is affected in dry eye syndrome (DED) (Craig and Tomlinson, 1997). DED is a symptomatic disorder characterized by a vicious cycle of TF instability and hyperosmolarity, which leads to exaggerated ocular surface inflammation and damage, as well as neurosensory abnormalities (Willcox et al., 2017). The classification of DED into "dry eye with reduced tear production (aqueous deficient DED -ADDE)" and "dry eye with increased evaporation of the tear film (hyperevaporative DED -EDE)" has proved useful on practical grounds. Around 10% of patients with DED have ADDE exclusively (Messmer, 2015), whereas mixed EDE/ADDE forms account for more than 80% of cases (Stern et al., 2013; Heiligenhaus et al., 1995; Tong et al., 2010). Although the composition of the tear film is altered in both these types of DED (Willcox et al., 2017), hyperevaporative disorders, are mostly caused by MG dysfunction.

Maintaining the homeostasis of the corneal epithelium requires establishing a balance between limbal stem function, tear quantity and quality, eyelid anatomy and function, and corneal sensitivity (Tseng and Tsubota, 1997). Blood derivatives, including autologous serum (Anitua et al., 2015a), platelet-rich plasma (PRP) (Alio et al., 2007), or serum derived from plasma rich in growth factors (PRGF eye drops) (Lopez-Plandolit et al., 2011; Suarez-Barrio et al., 2019) have been used to manage corneal epithelial defects in patients with DED. Furthermore, PRGF eye drops has been used successfully to treat other eye disorders (Rodriguez-Agirretxe et al., 2018). As preparations free of leukocytes and a low-density fibrin network, PRGF eye drops is a subtype of pure PRP (P-PRP) that provides a complex pool of mediators which may stimulate and accelerate tissue regeneration (Dohan Ehrenfest et al., 2014). Autologous PRGF eye drops has been approved for clinical use by the European Medical Agency (EMA) and the Food and Drug Administration (FDA) in the USA (Osborne et al., 2018; Cui et al., 2017; Hayashi and Takagi, 2015), and it has been used in ophthalmology in the form of eye drops (serum) and clots (Lopez-Plandolit et al., 2010, 2011; Merayo-Lloves et al., 2015; Sanchez-Avila et al., 2018). Platelets are anuclear cell fragments mainly involved in forming blood scaffolds to repair tissue damage. They act as biological dikes that prevent blood loss from vessels, although they also induce and coordinate the healing process (Anitua et al., 2004). Once activated, platelets secrete numerous proteins that drive tissue regeneration (McNicol and Israels, 1999; Blair and Flaumenhaft, 2009), including growth factors (Mazzucco et al., 2010). Thus, platelets are potential sources of growth factors that may consequently have a variety of applications in tissue engineering (Anitua et al., 2004).

Although the PRGF proteome has been defined (Anitua et al., 2015b, 2021), its lipid composition is yet to be studied. Given the altered lipid composition of the tear in EDE, it would be of interest to define the lipid components of this enriched plasma to assess if it resembles the lipid composition of the tear, and thus, if it may serve as a good tear supplement. Recently, we optimized a protocol to extract lipids from the tear and we characterized the tear lipid profile of healthy subjects (Acera et al., 2019). In this current study, we set out to analyze the PRGF eye drops lipidome and to compare it with that of the tear in order to determine if PRGF eye drops may represent an adequate treatment for both types of DED, EDE and ADDE.

2. Methods

2.1. Subjects

An observational, prospective study was carried out by medically qualified personnel after receiving approval from the Euskadi Ethics Committee. The study was performed following the tenets of the Helsinki Declaration on Biomedical Research and adhered to the ARVO statement involving Human Subjects. Before tear collection, written informed consent was obtained from all the participants once the nature and possible consequences of the study had been fully explained. Healthy donors (n = 10) were recruited from the Cornea and Ocular Surface Unit, Instituto Clinico Quirurgico de Oftalmologia (ICQO, Bilbao, Vizcaya, Spain). The mean age of the participants was 38.06 (±6.21) years old, and 60% were women and 40% men.

2.2. Tear fluid collection

Inclusion in the study was based on a clinical examination that included: Schirmer I test with anesthesia to measure basal secretion, slit-lamp examination of the lid margin and MGs, fluorescein staining results according to the Oxford scale, tear film break-up time (TFBUT), and tear osmolarity. The following exclusion criteria were applied: ocular surgery performed in the previous three months; a systemic condition (active allergy) or medication use (anti-inflammatory agents) that could interfere with the interpretation of the results; or the concomitant administration of topical medication other than artificial tears. Tear samples were collected from the lower conjunctival sac using 10 μl glass capillary tubes, as described previously (Soria et al., 2013). The samples were stored at −80 °C until lipid extraction.

2.3. PRGF eye drops preparation

Blood samples were centrifuged at 460g for 8 min at room temperature in 5 mL sterile tubes containing 0.5 mL of 3.8% sodium citrate. The resulting plasma was recovered and the platelets were activated with 22.8 mM calcium chloride. After the formation of a clot and the liberation of the growth factors, the supernatant was recovered and transferred to 1.5 mL sterilized Eppendorf tubes and the samples were stored at −80 °C until lipid extraction. All procedures were performed under highly sterile conditions, operating inside a laminar flow hood.

2.4. Chemicals and standards

Optima® LC/MS-grade water, methanol, acetonitrile, 2-propanol, formic acid and the Pierce LTQ Velos ESI Positive/Negative Ion Calibration Solutions were obtained from Fisher Scientific (Fair Lawn, NJ). Ammonium formate was purchased from Sigma-Aldrich (Sigma Chemical Co., St Louis, MO), while the Splash™ LipidoMix™ and the

ceramide/sphingoid internal standard mixtures were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). In-house synthesis of the OAHFA standard, 16-(oleoyloxy) hexadecanoic acid, was carried out as described previously (Acera et al., 2019). The WE standard −15,15,16, 16,17,17,18,18,18-d9 (WE 18:1(d9)/26:0)— was obtained by treating deuterated oleic acid with $SOCl_2$(Vrkoslav et al., 2010). Both compounds were characterized by 1H and 13C nuclear magnetic resonance (NMR) spectroscopy and high-resolution mass spectrometry (MS), and their purity was established as >95%. The crude products were purified by column chromatography.

2.5. Lipid extraction

Pooled tears (10 μl) and PRGF eye drops samples (10 μl, 10 replicates of each) were prepared in Eppendorf tubes, performing lipid extraction with the isopropanol protocol described elsewhere (Acera et al., 2019). UHPLC was performed on a Vasquish UHPLC system (ThermoFisher Scientific), equipped with a binary solvent delivery pump, an autosampler and a column oven. A reverse-phase column (Acquity UPLC C_{18} CSH™ 2.1 × 100 mm, 1.7 μm) and a pre-column (Acquity UPLC C18 CSH™ 2.1 × 5 mm, 1.7 μm: VanGuard) were used at 65 °C to separate individual lipids. The mobile phases consisted of acetonitrile and water (40:60, v/v) with 10 mM ammonium formate and 0.1% formic acid (phase A), or acetonitrile plus isopropanol (10:90, v/v) with 10 mM ammonium formate and 0.1% formic acid (phase B). The elution conditions employed were: 0–2 min, 40–43% B; 2–2.1 min, 43–50% B; 2.1–12, 50–54% B, 12–12.1 min, 54–70% B; 12.1–18 min, 70–100% B. After elution, the column was washed and reconditioned. The flow rate was 500 μl/min and the injection volume was 10 μl, and all samples were kept at 10 °C before analysis.

Untargeted lipidomic analysis was performed on a Q Exactive HF-X hybrid quadrupole-Orbitrap mass spectrometer (ThermoFisher Scientific, USA). All MS experiments were performed in positive and negative ion modes using a HESI (heated electrospray ionization) source, and optimizing the parameters using the Splash™ LipidoMix™ standard internal mixture. The flow rates of sheath gas, sweep gas and auxiliary gas for both polarities were adjusted to 35, 0 and 10 (arbitrary units). For both ionization modes, the capillary temperature and the heater temperature were maintained at 285 °C and 370 °C, respectively, while the spray voltage was 3.90 KV for positive and 3.20 kV for negative ionization. The S-lens RF level was set at 40. The Orbitrap mass spectrometer was operated at a resolving power of 120,000 in full-scan mode (scan range 250–2000 m/z, automatic gain control target $1xe^6$) and 7500 in Top15 data-dependent MS^2 mode (HCD fragmentation with a stepped normalized collision energy of 25 and 30 in positive mode, and 20, 30 and 40 in negative ion mode; injection time 11 ms; isolation window 1m/z; automatic gain control target $1xe^5$ with a dynamic exclusion setting of 6.0 s). The spectrometer was calibrated externally every three days within a mass accuracy of 1 ppm.

2.6. MS data processing

All the MS data were acquired and processed using the Xcalibur 4.1 software package, while the LipidSearch software version 4.2.2 (Mitsui Knowledge Industry, Tokyo, Japan) was used to identify and quantify the lipid species in these complex biological samples. The key processing parameters were: target database, General; precursor tolerance, 5 ppm; product tolerance, 5 ppm; product ion threshold, 1%; m-score threshold, 2; Quan m/z tolerance, ± 5 ppm; Quan RT (retention time) range, ±0.5 min; use of main isomer filters and ID quality filters A, B, C and D; Adduct ions H^+, Na^+ and $NH4^+$ for positive ion mode, and $H^−$ and $HCOO^+$ for negative ion mode. The lipid classes selected for the search were: LPC (lysophosphatidylcholine), PC (phosphatidylcholine), LPE (lysophosphatidylethanolamine), PE (phosphatidylethanolamine), LPG (lysophosphatidylglycerol), PG (phosphatidylglycerol), LPI (lysophosphatidylinositol), PI (phosphatidylinositol), LPS (lysophosphatidyl

serine), PS (phosphatidylserine), LPA (lysophosphatidic acid), PA (phosphatidic acid), SM (sphingomyelin), Cer (ceramide), Hex1Cer (hexosylceramide), Hex2Cer (dihexosylceramide), Hex3Cer (trihexosylceramide), CL (cardiolipin), MG (monoacylglycerol), DG (diacylglycerol), TG, ChE (cholesterol ester), OAHFA, and WE.

Quantification was carried out by normalization of the extracted monoisotopic ion peak area of each native lipid species to the intensity of the extracted monoisotopic ion peak area of the internal standard. The internal standards used in this study were chosen to avoid those present in native tear and plasma samples.

3. Results

3.1. Lipid profile of the PRGF eye drops and tear samples

This analysis identified 408 lipids in the PRGF eye drops obtained in ESI+ mode and 183 in ESI- mode (Table 1S), which were all grouped into 15 different classes of lipids from 4 lipid categories: glycerolipids (DG and TG); sphingolipids (SM, Cer, Hex1Cer and Hex2Cer); glycerophospholipids (LPC, LPE, LPI, LPG, LPS, PC, PE and PI); and sterol lipids (ChE). In tear samples, 112 lipid species were identified in ESI+ and 36 in ESI- mode (Table 2S), and quantified as individual lipid species. These lipids belonged to 12 lipid classes from six different lipid categories: FAs - OAHFAs; sphingolipids (SM and Cer); glycerophospholipids (LPC, LPE, LPI, LPG, PC and PG); glycerolipids (TG); sterol lipids (ChE); and WEs.

3.2. Relative abundance of the lipid species

The relative composition of the two lipid sources analysed indicated that most of the lipid species the TF were also found in the PRGF eye drops, although their relative abundance differed. In the case of PRGF eye drops (Table 1, Fig. 1), the relative abundance of most lipid species was much greater than in the TF (Table 1, Fig. 2). It is worth noting that ChE was the most abundant lipid class in PRGF eye drops (6514 μM) at a concentration 241-fold higher than in the tear (26.96 μM). Extracted ion chromatogram of the UHPLC-MS/MS separation of the ChE in PRGF and tear samples and relative concentrations showed in Fig. 3. Indeed, only LPE, LPI and LPG were present at a higher relative concentration in the tear than PRGF eye drops (1.07-, 4.5- and 105-fold, respectively). The presence of OAHFA and WE in the tear but not in the PRGF eye drops fractions was a significant difference between the two samples analysed. Their extracted ion chromatograms of the UHPLC-MS/MS were

Table 1
PRGF and tear lipid composition.

Class	PRGF Conc (μM)			Tear Conc (μM)		
	Mean	SD	CV	Mean	SD	CV
ChE	6514.05	680.16	10.4	26.96	1.97	7.31
PC	1747.82	234.37	13	1.86	0.13	7.19
TG	1237.52	76.07	6.15	8.57	0.28	3.27
SM	592.27	84.82	14	11.76	0.03	0.28
LPC	115.7	11.0	9.5	17.52	0.05	0.28
DG	103.23	27.10	26	nd	nd	nd
PI	97.11	18.37	19	nd	nd	nd
PE	64.65	7.05	11	nd	nd	nd
Cer	4.07	0.46	11	0.23	0.01	2.86
LPE	3.24	0.47	14	3.48	0.17	4.91
Hex1Cer	1.0	0.3	32.9	nd	nd	nd
LPI	0.61	0.08	13	2.75	0.67	24.51
Hex2Cer	0.3	0.1	24.2	nd	nd	nd
LPG	0.09	0.01	10	9.46	1.21	12.83
LPS	0.09	0.01	10	nd	nd	nd
WE	nd	nd	nd	21.28	0.76	3.59
OAHFA	nd	nd	nd	3.85	0.03	0.66
PG	nd	nd	nd	2.00	0.21	10.32

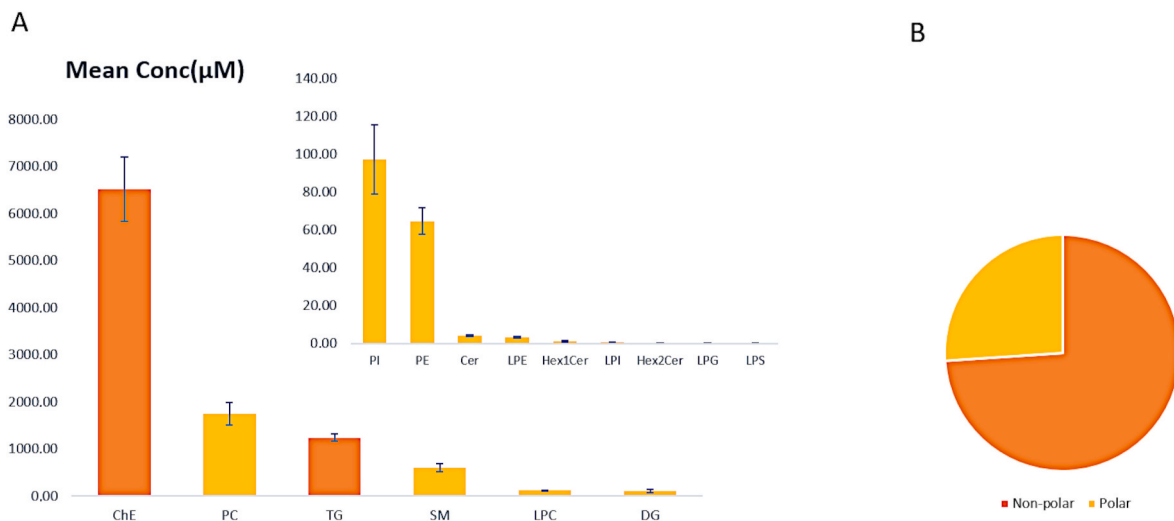SD (standard deviation); CV (coefficient of variation); nd (not detected).

Fig. 1. Major classes of lipids in PRGF eye drops. (A) Average concentration of the major lipid classes in PRGF eye drops, with a high concentration of neutral hydrophobic cholesterol esters (ChEs) and TGs (triacylglycerols). (B) General distribution represented as the percentages of non-polar and polar lipids in a ratio close to 75:25.
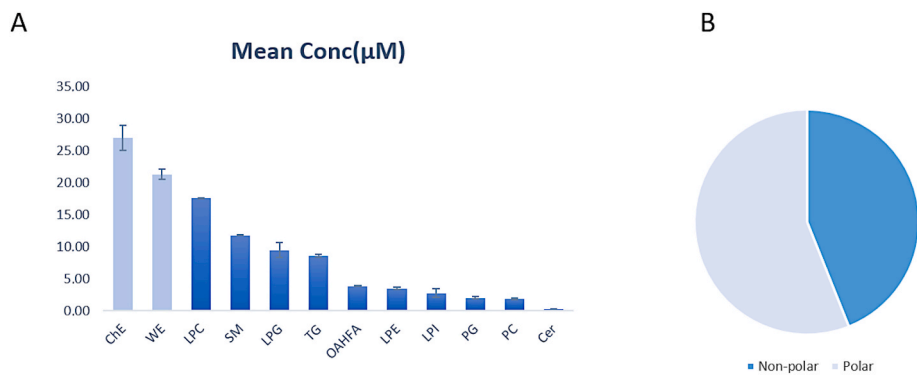

Fig. 2. Main classes of lipids present in tears. (A) Average concentration of the main lipid classes in tears, where the majority of the lipids are cholesterol esters (ChEs) and wax esters (WEs). (B) General distribution represented as the percentages of non-polar and polar lipids in a ratio close to 50:50, which allows the tear to modulate its properties and to form a stable multilayer film.
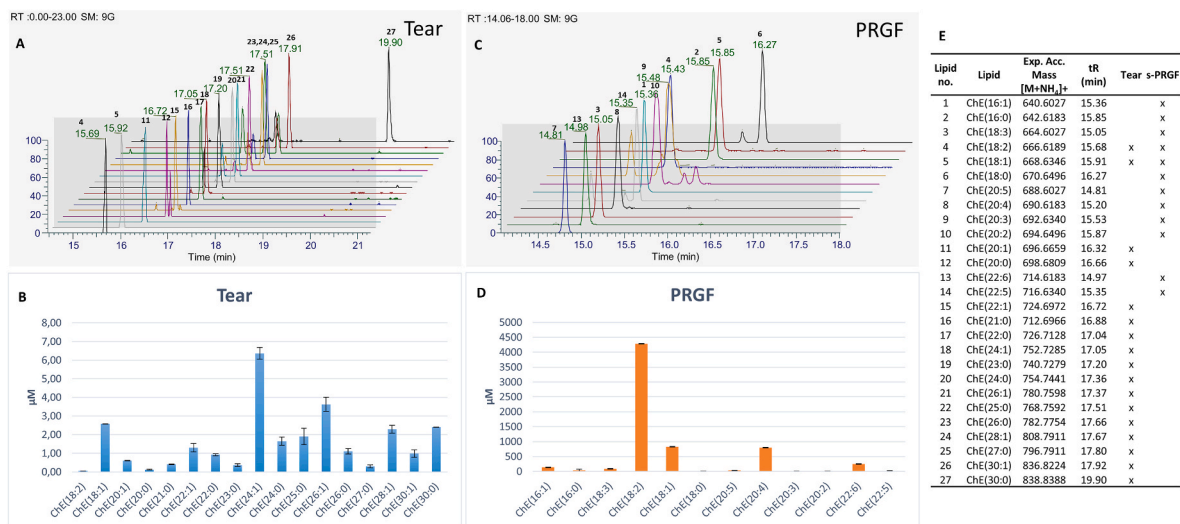

Lipid no.	Lipid	Exp. Acc. Mass [M+NH₄]+	tR (min)	Tear	s-PRGF
1	ChE(16:1)	640.6027	15.36		x
2	ChE(16:0)	642.6183	15.85		x
3	ChE(18:3)	664.6027	15.05		x
4	ChE(18:2)	666.6189	15.68	x	x
5	ChE(18:1)	668.6346	15.91	x	x
6	ChE(18:0)	670.6496	16.27		x
7	ChE(20:5)	688.6027	14.81		x
8	ChE(20:4)	690.6183	15.20		x
9	ChE(20:3)	692.6340	15.53		x
10	ChE(20:2)	694.6496	15.87		x
11	ChE(20:1)	696.6659	16.32	x	
12	ChE(20:0)	698.6809	16.66	x	
13	ChE(22:6)	714.6183	14.97		x
14	ChE(22:5)	716.6340	15.35		x
15	ChE(22:1)	724.6972	16.72	x	
16	ChE(21:0)	712.6966	16.88	x	
17	ChE(22:0)	726.7128	17.04	x	
18	ChE(24:1)	752.7285	17.05	x	
19	ChE(23:0)	740.7279	17.20	x	
20	ChE(24:0)	754.7441	17.36	x	
21	ChE(26:1)	780.7598	17.37	x	
22	ChE(25:0)	768.7592	17.51	x	
23	ChE(26:0)	782.7754	17.66	x	
24	ChE(28:1)	808.7911	17.67	x	
25	ChE(27:0)	796.7911	17.80	x	
26	ChE(30:1)	836.8224	17.92	x	
27	ChE(30:0)	838.8388	19.90	x	

Fig. 3. Extracted ion chromatogram of the UHPLC-MS/MS separation of the ChE in tear (A, B) and PRGF eye drops samples (C, D) and relative concentrations (E).
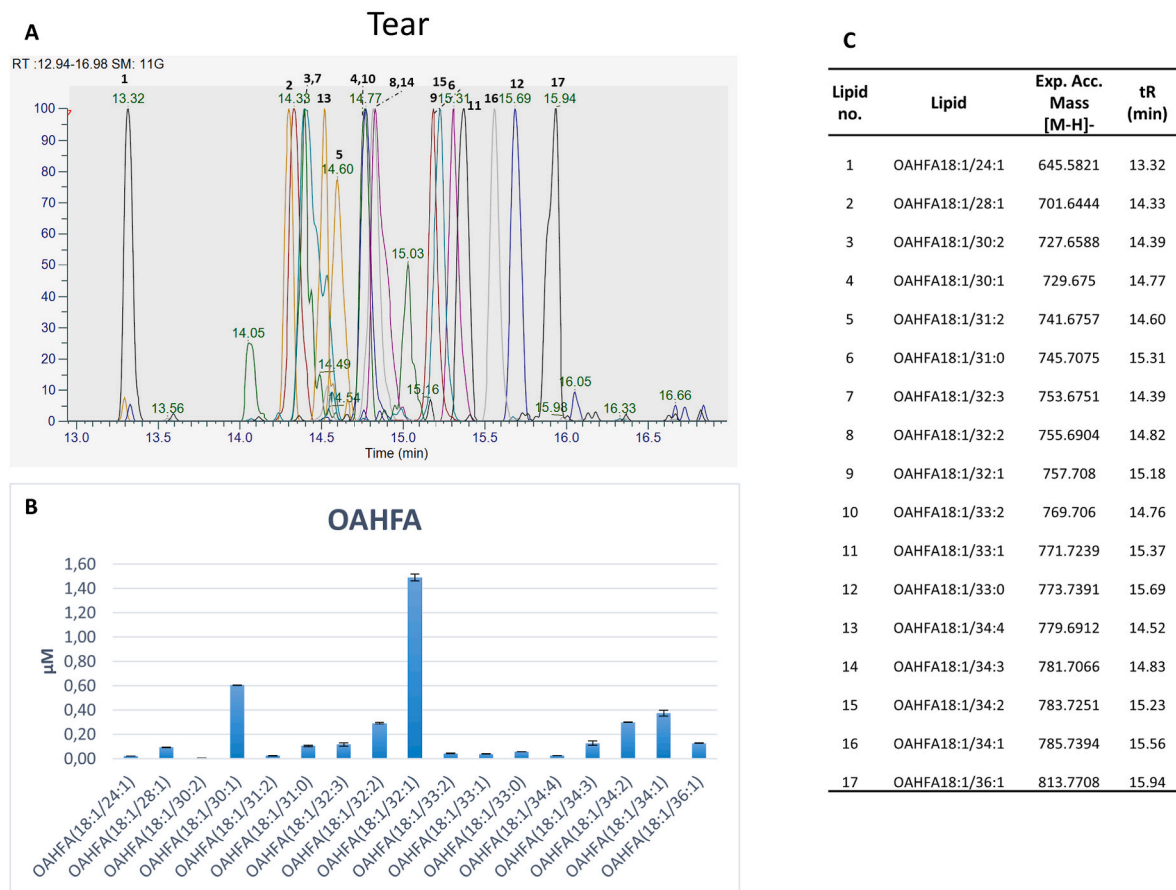
Fig. 4. Extracted ion chromatogram of the UHPLC-MS/MS separation of the OAHFA in tear samples (A) and relative concentrations (B, C).

observed in Figs. 4 and 5 respectively. Conversely, some lipid classes were detected in PRGF eye drops that were not found in the tear samples, such as DG, PE, PI, Hex1Cer and Hex2Cer. The PRGF eye drops samples obtained were rich in hydrophobic (ChE) and neutral hydrophobic (TG) lipids. They also contained several polar lipids with amphipathic properties, including SM, PC, PI, PE, LPC, LPE and LPI. While the distribution of hydrophobic and polar lipids with amphipathic traits was close to 50:50 in tears, they were found in a 75:25 ratio in PRGF eye drops. Hydrophobic lipids were evident in both PRGF eye drops (ChE and TG) and tears (ChE, WEs and TG), and in both samples some of these lipids carried unsaturated bonds in their acyl chains. In terms of polar lipids with amphipathic features, three classes were present in tears, namely OAHFAs, LPG and PC. These lipids contained unsaturated chains, whereas saturated chains were abundant in the lysophospholipid, Cer and SM classes (Fig. 6). Furthermore, polar lipids with amphipathic properties found in PRGF eye drops contained unsaturated chains (for example, PC) and saturated acyl chains, such as lysophospholipids, Cer and SM (Fig. 7).

4. Discussion

In this study we characterized the lipidomic profile of PRGF eye drops, comparing this with that of tears. In recent years, blood derivatives have been used more widely to treat ocular surface pathologies since plasma products may mimic natural tears due to the growth factors, cytokines, vitamins and nutrients they contain, all of which help maintain the homeostasis of the protective TF (Anitua et al., 2015a). However, it is important to define the lipid composition of these products for a more precise indication in treating EDE. An in depth study of the lipid profile of plasma was first reported by the LIPID MAPS Consortium (Quehenberger et al., 2010) and subsequently, a new analytical

method was used to quantify the wide range of lipids in plasma (Forest et al., 2018). In this study, the lipids in PRGF have been quantified and compared with tear composition.

Of all the hemoderived products that are used to treat distinct ocular pathologies, we chose to study the lipid profile of PRGF eye drops as it is that which is currently used most often in patients with EDE and ADDE. The high lipid content of PRGF eye drops suggests it might be suitable to treat EDE, a condition in which the lipid composition of the TF appears to be altered and in which the contribution of specific lipids like ChEs is likely to be beneficial. The physiological tear has a lipid content that does not permit excessive evaporation due to the non-polar lipids it contains, in particular WEs, as well as its specific amphipathic lipids that are associated with a low surface tension.

The TF forms a skinny coat that maintains a critical surface tension greater than the gravitational forces in order to prevent its collapse, and to enable the film to spread across the cornea. The low surface tension of the TF contributes to the decrease in surface tension of the cornea, and it is achieved by the combination of the amphiphilic lipids at the polar: non-polar interface and their contact with proteins in the water-soluble region through their polar heads. When considering the structural integrity of the lipid layer, it appears to fold like a curtain that then unfurls with the opening of the eyes. Any diffusion of lipids from the lipid layer to the epithelial surface is unlikely because polar lipids are predominantly long-chain OAHFAs that would remain firmly anchored in the lipid layer through their large hydrophobic acyl chains (Paananen et al., 2020).

The three-dimensional organization of the tear has been studied, supporting a model in which the lipid layer is a highly organized laminated structure with a polar:non-polar lipid interface that comes into contact with water/air (Kulovesi et al., 2010). Lipids organize into a sandwich-like structure, whereby phospholipids are situated adjacent to
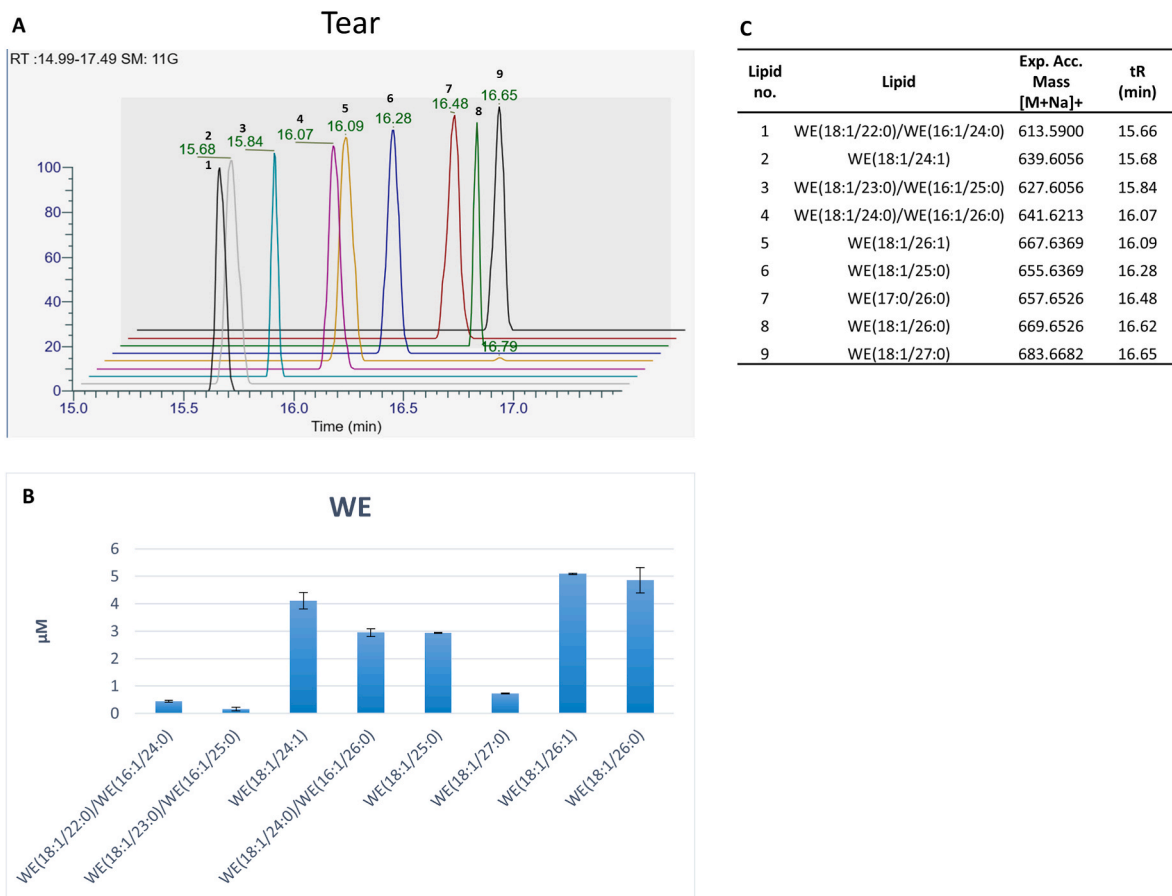
C

Lipid no.	Lipid	Exp. Acc. Mass [M+Na]+	tR (min)
1	WE(18:1/22:0)/WE(16:1/24:0)	613.5900	15.66
2	WE(18:1/24:1)	639.6056	15.68
3	WE(18:1/23:0)/WE(16:1/25:0)	627.6056	15.84
4	WE(18:1/24:0)/WE(16:1/26:0)	641.6213	16.07
5	WE(18:1/26:1)	667.6369	16.09
6	WE(18:1/25:0)	655.6369	16.28
7	WE(17:0/26:0)	657.6526	16.48
8	WE(18:1/26:0)	669.6526	16.62
9	WE(18:1/27:0)	683.6682	16.65

Fig. 5. Extracted ion chromatogram of the UHPLC-MS/MS separation of the WE in tear samples (A) and relative concentrations (B, C).
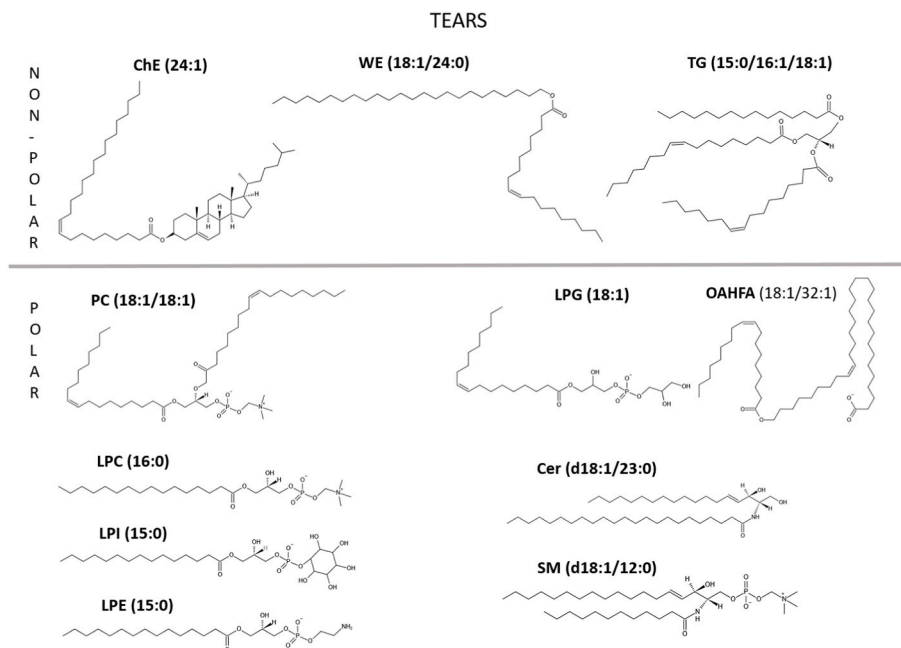

Fig. 6. Representative lipids classes in tears: Non-polar - ChE (cholesterol ester), TG (triacylglycerol) and WEs (wax esters); and Polar - PC (phosphatidylcholine), LPC (lysophosphatidylcholine), LPE (lysophosphatidylethanolamine), LPI (lysophosphatidylinositol), LPG (lysophosphatidylglycerol), Cer (ceramide), SM (sphingomyelin) and OAHFAs ((O-Acyl)-ω-hydroxy fatty acids).
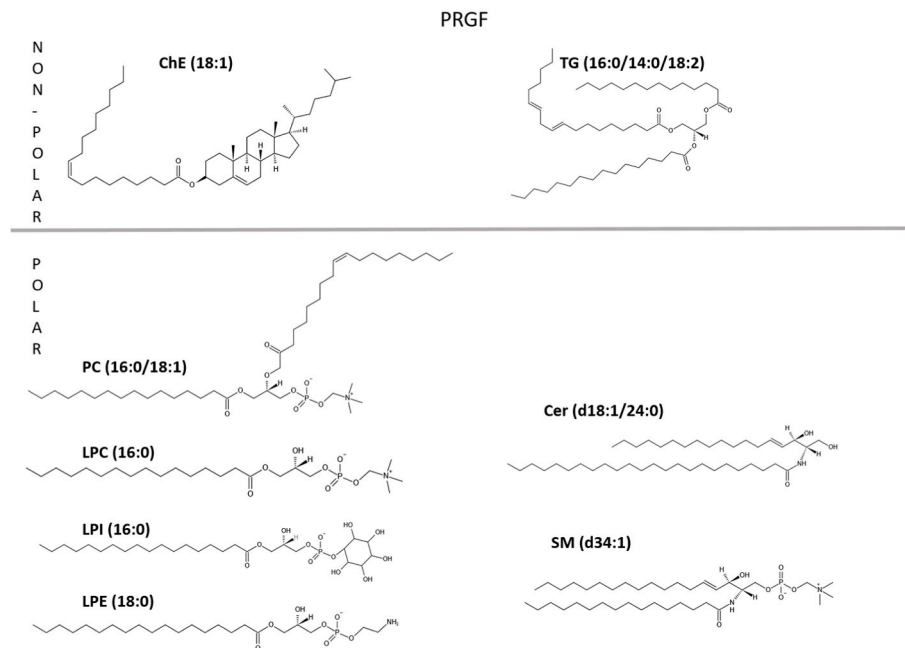
PRGF

Fig. 7. Representative lipid classes present in PRGF eye drops: Non-polar - ChE (cholesterol ester) and TG (triacylglycerol); and polar - PC (phosphatidylcholine), LPC (lysophosphatidylcholine), LPE (lysophosphatidylethanolamine), LPI (lysophosphatidylinositol), Cer (ceramide) and SM (sphingomyelin).

the aqueous phase, and non-polar lipids (mainly TG and ChE) are deposited on top of the phospholipid film. Some lipids are crucial to prevent the evaporation of the tear, including WEs in the part of the tear that is in contact with the air and that make the tear film waterproof, or the OAHFAs believed to reside primarily at the aqueous:lipid interface and that represent a surfactant essential for tear film stability (Millar and Schuett, 2015). Amphiphilic lipids like OAHFAs appear to be down-regulated in DED (Lam et al., 2011), reportedly due to the inactivation of the fatty acid ω-hydroxylase *Cyp4f39* (Miyamoto et al., 2020). Therefore, OAHFAs appear to establish an anchor in the non-polar tear layer that prevents excess evaporation. Recent biophysical studies performed in artificial tear film lipid layers (TFLL), made up of lipids mimicking the natural tear lipid composition in specific proportions, reported that phosphatidylcholine contributes to diminishing surface tension. Additionally, fatty acid esters of hydroxy fatty acids, such as palmitic acid-9-hydroxystearic acid (PAHSA), influence the rheological properties of the layer (Xu et al., 2022b). The importance of fatty acid esters of hydroxy fatty acids in maintaining unique rheological features of the natural tear lipid film, where (O-acyl)-ω-hydroxy fatty acids (OAHFAs) accumulates, resides in the possible configurational transitions adopted by these molecules when challenged with lateral pressure variations (Xu et al., 2022a). Other lipids with a unique presence in PRGF may also bestow optimal rheological properties to the film. Further biophysical studies should explore the contribution of the lipid elements (proportion and types of polar and non-polar lipids) in PRGF and their closeness to the distinctive properties of the natural tear film.

WEs are non-polar lipids that form the TF's most external layer, isolating the aqueous internal tear layer and prevents its evaporation (Butovich, 2017). Other lipids sit at the lipid:aqueous interphase, including LPI, LPG and OAHFAs, and these lipids may interact with the aqueous phase of the TF and help maintain its organization. The amphiphilic properties of OAHFAs (Willcox et al., 2017) are due to the capacity of the ester bond to form hydrogen bonds with water molecules in its vicinity. Their free carboxylic group has polar properties, whereas the rest of the molecule is very hydrophobic and directs its carbon chains toward the non-polar layer. LPI and LPG are also amphiphilic, containing larger polar groups than OAHFAs (inositol phosphate in LPI and glycerol phosphate in LPG), together with a free hydroxyl group at the sn-2 position of the glycerol molecule. The presence of specific lipids

with hydrophobic unsaturated acyl chains and lipids with hydrophobic saturated tails further reinforces the organizational capacity of polar and non-polar lipids, and favours the fluidity of the TFLL. In the present study we have seen that the proportion of polar lipids in TFLL is higher than reported to date (50:50) probably due to the method of tear lipid extraction or the method to obtain the tears. In a previous study of our group (Acera et al., 2019), we compared two methods of tear lipid extraction, the Bligh and Dyer (BD) method widely used to date in reported articles (Butovich, 2013; Lam et al., 2014) and a new method using isopropanol (IPA). In the previous article we were able to prove that the IPA extraction method allows the detection of a greater number of polar lipids. In addition, current analytical techniques are very sensitive and that is why we have managed to identify and quantify a large amount of polar lipids in the tear which, adding up their concentration, shows us a 50:50 ratio between the non-polar lipids found in the tear and the polar lipids. This ratio will probably change under pathological conditions that we will have to study in future projects. The main differences between the two methods analysed (BD and IPA) seem to be predominantly due to intrinsic differences in the lipid selectivity of the solvents used. Thus, polar lipids (PC, LPC, LPE, PE, SM and Cer), except OAHFAs, were better recovered with the IPA method than with the BD extraction method (Acera et al., 2019).

On the other hand, it is speculated that these and other differences could also be due to variations in tear sample collection. As for whole tear collection, Schirmer strips and capillary tubes are used. Logically, a Schirmer strip will be in contact with conjunctival epithelial cells, tear in the lacrimal lake, TFLL, lid margin and meibomian gland orifices, whereas careful use of the capillary tube would only bring it in contact with tear in the lacrimal lake, and TFLL.

The non-polar (ChE and TG) and polar lipids with amphipathic properties (lysophospholipids, PC, Cer and SM) in PRGF eye drops may to some extent arrange spontaneously when in contact with the surface of the eye, and they could adopt an organized lipid architecture similar to that of the TF (Fig. 8). These lipids would be distributed between a hydrophobic layer in contact with the air and a lipid:aqueous interphase where the polar heads of the amphipathic lipids would be situated, their hydrophobic chains extending towards the non-polar layer. Furthermore, the combination of lipids with unsaturated and saturated acyl chains in PRGF eye drops can provide stability to the TFLL.
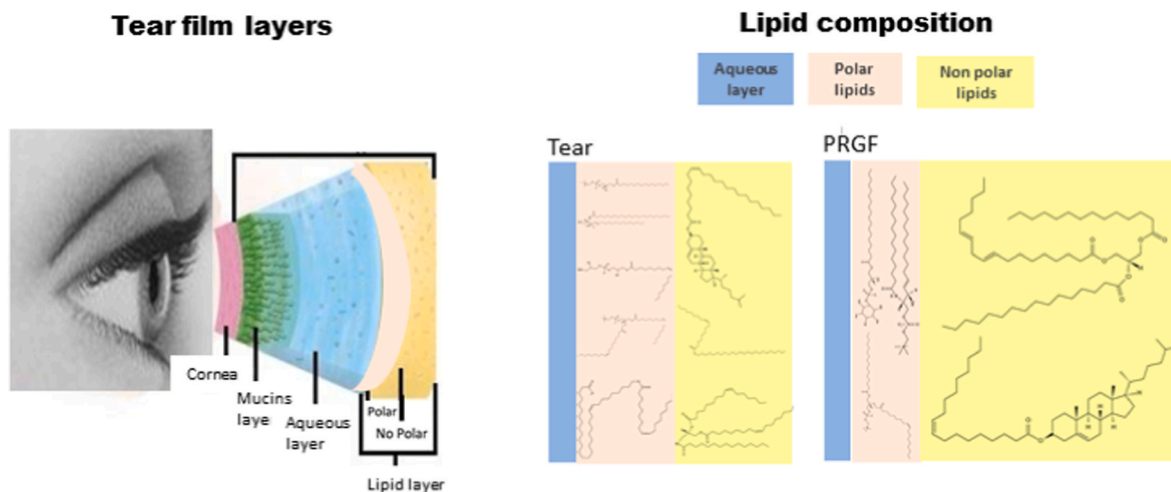
Fig. 8. Schematic representation of the tear film based on the results of the present study. Comparative hypothetical organization of the lipids in tears and PRGF eye drops. In PRGF eye drops there is a higher proportion of non-polar lipids than in tears, mainly TGs and ChEs. The most representative polar lipids in PRGF eye drops are PC, LPC, SM and Cer.

The lipid composition of the PRGF eye drops analysed has not yet been studied in detail. The lipid profile obtained suggests that it might be a good complement to the TF in order to prevent the high rates of evaporation of the TF in patients with EDE. This PRGF eye drops is likely to avoid rapid disruption of the TF given that its high concentration of non-polar lipids could ensure it acts as a barrier, isolating and protecting the corneal epithelium and conjunctiva, and that it maintains its cohesion and thyxotropic properties (McCulley and Shine, 1997). In addition, we established here a methodology that enables the different families of lipids and their compositions (acyl chains and their degree of unsaturation) to be defined exhaustively. However, apart from the specific lipids present and their concentrations, it is not possible to define the molecular dynamics or specific behaviour of the lipids detected in the PRGF eye drops. Nevertheless, the presence of polar and nonpolar lipids, although not exactly corresponding to those found in TF, provide clues as to how the lipid mixture of PRGF eye drop might behave in an aqueous medium such as the tear, suggesting that it would self-assemble and establish weak interactions with proteins and other polar molecules in the tear. One of the lipids identified, ceramide, showed higher levels in PRGF (4 μM) than in the tear (0.23 μM) which are similar levels to those found by Rantamäki et al., who identified five unique ceramides with a combined concentration in the tear lower than 1 μM (Rantamaki et al., 2011). However, analysing the lipid composition of PRGF, we understand that the concentration of ceramides in PRGF is residual compared to the rest of the lipids identified and these concentrations should not produce inflammation on the ocular surface, taking into account, the works indicating improvement in patients with severe dry eye where the inflammatory component is very important (Anitua et al., 2015a; Merayo-Lloves et al., 2015; Lopez-Plandolit et al., 2011). Moreover, PRGF eye drops provides other elements that could help protect the corneal epithelium, such as growth factors. The dynamic nature of the lipid layer allows it to respond to the shear stress induced by the changing area of the air-tear interface during blinking. The lipid layer should be compressible during the downward sweep (Kulovesi et al., 2010), but should extend rapidly after the upward sweep of the eyelid (Bron et al., 2004). Polar lipids decrease the surface tension of the air-tear interface and increase the propagation velocity of the lipid layer. Non-polar lipids increase the compressibility and stability of the lipid layer (Kulovesi et al., 2010). Imbalances in composition manifest themselves in the form of impaired viscoelastic properties of the tear. Therefore, it is important to maintain the ratio between polar and nonpolar lipids.

Both WEs and ChE are postulated as lipids that prevent evaporation

of the tear film by positioning themselves in the most hydrophobic part of the TFLL. PRGF does not contain WE and it can be thought that the high concentration of ChE may partly supplant the function of WEs. This premise, however, cannot be demonstrated in the present work which is only a descriptive work on the lipid composition of the two fluids. However, there is controversy in the literature on these aspects.

Kulovesi et al., Paananen et al. and Rantamäki et al., investigated the effects of lipid layers of the emulated tear film using individual WEs and lipid mixtures at different temperatures on evaporation (Kulovesi et al., 2014; Paananen et al., 2014; Rantamaki et al., 2013). While they showed that only a few WEs, could form films that reduced evaporation, they concluded that "In multicomponent lipid layers, evaporation retarding interactions between carbon chains decrease and, therefore, these lipid layers do not retard evaporation". Therefore, their data support the concept that isolated lipids do not retard evaporation, because meibum is a multicomponent lipid mixture (Butovich, 2009a). Borchman et al. (2013) reported a similar finding: an artificial lipid layer made of a mixture of a WE and a ChE can reduce evaporation, but it needed to be 500 times thicker than TFLL to have a noticeable effect (Borchman et al., 2013). Our results show the ChE concentration in PRGF is 241 times higher than in tear, perhaps, this composition could to retard tear evaporation, but we cannot conclude this premise.

However, we may think that the absence of WE in PRGF may be supplied by the high presence of non-polar lipids such as ChE, but it should be checked in future studies.

5. Summary

In order to establish how the lipids, present in PRGF eye drops behave, it will be necessary to study their assembly and molecular thermodynamics, and define the specific interaction that occur between the non-polar (ChEs) and polar or amphipathic lipids. It would not be surprising if the polar regions of the amphipathic lipid were to organize spontaneously in the aqueous medium, directing their polar regions towards the water while their hydrophobic tails interact with other non-polar structures. This type of organisation may be that undertaken by such lipids present in PRGF eye drops. It is certain that we do not know how this organization arises, yet the identification of numerous lipid families in PRGF eye drops here suggests that it would be worthwhile delving deeper into the composition and molecular interactions that occur among the lipid present in PRGF eye drops. Moreover, the data provided here support the controlled clinical use of PRGF eye drops in certain pathologies of the surface of the eye, as well as the further study

of the benefits related to such treatments.

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Declaration of competing interest

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Appendix A. Supplementary data

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References

Acera, A., Pereiro, X., Abad-Garcia, B., Rueda, Y., Ruzafa, N., Santiago, C., Barbolla, I., Duran, J.A., Ochoa, B., Vecino, E., 2019. A simple and reproducible method for quantification of human tear lipids with ultrahigh-performance liquid chromatography-mass spectrometry. Mol. Vis. 25, 934–948.

Alio, J.L., Colecha, J.R., Pastor, S., Rodriguez, A., Artola, A., 2007. Symptomatic dry eye treatment with autologous platelet-rich plasma. Ophthalmic Res. 39 (3), 124–129.

Anitua, E., Andia, I., Ardanza, B., Nurden, P., Nurden, A.T., 2004. Autologous platelets as a source of proteins for healing and tissue regeneration. Thromb. Haemostasis 91 (1), 4–15.

Anitua, E., Muruzabal, F., Pino, A., Prado, R., Azkargorta, M., Elortza, F., Merayo-Lloves, J., 2021. Proteomic characterization of plasma rich in growth factors and undiluted autologous serum. Int. J. Mol. Sci. 22 (22).

Anitua, E., Muruzabal, F., Tayebba, A., Riestra, A., Perez, V.L., Merayo-Lloves, J., Orive, G., 2015a. Autologous serum and plasma rich in growth factors in ophthalmology: preclinical and clinical studies. Acta Ophthalmol. 93 (8), e605–e614.

Anitua, E., Prado, R., Azkargorta, M., Rodriguez-Suarez, E., Iloro, I., Casado-Vela, J., Elortza, F., Orive, G., 2015b. High-throughput proteomic characterization of plasma rich in growth factors (PRGF-Endoret)-derived fibrin clot interactome. J. Tissue Eng. Regen. Med. 9 (11), E1–E12.

Blair, P., Flaumenhaft, R., 2009. Platelet alpha-granules: basic biology and clinical correlates. Blood Rev. 23 (4), 177–189.

Borchman, D., Yappert, M.C., Milliner, S.E., Duran, D., Cox, G.W., Smith, R.J., Bhola, R., 2013. 13C and 1H NMR ester region resonance assignments and the composition of human infant and child meibum. Exp. Eye Res. 112, 151–159.

Bron, A.J., Tiffany, J.M., 1998. The meibomian glands and tear film lipids. Structure, function, and control. Adv. Exp. Med. Biol. 438, 281–295.

Bron, A.J., Tiffany, J.M., Gouveia, S.M., Yokoi, N., Voon, L.W., 2004. Functional aspects of the tear film lipid layer. Exp. Eye Res. 78 (3), 347–360.

Butovich, I.A., 2009a. Cholesteryl esters as a depot for very long chain fatty acids in human meibum. J. Lipid Res. 50 (3), 501–513.

Butovich, I.A., 2009b. The Meibomian puzzle: combining pieces together. Prog. Retin. Eye Res. 28 (6), 483–498.

Butovich, I.A., 2013. Tear film lipids. Exp. Eye Res. 117, 4–27.

Butovich, I.A., 2017. Meibomian glands, meibum, and meibogenesis. Exp. Eye Res. 163, 2–16.

Butovich, I.A., Wojtowicz, J.C., Molai, M., 2009. Human tear film and meibum. Very long chain wax esters and (O-acyl)-omega-hydroxy fatty acids of meibum. J. Lipid Res. 50 (12), 2471–2485.

Chen, J., Green-Church, K.B., Nichols, K.K., 2010. Shotgun lipidomic analysis of human meibomian gland secretions with electrospray ionization tandem mass spectrometry. Invest. Ophthalmol. Vis. Sci. 51 (12), 6220–6231.

Cory, C.C., Hinks, W., Burton, J.L., Shuster, S., 1973. Meibomian gland secretion in the red eyes of rosacea. Br. J. Dermatol. 89 (1), 25–27.

Craig, J.P., Tomlinson, A., 1997. Importance of the lipid layer in human tear film stability and evaporation. Optom. Vis. Sci. 74 (1), 8–13.

Cui, Y., Xu, N., Xu, W., Xu, G., 2017. Mesenchymal stem cells attenuate hydrogen peroxide-induced oxidative stress and enhance neuroprotective effects in retinal ganglion cells. In Vitro Cell. Dev. Biol. Anim. 53 (4), 328–335.

Dohan Ehrenfest, D.M., Andia, I., Zumstein, M.A., Zhang, C.Q., Pinto, N.R., Bielecki, T., 2014. Classification of platelet concentrates (Platelet-Rich Plasma-PRP, Platelet-Rich Fibrin-PRF) for topical and infiltrative use in orthopedic and sports medicine: current consensus, clinical implications and perspectives. Muscles Ligaments Tendons J. 4 (1), 3–9.

Forest, A., Ruiz, M., Bouchard, B., Boucher, G., Gingras, O., Daneault, C., Robillard Frayne, I., Rhainds, D., iGenoMed, C., Consortium, N.I.G., Tardif, J.C., Rioux, J.D., Des Rosiers, C., 2018. Comprehensive and reproducible untargeted lipidomic workflow using LC-QTOF validated for human plasma analysis. J. Proteome Res. 17 (11), 3657–3670.

Hayashi, H., Takagi, N., 2015. Endogenous neuroprotective molecules and their mechanisms in the central nervous system. Biol. Pharm. Bull. 38 (8), 1104–1108.

Heiligenhaus, A., Koch, J.M., Kruse, F.E., Schwarz, C., Waubke, T.N., 1995. [Diagnosis and and differentiation of dry eye disorders]. Ophthalmologe 92 (1), 6–11.

Kulovesi, P., Rantamaki, A.H., Holopainen, J.M., 2014. Surface properties of artificial tear film layers: effects of wax esters. Invest. Ophthalmol. Vis. Sci. 55 (7), 4448–4454.

Kulovesi, P., Telenius, J., Koivuniemi, A., Brezesinski, G., Rantamaki, A., Viitala, T., Puukilainen, E., Ritala, M., Wiedmer, S.K., Vattulainen, I., Holopainen, J.M., 2010. Molecular organization of the tear fluid lipid layer. Biophys. J. 99 (8), 2559–2567.

Lam, S.M., Tong, L., Duan, X., Acharya, U.R., Tan, J.H., Petznick, A., Wenk, M.R., Shui, G., 2014. Longitudinal changes in tear fluid lipidome brought about by eyelid-warming treatment in a cohort of meibomian gland dysfunction. J. Lipid Res. 55 (9), 1959–1969.

Lam, S.M., Tong, L., Yong, S.S., Li, B., Chaurasia, S.S., Shui, G., Wenk, M.R., 2011. Meibum lipid composition in Asians with dry eye disease. PLoS One 6 (10), e24339.

Lopez-Plandolit, S., Morales, M.C., Freire, V., Etxebarria, J., Duran, J.A., 2010. Plasma rich in growth factors as a therapeutic agent for persistent corneal epithelial defects. Cornea 29 (8), 843–848.

Lopez-Plandolit, S., Morales, M.C., Freire, V., Grau, A.E., Duran, J.A., 2011. Efficacy of plasma rich in growth factors for the treatment of dry eye. Cornea 30 (12), 1312–1317.

Mathers, W.D., Lane, J.A., 1998. Meibomian gland lipids, evaporation, and tear film stability. Adv. Exp. Med. Biol. 438, 349–360.

Mazzucco, L., Borzini, P., Gope, R., 2010. Platelet-derived factors involved in tissue repair-from signal to function. Transfus. Med. Rev. 24 (3), 218–234.

McCulley, J.P., Shine, W., 1997. A compositional based model for the tear film lipid layer. Trans. Am. Ophthalmol. Soc. 95, 79–88 discussion 88-93.

McNicol, A., Israels, S.J., 1999. Platelet dense granules: structure, function and implications for haemostasis. Thromb. Res. 95 (1), 1–18.

Merayo-Lloves, J., Sanchez, R.M., Riestra, A.C., Anitua, E., Begona, L., Orive, G., Fernandez-Vega, L., 2015. Autologous plasma rich in growth factors eyedrops in refractory cases of ocular surface disorders. Ophthalmic Res. 55 (2), 53–61.

Messmer, E.M., 2015. The pathophysiology, diagnosis, and treatment of dry eye disease. Dtsch Arztebl Int. 112 (5), 71–81 quiz 82.

Millar, T.J., Schuett, B.S., 2015. The real reason for having a meibomian lipid layer covering the outer surface of the tear film - a review. Exp. Eye Res. 137, 125–138.

Miyamoto, M., Sassa, T., Sawai, M., Kihara, A., 2020. Lipid polarity gradient formed by omega-hydroxy lipids in tear film prevents dry eye disease. Elife 9.

Mudgil, P., Borchman, D., Gerlach, D., Yappert, M.C., 2016. Sebum/meibum surface film interactions and phase transitional differences. Invest. Ophthalmol. Vis. Sci. 57 (6), 2401–2411.

Nichols, K.K., Foulks, G.N., Bron, A.J., Glasgow, B.J., Dogru, M., Tsubota, K., Lemp, M.A., Sullivan, D.A., 2011. The international workshop on meibomian gland dysfunction: executive summary. Invest. Ophthalmol. Vis. Sci. 52 (4), 1922–1929.

Osborne, A., Sanderson, J., Martin, K.R., 2018. Neuroprotective effects of human mesenchymal stem cells and platelet-derived growth factor on human retinal ganglion cells. Stem Cell. 36 (1), 65–78.

Paananen, R.O., Rantamaki, A.H., Holopainen, J.M., 2014. Antievaporative mechanism of wax esters: implications for the function of tear fluid. Langmuir 30 (20), 5897–5902.

Paananen, R.O., Viitaja, T., Olzynska, A., Ekholm, F.S., Moilanen, J., Cwiklik, L., 2020. Interactions of polar lipids with cholesteryl ester multilayers elucidate tear film lipid layer structure. Ocul. Surf. 18 (4), 545–553.

Quehenberger, O., Armando, A.M., Brown, A.H., Milne, S.B., Myers, D.S., Merrill, A.H., Bandyopadhyay, S., Jones, K.N., Kelly, S., Shaner, R.L., Sullards, C.M., Wang, E., Murphy, R.C., Barkley, R.M., Leiker, T.J., Raetz, C.R., Guan, Z., Laird, G.M., Six, D. A., Russell, D.W., McDonald, J.G., Subramaniam, S., Fahy, E., Dennis, E.A., 2010. Lipidomics reveals a remarkable diversity of lipids in human plasma. J. Lipid Res. 51 (11), 3299–3305.

Rantamaki, A.H., Seppanen-Laakso, T., Oresic, M., Jauhiainen, M., Holopainen, J.M., 2011. Human tear fluid lipidome: from composition to function. PLoS One 6 (5), e19553.

Rantamaki, A.H., Wiedmer, S.K., Holopainen, J.M., 2013. Melting points–the key to the anti-evaporative effect of the tear film wax esters. Invest. Ophthalmol. Vis. Sci. 54 (8), 5211–5217.

Rodriguez-Agirretxe, I., Freire, V., Muruzabal, F., Orive, G., Anitua, E., Diez-Feijoo, E., Acera, A., 2018. Subconjunctival PRGF fibrin membrane as an adjuvant to nonpenetrating deep sclerectomy: a 2-year pilot study. Ophthalmic Res. 59 (1), 45–52.

Sanchez-Avila, R.M., Merayo-Lloves, J., Riestra, A.C., Fernandez-Vega Cueto, L., Anitua, E., Begona, L., Muruzabal, F., Orive, G., 2018. Treatment of patients with neurotrophic keratitis stages 2 and 3 with plasma rich in growth factors (PRGF-Endoret) eye-drops. Int. Ophthalmol. 38 (3), 1193–1204.

Soria, J., Duran, J.A., Etxebarria, J., Merayo, J., Gonzalez, N., Reigada, R., Garcia, I., Acera, A., Suarez, T., 2013. Tear proteome and protein network analyses reveal a novel pentamarker panel for tear film characterization in dry eye and meibomian gland dysfunction. J. Proteonomics 78, 94–112.

Stern, M.E., Schaumburg, C.S., Pflugfelder, S.C., 2013. Dry eye as a mucosal autoimmune disease. Int. Rev. Immunol. 32 (1), 19–41.

Suarez-Barrio, C., Etxebarria, J., Hernaez-Moya, R., Del Val-Alonso, M., Rodriguez-Astigarraga, M., Urkaregi, A., Freire, V., Morales, M.C., Duran, J.A., Vicario, M., Molina, I., Herrero-Vanrell, R., Andollo, N., 2019. Hyaluronic acid combined with serum rich in growth factors in corneal epithelial defects. Int. J. Mol. Sci. 20 (7).

Tiffany, J.M., 1987. The lipid secretion of the meibomian glands. Adv. Lipid Res. 22, 1–62.

Tong, L., Chaurasia, S.S., Mehta, J.S., Beuerman, R.W., 2010. Screening for meibomian gland disease: its relation to dry eye subtypes and symptoms in a tertiary referral clinic in Singapore. Invest. Ophthalmol. Vis. Sci. 51 (7), 3449–3454.

Tseng, S.C., Tsubota, K., 1997. Important concepts for treating ocular surface and tear disorders. Am. J. Ophthalmol. 124 (6), 825–835.

Vrkoslav, V., Urbanova, K., Cvacka, J., 2010. Analysis of wax ester molecular species by high performance liquid chromatography/atmospheric pressure chemical ionisation mass spectrometry. J. Chromatogr. A 1217 (25), 4184–4194.

Willcox, M.D.P., Argueso, P., Georgiev, G.A., Holopainen, J.M., Laurie, G.W., Millar, T.J., Papas, E.B., Rolland, J.P., Schmidt, T.A., Stahl, U., Suarez, T., Subbaraman, L.N., Ucakhan, O.O., Jones, L., 2017. TFOS DEWS II tear film report. Ocul. Surf. 15 (3), 366–403.

Xu, X., Kang, C., Sun, R., Zuo, Y.Y., 2022a. Biophysical properties of tear film lipid layer II. Polymorphism of FAHFA. Biophys. J. 121 (3), 451–458.

Xu, X., Li, G., Zuo, Y.Y., 2022b. Biophysical properties of tear film lipid layer I. Surface tension and surface rheology. Biophys. J. 121 (3), 439–450.

Yokoi, N., Bron, A.J., Georgiev, G.A., 2014. The precorneal tear film as a fluid shell: the effect of blinking and saccades on tear film distribution and dynamics. Ocul. Surf. 12 (4), 252–266.