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1 **Lipidomic Analysis of Human Corneal Epithelial Cells Exposed to Ocular Irritants Highlights**
2 **the Role of Phospholipid and Sphingolipid Metabolisms in Detergent Toxicity Mechanisms**

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19 **Highlights**

- 20 • A lipidomic analysis of HCE cells incubated with ocular irritant was performed
- 21 • Ocular irritants induced significant change in lipidome of HCE cells
- 22 • BAK, SLS and TXT all increased the content of ceramide
- 23 • BAK, SLS and TXT similarly decreased the level of DHA-containing plasmalogen
- 24 • Some lipid modifications are specific to each irritant, especially for phospholipids

25 **Abstract**

26 Detergent chemicals, widely used in household products, in pharmaceutical, medical, cosmetic
27 and industrial fields, have been linked to side effects and involved in several eye diseases. On the
28 ocular surface, detergents can interfere with the corneal epithelium, the most superficial layer of the
29 cornea, representing a line of defence against external aggression. Despite its major role in numerous
30 biological functions, there is still little data regarding disruption of lipid homeostasis induced by
31 ocular irritants. To this purpose, a lipidomic analysis using UPLC-HRMS/MS-ESI+/- was performed
32 on human corneal epithelial (HCE) cells incubated with three widely known ocular irritants:
33 benzalkonium chloride (BAK), sodium lauryl sulfate (SLS) and Triton X-100 (TXT). We found that
34 these ocular irritants lead to a profound modification of the HCE cell lipidome. Indeed, the cell content
35 of ceramide species increased widely while plasmalogens containing polyunsaturated fatty acid
36 species, especially docosahexaenoic acids, decreased. Furthermore, these irritants upregulated the
37 activity of phospholipase A₂. The present study demonstrates that BAK, SLS and TXT induced
38 disruption of the cell lipid homeostasis, highlighting that lipids mediate inflammatory and cell death
39 processes induced by detergents in the cornea. Lipidomics may thus be regarded as a valuable tool to
40 investigate new markers of corneal damage.

41

42 **Key words:** ocular irritants, detergents, lipid homeostasis, PLA₂, cell toxicology

43 1. Introduction

44 Detergent chemicals have been widely used for numerous decades to meet people's need for
45 hygiene and to fight against microorganisms. Their use is ubiquitous, not only in household products
46 but also in the pharmaceutical, medical, cosmetic and industrial fields. However, detergent exposure
47 has been linked to side effects, especially at dermatological, respiratory and ocular levels [1–3]. For
48 example, chronic exposure may lead to irritant contact dermatitis, worsening asthma or corneal
49 damage [1–3]. The most affected tissues are those directly exposed to external environments, as
50 detergents mainly act by surface contact. Indeed, exposure to detergents induces remarkable stress at
51 the cell membrane level, resulting in reorganization, cell activation and damage [4]. Regarding the
52 ocular surface, detergents can profoundly interfere with the corneal epithelium, the most superficial
53 layer of the cornea and the second line of defense against external aggression immediately under the
54 tear film [5].

55 The main sources of chronic exposure of the cornea to chemicals with detergent properties come
56 from eye drop medicine [3]. As recommended by Pharmacopoeias, ophthalmic preparations should
57 contain a preservative agent when prepared in multidose bottles and dispensers. Benzalkonium
58 chloride (BAK), a quaternary ammonium compound, is the preservative that is the most frequently
59 used in multidose eye drops, and is thus widely administered for chronic disorders requiring mid- to
60 long-term treatments, such as glaucoma, allergic reactions or dry eye diseases (DED) [3,6–8]. Since
61 the 90's, numerous investigations based on *in vitro* and *in vivo* models as well as clinical observations
62 have revealed that the chronic use of BAK is clearly responsible for induction or worsening of DED
63 [9–11]. DED is a chronic inflammatory pathology of the ocular surface, which leads to disabling
64 ocular symptoms, such as discomfort, foreign body sensation, irritations, burning and pain, or visual
65 disturbance [12–15]. DED has also been previously related to chronic eye exposure to detergents due
66 to occupational and domestic activities [16]. Indeed, changes in ocular surface tissues and tear film
67 were reported in workers in the dry-cleaning industries. Besides, acute exposure leading to corneal
68 damage has also been detected in children exposed to liquid laundry detergents pods [17].

69 To assess the ocular damage potency of chemicals and to understand the mechanisms underlying
70 ocular irritation, the Draize eye test performed in rabbits has been widely used in the past decades
71 [18]. Nevertheless, due to the increase in awareness of animal welfare and demands for non-animal-
72 based test methods, *in vitro* models have progressively replaced those using animals [19]. These *in*
73 *vitro* models are mainly based on corneal cells incubated with widely used ocular irritants, such as
74 BAK, sodium dodecyl sulfate (SLS) or Triton X-100 (TXT) [19,20]. Using *in vitro* models,
75 deleterious effects induced by detergents have been associated to numerous alterations of cell
76 signaling pathways such as oxidative stress, mitochondrial damage or lipid peroxidation. Irritation
77 processes also involve inflammation through the release of pro-inflammatory cytokines and cell death
78 through apoptosis or necrosis pathways [21,22].

79 Inflammation and cell death processes are tightly linked to lipid homeostasis [23,24]. Indeed,
80 lipids are not only molecules involved in energy storage, but they also play a critical role in cell
81 signaling. For example, phospholipids (PL) constitute a pool of fatty acids (FA), as they can be
82 hydrolyzed through the action of phospholipase A₂ (PLA₂) to generate free FA, which in turn are
83 oxidized in oxylipins involved in inflammatory processes [23,25,26]. Sphingolipids (SL) are also well-
84 known bioactive lipid species that induce the release of pro-inflammatory cytokines such as IL-1 β and
85 activate caspase 3 resulting in cell apoptosis [27,28]. Perturbation of lipid metabolism has been
86 previously described in corneal alteration [29–31]. Dysregulation of SL metabolism, especially
87 ceramide species, play a key role in corneal injury due to nitrogen mustard exposure [32]. Moreover, it
88 was reported that highly cytotoxic concentrations of ocular irritants in 3D-reconstructed cornea-like
89 models alter the content of ceramide and the gene expression of enzymes involved in lipid metabolism
90 [33]. Nevertheless, to our knowledge, little data is available regarding the effect of ocular irritants on
91 cell lipid content and the potential consequences on inflammatory and cell death processes.

92 The aim of the present study was to investigate the mechanisms of three widely used detergents
93 and to understand their effects on cell lipidome. To this purpose, human corneal epithelial (HCE) cells
94 were incubated with BAK, SLS or TXT respectively, cationic, anionic and non-ionic detergents. We

95 first determined values corresponding to subcytotoxic concentrations respective to each ocular irritant.
96 Using an untargeted LC/MS/MS lipidomic analysis, we then comprehensively determined the
97 lipidome of HCE cells and characterized lipid alterations induced by BAK, SLS and TXT. Finally, the
98 mechanisms underlying this lipidome change were investigated.

99 2. Materials and methods

100 2.1. Cell line, culture conditions and incubation solutions

101 Human corneal epithelial cell line (HCE) was obtained from the RIKEN biobank (Tsukuba, Japan)
102 [34]. HCE cells were grown in culture flasks using DMEM/F12 (1/1), 10% fetal bovine serum (FBS),
103 2 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin all from Gibco (Paisley, UK). At
104 confluence, every 3 days, cells were harvested with trypsin-EDTA 0.05% in Dulbecco's phosphate-
105 buffered saline (DPBS). The cells used in this study were from passages 2 to 10.

106 The 10⁻⁴% BAK solution was prepared with a stock solution of 0.1% (w/v) BAK (Sigma, Saint
107 Quentin Fallavier, France). For the 5.10⁻³% and 3.10⁻³% solution of SLS and TXT respectively, a stock
108 solution of 1% of SLS (w/v) or TXT (v/v) (Sigma, Saint Quentin Fallavier, France) was used. The
109 stock solutions were prepared in water, prior to dilution in the culture medium for incubation. HCE
110 cells were seeded in 96-well (20,000 cells/well) and 6-well (400,000 cells/well) plates to reach
111 subconfluence in 24 hours and then incubated for 24 hours with 1.10⁻⁴% BAK, 5.10⁻³% SLS or 3.10⁻³%
112 TXT containing 2% FBS. Cells were washed with DPBS prior to each assay.

113 2.2. Cell viability assay

114 The viability of HCE cells was assessed using the neutral red (NR) assay according to previously
115 published data [35]. Briefly, a NR solution (50 µg/mL) prepared in culture media without serum was
116 added to the cells after incubation with each ocular irritants. The NR solution was incubated for 3h at
117 37°C. Cells were washed with DPBS, lysed using a mixture solution containing water, ethanol, acetic
118 acid (49.5/49.5/1, v/v/v) and finally homogenized at room temperature for 15 minutes on a stirring
119 plate. Fluorescence intensity was measured using $\lambda_{\text{ex}} = 540 \text{ nm}$ and $\lambda_{\text{em}} = 600 \text{ nm}$ (Spark[®], TECAN,
120 Männedorf, Switzerland).

121 2.3. Reactive oxygen species production

122 ROS production was determined using the 2',7'-Dichlorohydrofluorescein diacetate (H₂DCFDA)
123 assay. The H₂DCFDA 0.1 M stock solution in DMSO (Thermo Fisher Scientific, Les Ulis, France)
124 was used to prepare a 20 µM solution in DPBS. HCE cells were incubated with the H₂DCFDA

125 working solution for 30 minutes at 37°C after incubation with each irritants and fluorescence intensity
126 was measured using $\lambda_{\text{ex}} = 485 \text{ nm}$ and $\lambda_{\text{em}} = 535 \text{ nm}$ (Spark®, TECAN).

127 **2.4. Caspase-1 activity**

128 Caspase-1 activity was evaluated using the Caspase-Glo® 1 Inflammasome Assay (Promega,
129 Madison, WI, USA). The assay was performed according to the manufacturer's instructions.
130 Luminescence, resulting in luciferase activity, was quantified using the Spark® microplate reader
131 (TECAN).

132 **2.5. P2X7 activation**

133 P2X7 activation was assessed with YO-PRO-1 probe as previously described [36]. The YO-PRO-
134 1 1 mM stock solution in DMSO (Thermo Fisher Scientific) was used to prepare a 2 μM solution in
135 DPBS. HCE cells were incubated with the YO-PRO-1 working solution for 10 minutes at room
136 temperature after incubation with each irritants and fluorescence intensity was measured using $\lambda_{\text{ex}} =$
137 485 nm and $\lambda_{\text{em}} = 531 \text{ nm}$ (Spark®, TECAN).

138 **2.6. Untargeted lipidomic analysis**

139 **2.6.1. Sample preparation**

140 HCE cells were incubated with BAK ($10^{-4} \%$), SLS ($5 \cdot 10^{-3} \%$) or TXT ($3 \cdot 10^{-3} \%$) for 24 hours and
141 were then washed with DPBS. Cells were harvested using trypsin-EDTA 0.05%, washed with DPBS,
142 centrifuged at 2000 rpm for 10 min. Dry cell pellets were adjusted to 3 million cells and stored at -
143 80°C until analytical process. After thawing, the cell pellets were resuspended in ultra-pure water (1
144 mL) and were sonicated for 5 minutes. The cell lysate was spiked with a mixture of internal standard
145 (**Table S1**) prior to lipid extraction using a chloroform/methanol/water (5:5:2, v/v/v) mixture
146 containing the antioxidant agent 3,5-di-tert-4-butylhydroxytoluene 0.01% (w/v). Samples were
147 subsequently centrifuged at 3000 rpm for 10 min, organic phase was collected, and solvents were
148 evaporated under reduced pressure at 45 °C. Dry residues were dissolved in 100 μL of a mixture

149 containing acetonitrile/isopropanol/chloroform/water (35:35:20:10, v/v/v/v) before injection into the
150 UPLC-MS/MS system.

151 **2.6.2.Data-dependent LC-ESI-HRMS/MS analysis**

152 Liquid chromatography in both positive and negative electrospray ionization mass spectrometry
153 analysis of lipid extracts was performed on a UPLC system (Waters, Manchester, UK) combined with
154 a Synapt[®]G2 (Q-TOF) mass spectrometer (Waters). Chromatographic separation was achieved on an
155 Acquity[®] CSH C18 column (100 mm × 2.1 mm; 1.7 μm). Elution was performed using a binary
156 gradient system consisting in 10 mM ammonium acetate in acetonitrile/water mixture (40:60, v/v) as
157 solvent A and 10 mM ammonium acetate in acetonitrile/isopropanol mixture (10:90, v/v) as solvent B.
158 The eluent increased from 40% B to 100% B in 10 min, was held at 100% B for 2 min before
159 returning to 40% B. The flow rate was kept at 0.4 mL.min⁻¹, the column oven was set at 50°C and the
160 injection volume was 5 μL. The source parameters were as follows: capillary voltage 2,400 V, cone
161 voltage 45 V, source temperature 120°C, desolvation temperature 550°C, cone gas flow 20 L.h⁻¹, and
162 desolvation gas flow 1,000 L.h⁻¹. Leucine enkephalin (2 ng.mL⁻¹) was used as an external reference
163 compound (Lock-Spray[™]) for mass correction. In a data-dependent acquisition mode (DDA), MS full
164 scans were followed by 5 MS/MS scans performed on the most intense ions above an absolute
165 threshold of 1,000 counts. Selected parent ions were fragmented at collision energy from 20 to 40 eV.
166 Scan duration for both MS1 and MS2 was 0.2 s. In the full scan mode, the data were acquired between
167 *m/z* 50 and 1200 using a resolution of 20,000 FWHM at *m/z* 500. Data acquisition was managed using
168 Waters MassLynx[™] software (version 4.1; Waters MS Technologies). Sample injections order was
169 orthogonalized to sample status into UPLC/MS/MS system to avoid any bias. A mixture of 65
170 standard lipids belonging to 9 of the main lipid classes : Fatty acid (FA), phosphatidic acid (PA),
171 phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylcholine (PC),
172 phosphatidylglycerol (PG), ceramide (Cer), sphingomyelin (SM), HexosylCeramide (HexCer),
173 monoacylglycerol (MG), diacylglycerol (DG) and triacylglycerol (TG) at a final individual
174 concentration of 1 μM was also periodically injected throughout the analytical batch (**Table S2**).

175 **2.6.3.Data-preprocessing parameters**

176 For both ion modes, raw data files were converted into universal open source mzXML file with
177 MSConvert 3.0 and were then processed using MZmine 2.5 software. MS and MS/MS spectra were
178 extracted with a mass detection noise level set at 1E2 and 0E0, respectively. Chromatograms were
179 built with the ADAP algorithms [37] (minimum group size of 5 scans, a group intensity threshold of
180 1,000, and an m/z tolerance of 10 ppm). The ADAP wavelets chromatogram deconvolution algorithm
181 was used with the following settings: signal to noise ratio = 10, coefficient/area ratio = 60, peak
182 duration range = 0.05-0.5 min, retention time wavelet range = 0.0-0.1. Chromatograms were de-
183 isotoped using the isotopic peaks grouper algorithm using a m/z and t_R tolerance of respectively 10
184 ppm and 0.1 min. Peak alignment was performed through the join aligner method using the following
185 parameters: m/z and t_R tolerance of respectively 10 ppm and 0.15 min. Each MS/MS scans were
186 associated with the corresponding MS scans using a m/z and t_R tolerance of respectively 15 ppm and
187 0.15 min. The peak list was finally gap-filled using the so-called module "same RT and m/z range gap
188 filler" with m/z tolerance of 5 ppm. Lipids were then annotated based on exact mass measurement and
189 retention time with the "custom database" module using an in-house database. These preprocessing
190 steps led to two matrixes, both in ESI+ and ESI- mode, listing for annotated lipid m/z , t_R and peak area
191 values. These matrixes were normalized and filtered as previously described [38].

192 **2.6.4.Lipid structure assignment**

193 The structure assignment of lipid species was based on MS and MS/MS data, using a tolerance
194 window of 5 and 15 ppm, respectively (**Table S3-S11**). Identification was supported by t_R values
195 through comparison of experimental to expected values calculated using t_R prediction models. Indeed,
196 the UPLC-ESI-MS analysis of the standard lipid mixture allowed determining for each lipid class (FA,
197 PA, PE, PS, PC, PG, PI, Cer, SM, HexCer, MG, DG, TG) the linear relationship between retention
198 time and equivalent carbon number (ECN) [38,39]. MS/MS data was used to fully determine
199 phospholipid fatty acid composition (**Table S4-S10**). Fatty acyl sn₁ and sn₂ position was determined in

200 negative ion mode using the relative intensity of ions corresponding to carboxylates as previously
201 described [40,41].

202 **2.6.5. Estimation of lipid amounts**

203 The cell lipid amounts, expressed as mol%, were estimated using internal standard lipid mixture
204 spiked in cell suspension samples before extraction [42]. Lipid species intensities were individually
205 normalized to the one of the corresponding internal standard chosen in the same subclass. The
206 standard lipid mixture includes one lipid species representative of each investigated subclass (**Table**
207 **S2**). Lipid species used as internal standard contain a fatty acid side chain with total odd carbon
208 number or are deuterated derivatives and thus cannot overlap with endogenous lipid species present in
209 HCE cells.

210 **2.7. PLA₂ activity assay**

211 The global PLA₂ activity was determined using Red/Green BODIPY based EnzCheck
212 Phospholipase A₂ assay kit (Thermo Fisher Scientific) according to manufacturer's procedure. Briefly,
213 cells were incubated for 24 hours with BAK or HO. Culture media was removed, and cells were
214 washed with DPBS. Harvested cells were centrifugated, DPBS removed and the cell pellet was
215 suspended in 100 µL of PLA₂ reaction buffer with protease inhibitor and finally sonicated for 10 sec.
216 A volume of 50 µL of cell lysate was then transferred in a 96 well plate and mixed with liposomes
217 prepared with the EnzChek Phospholipase A₂ substrate at a ratio of 1:1. Following a 30-minute
218 incubation at 37°C, PLA₂ activity was determined as a FRET ratio ($\lambda_{\text{ex}} = 460 \text{ nm}$, $\lambda_{\text{em}} = 515/575 \text{ nm}$)
219 using the Spark® spectrofluorometer.

220 **2.8. Statistical analysis**

221 Unsupervised multivariate analyses were performed for lipidomic data using SIMCA-P+ software
222 version 13.0.3 (Umetrics, Umeå, Sweden) as previously described [38,43,44]. A pareto scaling was
223 applied to the variables prior to unsupervised principal component analyses (PCA).

224 Each experiment was performed independently five times. Results are expressed in mol% or fold
225 change compared to control and are displayed as means \pm standard deviation (SD). ANOVA, Dunnett
226 test and Student t-test were performed using GraphPad Prism 8 software (GraphPad Software, La
227 Jolla, CA) with a risk set at 0.05 (*p < 0.05, **p < 0.01, ***p < 0.001 compared to control).

228 3. Results

229 3.1. Cytotoxic effects of ocular irritants

230 Human corneal epithelial (HCE) cells were incubated for 24 hours with BAK, SLS or TXT in
231 concentrations ranging from 1.10^{-5} to $5.10^{-1}\%$. All the ocular irritants altered HCE cell viability in a
232 concentration-dependent manner (Figure 1A). BAK $1.10^{-4}\%$, SLS $5.10^{-3}\%$ and TXT $3.10^{-3}\%$ induced a
233 loss of cell viability of about 20% compared to control, indicative of a sub-cytotoxic effect (Figure
234 1B). The above concentrations were therefore used in the rest of this study.

235 BAK $1.10^{-4}\%$, SLS $5.10^{-3}\%$ and TXT $3.10^{-3}\%$ led to a 2.0-, 2.05- and 2.4-fold increase in ROS
236 production, respectively (Figure 1C) and a 1.12-, 1.52- and 1.15-fold increase in caspase-1 activity
237 (Figure 1D). SLS was the only irritant to trigger P2X7 receptor activation (Figure 1E). In contrast,
238 BAK and TXT did not induce any P2X7 receptor activation.

239 3.2. Lipidome modifications induced by ocular irritants

240 To investigate the impact of incubation with ocular irritant on HCE cell lipidome, we performed a
241 lipidomic analysis using liquid chromatography hyphenated to tandem mass spectrometry (UPLC-
242 MS/MS). In positive ion mode, a typical chromatogram showed that lysophospholipids (LP) were first
243 eluted before 3 min, phospholipids (PC, PE, PI, PG and PS) and sphingolipids (SM and Cer) were
244 eluted between 5 and 8 min, and DG or TG were lastly eluted after 7 min. In negative ion mode, the
245 elution was for FA before 6 min, and for Cer and phospholipids (PC, PE, PI, PG, and PS) between 6
246 and 9 min.

247 A PCA was performed on the data relative to control and treated cells in both positive and
248 negative ion mode. Either in ESI+ and ESI- ion modes, the built PCA models showed good quality
249 parameters and the score plots exhibited four clusters corresponding to BAK-, SLS- and TXT-treated
250 and control cells (Figure 2A). These clustering indicate alterations of the HCE cell lipidome following
251 incubation with ocular irritants. To identify the impacted lipids, the lipidome of HCE cells was first
252 established thanks to an in-house database including lipid species representative of phospholipid (PL),
253 glycerolipid (GL), sphingolipid (SL) and fatty acid (FA) classes. A total of 300 unique lipids,

254 belonging to 11 subclasses, was identified. Lipid species in HCE cells impacted by ocular irritant were
255 then sought out.

256 The three investigated ocular irritants led to major changes in the lipidome of HCE cells. Indeed,
257 BAK, SLS and TXT significantly modified the cell content of 104, 202 and 165 lipid species
258 respectively, as shown on volcano plot (Figure 2C, 2D and 2E). Regarding the lipid classes, PL were
259 significantly decreased in HCE cells incubated with BAK while a striking increase in GL was
260 observed following incubation with SLS and TXT (Figure 2F). Moreover, SLS induced a decrease in
261 SL level. Finally, no changes in FA class were displayed whatever the ocular irritant. Regarding the
262 discriminant lipid species, 30 lipids were modified in the same way, whatever the ocular irritant, as
263 displayed on the Venn diagram (Figure 2G). Indeed, 12 lipids increased while 18 decreased following
264 incubation with BAK, SLS or TXT. These lipid species belonged to the PC, PE, DG, TG and Cer
265 subclasses. Nine PC, including seven ether PC, decreased by about 40% (Figure 2H). LPE (18:0)
266 content was increased by 2.2, 1.5 and 3.1-fold compared to control following incubation with BAK,
267 SLS or TXT, respectively. PG (18:1/18:0) and PI (16:0/16:0) increased widely whatever the stressor.
268 Two DG species exhibited a decrease while ten TG species increased. Finally, six ceramides,
269 containing saturated FA, also increased.

270 **3.3. Effect of ocular irritants on phospholipid content**

271 Regarding effects at the subclass level, while the incubation with BAK and TXT led to a decrease
272 in overall PC content, in contrast, SLS induced a significant increase in this subclass (Figure 3). No
273 change in PE was observed following incubation with BAK or SLS while TXT induced a significant
274 increase in this subclass (Figure 3). Regarding FA PL content, PL with saturated fatty acids (SFA)
275 decreased following incubation with BAK but increased with SLS or TXT (Figure 3B). A decrease in
276 mono-unsaturated fatty acid (MUFA) PL was induced by TXT and a decrease in poly-unsaturated
277 fatty acid (PUFA) PL was displayed for SLS (Figure 3B and 3C).

278 While the overall ether PL (ePL) containing arachidonic acid (AA) displayed no change (Figure
279 4B), at the molecular species level, four AA-containing ePC at sn₂ position were impacted (Figure

280 2H). For example, PC (P-18:0/20:4) decreased by 35%, 65% and 45% following incubation with
281 BAK, SLS or TXT, respectively (Figure 2H). In contrast, the overall content of ePL with
282 docosahexaenoic acid (DHA) displayed a significant decrease of 55%, 95% and 45% in HCE cells
283 incubated with BAK, SLS or TXT, respectively (Figure 4A). To investigate the consequences at the
284 PL metabolism level, we determined PLA₂ activity. BAK, SLS and TXT significantly induced an
285 increase in PLA₂ activity of 1.4-, 1.8- and 1.75-fold change compared to control, respectively (Figure
286 4C).

287 **3.4. Effect of ocular irritants on sphingolipid content**

288 The ocular irritants assessed in the present study significantly increased the overall Cer content in
289 HCE cells following a 24-hour incubation (Figure 5A). In contrast, a significant decrease in SM was
290 observed with SLS and TXT but not with BAK (Figure 5B). The SM/Cer ratio was calculated and it
291 decreased substantially following incubation with SLS or TXT (Figure 5C). At the molecular species
292 level, Cer (d18:1/16:0) increased while SM (d18:1/16:0) decreased because of SLS and TXT (Figure
293 5E). The increase in Cer at the expense of SM species was observed for 5 other sphingosine based-
294 species (Figure 5E).

295 **3.5. Effect of ocular irritants on glycerolipid content**

296 Incubation with BAK, SLS or TXT decreased the overall HCE cell content of DG (Figure 6A).
297 TG content displayed a striking increase following incubation with SLS and TXT while BAK induced
298 no significant change (Figure 6B).

299 4. Discussion

300 Intensive use of detergents in household cleaning products, cosmetics and medical products for
301 several decades has led to a chronical exposure of most of the population to these chemicals.
302 Detergents are responsible for numerous deleterious effects, especially at the cutaneous and
303 respiratory levels [1,2]. Detergents have also been involved in several eye diseases as they may
304 interact with proteins and lipids at the ocular surface. One of the major ocular pathologies, namely dry
305 eye disease, is characterized by irritation, itching, and/or burning sensations [12,15,45,46]. DED is
306 favored by the used of preserved eye drops containing detergents such as BAK. To assess the irritant
307 potential of surfactants, *in vitro* investigations of cytotoxicity and inflammatory cytokine production
308 have been performed [20]. Quite amazingly, although lipids are known to play key roles in
309 inflammatory processes and are targeted by detergents, to our knowledge, no studies have aimed at
310 describing the effects of detergents on corneal cell lipids. Our purpose was therefore to investigate the
311 change in lipidome of human corneal epithelial cells incubated with BAK, SLS or TXT, three
312 chemicals known to induce ocular irritation.

313 To investigate these alterations, we first determined sub-cytotoxic concentrations, corresponding
314 to a decrease in HCE cell viability of approximately 20%, to compare the three agents based on the
315 same level of cytotoxicity. The use of sub-cytotoxic concentrations is highly valuable to investigate
316 the effect of toxics on cell signaling pathways [36]. Sub-cytotoxic concentrations were observed at
317 $1.10^{-4}\%$ BAK, $5.10^{-3}\%$ SLS and $3.10^{-3}\%$ TXT. In preserved eye drops, BAK concentrations, ranging
318 from $1.10^{-2}\%$ to $5.10^{-3}\%$ [3], are between 50 to 200 times higher than the one used in the present study.
319 In addition, it must be emphasized that BAK induced a similar decrease in cell viability at a
320 concentration 30 to 50-fold lower than SLS or TXT did. This result is in accordance with previous
321 published studies indicating that cationic detergents are more cytotoxic than other detergents [47]. To
322 assess whether the ocular irritants induce alterations in HCE lipidome, we performed a comprehensive
323 lipidomic analysis to characterize changes in cell lipid following incubation with BAK, SLS or TXT.
324 We focused the analysis on PL, GL, SL and FA classes. Some of the lipid alterations are common to
325 the three detergents: BAK, SLS and TXT similarly regulated the cell content of 30 lipid species,

326 which represent about 10% of total identified lipids. Among them, BAK, SLS and TXT induced a
327 decrease in the abundance of few PL species, especially eight ether-PC. These lipid species contain an
328 ether link in sn₁ position and are thus prone to oxidation [48,49]. Oxidative stress being a major
329 mechanism of the deleterious effects induced by these irritants [50–52], the increase in ROS
330 production following BAK, SLS and TXT incubation reinforced the aforementioned observations.
331 Oxidative products of plasmalogens include both hydroxyaldehyde derivatives and lysophospholipids
332 containing AA or DHA located in sn₂ position [49,53,54]. LPC containing AA or DHA were not
333 detected in HCE cells using our methods, probably as they are produced in small amount and readily
334 metabolized. Hydroxyaldehyde derivatives, such as hydroxy fatty aldehydes, may be regarded as
335 interesting markers of plasmalogen degradation due to oxidative stress. Indeed, in systemic lupus
336 erythematosus, a pathology for which oxidative stress also induced a decrease in plasmalogen species,
337 an increase in hydroxyaldehyde derivatives has been previously reported [55]. Nevertheless, these
338 compounds are highly reactive exhibiting very short half-life in cells and are therefore difficult to
339 analyze [56].

340 Plasmalogens are known to be a major polyunsaturated fatty acids (PUFA) pool. Indeed, most ePL
341 contain AA and DHA in sn₂ position [48,49,57]. To determine the nature and the position of FA side
342 chain in PL, we used MS/MS data acquired in DDA negative ion mode as it represents a reliable and
343 powerful tool [39]. At molecular level, the three investigated irritants strikingly reduced the total cell
344 content of DHA-containing plasmalogens in sn₂ position. This result is in accordance with the increase
345 in global PLA₂ activity we observed in HCE cells following incubation with irritants. The increase in
346 PLA₂ activity obtained with the assay used in this study cannot unequivocally prejudge if the activity
347 of the enzymatic isoform involved in the cleavage of the plasmalogens is actually increased. Indeed,
348 previous studies indicated that plasmalogen species were substrates of Plasmalogen-Selective PLA₂
349 specific isoforms such as Pls-Etn PLA₂ [58–60]. The drop in DHA-containing plasmalogens may be
350 however regarded as in agreement with the increase in overall PLA₂ activity mentioned in the present
351 study. A specific assay targeting Pls-Etn PLA₂ activity was previously proposed and could be applied
352 to selectively assess this isoform of PLA₂ [58]. PLA₂, the enzyme involved in sn₂ cleavage of PL,

353 leads to the release of free FA in the cytoplasm [23,61]. Free DHA can produce oxylipins through the
354 lipo-oxygenase (LOX) and the cyclo-oxygenase (COX) pathways leading to the release of numerous
355 lipid mediators such as resolvins, maresins or protectins [30,62]. These oxidized metabolites of DHA
356 have been previously described as second messengers in the resolution of inflammation [30,63]. Our
357 results thus suggest that the activation of PLA₂ decreases the total pool of DHA in plasmalogen
358 species. Depletion in DHA content has been associated with pro-inflammatory profile [64–66], ocular
359 irritants and especially detergents may thus partially exert their toxic effect in part through this
360 pathway. A targeted LC-MS/MS analysis focusing on DHA-derived metabolites could be valuable to
361 define which species are especially impacted, for further experiments.

362 Regarding sphingolipid metabolism, our study indicated a significant increase in the total
363 ceramide abundance in HCE cells following incubation with BAK, SLS or TXT. Some species were
364 increased by the three investigated stressors, it includes Cer (d18:1/14:0), Cer (d18:1/16:0), Cer
365 (d18:1/18:0) and Cer (d18:1/24:1). Cer (d18:1/24:1) has been previously described as a targeted lipid
366 of BAK, SLS and TXT in an *in vitro* 3D reconstructed cornea-like model [33]. In biological cell
367 processes, ceramides play signaling roles promoting inflammation through IL-1 β release and inducing
368 apoptosis through caspase 3 activation [27,67,68]. In addition, dysregulation of ceramide metabolism
369 is now recognized as a key feature in many inflammatory diseases such as atherosclerosis,
370 inflammatory bowel disease, non-alcoholic steatohepatitis, or multiple sclerosis [69–71]. In the frame
371 of toxic mechanisms mediated by ocular irritants, ceramides may be regarded as important mediators.
372 They could furthermore be suggested as relevant markers to identify ocular irritants, and they have
373 previously been described as good candidates to determine ocular irritant potency in *in vitro* models
374 [33,72].

375 Linked to the accumulation of Cer, our results reveal that SLS and TXT decreased the total
376 content in SM of HCE cells. Indeed, as exemplified by Cer (d18:1/16:0) and SM (d18:1/16:0),
377 ceramides increased while the corresponding SM decreased in the presence of SLS or TXT.
378 Ceramides are produced by different biosynthetic pathways such as *de novo* or salvage pathways
379 [25,27,73]. The latter involved the hydrolysis of SM in ceramide through the action of

380 sphingomyelinase (SMase), an enzyme located at the cell membrane as well as in the lysosomes [27].
381 The decrease in SM in favor of Cer species thus suggests that SLS and TXT increased SMase activity.
382 This activation has been previously described in numerous pro-inflammatory processes such as those
383 induced by TNF α or UV irradiation [74–76] and is also known to be involved in apoptosis and to
384 contribute to cell death processes. Furthermore, SMase activation has been involved in endoplasmic
385 reticulum stress, which is part of cell apoptosis [77]. It must be emphasized that SMase activation is
386 required to induce the accumulation of TG species [78] and may thus be related to the increase in TG
387 in HCE cells incubated with SLS or TXT. Nevertheless, in the case of the incubation with SLS or
388 TXT, the exact role of SMase in inflammatory and cell death processes needs to be elucidated.

389 Beside lipid homeostasis alteration, BAK, SLS and TXT induced ROS overproduction and
390 caspase 1 activation. P2X7 receptor activation, related to pore permeation, was also observed but only
391 with SLS. Activation of P2X7 receptor were previously reported in ocular stresses [79]. P2X7 receptor
392 activation and oxidative stress can trigger caspase-1 activation, leading to the release of pro-
393 inflammatory cytokines such as IL-1 β [80]. Ceramide accumulation, especially through SMase, also
394 leads to caspase-1 activation [81]. This suggests that, through caspase-1 activation, Cer accumulation
395 leads to and thus contributes to inflammatory processes and cell death. In contrast to ceramides, other
396 lipid species, especially DHA and DHA-derived oxylipins, are known to inhibit caspase-1. Indeed,
397 DHA can counteract caspase-1 cleavage, an essential step to mediate its pro-inflammatory properties
398 [82,83]. Interestingly, SLS induced the highest activation of caspase-1 and P2X7 receptor and the
399 most important decrease in DHA-containing ePL. DHA released from plasmalogens may be therefore
400 considered as a cell response to counteract the inflammatory processes mediated by BAK, SLS and
401 TXT, through caspase-1 modulation. The crosstalk between SL and PL metabolisms, and caspase-1
402 activation nevertheless needs further investigations to be fully understood.

403 On the one hand, the three compounds shared some lipid alterations underlying similar
404 mechanisms; on the other hand, other lipid alterations appeared to be specific and could discriminate
405 cationic from anionic and non-ionic detergents (Figure 7). These alterations mainly concerned PL

406 species: BAK decreased the overall SFA-containing PL species, contrary to TXT that decreased
407 MUFA-containing PL species in favor of those containing SFA. This result clearly supports a possible
408 cell membrane stiffening induced by TXT [84]. The anionic agent SLS significantly decreased the
409 PUFA-containing PL, mainly through the alteration of PI species. Interestingly, SLS is the only
410 compound both activating P2X7 receptor and decreasing PI content. The phosphatidylinositol-3-
411 kinase pathway, which is involved in P2X7 receptor activation [85], may thus be linked to the
412 observed alteration of PI homeostasis. Further studies are needed to assess if the specific lipid changes
413 observed for cationic, anionic and non-ionic surfactants may be extended to the entire classes of these
414 three detergents.

415 **5. Conclusions**

416 This study highlights the role of lipid metabolism in corneal cells incubated with ocular irritants,
417 especially detergents. The three investigated ocular irritants led to lipid homeostasis alteration, mainly
418 involving SL and PL, two lipid subclasses related to inflammatory and cell death processes. PL
419 metabolism perturbation, especially regarding species containing PUFA, is mainly mediated by PLA₂
420 activation. The exact role and the link between lipid alteration and inflammation induced by ocular
421 irritants together with targeted signaling pathways need to be further explored. However, our results
422 indicate that lipid markers may be helpful to determine and discriminate other well-known chemicals,
423 including mild to moderate ocular irritants. The identification of lipid species at the fatty acid levels,
424 including for PL and SL, is essential to understand the changes induced by stressors and to
425 characterize toxicity mechanisms. Finally, the use of a lipidomic approach may thus be regarded as a
426 valuable tool to investigate new markers of corneal damage.

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433 **Conflict of interest**

434 The authors declare that they have no conflicts of interest.

435 **Contribution**

- 436 Romain Magny: Conception, Collection of data, Analysis of data, Writing – original draft
- 437 Nicolas Auzeil: Conception, Collection of data, Analysis of data, Writing – original draft
- 438 Elodie Olivier: Conception, Collection of data, Analysis of data, Writing – original draft
- 439 Karima Kessal: Conception, analysis of data, Writing – original draft
- 440 Anne Regazzetti: Conception, analysis of data, Writing – original draft
- 441 Melody Dutot: Conception, analysis of data, Writing – original draft
- 442 Stéphane Melik-Parsadaniantz: Conception, Supervision, Review of the manuscript
- 443 Patrice Rat: Conception, Supervision, Review of the manuscript
- 444 Christophe Baudouin: Conception, Supervision, Review of the manuscript
- 445 Olivier Laprêvôte: Conception, Supervision, Review of the manuscript
- 446 Françoise Brignole-Baudouin: Conception, Supervision, Analysis of data, Writing – original draft

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- 703

704 **7. Figures**

705 **Figure 1. Cytotoxic effects induced by BAK, SLS or TXT on HCE cells.** (A) Neutral Red cell
706 viability assay: concentration curves. (B) Concentrations inducing a 20% viability decrease. (C)
707 Reactive oxygen species (ROS) production, (D) Caspase-1 activity and (E) P2X7 receptor activation
708 following incubation with BAK, SLS or TXT. Results are expressed as the mean \pm SD fold change
709 compared to control cells (ANOVA with Tukey's multiple comparison test. n=5. *p < 0.05; **p <
710 0.01; ***p < 0.001).

711 **Figure 2. Changes in lipidome of HCE cells induced by BAK, SLS or TXT on HCE cells.** (A)
712 Positive and (B) negative ionization mode of PCA score plots generated in UPLC-MS/MS. (C, D, E)
713 Volcano plots of discriminant lipid species. (F) Content of HCE lipid classes. (G) Venn diagram of
714 common and specific modulated lipid species (H) Heat-map representation of discriminant lipids
715 impacted by the three ocular irritants. Results are expressed as fold changes compared to control. Blue
716 and red color corresponds to, respectively, a decrease and an increase in the cell content compared to
717 control. PL: Phospholipids, SL: Sphingolipids; GL: Glycerolipids; FA: Fatty acid.

718 **Figure 3. Alteration of phospholipid metabolism induced by BAK, SLS or TXT on HCE**
719 **cells.** Content of the different phospholipid subclasses according to the incubation (A) Pie chart
720 displaying the relative proportion of each phospholipid subclass in control HCE cells. Change in cell
721 content of phospholipids containing (B) SFA, (C) MUFA, (D) PUFA following incubation of HCE
722 cells with BAK, SLS or TXT. Results are expressed as mean mol% \pm SD (ANOVA with Tukey's
723 multiple comparison test. n=5. *p < 0.05; **p < 0.01; ***p < 0.001).

724 **Figure 4. Alteration of plasmalogen metabolism induced by BAK, SLS or TXT on HCE cells.**
725 Change in cell content of (A) ePL containing DHA, (B) ePL containing AA following incubation of
726 HCE cells with BAK, SLS or TXT. Results are expressed as mean mol% \pm SD (C) Change in PLA₂
727 activity induced by BAK, SLS or TXT. Results are expressed as fold change \pm SD (ANOVA with
728 Tukey's multiple comparison test. n=5. *p < 0.05; **p < 0.01; ***p < 0.001).

729 **Figure 5. Alteration of sphingolipid metabolism induced by BAK, SLS or TXT on HCE cells.**
730 Change in cell content of (A) total Cer, (B) total SM, (C) SM/Cer ratio following incubation of HCE
731 cells with BAK, SLS or TXT. (D) Metabolic pathway of Cer species. (E) Change in cell content of
732 Cer and SM containing the same fatty acid chain following incubation with BAK, SLS or TXT.
733 Results are expressed as mean mol% \pm SD (ANOVA with Tukey's multiple comparison test. n=5. *p
734 < 0.05; **p < 0.01; ***p < 0.001).

735 **Figure 6. Alteration of glycerolipid metabolism induced by BAK, SLS or TXT on HCE cells.**
736 Change in cell content of (A) total DG and (B) total TG species following incubation of HCE cells
737 with BAK, SLS or TXT. Results are expressed as mean mol% \pm SD (ANOVA with Tukey's multiple
738 comparison test. n=5. *p < 0.05; **p < 0.01; ***p < 0.001).

739 **Figure 7. Similar and specific lipid change induced by BAK, SLS or TXT.** (A) Bar code
740 representation underlying specific and similar changes of the different lipid species. Red and blue
741 color indicated respectively an increase and a decrease in the cell content of HCE cells. (B)
742 Discriminating factors between the three irritants based on our results.