



# Comparative lipidomic analysis of inflammatory mediators in the aqueous humor and tear fluid of humans and rabbits

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## Abstract

**Introduction** Ocular inflammation is a key pathogenic factor in most blindness-causing visual disorders. It can manifest in the aqueous humor (AH) and tear fluid (TF) as alterations in polyunsaturated fatty acids (PUFAs) and their metabolites, oxylipins, lipid mediators, which are biosynthesized via enzymatic pathways involving lipoxygenase, cyclooxygenase or cytochrome P450 monooxygenase and specifically regulate inflammation and resolution pathways.

**Objectives** This study aimed to establish the baseline patterns of PUFAs and oxylipins in AH and TF by their comprehensive lipidomic identification and profiling in humans in the absence of ocular inflammation and comparatively analyze these compounds in the eye liquids of rabbits, the species often employed in investigative ophthalmology.

**Methods** Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was used for qualitative and quantitative characterization of lipid compounds in the analyzed samples.

**Results** A total of 28 lipid compounds were identified, including phospholipid derivatives and PUFAs, as well as 22 oxylipins. Whereas the PUFAs included arachidonic, docosahexaenoic and eicosapentaenoic acids, the oxylipins were derived mainly from arachidonic, linoleic and  $\alpha$ -linolenic acids. Remarkably, although the concentration of oxylipins in AH was lower compared to TF, these liquids showed pronounced similarity in their lipid profiles, which additionally exhibited noticeable interspecies concordance.

**Conclusion** The revealed correlations confirm the feasibility of rabbit models for investigating pathogenesis and trialing therapies of human eye disorders. The identified metabolite patterns suggest enzymatic mechanisms of oxylipin generation in AH and TF and might be used as a reference in ocular inflammation studies.

**Keywords** Aqueous humor · Tear fluid · UPLC-MS/MS · PUFAs · Oxylipins · COX · LOX · CYP450

## Abbreviations

AA	Arachidonic acid
AEA	N-arachidonylethanolamine
AH	Aqueous humor
ALA	$\alpha$ -Linolenic acid

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COX	Cyclooxygenase
CYP450	Cytochrome P450 monooxygenase
DES	Dry eye syndrome
DHA	Docosahexaenoic acid
DiHODE	Dihydroxyoctadecadienoic acid
DiHOME	Dihydroxyoctadecamonoenoic acid
EPA	Eicosapentaenoic acid
EpODE	Epoxyoctadecadienoic acid
EpOME	Epoxyoctadecamonoenoic acid
HDoHE	Hydroxydocosahexaenoic acid
HEPE	Hydroxyeicosapentaenoic acid
HETE	Hydroxyeicosatetraenoic acid
HODE	Hydroxyoctadecadienoic acid
HOTrE	Hydroxyoctadecatrienoic acid
HpODE	Hydroperoxyoctadecadienoic acid
HpOTrE	Hydroperoxyoctadecatrienoic acid
LA	Linoleic acid
LOX	Lipoxygenase
LT	Leukotriene
Lyso-PAF	Lyso-platelet-activating factor
OEA	Oleoylethanolamide
PG	Prostaglandin
PUFAs	Polyunsaturated fatty acids
TF	Tear fluid
UPLC-MS/MS	Ultra-performance liquid chromatography-tandem mass spectrometry

## 1 Introduction

Inflammation of the eye tissues is a pathogenic factor for a number of visual disorders. It plays a crucial role in development of dry eye syndrome (DES) and aggravates corneal abrasions, recurrent corneal erosions and persistent corneal epithelial defects of different etiologies (Wei and Asbell 2014; Zernii et al. 2019). Intraocular inflammation exacerbated by ageing, vascular, metabolic or genetic conditions can affect the retina thereby contributing to age-related macular degeneration, diabetic retinopathy and retinitis pigmentosa (Whitcup et al. 2013). Neuroinflammation was recognized as a key process in the development of glaucoma (Williams et al. 2017). Intraocular inflammation affecting the uveal (vascular) tract of the eye represents uveitis, a group of diseases, which is induced by infectious, autoimmune and/or systemic factors and can involve the cornea and the retina/optic nerve as well as the lens and vitreous (Forrester et al. 2018). Inflammation is also commonly associated with iatrogenic visual disorders, which occur as a complication of the surgical treatment of visual impairments (Zernii et al. 2018a, b; Johnson 2009; Wolffe 2016; Baksheeva et al. 2018) or may develop as a consequence of prolonged general anesthesia (Zernii et al. 2016b, c, 2017).

Signaling mechanisms underlying induction and resolution of ocular surface and intraocular inflammation remain poorly recognized. Meanwhile, they can be clarified by analysis of the proinflammatory and anti-inflammatory signaling molecules present in eye liquids, tear fluid (TF) and aqueous humor (AH). In the absence of the blood supply, TF and AH have nourishing and protective functions in relation to cornea and TF is known to support the immune response of the ocular surface. Thus, it contains a number of inflammatory molecules, including immunoglobulins, cytokines and chemokines, some of which become upregulated in ocular surface inflammation-based conditions, such as keratitis and DES (Enriquez-de-Salamanca et al. 2010; Pflugfelder et al. 2013; Zernii et al. 2017; Coyle and Sibony 1986). Elevation of concentration of immunoglobulins, cytokines and chemokines in AH can accompany intraocular inflammation affecting both the anterior (in anterior uveitis (Chen et al. 2015)) and posterior (in diabetic retinopathy (Feng et al. 2018), macular edema (Hillier et al. 2017), age-related macular degeneration (Terao et al. 2018) and glaucoma (Takai et al. 2012)) segments of the eye. Remarkably, in some disorders, such as fungal keratitis (Zhang et al. 2018; Vasanthi et al. 2007) and uveitis (Zhao et al. 2015; Carreno et al. 2017; Valentincic et al. 2011; Min et al. 2006), there are positive correlations between the content of inflammatory molecules in TF and AH, suggesting that specific diagnosis of some kinds of ocular inflammation can be accomplished via the non-invasive analysis of TF.

One of the most important classes of inflammatory molecules is oxylipins, derivatives of omega-3 and omega-6 polyunsaturated fatty acids (PUFAs) exerting both proinflammatory and pro-resolving properties. Generally, eicosapentaenoic (EPA) and docosahexaenoic (DHA) omega-3 PUFAs, as well as their derivative oxylipins, hydroxyeicosapentaenoic acids (HEPEs) and hydroxydocosahexaenoic acids (HDoHEs), are regarded as anti-inflammatory mediators. They inhibit the generation of prostaglandins (PGs), leukotrienes (LTs) and hydroxyeicosatetraenoic acids (HETEs), pro-inflammatory oxylipins derived from omega-6 PUFA, arachidonic acid (AA). In addition to these, oxylipins called eicosanoids (metabolites of 20 carbon-length PUFAs), which are inflammatory lipid mediators, comprise derivatives of linoleic (LA) and  $\alpha$ -linolenic (ALA) acids. The LA oxylipins include hydroperoxyoctadecadienoic (HpODEs) and hydroxyoctadecadienoic (HODEs) acids as well as epoxyoctadecamonoenoic (EpOMEs) and dihydroxyoctadecamonoenoic (DiHOMEs) acids. ALA can be transformed into hydroperoxyoctadecatrienoic (HpOTrEs) and hydroxyoctadecatrienoic (HOTrEs) acids, as well as epoxyoctadecadienoic (EpODEs) and dihydroxyoctadecadienoic (DiHODEs) acids. The conversion of PUFAs into oxylipins occurs via three major pathways involving cyclooxygenases (COX), lipoxygenases (LOX) and cytochrome P450

monooxygenases (CYP450) [for review, see (Gabbs et al. 2015)]. The discrimination which of these pathways contributes to development of different visual disorders by analyzing the respective lipid products is an important task, as these enzymes represent attractive targets for the specific attenuation of ocular inflammation (Lim et al. 2015).

There have been several attempts to measure normal and pathological levels of PUFAs and oxylipins in human TF (Shim et al. 2012; Walter et al. 2016; Mieyal et al. 2001; Aragona et al. 2005; Nathan et al. 1994; Sack et al. 2010; Thakur and Willcox 1998; Proud et al. 1990; Irkec et al. 1999). The concentration of AA and the eicosanoids PGE<sub>2</sub>, PGD<sub>2</sub> and 12-HETE was found to be significantly increased upon ocular surface inflammation in concordance with its clinical score (Shim et al. 2012; Walter et al. 2016; Mieyal et al. 2001). Furthermore, the therapies focused on the modulation of oxylipins or involving their precursor PUFAs produced prominent anti-inflammatory effects in the respective ocular surface diseases (Nathan et al. 1994; Aragona et al. 2005; Lim et al. 2015; Rosenberg and Asbell 2010; Pinazo-Duran et al. 2013). Accordingly, the detection of oxylipin patterns in TF may allow the diagnosis and monitoring of the course of these diseases. So far, the studies have been confined to the detection of TF PUFAs or single lipids belonging to the eicosanoid subgroup. Recently, an extended panel of TF eicosanoids was identified in patients with meibomian gland dysfunction by means of UPLC-MS/MS (Ambaw et al. 2018). However, a full-scale lipidomic characterization of human TF oxylipins, including derivatives of LA and ALA, has never previously been conducted, to the best of our knowledge.

In contrast to TF, AH can be sampled only by the invasive procedure, which renders its biochemical examination more complicated in humans. Meantime, animal studies report reliable correlations between the concentration of certain lipid mediators in AH and the clinical characteristics of intraocular inflammatory diseases (Csukas et al. 1990; Kulkarni 1991; Mahlberg et al. 1987). Thus, the understanding of the normal and pathologic content of lipid mediators in human AH may also be of diagnostic relevance. The normal levels of AH oxylipins can be assessed in patients who have undergone cataract surgery, as this is commonly performed for the analysis of the other inflammatory molecules, including cytokines, chemokines and immunoglobulins (Fielder and Rahi 1979; Takai et al. 2012; Wang et al. 2016; Mastropasqua et al. 2018). Although single eicosanoids were detected in such AH samples (Matsuo 2004; Wang et al. 2016), the complete patterns of PUFAs and oxylipins in human AH remain unidentified.

The goal of this study was to reveal the baseline patterns of PUFAs and oxylipins in AH and TF by their profiling in individuals without ocular inflammation by means of modern lipidomic technique (Ibanez et al. 2017). Along with

establishing the reference parameters of the eye liquids, these data suggest mechanisms of oxylipin generation in the eye and unravel correlations between the lipid content of the AH and TF obtained from the same individual. Furthermore, we performed comparative lipidomic analysis of PUFAs and oxylipins in the eye liquids of rabbits, the species widely employed in investigative ophthalmology (Zernii et al. 2016a). Remarkably, we found for the first time a pronounced similarity in the lipid metabolomic profiles of AH and TF and demonstrated that their oxylipin content exhibited noticeable interspecies concordance. The revealed correlations confirm the feasibility of rabbit models for studies of pathogenesis and therapies of human ocular disorders.

## 2 Experimental

### 2.1 Chemicals

The Schirmer test tear strips were from Haag-Streit (Switzerland) or Contacare Ophthalmics & Diagnostics (India) for collecting TF from humans and rabbits, respectively. The oxylipins standards were as follows: 6-keto PGF<sub>1α</sub>-d<sub>4</sub> (cat. no. 315210), TXB<sub>2</sub>-d<sub>4</sub> (cat.no. 319030), PGF<sub>2α</sub>-d<sub>4</sub> (cat. no. 316010), PGE<sub>2</sub>-d<sub>4</sub> (cat.no. 314010), PGD<sub>2</sub>-d<sub>4</sub> (cat. no. 312010), Leukotriene (LT) C<sub>4</sub>-d<sub>5</sub> (cat.no. 10006198), LTB<sub>4</sub>-d<sub>4</sub> (cat.no. 320110), 5(S)-HETE-d<sub>8</sub> (cat.no. 334230), 12(S)-HETE-d<sub>8</sub> (cat.no. 334570), 15(S)-HETE-d<sub>8</sub> (cat. no. 334720), Oleoyl Ethanolamide-d<sub>4</sub> (cat.no. 9000552), EPA-d<sub>5</sub> (cat.no. 10005056), DHA-d<sub>5</sub> (cat.no. 10005057), AA-d<sub>8</sub> (cat. No. 390010) (Cayman Chemical, Ann Arbor, MI, USA). Oasis® PRIME HLB solid-phase lipid extraction cartridge (60 mg, 3 cc, cat.no. 186008056) was obtained from Waters, Eschborn, Germany.

### 2.2 Human participants

The study recruited 23 individuals without eye pathologies (except senile and congenital cataract) and signs and symptoms of ocular inflammation according to the results of a complete ocular examination performed by an experienced clinical ophthalmologist. The additional exclusion criteria for the study participants were previous treatment with corticosteroids or nonsteroidal anti-inflammatory drugs, systemic disease (e.g. diabetes, thyroid disease), pregnancy, or breast-feeding during the study visit. Four persons had no history of ophthalmological diseases/treatments (absolute controls), whereas the other 19 persons underwent cataract surgery. TF was collected from all 23 participants, whereas AH samples were obtained from 16 cataract patients. The demographic data and diagnoses of the participants are summarized in Table S1.

## 2.3 Animals

The study involved a total of 16 male pigmented Dutch rabbits (6 months old, 2.3 to 3 kg) purchased from a certified farm (Krolinfo, Russia). The animals were housed individually in  $795 \times 745 \times 1776 \text{ mm}^3$  cages at a 12 h light–dark cycle (8:00–20:00) at a temperature of 22–25 °C and humidity of 55–60% with free access to food (BioPro, Russia) and water. The health status of all animals was monitored daily and no adverse events were observed during the course of the study.

## 2.4 Collection of TF and AH from human participants

TF was obtained using gauged Schirmer's test paper strips (width 6 mm, thickness 0.23 mm) without anesthesia and tear stimulation. The sampling was performed in all participants under the same conditions, which included the fasted state, measuring time at least two hours after awakening and similar environments (medical personnel, air quality, and light conditions). The strip was placed under the lower eyelid and allowed to reach the moistened fragment of exactly 10 mm. The moistened fragment of the strip (total volume =  $10 \text{ mm} \times 6 \text{ mm} \times 0.23 \text{ mm} = 13.8 \text{ mm}^3$ ) was cut off, transferred to a plastic tube with 1 ml of anhydrous methanol containing 0.1% v/v butylated hydroxytoluene (BHT) and stored at  $-80 \text{ }^\circ\text{C}$ .

AH was taken by a surgeon during phacoemulsification under peribulbar (2% lidocaine) and topical (proxymetacaine 0.5% eye drops) anesthesia. Briefly, a paracentesis was made using 1.2 mm long single-use knife and 50  $\mu\text{l}$  of AH was aspirated using insulin syringe, mixed with 1  $\mu\text{l}$  of 0.05% BHT, aliquoted by 15  $\mu\text{l}$ , and stored at  $-80 \text{ }^\circ\text{C}$ .

## 2.5 Collection of TF and AH from animals

To collect TF from rabbits, the gauged Schirmer's test strip (width 5 mm, thickness 0.18 mm) was placed under the lower eyelid of the restrained animal and allowed to get moistened reaching exactly 15 mm. The procedure was repeated with 3 strips. Fifteen millimeters-long fragments of each strip (total volume =  $15 \text{ mm} \times 5 \text{ mm} \times 0.18 \text{ mm} = 13.5 \text{ mm}^3$ ) were cut off, transferred to a plastic tube with 1 ml of anhydrous methanol containing 0.1% v/v BHT and stored at  $-80 \text{ }^\circ\text{C}$ .

To obtain AH, the animals was placed in prone position in a restraining device and subjected to intramuscular (with 50 mg/ml tiletamine and 50 mg/ml zolazepam) and topical (with proxymetacaine 0.5% eye drops) anesthesia. AH (50  $\mu\text{l}$ ) was collected from the limbus by insulin syringe

equipped with 30-gauge needle, mixed with 1  $\mu\text{l}$  of 0.05% BHT, aliquoted by 15  $\mu\text{l}$ , and stored at  $-80 \text{ }^\circ\text{C}$ .

## 2.6 Lipid extraction

Frozen AH samples (15  $\mu\text{l}$ ) were thawed and diluted with anhydrous methanol or water–methanol solutions yielding 4%, 25% or 95% v/v MeOH. The obtained probes were mixed with 2 ng of deuterated internal standard solutions, centrifuged ( $12,000 \times g$ , 3 min), mixed with 6 ml of 0.1% acetic acid and loaded onto solid-phase lipid extraction cartridge Oasis® PRIME HLB. The cartridge was washed with 2 ml of 15% methanol containing 0.1% formic acid and the lipids were sequentially eluted with 500  $\mu\text{l}$  of anhydrous methanol and 500  $\mu\text{l}$  of acetonitrile. Preliminary UPLC-MS/MS analysis (for the procedure, see Sect. 2.7) demonstrated that efficacy of extraction of various oxylipins from AH was maximal upon employing 95% v/v water–methanol solution (Fig. S1), which was therefore used in all subsequent experiments. To maintain the identity of the sampling, extraction of the lipids from TF was performed exactly as described for AH. The obtained AH or TF extracts were concentrated by evaporation of the solvent under gentle stream of nitrogen, reconstituted in 50  $\mu\text{l}$  of 90% methanol and stored at  $-80 \text{ }^\circ\text{C}$  until UPLC-MS/MS analysis (see Sect. 2.7).

To assess the impact of the extraction methods on lipid detection, rabbit TF (used as a matrix) was collected employing capillaries, mixed with deuterated lipid standards and treated according to the procedures developed for AH or TF. In the first case, 15  $\mu\text{l}$  of the matrix were mixed with 0.1% v/v BHT, frozen at  $-80 \text{ }^\circ\text{C}$ , thawed and mixed with 1 ml of 95% v/v water–methanol solution. In the second case, the matrix was collected from the tube using tear strip reaching exactly 15 mm, the moistened fragment was cut off, transferred to a plastic tube with 1 ml of anhydrous methanol containing 0.1% v/v BHT, frozen at  $-80 \text{ }^\circ\text{C}$  and thawed. The obtained probes were next identically extracted as indicated above and subjected to UPLC-MS/MS analysis (for the procedure, see Sect. 2.7). Only 2 out of 14 analyzed standards (for the list, see Sect. 2.7), namely DHA-d5 and AA-d8, showed a decrease in concentration in the case of TF-type extraction (Fig. S2), which was taken into account in the subsequent studies.

## 2.7 UPLC-MS/MS analysis

TF and AH-derived lipid extracts were analyzed using 8040 series UPLC-MS/MS mass spectrometer (Shimadzu, Japan) in multiple-reaction monitoring mode at a unit mass resolution for both the precursor and product ions (Chistyakov et al. 2018). The methods used are in line with the proposed minimum reporting standards published in 2007 by (Sumner et al. 2007). Briefly, the lipid compounds were



separated by reverse-phase UPLC (injection volume 20  $\mu$ l) using Phenomenex C8 column (2.1 mm  $\times$  150 mm  $\times$  2.6  $\mu$ m) with the flow rate of 0.4 ml/min and the sample cooler and the column temperature of 5  $^{\circ}$ C and 40  $^{\circ}$ C, respectively. The elution was performed using acetonitrile gradient in 0.1% (v/v) formic acid. The changes in concentrations of acetonitrile were as follows: 10% (0 min)—25% (5 min)—35% (10 min)—75% (20 min)—95% (20.1 min)—95% (25 min)—10% (25.1 min)—10% (30 min). The separated lipids were subjected to MS analysis employing an electrospray ionization source operating in both positive and negative ion modes using nitrogen as the nebulizer gas. The selected molecular ions were fragmented in the gas phase by collision-induced dissociation and analyzed by tandem (MS/MS) mass spectrometry. The examples of calibration graphs and the plots described extraction recovery rates (detected using TF as a matrix) are presented in Fig. S3A and B, respectively. The calibration data showed linearity with  $r > 0.91$  for all analyzed lipids (Table S2). The parameters of mass spectrometry were set as follows: nebulizer gas flow—3 l/min; drying gas—10 l/min; heat block temperature—400  $^{\circ}$ C; desolvation line temperature—250  $^{\circ}$ C; collision induced dissociation pressure—230 kPa. The target PUFAs and oxylipins were identified and quantified by comparing their MS, MS/MS and UPLC (retention times, peak areas) data with the data obtained for deuterated internal standard compounds of the same classes (6-keto PGF1 $\alpha$ -d4, TXB2-d4, PGF2 $\alpha$ -d4, PGE2-d4, PGD2-d4, Leukotriene (LT) C4-d5, LTB4-d4, 5(S)-HETE-d8, 12(S)-HETE-d8, 15(S)-HETE-d8, Oleoyl Ethanolamide-d4, EPA-d5, DHA-d5, AA-d8) using a commercial software method package Lipid Mediator Version 2 (Shimadzu, Japan) according to the manufacturer's instructions. This assay covers 158 lipid mediator compounds derived from PUFAs (for the list, see <https://www.shimadzu.nl/lcmsms-method-package-lipid-mediators-ver-2>).

## 2.8 Data analysis

The data were analyzed using the 2-sample two-sided t-test followed by Bonferroni-Holm correction for multiple comparisons. Statistical associations were determined by calculation of the Pearson correlation coefficient.  $p < 0.05$  was considered as statistically significant.

## 3 Results

### 3.1 Profiling of PUFAs and oxylipins in the TF and AH of humans

To examine the baseline (normal) content of PUFAs and their metabolites in TF and AH, we employed an experimental

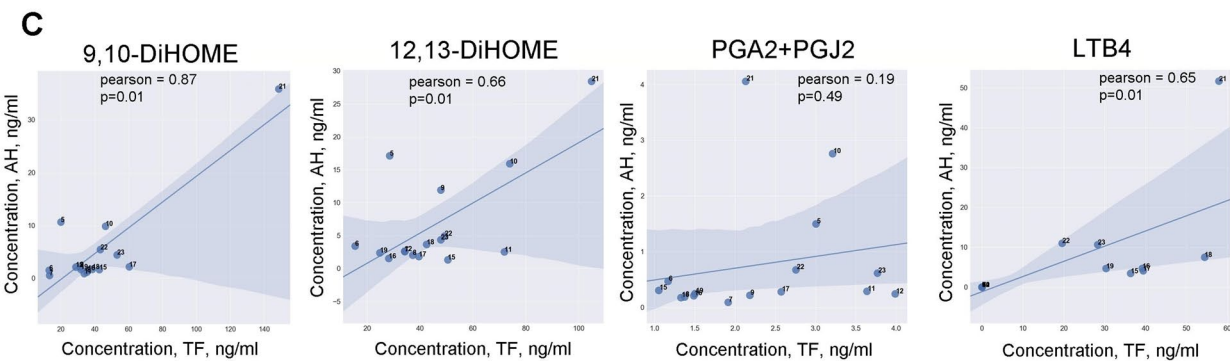
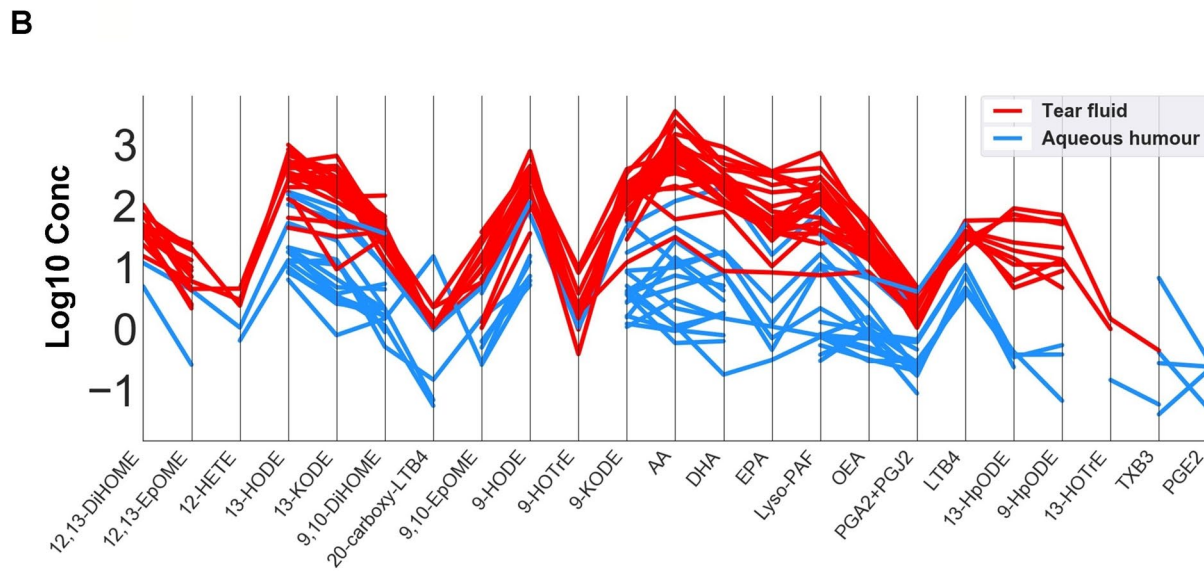
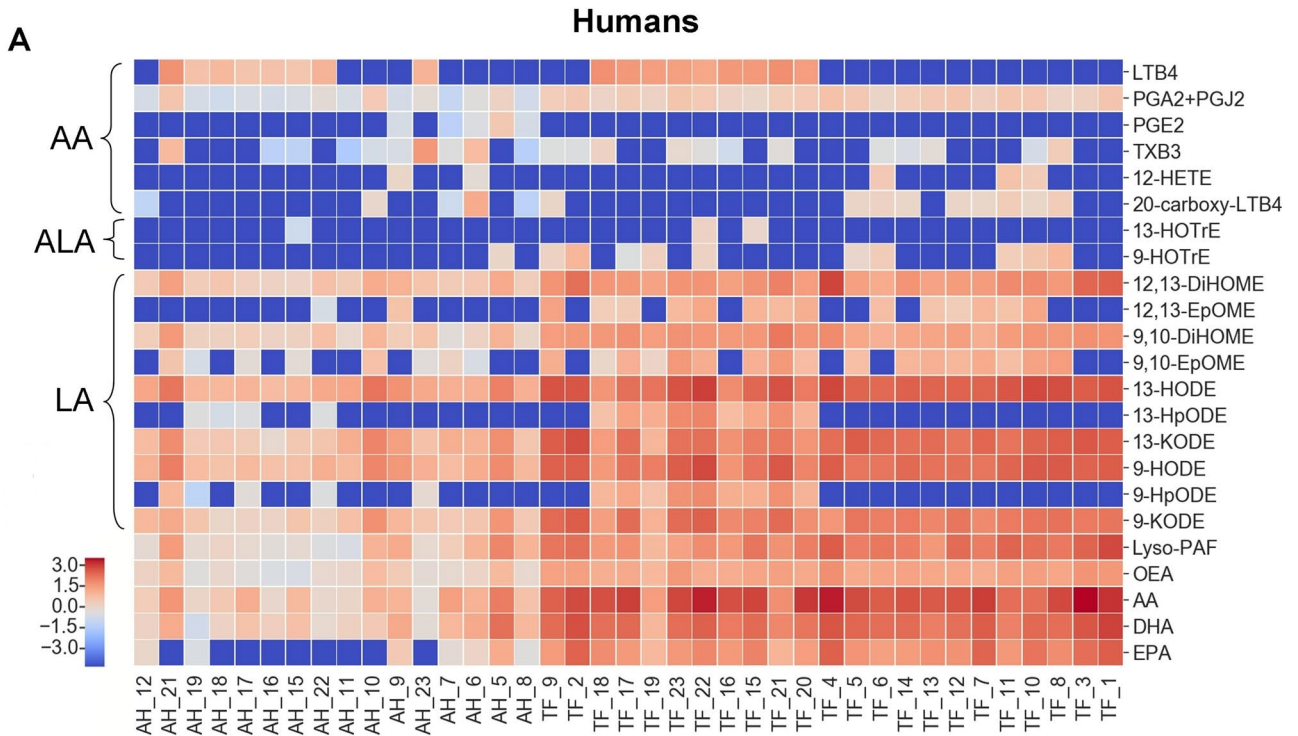
group that included cataract surgery patients of both sexes covering a wide age range, from 39 to 84 years (Table S1). For comparison, we additionally analyzed TF samples obtained from ophthalmologically healthy, young (from 20 to 29 years) participants to be used as an absolute control. UPLC-MS/MS analysis of the eye liquids identified a total of 3 PUFAs and 20 other lipids, including 18 oxylipins (Fig. 1a, Table 1). Interestingly, although the determined PUFAs were AA, DHA and EPA, we detected oxylipins that were derived mainly from AA (PGA2-PGJ2, PGE2, LTB4, 20-carboxy-LTB4, TBX3, 12-HETE), LA (13-HpODE/13-HODE/13-KODE, 9-HpODE/9-HODE/9-KODE, 9,10-DiHOME/9,10-EpOME, 12,13-DiHOME/12,13-EpOME) and ALA (9-HOTrE, 13-HOTrE). In addition, we found two derivatives of phospholipids, including (Z)-N-(2-Hydroxyethyl)octadec-9-enamide (oleoylethanolamide, OEA) and 1-O-hexadecyl-sn-glycerol-3-phosphorylcholine (Lyso-Platelet-Activating Factor, Lyso-PAF). Despite significant amounts of DHA and EPA, we did not detect their derivatives in both TF and AH samples. For quantitative data regarding the detected compounds, see Figs. S4–S8).

The comparison of the lipid metabolomic patterns of human AH and TF revealed almost equal panels of PUFAs and their metabolites in these liquids, consisting of 22 compounds in TF and 23 compounds in AH: the former did not contain PGE2 (AA derivative). Furthermore, these eye liquids exhibited similar quantitative profiles of the detected metabolites (Fig. 1b). For instance, they statistically correlated in terms of containing high (compared to other oxylipins) concentrations of 12,13-DiHOME, but low concentrations of 9,10-DiHOME (LA derivatives), similar concentrations of PGA2-PGJ2 and relatively high levels of LTB4 (AA derivatives), etc. (Fig. 1c). Overall, our data provide a reference range for PUFAs/oxylipins in AH and TF in the absence of ocular inflammation and suggest that there are relationships between the content of lipid mediators in these eye liquids.

It should be added that no differences between the lipid patterns of TF and AH were found in respect to participant sex differences, but this finding cannot be recognized as statistically significant due to low sample size. Although a few compounds, including OEA, DHA, EPA, 13-KODE and 12,13-DiHOME, exhibited a negative correlation with age (Table S3), in general, there were no pronounced differences in the lipid composition of TF between cataract patients and the absolute control group. These observations confirm our suggestion that individuals undergoing cataract surgery can be used as a control in ocular inflammation studies.

### 3.2 Profiling of PUFAs and oxylipins in the TF and AH of healthy rabbits

To investigate whether the revealed similarities in the lipid composition of AH and TF are unique to humans or



**Fig. 1** Patterns of PUFAs/oxylipins in TF and AH of human participants without ocular inflammation. **a** Heat map visualization of the lipids contents. log10 of concentration (ng/ml) of each compound is indicated in colors, ranging from blue (the absence of the compound) to red ( $10^3$  ng/ml or more). The methodologically detectable compounds that were absent in both liquids are not included in the heat map. **b** Comparison of oxylin concentration, measured in TF (red) and AH (blue) collected from the same patient. **c** Joint plots representing associations between the concentrations of several LA (9,10-DiHOME and 12,13-DiHOME) and AA (PGA2+PGJ2 and LTB4) derivatives in TF and AH collected from the same individual participant (Color figure online)

possess interspecies analogy in mammals, we analyzed the same panel of compounds in the AH and TF collected from healthy rabbits. In order to maintain the integrity of the study, the collection and analysis of the rabbit specimens were performed exactly as in humans. A total of 24 lipid compounds were identified in rabbit AH and TF (Fig. 2a, Table 1). Among them, there were three PUFAs (AA, EPA, DHA), three derivatives of phospholipids (N-arachidonoyl ethanolamine (AEA), OEA and Lyso-PAF), as well as 18 oxylipins, including eight derivatives of AA (PGA2+PGJ2, PGD2, PGE2, LTB4, TXB2, 5-HETE, 11-HETE, 12-HETE), eight derivatives of LA (9-HODE/9-KODE, 13-HODE/13-KODE, 9,10-DiHOME/9,10-EpOME, 12,13-DiHOME/12,13-EpOME) and two derivatives of ALA (9-HOTrE and 13-HOTrE). For quantitative data regarding the detected compounds, see Figs. S4–S8).

Generally, the concentration of PUFAs and oxylipins in TF was greater than in AH. Nevertheless, just like in humans, in rabbits we observed prominent similarity between the overall metabolomic composition of TF and AH. Thus, the comparative analysis of these liquids obtained from each individual animal revealed positive and significant correlation in their overall lipid content (Fig. S9). An exception is 11-HETE and EPA, the concentration of which was high in TF but relatively low in AH (Fig. 2b). Accordingly, our data confirm the existence of relationships between the TF and AH lipid mediator content in mammals.

### 3.3 Comparison of the lipid mediator metabolomes of TF and AH in humans and rabbits

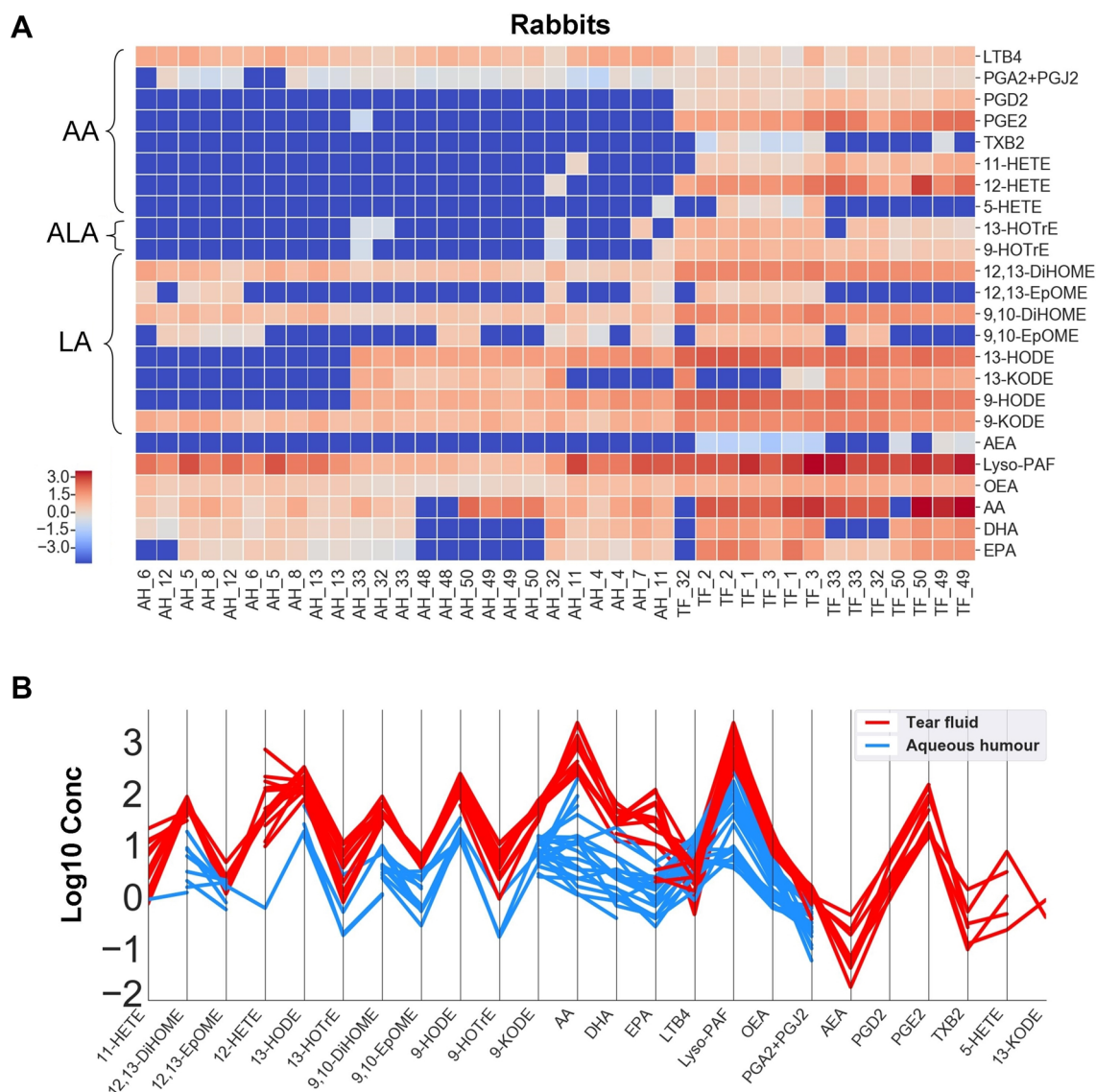
Overall, a total of 28 lipid compounds were detected in the eye liquids of humans and rabbits (UPLC-MS/MS parameters of the identified compounds are summarized in Table S4). Based on the obtained lipidomic data, we next searched for differences and similarities in the lipid composition of TF and AH in humans and rabbits, yielding several important observations. As can be seen from Fig. 3a, TF samples obtained from rabbits were characterized by a lower dispersion of oxylin concentration as compared to human TF. However, the type of PUFAs and oxylipins in rabbit TF largely reproduced the composition of human

**Table 1** Characteristics of the lipid compounds detected in AH and TF of humans and rabbits

#	Compound	Precursor PUFA	Metabolic pathway	Presence in eye liquids	
				Humans	Rabbits
PUFAs					
1	AA	–	–	AH, TF	AH, TF
2	DHA	–	–	AH, TF	AH, TF
3	EPA	–	–	AH, TF	AH, TF
Oxylinins					
4	LTB4	AA	LOX	AH, TF	AH, TF
5	20-carboxy-LTB4	AA	LOX	AH, TF	–
6	5-HETE	AA	LOX	–	AH, TF
7	11-HETE	AA	LOX	–	AH, TF
8	12-HETE	AA	LOX	AH, TF	AH, TF
9	PGA2+PGJ2	AA	COX	AH, TF	AH, TF
10	PGD2	AA	COX	–	TF
11	PGE2	AA	COX	AH	AH, TF
12	TXB2	AA	COX	–	TF
13	TXB3	AA	COX	AH, TF	–
14	9-HODE	LA	LOX	AH, TF	AH, TF
15	9-KODE	LA	LOX	AH, TF	AH, TF
16	9-HpODE	LA	LOX	AH, TF	–
17	13-HODE	LA	LOX	AH, TF	AH, TF
18	13-KODE	LA	LOX	AH, TF	AH, TF
19	13-HpODE	LA	LOX	AH, TF	–
20	9,10-DiHOME	LA	CYP	AH, TF	AH, TF
21	9,10-EpOME	LA	CYP	AH, TF	AH, TF
22	12,13-DiHOME	LA	CYP	AH, TF	AH, TF
23	12,13-EpOME	LA	CYP	AH, TF	AH, TF
24	9-HOTrE	ALA	LOX	AH, TF	AH, TF
25	13-HOTrE	ALA	LOX	AH, TF	AH, TF
Phospholipids					
26	AEA	–	–	–	TF
27	Lyso-PAF	–	–	AH, TF	AH, TF
28	OEA	–	–	AH, TF	AH, TF

TF: we detected 19 common compounds (Table 1). Moreover, although rabbit TF did not contain the LA products 9-HpODE and 13-HpODE found in human TF, it contained 9-HODE/9-KODE and 13-HODE/13-KODE, representing further derivatives of the missing oxylinins. Yet, some AA-derived compounds, such as TXB3 in human TF and 5-HETE, 11-HETE and TXB2 in rabbit TF were unique for the respective species. Generally, in both cases the composition of TF was represented by LA and AA (mainly) and ALA oxylinins, whereas derivatives of EPA and DHA were absent (Fig. 3b, c).

The AH of rabbits was more broadly congruent with the human counterpart in relation to the concentrations of



**Fig. 2** Patterns of PUFAs/oxylipins in TF and AH of healthy rabbits. **a** Heat map visualization of the lipids contents. log<sub>10</sub> of concentration (ng/ml) of each compound is indicated in colors, ranging from

blue (the absence of the compound) to red (10<sup>3</sup> ng/ml or more). **b** Comparison of oxylipin concentration, measured in TF (red) and AH (blue) collected from the same animal (Color figure online)

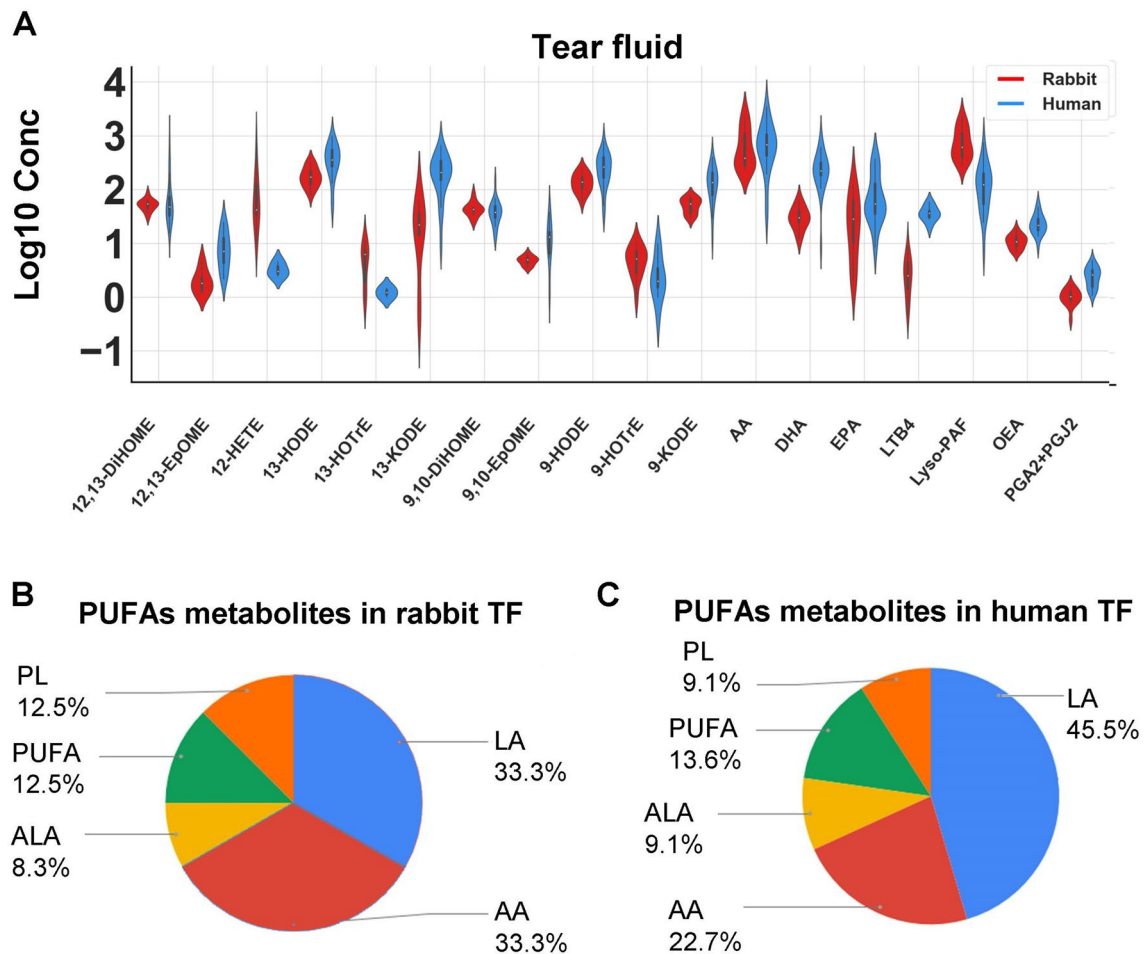
phospholipid derivatives, PUFAs and LA oxylipins (Fig. 4a). However, this is not entirely true for AA derivatives, which in rabbits were less represented in AH as compared to TF (Figs. 3b, 4b), but demonstrated the opposite trend in humans (Figs. 3c, 4c). In addition, the exceptions made for the above-mentioned species-specific AA oxylipins in TF were equally true for AH. Overall, the AH of both organisms contained derivatives of LA, AA and ALA, but the diversity of these compounds in this eye liquid was slightly less pronounced (Fig. 4b, c) than in TF (Fig. 3b, c). We were able to detect three common PUFAs (AA, EPA and DHA) in the AH of humans and rabbits, but their concentration in rabbits was lower.

We concluded that despite some differences, the overall profiles of lipid mediators, particularly PUFAs and oxylipins, in TF and AH in the absence of ocular inflammation are similar in humans and rabbits.

### 4 Discussion

PUFAs and their derivative oxylipins constitute an important class of lipid mediators involved in many aspects of ocular immunity. Together with the other signaling molecules, such as cytokines and chemokines, they regulate both ocular surface and intraocular inflammation by triggering





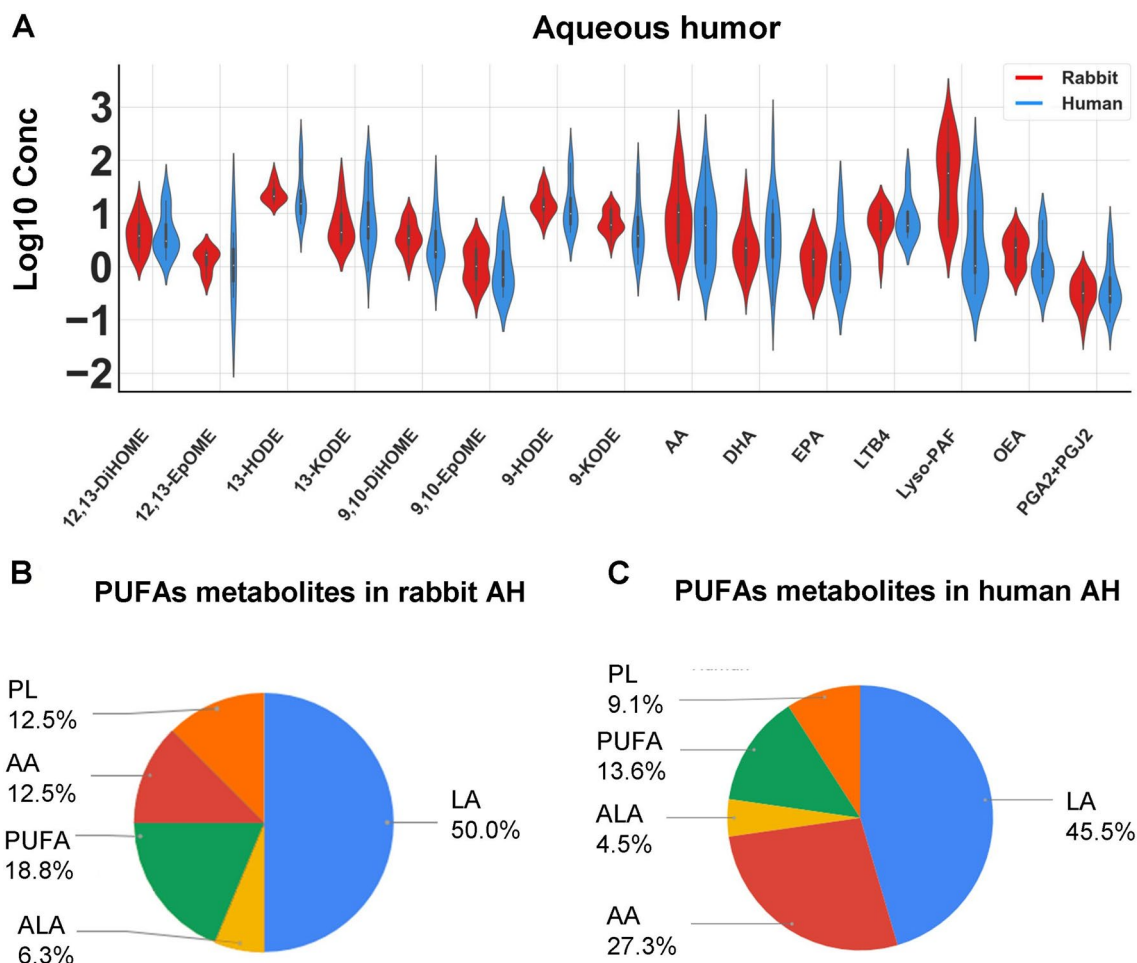
**Fig. 3** Comparison of PUFAs/oxylipins composition of TF in rabbits and humans. **a** Violin plot (box plot combined with kernel density trace) demonstrating patterns of TF concentrations (log10) of PUFAs/oxylipins in rabbits (red) and humans (blue). The data are presented for 18 compounds detected in TF of both species. **b** Pie plots show-

ing the source distribution of PUFAs/oxylipins detected in TF of rabbits (left) and humans (right). The plot considers the presence of the compounds in TF without regard to their concentration (Color figure online)

multiple cellular responses in the eye tissues (Tripathi and Alizadeh 2014; Elmasry et al. 2019). It was reported that ocular inflammation can be associated with alterations in the oxylipin content of TF and AH, but their detection for a long time was methodologically limited to certain individual molecules (Wang et al. 2016; Matsuo 2004). Here, we performed for the first time lipidomic profiling of AH for the presence of PUFAs metabolites in humans (cataract surgery patients) and rabbits without ocular inflammation and compared their patterns in AH and TF. The revealed lipid profiles of human AH generally agreed with the results of previous studies. For instance, as in our case, these studies did not detect PGF2 $\alpha$ , but found PGE2 and several PUFAs in the AH of cataract surgery patients (Wang et al. 2016; Matsuo 2004; Nussdorf et al. 2013). AH of healthy rabbits was previously found to contain PGF2 $\alpha$ , PGE2 and PGD2 (Liu 2000; Acar et al. 2017), with the two latter metabolites

were also detected in our experiments. It should be noted however, that in our study we identified at total of 23 lipids in human AH and 21 lipids in rabbit AH (see Table 1) and therefore it represents the most comprehensive characterization of the inflammatory lipid mediator metabolome of this eye liquid for the moment.

Lately, there have been several attempts to characterize the metabolome of human TF by similar LS-MS/MS lipidomic approaches (Ambaw et al. 2018; Panthi et al. 2019; English et al. 2017) and our data generally overlap and complement the results of these studies. The most comprehensive work, focused on TF eicosanoids, reported the detection of four PUFAs and more than thirty of their derivatives, including all of the AA oxylipins identified in our study (Ambaw et al. 2018). The wider coverage of eicosanoids in that work is likely associated with a different sample volume as well as different extraction procedure for TF. We did not employ



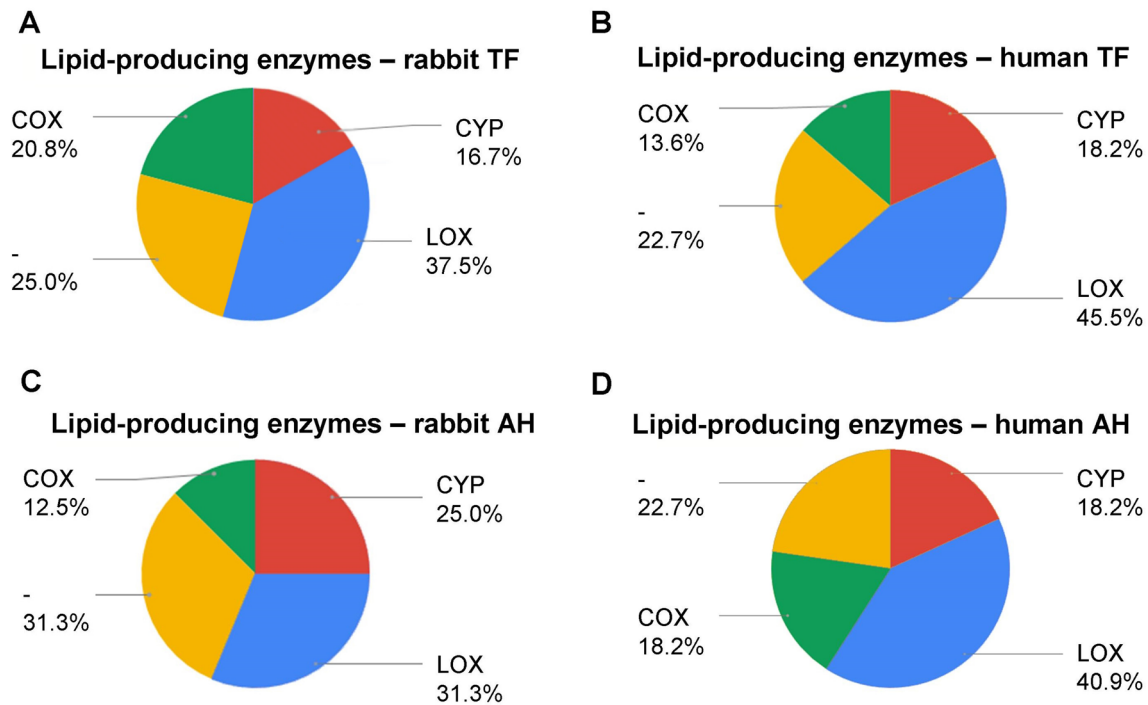
**Fig. 4** Comparison of PUFAs/oxylipins composition of AH in rabbits and humans. **a** Violin plot demonstrating patterns of AH concentrations (Log10) of PUFAs/oxylipins in rabbits (red) and humans (blue). The data are presented for 18 compounds detected in TF of both spe-

cies. **b** Pie plots showing the source distribution of PUFAs/oxylipins detected in AH of rabbits (left) and humans (right). The plot considers the presence of the compounds in AH without regard to their concentration (Color figure online)

such procedure since it was less suitable for sampling human AH (see Sect. 2.6), which was used in our experiments for comparative analysis. The other studies on the subject also dealt with eicosanoids, as well as PUFAs and several EPA and DHA-derived pro-resolving mediators (English et al. 2017; Panthi et al. 2019; Walter et al. 2016). Yet, we additionally identified LA and ALA derivatives, thereby significantly expanding the understanding of the composition of lipid mediators in human TF. Furthermore, our study is the first to comprehensively characterize PUFAs metabolites in rabbit TF and has demonstrated that their basic distribution shares common features with AH, as well as with both eye liquids of humans. These observations point on existence of relationships between the sources for these liquids and suggest that their baseline patterns are similar in different

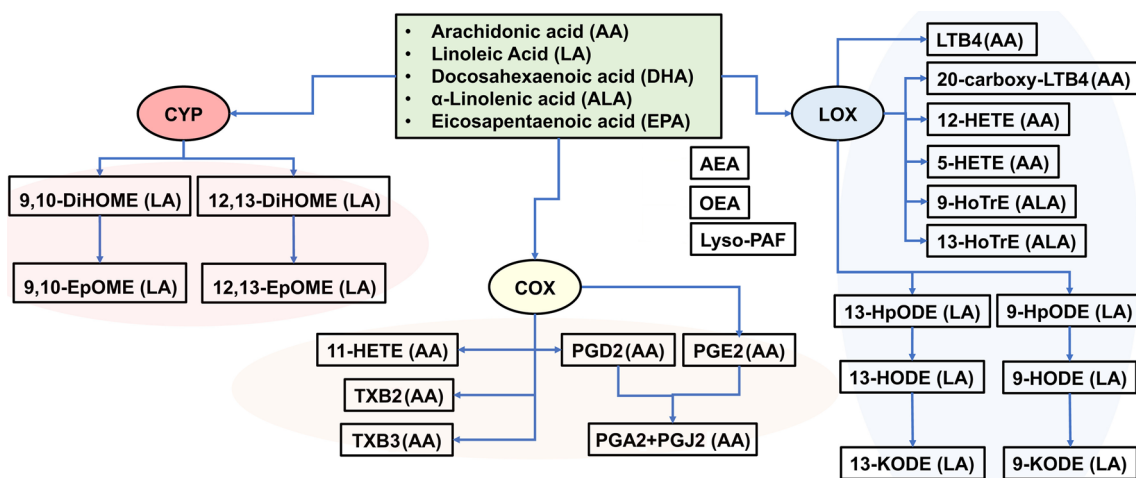
mammals, which provides more proof of the feasibility of the use of rabbits as experimental animals for ophthalmological and vision research studies. In addition, the revealed correlations raise a question whether the composition of AH, which is collected only by an invasive procedure, can be assessed by a non-invasive analysis of TF (and vice versa) (Pieragostino et al. 2015).

Our data allowed the revealed AH and TF oxylipins to be clustered according to the enzymatic pathways responsible for their production (Figs. 5, 6). In general, we detected the products of metabolic reactions catalyzed by the enzymes of all three classes (COX, LOX and CYP450), but the nomenclature and concentrations of these products dramatically differed. As can be seen from Fig. 5b and d, in humans, both eye liquids contained mostly LOX derivatives



**Fig. 5** Clustering of oxylipins of rabbit (a, c) and human (b, d) eye liquids according to the biosynthetic mechanisms. Pie plots showing patterns of the lipid compounds detected in TF (a, b) and AH (c, d), which were generated via PUFAs metabolic cascades involving LOX

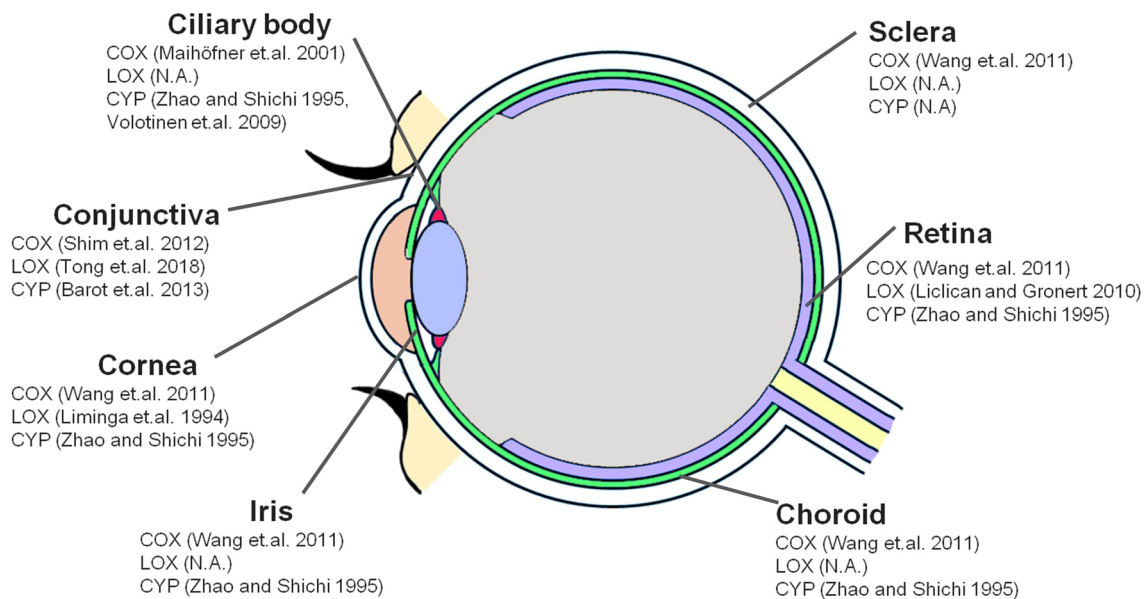
(blue), CYP (red) and COX (green) enzymes as well as other (yellow) pathways. The plot considers the presence of the compounds in eye liquids without regard to their concentration (Color figure online)



**Fig. 6** Mechanisms of biosynthesis of lipid mediators. Metabolites are divided into the groups synthesized via lipoxygenase (LOX), cyclooxygenase (COX) or cytochrome P450 monooxygenase (CYP) pathways

of LA (12-HETE, 20-carboxyl-LTB4, 9-HOTrE13-HODE, 13-KODE, 9-HODE, 9-KODE). CYP derivatives of LA run second and include 9,10-EpOME and 12,13-EpOME as well as their derivatives, 9,10-Di-HOME and 12,13-DiHOME, produced by soluble epoxide hydrolase (Gabbs et al. 2015). Notably, the concentration of the latter derivatives was two to three orders of magnitude higher than the level of their

parent compounds, indicative of high soluble epoxide hydrolase activity in AH-producing tissues. In contrast, COX-dependent compounds were limited to few prostaglandins, among which PGA2 + PGJ2 were detected in both AH and TF, whereas PGE2 was found exclusively in AH. The analogous clustering of oxylipins in rabbits revealed very similar patterns (Fig. 5a, c), thereby confirming intraspecies (TF



**Fig. 7** Expression of metabolic enzymes involved in three main pathways of oxylipin biosynthesis by the eye tissues under normal conditions (N.A.—data not available)

versus AH) and interspecies (human versus rabbit) similarity of PUFAs/oxylipins metabolomes.

The observed relationships between the baseline content of AH and TF lipid mediators may be considered in the light of the mechanisms of their biosynthesis. Metabolic enzymes governing oxylipin generation are widely distributed in different eye tissues (Fig. 7), but which of these tissues participates in the establishment of the baseline lipid patterns of AH and TF remains an open question. AH is produced by active secretion from the non-pigmented epithelium of the ciliary body, circulates around the lens and through the pupil into the anterior chamber, thereby reaching the iris and posterior cornea, and leaves the eye through the trabecular meshwork and Schlemm's canal, or uveoscleral drainage (Goel et al. 2010). The majority of the above-mentioned tissues express oxylipin-producing enzymes (Fig. 7) (Wang et al. 2011; Maihofner et al. 2001; Zhao and Shichi 1995; Volotinen et al. 2009; Liminga et al. 1994; Liclican and Gronert 2010; Shim et al. 2012; Tong et al. 2018; Barot et al. 2012) and can, in principle, contribute to the lipid composition of AH. In addition, AH is at least partially produced via plasma ultrafiltration (Goel et al. 2010). TF, in turn, is secreted mainly by various glands surrounding the eye, and contacts only ocular surface epithelia, including anterior cornea and the conjunctiva (Dartt 1992). Yet, despite these differences, some of the detected oxylipins can be secreted into both eye liquids from the same ocular tissues (Thorig et al. 1985), such as the cornea.

Overall, we propose that the baseline levels of PUFAs and oxylipins in AH and TF are similar, although these eye

liquids are generally produced by different mechanisms. However, this setup may change upon the development of ocular inflammation, thereby generating a specific pattern of lipid mediators in eye liquids. Further studies are required to recognize such lipid “signatures” and the corresponding signaling mechanism underlying inflammation in each particular ocular disease in order to propose the appropriate therapy, such as the inhibition of the respective metabolic enzymes.

## 5 Conclusion

This study is the first to perform quantitative profiling of signaling lipid mediators in AH of humans and rabbits in the absence of ocular inflammation and to compare their baseline patterns with those in TF. A total of 23 and 24 compounds were identified in human and rabbit specimens, respectively. These compounds included phospholipid derivatives and PUFAs as well as oxylipins biosynthesized via three main enzymatic pathways, the contribution of which decreased in the order LOX > CYP450 > COX. Whereas the detected PUFAs included AA, DHA and EPA, the oxylipins were derived mainly from AA and LA, as well as ALA. Although the concentration of oxylipins in AH was lower compared to TF, these liquids showed pronounced similarity in their metabolomic profiles and their oxylipin content exhibited noticeable interspecies concordance. The revealed correlations confirm the feasibility of rabbit models for investigating pathogenesis and



trials therapies of human eye disorders. The identified metabolite patterns unravel the enzymatic mechanisms of oxylipin generation in AH and TF and might be used as a reference in any study addressing the signaling lipids involved in ocular inflammation.

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**Author contributions** DVC, MGS, AAZ, PPP and EYZ conceived and designed research. VIK, EVF, OAK, AMB and ENI performed studies involving human participants. VVT, VEB and IIS conducted experiments with animals. DVC, NVA, AAA, SVG and VVC carried out mass-spectrometry analysis. SVG and VVC contributed new reagents or analytical tools. DVC, MGS, AAZ, PPP and EYZ analyzed data. DVC, NVA and EYZ visualized the results. EYZ wrote the manuscript. All authors read and approved the manuscript.

## Compliance with ethical standards

**Conflict of interest** Authors declare that there is no conflict of interest.

**Informed consent** All participants signed a written declaration of consent approved by the Ethics Committee of Helmholtz National Medical Research Center of Eye Diseases (Moscow, Russia).

**Research involving human participants and/or animals** The experimental procedures followed the tenets of The Code of Ethics of the World Medical Association (Declaration of Helsinki). The treatment of the animals was performed according to the 8th edition “Guide for the Care and Use of Laboratory Animals” of the National Research Council and “Statement for the Use of Animals in Ophthalmic and Visual Research” of The Association for Research in Vision and Ophthalmology (ARVO). The study protocols were approved by the Belozersky Institute of Physico-chemical Biology Animal Care and Use Committee (Protocol Number 1/2016).

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