

1 **Effect of oxidation and *in vitro* intestinal hydrolysis on phospholipid toxicity towards**  
2 **HT29 cell line serving as a model of human intestinal epithelium**

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4 Karol Parchem<sup>a\*</sup>, Monika Baranowska<sup>a</sup>, Anna Kościelak<sup>a</sup>, Ilona Kłosowska-Chomiczewska<sup>b</sup>, Maria  
5 Rosário Domingues<sup>c,d</sup>, Adam Macierzanka<sup>b</sup>, Agnieszka Bartoszek<sup>a</sup>

6

7 <sup>a</sup> Department of Food Chemistry, Technology and Biotechnology, Faculty of Chemistry, Gdańsk

8 University of Technology, 11/12 Gabriela Narutowicza St., 80-233 Gdansk, Poland,

9 [karol.parchem@pg.edu.pl](mailto:karol.parchem@pg.edu.pl) or [parchem.karol@gmail.com](mailto:parchem.karol@gmail.com) (K.P.) [bbmmonik@gmail.com](mailto:bbmmonik@gmail.com) (M.B.)

10 [anna.koscielak@pg.edu.pl](mailto:anna.koscielak@pg.edu.pl) (A.K.), [agnieszka.bartoszek@pg.edu.pl](mailto:agnieszka.bartoszek@pg.edu.pl) (A.B.)

11

12 <sup>b</sup> Department of Colloid and Lipid Science, Faculty of Chemistry, Gdańsk University of Technology,

13 11/12 Gabriela Narutowicza St., 80-233 Gdansk, Poland, [ilona.chomiczewska@pg.edu.pl](mailto:ilona.chomiczewska@pg.edu.pl) (I.K-C),

14 [adam.macierzanka@pg.edu.pl](mailto:adam.macierzanka@pg.edu.pl) (A.M.)

15

16 <sup>c</sup> Mass Spectrometry Centre, LAQV-REQUIMTE, Department of Chemistry, University of Aveiro,

17 Santiago University Campus, 3810-193 Aveiro, Portugal, [mrd@ua.pt](mailto:mrd@ua.pt) (M.R.D)

18

19 <sup>d</sup> Centre for Environmental and Marine Studies, CESAM, Department of Chemistry, University of

20 Aveiro, Santiago University Campus, 3810-193 Aveiro, Portugal, [mrd@ua.pt](mailto:mrd@ua.pt) (M.R.D)

21

22 \*Corresponding author:

23 *E-mail address:* [karol.parchem@pg.edu.pl](mailto:karol.parchem@pg.edu.pl) or [parchem.karol@gmail.com](mailto:parchem.karol@gmail.com) (K. Parchem)

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27 **Abstract**

28 Oxidation of food-derived phospholipids (PLs) can influence nutrient digestion and induce oxidative  
29 stress in gastrointestinal epithelium. In this study, hen egg yolk PL fraction was used to evaluate the  
30 effect of lipoxygenase (LOX)-induced PL oxidation on the hydrolysis of PLs catalyzed by pancreatic  
31 phospholipase A<sub>2</sub> (PLA<sub>2</sub>) in the presence of bile salts (BSs). Then, PL/BS solutions containing native  
32 or oxidized PLs were used in *in vitro* intestinal digestion to assess the effect of PL oxidation and  
33 hydrolysis on the toxicity towards HT29 cell line. Based on the obtained results, we suggest that  
34 hexanal and (*E*)-2-nonenal, formed by the decomposition of PL hydroperoxides, inhibited PLA<sub>2</sub>  
35 activity. The cell exposure to simulated intestinal fluid (SIF) containing BSs decreased HT29 cell  
36 viability and significantly damaged cellular DNA. However, the genotoxic effect was reversed in the  
37 presence of all tested PL samples, while the protective effect against the BS-induced cytotoxicity was  
38 observed for native non-hydrolyzed PLs, but was not clearly visible for other samples. This can result  
39 from an overlap of other toxic effects such as lipotoxicity or disturbance of cellular redox homeostasis.  
40 Taking into account the data obtained, it was proposed that the PLA<sub>2</sub> activity decline in the presence  
41 of PL oxidation products may be a kind of protective mechanism against rapid release of oxidized FAs  
42 characterized by high cytotoxic effect towards intestinal epithelium cells.

43  
44 **Key words:** oxidized phospholipids; phospholipid hydrolysis; pancreatic phospholipase A<sub>2</sub>; bile salts;  
45 intestinal oxidative stress;

46  
47 **Abbreviations:** BS – bile salts; CLD – cytosolic lipid droplets; ER – endoplasmic reticulum; FA – fatty  
48 acid; FAME – fatty acid methyl ester; GPx2 – gastrointestinal glutathione peroxidase 2; GSH –  
49 glutathione; GSSG – glutathione disulfide; IC – inhibitory concentration IEC – intestine epithelial cell;  
50 LOH – lipid hydroxide; LOOH – lipid hydroperoxide; LOP – lipid oxidation products; LOX –  
51 lipoxygenase; LPL – lysophospholipid; MDA – malondialdehyde; O/W – oil-in water emulsion; oxPL –  
52 oxidized phospholipid; PC – phosphatidylcholine; PDI – polydispersity index; PE –  
53 phosphatidylethanolamine; PL – phospholipid; PLA<sub>2</sub> – phospholipase A<sub>2</sub>; PUFA – polyunsaturated  
54 fatty acid; PV – peroxide value; RNS – reactive nitrogen species; ROS – reactive oxygen species;  
55 SFA – saturated fatty acid; SIF – simulated intestinal fluid; TAG – triacylglycerol; TBARS –  
56 thiobarbituric acid reactive substances; UFA – unsaturated fatty acid.

57

## 58 1. Introduction

59 Phospholipids (PLs) are important biomolecules, which apart from building cell and organelle  
60 membranes, also play a role of endogenous signalling molecules. The biophysical properties of  
61 membranes, including their fluidity and permeability as well as the distribution and activity of  
62 embedded proteins, are related to the composition of lipid bilayer, including PL structures or  
63 cholesterol to PL ratio (KüllenberG de Gaudry, Taylor, Schneider, & Massing, 2012). Moreover,  
64 polyunsaturated fatty acids (PUFAs) present in the structure of membrane PLs can be oxidatively  
65 modified under oxidative stress conditions. Such modifications change hydrophobicity of the fatty  
66 acids (FAs) build in the PL structure, which perturbs the assembly of the cellular membranes and  
67 alters their functional properties, e.g., by disturbing ion and nutrient transport (Catalá, 2012; Catalá &  
68 Díaz, 2016). Additionally, endogenous oxidized PLs (oxPLs) may be recognized by specific receptors  
69 leading to the activation of signaling pathways that mediate the inflammatory response or cell  
70 apoptosis (Fruhwith, Loidl, & Hermetter, 2007). Therefore, elevated levels of cellular or plasma oxPLs  
71 are believed to be implicated in the pathogenesis of some diseases and disorders (Parchem, Sasson,  
72 Ferreri, & Bartoszek, 2019). The endogenous abundance of oxPLs increases i.a., under the conditions  
73 of cellular oxidative stress, which results from imbalance between the prooxidant and antioxidant  
74 systems, and consequently the excessive production of reactive oxygen and nitrogen species  
75 (ROS/RNS).

76 Diet is one of the most relevant factors that can modulate cellular and tissue levels of ROS/RNS. It  
77 has been demonstrated that an overload of nutrients, especially caused by high-fat or/and high-  
78 glucose diets can be implicated in the disruption of cellular redox homeostasis. One of the  
79 mechanisms involved in oxidative stress induced by high-energy-dense diets is lipotoxicity, which  
80 result from inadequate lipid storage capacity in adipose tissue and/or abnormal adipose tissue lipolysis  
81 (Montgomery, De Nardo, & Watt, 2019). This leads to chronically elevated levels of plasma non-  
82 esterified FAs and increased ectopic lipid deposition. As a consequence, lipotoxic cellular outcomes  
83 including endoplasmic reticulum (ER) stress, inflammation, mitochondrial dysfunction and finally cell  
84 apoptosis can occur (Montgomery et al., 2019). Diet-related oxidative stress may result in dysfunction  
85 of various gastrointestinal cell types such as hepatocytes (Lian, Zhai, Li, & Wang, 2020), pancreatic  $\beta$ -  
86 cells (Sasson, 2017) or intestine epithelial cells (IECs) (Gulhane et al., 2016). The latter can be  
87 exposed to overhigh FA concentration in both, basolateral and apical sides (Bashllari et al., 2020; Van

88 Rijn et al., 2019). Another factor that can trigger cellular oxidative stress is an exposition of IECs to  
89 food-delivered lipid oxidation products (LOPs) (Awada et al., 2012). PUFAs present in the structure of  
90 dietary lipids are highly prone to oxidative modifications during food processing and storage  
91 (Wąsowicz et al., 2004) as well as in the gastrointestinal digestion (Nieva-Echevarría, Goicoechea, &  
92 Guillén, 2018). Importantly, previous studies indicated that for foods such as fish or meat, oxidative  
93 changes first occurred in PLs and then in triacylglycerols (TAGs) (Igene, Pearson, Dugan, & Price,  
94 1980; Jittrepotch, Ushio, & Ohshima, 2006). This order result from higher unsaturation of FAs in the  
95 structures of muscle PLs compare to TAGs from the same origin (Cui & Decker, 2016; Guyon,  
96 Meynier, & de Lamballerie, 2016). Additionally, it seams that PLs, due to their presence in cellular  
97 membranes characterized by large surface area, are more exposed to prooxidant molecules (Cui &  
98 Decker, 2016).

99 PLs, next to bile salts (BS), are surface-active compounds that facilitate solubilization of ingested oils  
100 and fats, leading to the formation of an oil-in water (O/W) emulsion. As a result of pancreatic lipolytic  
101 enzyme activity, dietary lipids are hydrolyzed, and the formed products are removed form the O/W  
102 surface. However, the presence of oxidized (phospho)lipids in the colloidal structures subjected to  
103 intestinal digestion can change the kinetics of lipolysis. So far, this topic has only been covered in few  
104 research studies. For example, Baba et al., observed that porcine pancreatic PLA<sub>2</sub> preferentially  
105 released 13(S)-hydroperoxy-(9Z,11E)-octadecadienoic acid from the *sn*-2 position of  
106 phosphatidylcholine (PC) molecule in the presence of sodium deoxycholate compared to non-oxidized  
107 PC species (Baba, Mikami, Shigeta, Nakajima, & Matsuo, 1993). In another study, the authors  
108 monitored the activity of porcine pancreatic PLA<sub>2</sub> towards UV-irradiated hen egg yolk PC species in  
109 PC/sodium deoxycholate mixed micelle structures compared to the non-irradiated sample (Litvinko,  
110 Skorostetskaya, & Gerlovsky, 2018). The obtained data revealed the increase in the rate of PLA<sub>2</sub>-  
111 catalyzed hydrolysis of PC fraction exposed up to 40 min to the UV-irradiation, while after this time the  
112 enzymatic activity declined. It was suggested that the presence of primary product of PC oxidation (PC  
113 hydroperoxides) lead to PLA<sub>2</sub> activation and efficient removal of oxidatively modified FA from the PC  
114 structure. After 40 min of PC irradiation, a significant increase in malondialdehyde (MDA) level was  
115 observed. Probably, MDA and other highly reactive secondary lipid oxidation products were  
116 responsible for the inhibition of PLA<sub>2</sub> activity (Litvinko et al., 2018).

117 As mentioned earlier, increased absorption and accumulation of oxidized lipids and products of their

118 decomposition, such as 4-hydroxy-2-alkenals, by IECs can contribute to the disruption of cellular  
119 redox homeostasis. However, exposure of human colon adenocarcinoma Caco-2/TC7 cells to a  
120 relatively low LOP concentration has been shown to induce cellular antioxidant systems including  
121 overexpression of gastrointestinal glutathione peroxidase 2 (GPx2) (Awada et al., 2012). This enzyme  
122 is responsible for detoxification and degradation of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and lipid hydroperoxides  
123 (LOOHs) to water and lipid hydroxides (LOH), respectively (Awada et al., 2012). Elevated LOP  
124 concentration and/or chronic cell exposure to these products can disrupt cellular antioxidant systems.  
125 The previous *in vivo* study in rats indicated that chronic exposure to subtoxic level of oxidized fish oil  
126 led to a decrease in the intestinal tissue glutathione (GSH) in favour of glutathione disulfide (GSSG)  
127 concentration (Tsunada et al., 2003). Moreover, the disturbance of intestinal GSH/GSSG ratio  
128 resulting from cell exposure to oxidized lipids, induced cellular transitions from proliferation to growth  
129 arrest and apoptosis (Tsunada et al., 2003). This example clearly indicated the effects of redox  
130 imbalance on intestinal cell dysfunction.

131 The main cellular structures that can be modified due to oxidative stress include membrane lipids,  
132 proteins and nucleic acids. Concerning ROS-induced modifications of (phospho)lipids, these include  
133 hydroxylation, peroxidation or carbonylation of unsaturated FAs (UFAs) as well as the cleavage of the  
134 C-C bond along FA acyl chain to form truncated (phospho)lipids (T. Y. Wang, Libardo, Angeles-Boza,  
135 & Pellois, 2017). Such oxidative alterations of UFAs present within membrane PLs prevent their  
136 immersion in the interior of the bilayer and lead to the translocation of oxidized FAs onto membrane  
137 surface in the form of so-called “whiskers” (Catalá, 2015). The accumulation of such aberrant  
138 structures can alter plasma, mitochondrial or ER membrane properties and thus their functions.  
139 Therefore, the modified membranes require reconstruction to maintain their original biophysical and  
140 functional properties. Oxidatively modified acyl chains of membrane PLs can be removed from the  
141 backbone upon hydrolysis catalyzed by intracellular or plasma phospholipases A<sub>2</sub> (PLA<sub>2</sub>) (Fruhwrth et  
142 al., 2007), followed by re-acylation of membrane PLs to restore their original structure. It has been  
143 suggested that the dynamic pool of undamaged UFAs for the membrane PL re-synthesis is provided  
144 by cellular TAGs stored in cytosolic lipid droplets (CLDs) (Girón-Calle, Schmid, & Schmid, 1997;  
145 Pamplona, 2008). The accumulation and FA composition of TAGs in CLDs strictly depend on the  
146 amount and the type of ingested lipids, especially in the case of IECs. The products of the intestinal  
147 lipolysis are re-synthesized to TAGs or PLs at the ER membrane after their absorption by enterocytes.

148 Next, in the ER lumen, the formed lipids are used for assembling of chylomicrons, which are finally  
149 secreted *via* lymphatic system to bloodstream (Beilstein, Carrière, Leturque, & Demignot, 2016). In  
150 order to avoid postprandial hypertriglyceridemia, the re-synthesized lipids may be temporary stored in  
151 the CLDs of enterocytes. In this period, enterocytes can hydrolyze esterified lipids and use the  
152 released FAs for their own needs e.g., energy production or membrane biosynthesis. In the  
153 interprandial phase, the storage lipids can be mobilized for chylomicron formation (Beilstein et al.,  
154 2016).

155 Another way of repairing damaged cellular membranes implies a direct incorporation of orally  
156 administrated PLs into cellular lipid bilayers. Such an approach, called lipid replacement therapy was  
157 proposed by Nicolson and involves a delivery of PLs to tissues and cells with limitations of their  
158 oxidation during the storage, ingestion, digestion and uptake as well as prevention of the intestinal  
159 hydrolysis of orally-administered PLs (Nicolson & Ash, 2014). Interestingly, some literature data  
160 indicated that even 20 % of dietary PLs can be absorbed by enterocytes in undigested form  
161 (Küllenberg de Gaudry et al., 2012) and potentially directly affect the membrane lipid remodelling.  
162 However, most of the consumed PLs are hydrolyzed in the small intestine by pancreatic  
163 phospholipase A<sub>2</sub> (PLA<sub>2</sub>) to lysophospholipids (LPLs) and FAs and then re-synthesized at the ER  
164 membrane.

165 Food-delivered PLs are known for their high nutritional value and beneficial health effects, not only as  
166 a source of essential FAs, but also due to their anti-inflammatory, antioxidant, antifibrogenic,  
167 membrane-protective and lipid-regulating properties (Blesso, 2015; Gundermann, Gundermann,  
168 Drozdziak, & Mohan Prasad, 2016; Küllenberg de Gaudry et al., 2012). However, PLs, especially those  
169 rich in PUFAs, are susceptible to oxidative modifications during the food processing and storage as  
170 well as the gastrointestinal digestion. Nevertheless, to date, data on the influence of PL oxidation on  
171 their intestinal digestion and the biological effect of the resulting products on IECs are limited.

172 Therefore, in our study, we proposed several research models to understand step-by-step the role of  
173 PL oxidation and intestinal hydrolysis on IEC physiology and pathophysiology. The aim of this study  
174 was threefold: (i) to evaluate the effect of lipoxygenase (LOX)-induced oxidative modification of egg  
175 yolk PL fraction on the parameters of colloidal structure found in the simulated *in vitro* intestinal fluid  
176 containing bile salts (BSs); (ii) to determine the effect of PL oxidation on the kinetics of their *in vitro*  
177 intestinal hydrolysis catalyzed by pancreatic PLA<sub>2</sub> in the presence of BSs, and (iii) to assess the

178 impact of native and oxidized PLs, both before and after *in vitro* intestinal hydrolysis, on HT29 cell  
179 physiology, serving as a model of intestinal tissue exposed to direct contact with ingested food  
180 ingredients. This cell line is a human intestinal epithelial cell line derived from human colorectal  
181 adenocarcinoma. However, HT29 cells express features of both absorptive enterocytes and intestinal  
182 secretory cells producing a gelatinous mucus-like substance (Fedi et al., 2021). Additionally, its  
183 differentiated phenotype is similar to small intestine enterocytes in terms of their structure and the  
184 presence of brush border-associated hydrolases (Martínez-Maqueda, Miralles, & Recio, 2015).  
185 Therefore, this cell line has been widely used in *in vitro* studies of cellular absorption, bioavailability  
186 and metabolism of food molecules (Martínez-Maqueda et al., 2015). Nevertheless, it should be noted  
187 that this cellular model has some disadvantage arising from cancerous and non-small intestinal origin,  
188 therefore differences in the gene expression of transporters and metabolic enzymes can be observed  
189 compare to the normal small intestinal cells (Fedi et al., 2021; Martínez-Maqueda et al., 2015).  
190 However, the aim of this research was to evaluate the effect of PL oxidation and hydrolysis on  
191 physiology of IECs and understanding the mechanism of toxicity of formed products, therefore we  
192 found this model to be appropriate.

## 193 **2. Materials and methods**

### 194 **2.1. Chemicals and materials**

195 Acetic acid, acetone, chloroform, hexane, methanol, ethanol (96 %), boric acid, disodium tetraborate  
196 decahydrate, sulphuric acid, hydrochloric acid (HCl), potassium iodine, sodium thiosulfate, sodium  
197 chloride (NaCl), calcium chloride (CaCl<sub>2</sub>), 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris-base), 0.1  
198 M sodium hydroxide solution (NaOH), ethylenediaminetetraacetic acid (EDTA) (all ACS grade) and  
199 acetonitrile (HPLC gradient grade) were purchased from Merck KGaA (Darmstadt, Germany).  
200 Ammonium formate (LC-MS grade) was obtained from VWR International (Leicestershire, UK).  
201 Lipoxidase from *Glycine max* (soybean) type I-B (LOX-1), sodium deoxycholate (≥98%), molybdenum  
202 (≥99.9), molybdenum (VI) oxide (≥99.9), fatty acid methyl ester (FAME) standards, trichloroacetic acid  
203 (TCA), thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), olive oil highly refined, bile salt (BS)  
204 mixture (United States Pharmacopeia Reference Standard), pancreatic phospholipase A<sub>2</sub> (PLA<sub>2</sub>),  
205 Varespladib (≥98%), thiazolyl blue tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), tablets of  
206 phosphate buffered saline (PBS), low melting point agarose (LMP agarose), Sybr Green I nucleic acid  
207 gel stain, Triton X100, formaldehyde, Nile Red were derived from Sigma-Aldrich, Merck KGaA

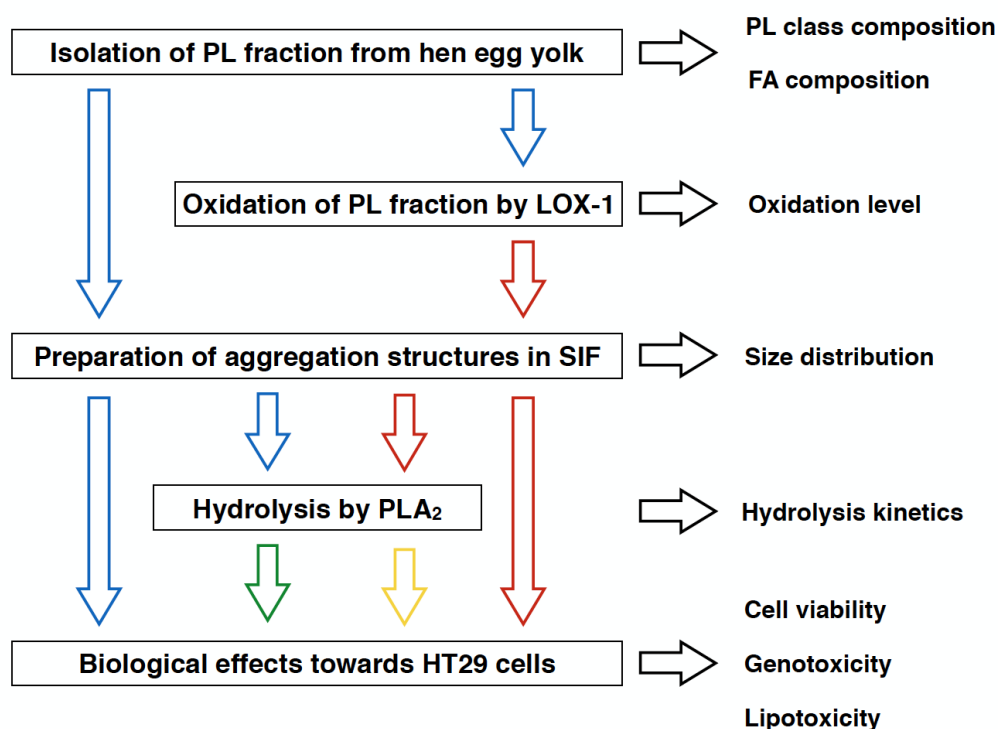
208 (Darmstadt, Germany). Normal melting point agarose (NMP agarose) was obtained from Bioline  
209 (London, United Kingdom). High purity water was produced using Millipore Elix 5 purification system  
210 (Darmstadt, Germany). Fresh free-range hen eggs with a mass in the range of 63-73 g were  
211 purchased in a local market (Gdansk, Poland).

## 212 **2.2. Study design**

213 PL fraction was isolated from fresh hen eggs and its composition was characterized by  
214 chromatography taking into account PL classes and FA composition (Fig. 1). In order to determine the  
215 effect of oxidative modification of PL on the kinetics of their hydrolysis and potential toxicity towards  
216 intestinal cells, part of the native PL fraction was enzymatically oxidized by LOX. The degree of PL  
217 oxidation was assessed by determination of peroxide value (PV) and thiobarbituric acid reactive  
218 substances (TBARS). In the next step, native and oxidized PLs were used to prepare aggregation  
219 structures (mixed micelles solutions and emulsions) in simulated intestinal fluid (SIF) containing BS  
220 whose concentration matched physiological levels. The BS concentration in the prepared solutions  
221 was calculated based the average molecular mass of commercially available BS mixture (c.a. 519.0  
222 g/mol) and was about 9.6 mM. While the physiological BS concentration in the postprandial state  
223 range from 4 – 20 mM (Maldonado-Valderrama, Wilde, Maclerzanka, & MacKie, 2011). The obtained  
224 aggregation structures were characterized in terms of size distribution and dispersity and then  
225 subjected to *in vitro* intestinal digestion. The kinetics of PL hydrolysis catalyzed by porcine pancreatic  
226 PLA<sub>2</sub> was monitored using pH-stat titration (pH=8). The samples of native and oxidized PL/BS mixed  
227 micelle solutions before and after hydrolysis were then used for comparisons of their biological effects.  
228 One of the cell lines recommended by the Replacement, Refinement and Reduction of Animals in  
229 Research for nutritional studies is HT29 (human colon adenocarcinoma) cell line, which was employed  
230 in this study (Krishna & Gopalakrishnan, 2016). The PL/BS mixed micelle solutions were added to cell  
231 culture medium in the amount of 10 % v/v. The impact of PL oxidation and hydrolysis on cell  
232 physiology was evaluated by determination of cell viability by MTT test, genotoxicity by comet assay  
233 and ability of cells to neutral lipid accumulation using fluorescence microscopy.







234

235 **Fig. 1.** Study design. (Abbreviations: FA – fatty acid; LOX-1 – lipoxygenase type 1; PL – phospholipid;  
 236 PLA<sub>2</sub> – phospholipase A<sub>2</sub>; SIF – simulated intestinal fluid)

### 237 2.3. Isolation of hen egg yolk PL fraction

238 Hen egg yolk PL fraction was isolated as described previously by Gładkowski et al. (Gładkowski,  
 239 Chojnacka, Kielbowicz, Trziszka, & Wawrzeńczyk, 2012) with further modifications (Parchem,  
 240 Kusznierewicz, Chmiel, Maciołek, & Bartoszek, 2019). Briefly, two egg yolks were combined with 120  
 241 mL of methanol, stirred for 10 min and left at room temperature until the alcohol-insoluble fraction  
 242 precipitated. Then, the supernatant was filtered and the precipitate was re-extracted twice with 120 mL  
 243 of methanol. The extracts were combined and the solvent was evaporated under reduced pressure at  
 244 40 °C using a rotary evaporator. The residue was dissolved in 20 mL of hexane and transferred to a  
 245 glass flask placed in an ice bath. Afterwards, under constant stirring, 120 mL of cold acetone at –20  
 246 °C was carefully added until PL fraction precipitated. The precipitate was then carefully washed 3  
 247 times with 40 mL of cold acetone at –20 °C and the solvent was removed each time by decantation.  
 248 Finally, the PL fraction was dried in a stream of nitrogen, frozen overnight at –20 °C and freeze-dried  
 249 for 48 h using laboratory freeze dryer Alpha 1-4 (Christ, Germany). The samples were stored at –80  
 250 °C in nitrogen atmosphere until used for experiments.

### 251 2.4. Lipoxygenase-catalyzed oxidation of egg yolk PLs

252 The enzymatic peroxidation was carried out as described previously by Butler O'Connor et al. (Butler  
253 O'Connor et al., 2010) with further modifications (Parchem, Kusznierevicz, et al., 2019). The  
254 methanolic solution of egg yolk PL fraction (20 mg/mL) was perpetrated in a round bottom flask.  
255 Methanol was evaporated under reduced pressure at 40 °C using a rotary evaporator and the PL  
256 fraction was dried at room temperature for 1 h in a stream of nitrogen. The obtained PL film was then  
257 hydrated with borate buffer (0.2 M, pH=9) containing 10 mM sodium deoxycholate. Prior to the  
258 addition of bile salt, the buffer was saturated with oxygen at room temperature for 30 min. Enzymatic  
259 peroxidation of PL was initiated by adding freshly prepared soybean lipoxidase type 1 (LOX-1) solution  
260 in the borate buffer. The final PL concentration was 5 mg/mL and the LOX-1 activity was 70 kU/mL.  
261 The reaction was carried out at 37 °C under oxygen atmosphere for 1 h. The peroxidation was  
262 stopped by the addition of chloroform/methanol mixture (2:1 v/v) in a volume equal to the volume of  
263 the reaction mixture. After intense shaking, the mixture was distributed to glass tubes and centrifuged  
264 (1600 × g, 5 min). The upper organic layer was transferred with a glass Pasteur pipettes to a round  
265 bottom flask. Then, the solvent was evaporated under a stream of nitrogen at 40 °C, frozen overnight  
266 at -20 °C, followed by freeze-drying for 48 h using laboratory freeze dryer Alpha 1-4 (Christ,  
267 Germany). The samples were stored at -80 °C in nitrogen atmosphere for further experiments.

## 268 **2.5. Characterization of egg yolk PL fraction**

### 269 **2.5.1. Chromatographic analysis of PL classes**

270 Hen egg yolk PL classes were analyzed using hydrophilic interaction liquid chromatography (HILIC) as  
271 described previously by Lisa et al. (Lísa, Cífková, & Holčápek, 2011) with further modifications  
272 (Parchem, Kusznierevicz, et al., 2019). The separation of individual PL classes was performed using  
273 a Dionex UltiMate 3000 LC system (Thermo Scientific, USA) equipped with a Rx-SIL column (250 ×  
274 4.5 mm, 5 µm) (Agilent Technology, USA). The mobile phase consisted of acetonitrile (A) and 5 mM  
275 aqueous ammonium formate (B). The gradient program was as follows: 0 – 30 min, 6 – 77 % B, 30 –  
276 35 min, 77 % B, finally the column was re-equilibrated to the initial conditions for 20 min. The  
277 separation was carried out at of 40 °C with a flow rate of 1 mL/min. The injection volume was 20 µL.  
278 The eluted PL classes were monitored using a Corona Ultra RS CAD (Thermo Scientific, USA) with  
279 following parameters: acquisition range of 100 pA, digital filter set to “none”, gas flow (air) of 12 L/min  
280 and nebulizer pressure of 35 psi. Individual PL classes were identified by comparison of their retention  
281 times with those of standards (more details: (Parchem, Kusznierevicz, et al., 2019)). Results are

282 expressed as a mean of three independent determinations of the percentage of the individual PL  
283 classes in total PLs identified in the sample.

### 284 **2.5.2. Analysis of FA composition in the structure of PC and PE fractions**

285 The methanolic solution of PLs (20 mg/mL) in a volume of 100  $\mu$ L was applied onto a preparative TLC  
286 glass silica gel plate (20  $\times$  20 cm, 1 mm, 10-12  $\mu$ m) (Merck, Germany) as a 20 cm band using a hand-  
287 held applicator equipped with a 100  $\mu$ L syringe. PL classes were separated with  
288 chloroform/methanol/acetic acid/water mixture (35:25:4:14:2 v/v/v/v) (Palusinska-Szyszk, Kania,  
289 Turska-Szewczuk, & Danikiewicz, 2014). After separation, the plate was dried using a stream of  
290 nitrogen and sprayed with a molybdenum reagent (Dittmer & Lester, 1964) over a width of 2 cm in  
291 order to locate individual PL classes. Then, silica gel from areas corresponding to the two main PL  
292 classes present in egg yolk (PCs and phosphatidylethanolamines (Pes)) was scraped off from the  
293 non-sprayed part of the plate using metal spatula. PC and PE fractions were extracted from the  
294 appropriate portions of silica gel using 10 mL of chloroform/methanol (2:1 v/v) mixture. The extraction  
295 was repeated twice. The obtained extracts corresponding to individual PL classes were combined and  
296 the solvent was evaporated under reduced pressure at 40  $^{\circ}$ C using rotary evaporator.

297 Fats present in the structure of PCs and Pes were esterified to fatty acid methyl esters (FAMES)  
298 according to the European Standard [EN:ISO 5509:2000]. FAMES were analyzed using high-  
299 performance gas chromatography (HP-GC). The separation of FAMES was performed using Perkin  
300 Elmer Autosystem XL gas chromatograph (Perkin Elmer, USA) equipped with split injector 60:1 and  
301 CP-Sil 88 column (50 m  $\times$  0,25 mm  $\times$  0,2  $\mu$ m, Agilent Technology, USA). FAMES were detected using  
302 flame ionization detector (FID). The injector and FID temperatures were maintained at 250  $^{\circ}$ C. The  
303 initial column temperature was 170  $^{\circ}$ C and held for 30 min, followed by an increase of 10  $^{\circ}$ C/min up to  
304 200  $^{\circ}$ C and held for 15 min. Finally, the temperature was raised to 210  $^{\circ}$ C at a rate of 5  $^{\circ}$ C/min and  
305 held for 10 min. Qualitative and quantitative analyses of Fats in the samples were performed using  
306 FAME standards. Results are expressed as means of three independent determinations of the  
307 percentage of the individual Fats in total Fats identified in the sample.

### 308 **2.5.3. Determination of peroxide value of PL fraction**

309 The peroxide value (PV) of native and oxidized PL fractions was determined according to the method  
310 described previously (Fruehwirth et al., 2020) with some modifications. The appropriate amount of PL

311 fraction (1 g and 50 mg for native and oxidized PL fraction, respectively) was dissolved in 5 mL of  
312 acetic acid/chloroform (3:2 v/v) mixture. The differences in weighted amounts between the native and  
313 oxidized samples resulted from a significant difference in the content of PL hydroperoxides. Therefore,  
314 the sample amount was optimized such that the volume of titrant added was between 2 and 18 mL  
315 using a 20 mL burette. Then, 200  $\mu$ L of a saturated potassium iodide solution was added, the sample  
316 was shaken for 1 min and placed in darkness for 5 min. Afterwards, 15 mL of distilled water and few  
317 droplets of 1 % w/w starch solution were added. The samples were titrated using 0.002 N sodium  
318 thiosulfate solution until complete discoloration was reached. The blank test was performed without PL  
319 fraction. The samples were analyzed in triplicate.

#### 320 **2.5.4. Determination of TBARS content**

321 The amount of thiobarbituric acid reactive substances (TBARS) in native and oxidized PL fraction was  
322 determined by method described by Fenaille et al. (Fenaille, Mottier, Turesky, Ali, & Guy, 2001) with  
323 modifications. In brief, 1 mL of aqueous solutions of PL fraction (10 and 2.5 mg/mL for native and  
324 oxidized PL fraction, respectively) was combined with 1 mL of reaction mixture containing equal  
325 volumes of 15 % trichloroacetic acid (TCA), 0.25 M HCl and 0.375 % thiobarbituric acid (TBA),  
326 followed by the addition of 0.1 mL of 0.4% solution of butylated hydroxytoluene (BHT) in 96% ethanol.  
327 The differences in weighted amounts between the native and oxidized samples resulted from a  
328 significant difference in the content TBARS. Therefore, the sample amount was optimized such that  
329 the absorbance was below 1.5 as recommended by manufacturer. The sample was shaken for 1 min  
330 and then centrifuged ( $4700 \times g$ , 5 min). The supernatant was collected to the new tube, while the  
331 precipitate was mixed with another 1 mL of reaction mixture, shaken and centrifuged. The combined  
332 supernatants were incubated at 95 °C under nitrogen atmosphere for 30 min. Then, the reaction was  
333 stopped by cooling the sample in an ice bath. The mixture was made up to 12 mL with distilled water  
334 and centrifuged ( $4700 \times g$ , 5 min). The absorbance of the supernatant was measured at wavelengths  
335 of 532 and 600 nm as a background. The TBARS content was calculated based on molar extinction  
336 coefficient ( $\epsilon$ ) for MDA-TBA<sub>2</sub> adduct ( $1.55 \times 10^5 \text{ M}^{-1} \times \text{cm}^{-1}$ ) (Meca et al., 2021). The samples were  
337 analyzed in triplicate.

#### 338 **2.6. Preparation and characterization of PL/BS mixed micelles and PL/BS/TAG emulsions**

339 The PL/BS mixed micelle solution was prepared by dispersing 1 % w/v egg yolk PL fractions in the  
340 simulated intestinal fluid (SIF) containing 150 mM NaCl, 6 mM CaCl<sub>2</sub>, 0.28 mM Tris-base and 5 mg/mL  
341 of BS mixture. The composition of used BS mixture is shown in Supplementary Materials (Table A1).  
342 The solution was then sonicated on ice (20 kHz, 4 min) to reduce the particle size and to homogenize  
343 the mixed micelles using Vibra-Cell VC 750 ultrasound sources fitted with a 3 mm diameter titanium  
344 micro-tip VCX500 (Sonics, USA). Power delivery was controlled as amplitude percentage, which was  
345 set as 40 %. A pulsed duty cycle of 8 sec on and 2 sec off was used for all experiments. The  
346 PL/BS/TAG emulsion was prepared by dispersing 1 % w/v egg yolk PL fractions and 1.5 % w/v highly  
347 refined olive oil in SIF. The solution was then sonicated using the conditions outlined above.

348 The mixed micelle and emulsion droplet size distribution and dispersity (polydispersity index, PDI)  
349 were determined using a non-invasive black scatter (NIBS) technique (Kupper, Kłosowska-  
350 Chomiczewska, & Szumala, 2017). For this purpose, a Zetasizer Nano ZS (Malvern, United Kingdom)  
351 equipped with a helium-neon laser diffraction operating at 633 nm was employed. For the mixed  
352 micelles, the samples were analyzed directly without dilution, while the emulsion samples were diluted  
353 with the buffer (150 mM NaCl, 6 mM CaCl<sub>2</sub>, 0.28 mM Tris base, pH=8) in a ratio of 1:1000. All  
354 measurements were performed at 37 °C. The samples were analyzed in quintuplicate.

### 355 **2.7. *In vitro* intestinal PL hydrolysis**

356 Four digestive systems, consisting of native or oxidized PL fraction without or with TAGs, have been  
357 proposed to understand the effect of PL oxidation and the aggregation structure in which PL are  
358 embedded on kinetics of their hydrolysis catalyzed by PLA<sub>2</sub>. The kinetics of PL hydrolysis was  
359 monitored using a pH-stat method, in which enzymatically released FAs were continually titrated with  
360 0.1 M NaOH to keep the pH constant at 8.0. The mixed micelle solution or emulsion in the volume of  
361 15 mL was placed in a titration vessel of the T70 titrator (Mettler Toledo, USA). The sample was pre-  
362 incubated at 37 °C for 15 min until the temperature equilibration and then pH value was adjusted to  
363 8.0 using 0.1 M NaOH and 0.1 M HCl. The reaction was initiated by the addition of porcine pancreatic  
364 phospholipase A<sub>2</sub> (PLA<sub>2</sub>) to reach the final activity of 35 U/mL. The PL hydrolysis was carried out at 37  
365 °C with continuous stirring (40 % of the maximum stirring speed). Unless otherwise stated, the  
366 hydrolysis reaction was carried out for 1 h. Hydrolysis reactions were carried out in triplicate for each  
367 sample type.

## 368 **2.8. PLA<sub>2</sub> activity in the presence of aldehydes**

369 The native PL/BS mixed micelles solution was prepared as described in section 2.6. The samples was  
370 placed in the titration vessel of the T70 titrator (Mettler Toledo, USA) and pre-incubated at 37 °C for 15  
371 min. Then, appropriate volumes of low-molecular aldehydes (hexanal or €-2-nonenal), corresponding  
372 to the following final concentrations: 0, 5, 10, 15, 20 mM, were added. For this range, a negative linear  
373 correlation was observed between the concentration of tested aldehydes and the PLA<sub>2</sub> activity, which  
374 allowed for determination of IC<sub>10</sub> and IC<sub>30</sub> values. The pH value was adjusted to 8.0 followed by  
375 addition of porcine pancreatic PLA<sub>2</sub>) with the final activity of 30 U/mL. PLA<sub>2</sub> activity was calculated  
376 based on the volume of 0.1 M NaOH added after 20 min from the start of the reaction according to the  
377 Eq. 1.

$$378 \text{ PLA}_2 \text{ activity} = (V_{\text{NaOH}} \times C_{\text{NaOH}}) / t \text{ (Eq.1)}$$

379 where:

380 *PLA<sub>2</sub> activity* – phospholipase A<sub>2</sub> activity [mol of FA released/min],

381 *V<sub>NaOH</sub>* – volume of consumed NaOH solution [dm<sup>3</sup>],

382 *C<sub>NaOH</sub>* – concentration of used NaOH solution [mol/dm<sup>3</sup>],

383 *t* – hydrolysis time [min].

384 Hydrolysis reactions were carried out in triplicate for each concentration points for both aldehydes.

## 385 **2.9. Cell culture**

386 HT29 (human colon adenocarcinoma) cell line was purchased from American Type Culture Collection  
387 (ATCC). The HT29 cells were maintained in the McCoy's 5A medium supplemented with fetal bovine  
388 serum (100 mL/L) and antibiotics: penicillin (100 g/L) and streptomycin (100 U/mL). All reagents for  
389 cell culture were purchases from Sigma-Aldrich, Merck KgaA (Darmstadt, Germany). The cells were  
390 cultured at 37 °C under humidified atmosphere containing 5% CO<sub>2</sub> in a cell incubator (Heal Force,  
391 China). For the experiments, the cells derived from passages between 6 and 11 were used. The cells  
392 were regularly tested for mycoplasma contamination using Universal Mycoplasma Detection Kit from  
393 ATCC.

### 394 **2.9.1. Preparation of native and oxidized PL/BS mixed micelle solution for cell treatment**

395 The solutions of native and oxidized PL in SIF (native and oxidized PL/BL mixed micelles) were  
396 prepared just before cell treatment. In the case of non-hydrolyzed PL, the samples were incubated in  
397 37 °C without addition of PLA<sub>2</sub>, while the digested samples were subjected to hydrolysis catalyzed by  
398 PLA<sub>2</sub> (Section 2.7). For the cell treatment purposes, the hydrolysis reaction was stopped after  
399 releasing 40 % of the Fas present at the *sn*-2 position of glycerol backbone. Based on the hydrolysis  
400 reaction stoichiometry and taking into account the average molecular mass of egg yolk PL (c.a. 752.3  
401 g/mol), the mentioned PL hydrolysis degree was achieved after adding 0.8 mL of 0.1 M NaOH solution  
402 during pH-stat titration. The PL hydrolysis was terminated by the addition of secretory PLA<sub>2</sub> inhibitor –  
403 Varespladib (Dennis, Cao, Hsu, Magrioti, & Kokotos, 2011). Based on the preliminary study,  
404 Varespladib at the concentration of 5 µM was found to be appropriate for the complete inhibition of  
405 porcine pancreatic PLA<sub>2</sub>. For this purpose, 15 µL of 5 mM Varespladib dissolved in DMSO was added  
406 to 15 mL of digestion mixture. To eliminate the differences between samples, this inhibitor was added  
407 to non-hydrolyzed samples as well. Additionally, the effect of Varespladib alone on the viability of  
408 HT29 cells was determined. The cells were treated with inhibitor at the concentration of 0.5 µM (the  
409 final concentration in cell culture medium after addition digestion mixtures in the proportion 9:1. The  
410 treatment was carried out for 6, 24, 48 and 72 h. To obtain the PL solutions with different  
411 concentrations, non-hydrolyzed and hydrolyzed samples were diluted using SIF (150 mM NaCl, 6 mM  
412 CaCl<sub>2</sub>, 0.28 mM Tris-base, 5 mg/mL BS, pH=8) containing 5 µM Varespladib. The final concentrations  
413 of PLs and products of their hydrolysis (LPLs and Fas) in individual samples are shown in  
414 **Supplementary Materials (Table A2).**

#### 415 **2.9.2. Determination of cytotoxicity towards HT29 cells by MTT test**

416 To determined the effect of native and oxidized PL before and after hydrolysis on cell viability, the  
417 MTT test was applied as described previously (Baranowska et al., 2018, 2020, 2021). In brief, the  
418 exponentially growing HT29 cells were seeded in 96-well cell culture plates (5 × 10<sup>3</sup> cells per well in  
419 180 µL of medium) and were allowed to settle for 24 h at 37 °C under 5 % CO<sub>2</sub> atmosphere. Then, the  
420 cells were treated for 6, 24, 48 and 72 h with 20 µL of different concentrations of native or oxidized PL,  
421 before or after *in vitro* intestinal hydrolysis (Table A2). Control cells were treated with the buffer (150  
422 mM NaCl, 6 mM CaCl<sub>2</sub>, 0.28 mM Tris-base, pH=8) or SIF (containing 5 mg/mL of BS mixture). After 6,  
423 24, and 48 h of exposure, the medium was aspirated from the wells and replaced with 200 µL of fresh  
424 medium. Then, the cells were incubated at 37 °C under 5 % CO<sub>2</sub> until 72 h of the total incubation time.

425 For the 72 h exposure, the medium was replaced for fresh one at the endpoint. After treatment, 50  $\mu$ L  
426 of MTT solution (4 g/L) was added to all wells and the cells were incubated for another 4 h at 37 °C  
427 under 5 % CO<sub>2</sub>. Finally, the medium was carefully removed from wells and the formazan crystals  
428 formed by metabolically active cells were dissolved in 50  $\mu$ L of DMSO. The absorbance of the  
429 obtained solutions was measured at wavelength of 540 nm and a reference wavelength 690 nm using  
430 TECAN Infinite M200 plate reader (Tecan Group Ltd., Switzerland). The differences in absorbance  
431 between formazan specific A<sub>540</sub> and the background A<sub>690</sub> for individual wells were used for further  
432 calculations. Cell treatments were performed as four technical repetitions and three independent  
433 repetitions. Cytotoxic effect of tested lipids was expressed as per cent of cell viability compared to  
434 control cells treated for 6, 24, 48 or 72 h with 20  $\mu$ L of the buffer (150 mM NaCl, 6 mM CaCl<sub>2</sub>, 0.28 mM  
435 Tris-base, pH=8) containing 5  $\mu$ M Varespladib (final concentration in culture medium was 0.5  $\mu$ M),  
436 whose viability was regarded as 100 %. The second type of control constituted cells treated for 6, 24,  
437 48 or 72 h with 20  $\mu$ L of SIF with 5 mg/mL of BS mixture containing 5  $\mu$ M Varespladib. Results are  
438 expressed as means of three independent experiments carried out as four technical replicates.

### 439 **2.9.3. Determination of genotoxic effect by comet assay**

440 To determine the genotoxic effect of native and oxidized PL before and after hydrolysis in HT29 cells,  
441 the comet assay was applied as described previously (Baranowska et al., 2018, 2020, 2021). Briefly,  
442 the exponentially growing HT29 cells were seeded in 24-well cell culture plates (10<sup>5</sup> cells per well in  
443 1.8 mL of medium) and were allowed to settle for 24 h at 37 °C under 5 % CO<sub>2</sub> atmosphere. Then, the  
444 cells were treated for 24 h with 200  $\mu$ L of two concentrations of native or oxidized PL, before or after *in*  
445 *vitro* intestinal hydrolysis (125 and 250  $\mu$ g/mL) (Table A2). After exposure, the medium was aspirated  
446 from the wells and the cells were washed with 0.5 mL of cold PBS, followed by cell detachment with  
447 0.2 mL of trypsin solution (0.5 g/L). The addition of 1.8 mL of fresh medium to wells stopped the  
448 enzymatic activity of trypsin. Then, 1 mL portions of the cell suspensions were transferred into 1.5 mL  
449 tubes and centrifuged (100  $\times$  g, 5 min, 4 °C). The cell pellets were washed with 1 mL of cold PBS and  
450 centrifuged again. PBS was discarded and the cells were resuspended in 150  $\mu$ L of 0.5 % low-melting  
451 point (LMP) agarose. Then, 40  $\mu$ L of the cell suspension was placed as two spots on a microscope  
452 slides covered with normal-melting point (NMP) agarose. The slides were covered with coverslips and  
453 placed on an ice-cold tray for at least 5 min to solidify the agarose. After overnight lysis in a high salt  
454 alkaline buffer (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 1 % Triton X100, pH 10), the slides were placed



455 on a Bio-Rad subcell GT electrophoresis platform (Bio-Rad UK), covered with cold electrophoresis  
456 buffer (0.3 M NaOH, 1 mM EDTA, pH 13) and chromatin was allowed to unwind for 25 min. Then, the  
457 electrophoresis was conducted at 26 V (0.75 V/cm) and 300 mA for 30 min in darkness at 4-8 °C. The  
458 slides were transferred to the neutralizing buffer (0.4 M Tris, pH 7.5) for 5 min. This step was repeated  
459 twice, then the slides were washed using distilled water and fixed in 70 % ethanol. The DNA was  
460 stained using SybrGreen in TE buffer (0.1 Tris-base, 1 mM 1 mM EDTA, pH 10) for 30 min. After  
461 staining, the slides were washed in distilled water for 5 min. Finally, the DNA comets were analyzed  
462 using fluorescence microscopy (Zeiss ImagerZ2, USA) coupled with a computerized slide scanning  
463 system (Metafer4, Germany). Comet analysis involved counting 200 consecutive nuclei per sample.  
464 Three slides with two repetitions on each were prepared for each concentration of tested lipid mixture.  
465 Genotoxicity of analyzed samples was expressed as the % of DNA in the comet tail relative to total  
466 cellular DNA. The control cells were treated for 24 h with 200 µL of the buffer (150 mM NaCl, 6 mM  
467 CaCl<sub>2</sub>, 0.28 mM Tris-base, pH=8) containing 5 µM Varespladib (final concentration in culture medium  
468 was 0.5 µM) or SIF with 5 mg/mL of BS mixture containing 5 µM Varespladib. As the positive control  
469 served the cells exposed to 150 µM hydrogen peroxide for 1 h.

#### 470 **2.9.4. Visualization of cytosolic lipid droplets**

471 To determine the ability of HT29 cells to accumulate neutral lipids, the cytosolic lipid droplets (CLDs)  
472 were visualized according to the method described earlier (Listenberger & Brown, 2007). The  
473 exponentially growing cells were seeded in 6-well cell culture plates (4 × 10<sup>5</sup> cells per well in 3.6 mL of  
474 medium) on the bottom of which, the sterile 22 × 22 mm coverslips were placed beforehand. The cells  
475 were allowed to settle for 24 h at 37 °C under 5 % CO<sub>2</sub> atmosphere. Then, the cells were treated for  
476 24 h with 400 µL of 125 µg/mL test solutions containing native or oxidized PL, before or after *in vitro*  
477 intestinal hydrolysis (Table A2). After incubation time, the medium was aspirated from the wells and  
478 the cells were washed twice with 2 mL of cold PBS. Then, 2 mL of 4 % formaldehyde in PBS was  
479 added to wells and the cells were fixed for 30 min at room temperature. Next, the cells were washed  
480 twice with 2 mL of PBS. Immediately before CLD staining, 1 µL of Nile Red stock solution (1 mg/mL) in  
481 DMSO was diluted in 10 mL of 150 mM NaCl solution. Next, 1 mL of diluted Nile Red solution was  
482 added to all wells. The plates were kept in darkness for 10 min in room temperature, followed by cell  
483 washing three times with 2 mL of PBS each. In the next step, the coverslips were mounted onto glass  
484 slides using 30 µL of ProLong™ Diamond antifade mountant. Finally, the cytosolic lipid droplets were

485 visualized using fluorescence microscopy (Zeiss ImagerZ2, USA). The control cells were treated for  
486 24 h with 400  $\mu$ L of the buffer (150 mM NaCl, 6 mM CaCl<sub>2</sub>, 0.28 mM Tris-base, pH=8) containing 5  $\mu$ M  
487 Varespladib (final concentration in culture medium was 0.5  $\mu$ M) or SIF with 5 mg/mL of BS mixture  
488 containing 5  $\mu$ M Varespladib.

## 489 2.10. Statistical analysis

490 Results are expressed as means  $\pm$  standard deviation (SD) values of three independent experiments  
491 unless otherwise stated. An unpaired Student's t-test ( $p \leq 0.05$ ) was used to assess the statistical  
492 significance of FA composition in PC and PE fractions, PVs and TBARS contents in native and  
493 oxidized PL fractions. The statistical significance of the PLA<sub>2</sub>-catalysed PL hydrolysis rate in different  
494 systems and Comet assay results was examined using one-way ANOVA with Tukey's post hoc test.  
495 The statistical analysis was performed using the Prism 6.0 software package (GraphPad Software,  
496 Inc., USA).

## 497 3. Results

### 498 3.1. Characterization of native and oxidized egg yolk PL fractions

#### 499 3.1.1. PL class composition

500 A high purity PL fraction was obtained from hen egg yolk by methanol extraction followed by  
501 precipitation of PLs in acetone cooled to  $-20$  °C. The obtained PL fraction contained approximately 5  
502 % of non-PL compounds, such as TAGs, Fas and cholesterol, based on total peak areas determined  
503 during HILIC-CAD analysis. The same technique confirmed that the most abundant PL classes in hen  
504 egg yolk were PCs and Pes, whose amounts were 52.9 % and 30.4 % of total PLs, respectively (Table  
505 1). Other PL classes identified were phosphatidylinositols (Pis) and phosphatidylglycerols (PGs),  
506 constituting approximately 5 % of total PLs. LPLs, namely lysophosphatidylcholines (LPCs) and  
507 lysophosphatidylethanolamines (LPEs) abundance amounted to 2.0 % and 1.5 % of total PLs,  
508 respectively. Additionally, sphingomyelins (SMs) were detected in hen egg yolk in the amount of 2.7 %  
509 of total PLs.

510 **Table 1. Composition of PL classes present in PL fraction isolated from hen egg yolk.**

PL class (abbreviation)	Composition [% of total peak areas]*
Lysophosphatidylethanolamines (LPEs)	1.54 $\pm$ 0.21
Lysophosphatidylcholines (LPCs)	2.03 $\pm$ 0.14
Phosphatidylethanolamines (PEs)	30.4 $\pm$ 0.6
Phosphatidylglycerols (PGs)	5.08 $\pm$ 0.42

Phosphatidylinositols (PIs)	5.32 ± 0.11
Phosphatidylcholines (PCs)	52.9 ± 0.5
Sphingomyelins (SMs)	2.68 ± 0.18

511 \* Results are the means of three independent analyses (n=3) ± standard deviation (SD) values.

512 Abbreviation: PL – phospholipid.

### 513 3.1.2. FA composition in PC and PE fractions

514 For a deeper structural analysis of hen egg yolk PLs, the FA composition in the two main PL classes  
515 (PCs and PEs), representing over 80 % of total PLs, was determined. For this purpose, the PC and  
516 PE classes were separated and isolated by means of preparative TLC, derivatized to FAMES, and  
517 then the FA compositions in each class were determined using GC-FID technique. The results showed  
518 statistically significant differences in FA composition between the two analyzed fractions. Saturated  
519 FAs (SFAs) accounted for approximately 48 % and 53 % of total FA peak areas in the PC and PE  
520 classes, respectively (Table 2). The most abundant FAs in PC species were palmitic acid (16:0) and  
521 stearic acid (18:0), whose amounts were 30.3 % and 17.2 % of total FAs, respectively. In contrast, PE  
522 fraction was characterised by higher content of stearic acid and lower of palmitic acid. Additionally, in  
523 both fractions, myristic (14:0), arachidic (20:0) and behenic (22:0) acids were identified, however, their  
524 concentrations were below 1 % of total FAs identified in hen egg yolk PLs.

526 Among the UFAs, in both PC and PE fractions, oleic acid (18:1; n-9) was the most abundant (Table 2).  
527 Another notable UFA present in both fractions at significant level was linoleic acid (18:2; n-6), whose  
528 concentrations were around 17.4 % and 11.7 % of total FAs, respectively for PC and PE species.

529 Substantial difference between the two fractions was observed for arachidonic (20:4; n-6), and  
530 docosahexaenoic acid (22:6; n-3), both of which were more abundant in PE species. In addition, in the  
531 structures of both classes, palmitoleic (16:1) and α-linolenic (18:3) were found.

532 **Table 2. Composition of FAs build-in the structure of PC and PE species.**

FA	FA abbreviation	PC fraction	PE fraction
<b>Saturated fatty acids (SFAs) [% of total peak areas]*</b>			
Myristic	14:0	0.14 ± 0.02 <sup>a</sup>	0.41 ± 0.03 <sup>b</sup>
Palmitic	16:0	30.3 ± 0.3 <sup>a</sup>	18.4 ± 0.8 <sup>b</sup>
Stearic	18:0	17.2 ± 1.8 <sup>a</sup>	33.6 ± 2.2 <sup>b</sup>
Arachidic	20:0	0.14 ± 0.01 <sup>a</sup>	0.28 ± 0.01 <sup>b</sup>
Behenic	22:0	0.21 ± 0.01 <sup>a</sup>	0.56 ± 0.03 <sup>b</sup>
<b>Total SFAs</b>		<b>48.0 ± 1.5<sup>a</sup></b>	<b>53.2 ± 1.5<sup>b</sup></b>
<b>Unsaturated fatty acids (UFAs) [%of total peak areas]*</b>			
Palmitoleic	16:1 (n-7)	0.63 ± 0.01 <sup>a</sup>	0.19 ± 0.01 <sup>b</sup>

Oleic	18:1 (n-9)	27.6 ± 0.3 <sup>a</sup>	18.4 ± 0.8 <sup>b</sup>
Linoleic	18:2 (n-6)	17.4 ± 1.7 <sup>a</sup>	11.7 ± 0.3 <sup>b</sup>
α-Linolenic	18:3 (n-3)	0.12 ± 0.02 <sup>a</sup>	0.11 ± 0.01 <sup>a</sup>
Arachidonic	20:4 (n-6)	4.1 ± 0.7 <sup>a</sup>	12.0 ± 0.6 <sup>b</sup>
Docosahexaenoic	22:6 (n-3)	1.7 ± 0.4 <sup>a</sup>	3.9 ± 0.2 <sup>b</sup>
<b>Total UFAs</b>		<b>51.6 ± 1.8<sup>a</sup></b>	<b>46.4 ± 1.5<sup>b</sup></b>

533 \* Results are the average of three independent analyses (n=3) ± standard deviation (SD) values.  
 534 Different letters indicate significantly different values examined using unpaired Student's test (p ≤  
 535 0.05).

536 Abbreviations: FA – fatty acid; PC – phosphatidylcholine; PE – phosphatidylethanolamine; SFA –  
 537 saturated fatty acid; UFA – unsaturated fatty acid.

### 538 3.1.3. Oxidation level of PL fractions

539 Hen egg yolk PLs were oxidized enzymatically in the presence of sodium deoxycholate using soybean  
 540 LOX-1. Then, PL oxidation level was estimated using PV and TBARS assays. The first one provides  
 541 quantitative information on the content of primary lipid oxidation products – lipid hydroperoxides. The  
 542 other allows to monitor decomposition of lipid peroxidation products. As a result of lipid  
 543 hydroperoxides decomposition by the C-C bond cleavage, truncated lipids and low-molecular weight  
 544 volatile aldehydes such as 4-hydroxyhexanal (4-HHE), 4-hydroxynonenal (4-HNE) or MDA are formed.  
 545 In acidic environment and increased temperature, MDA reacts with thiobarbituric acid (TBA) leading to  
 546 the formation of MDA-TBA<sub>2</sub> adduct that can be quantified. Unfortunately, some other fatty peroxide-  
 547 derived breakdown products may also react with TBA, giving rise increased result due to false TBA-  
 548 positive substances (Catalán, Frühbeck, & Gómez-Ambrosi, 2018). The mentioned-above methods  
 549 were employed for the characterization of PL fraction, both before and after LOX-catalyzed PL  
 550 oxidation.

552 The enzymatic oxidation catalyzed by LOX is known to cause oxidative modification of UFAs.  
 553 Accordingly, PV determined for FAs present in the structures of hen egg yolk PLs was statistically  
 554 significantly higher (p ≤ 0.0001) compared to native PL fraction (Table 3). In general, PV of fresh lipid  
 555 fraction (e.g., edible oils) should be below 10 *mEq O<sub>2</sub>/kg* (Gilbraith, Carter, Adams, Booksh, &  
 556 Ottaway, 2021), while this parameter determined for oxidized PL fraction exceeded around 30 times  
 557 the permissible level. This value measured for native fraction indicated that PLs were only slightly  
 558 modified. The level of TBARS in non-oxidized sample was 0.22 mg/kg, also confirming high quality of  
 559 the obtained PL fraction. In contrast, the amount of TBARS determined in oxidized PL sample was  
 560 around 70 times higher (p ≤ 0.0001) compared to the native PL fraction.

561 **Table 3. Peroxide values and TBARS contents in native and oxidized PL fractions.**

Parameters	Native PL fraction	Oxidized PL fraction
Peroxide value [ <i>mEq</i> O <sub>2</sub> /kg]*	1.3 ± 0.2 <sup>a</sup>	323 ± 22 <sup>b</sup>
TBARS [mg/kg]*	0.22 ± 0.04 <sup>a</sup>	15.3 ± 1.2 <sup>b</sup>

562 \* Results are the means of three independent analyses (n=3) ± standard deviation (SD) values.

563 Different letters indicate significantly different values examined using unpaired Student's test (p ≤  
564 0.05).

565 Abbreviations: PL – phospholipid; TBARS – thiobarbituric acid reactive substances.

### 566 **3.2. Characterization of aggregation structures containing native and oxidized PL**

567 In aqueous environment PLs can form aggregation structures with different sizes, such as liposomes,  
568 mixed micelles and vesicles or lipid droplets. Because lipid digestion occurs at the oil/water interface,  
569 an important factor affecting the kinetics of lipid hydrolysis is the ratio between the surface area of  
570 aggregation structures and their volume. Therefore, it is important to characterize the hydrodynamic  
571 diameters of obtained aggregation structures before the enzymatic hydrolysis. For this purpose, non-  
572 invasive black scatter (NIBS) technique was employed. Its application enabled the determination of  
573 aggregation structure size distribution based both on the volume and number of analyzed objects.

574 Additionally, the obtained population was characterized by polydispersity index (PDI) that assess the  
575 heterogeneity of the objects based on their size.

576 As a result of ultrasound treatment of PLs dissolved in SIF containing 5 mM BS, a homogeneous  
577 population of aggregation structures with an average diameter of 93 nm was obtained (Fig. A1A, Table  
578 4). The particles were characterized by a slightly smaller diameter of 67 nm when this parameter was  
579 determined based on their number (Table 4). The PDI value of 0.15 confirmed the high homogeneity  
580 of the native PL sample.

582 Also for oxidized PLs, using as a basis the object number, the presence of a homogeneous  
583 aggregation structure population with an average diameter of 77 nm was found (Table 4). However,  
584 different size distribution was observed when the particle diameter was determined by volume (Fig.  
585 A1B). In this case, apart from the main object population with diameter of 122 nm, a second  
586 population was found with a diameter of 2.8 μm. The latter population accounted for over 35 %  
587 aggregation structures analyzed by volume. For this sample, the PDI value was 0.39 that suggest a  
588 significantly higher heterogeneity.

590 Furthermore, based on the obtained results, it can be stated that the presence of olive oil TAGs in  
591 aggregation structures containing both native and oxidized PLs influenced their size distribution. Using

592 the described earlier conditions, the population with hydrodynamic diameter around 56 nm was found  
 593 for structures containing native PL fraction. Additionally, based on the object number, small population  
 594 (~ 1 %) of aggregation structures with a size approximately 272 nm was observed (Table 4). A  
 595 significantly different situation was observed in the size distribution determined by the object volume  
 596 (Fig. A1C), where the population with a similar hydrodynamic diameter (~ 66 nm) represented only 33  
 597 %. The main population consisted of aggregation structures with a diameter of approximately 408 nm  
 598 and accounted for 55 % of the total. Moreover, in the analyzed system, a small population with  
 599 hydrodynamic diameter of 5,6  $\mu\text{m}$  was detected. The PDI value for the native PL/BS/TAG emulsion  
 600 was 0.50, which showed the significant influence of TAGs on the increase of heterogeneity of the  
 601 sample compared to native PL/BS mixed micelle solution (PDI 0.15).  
 602 Also, the average diameter distribution (based on the object number) of aggregation structures  
 603 composed of oxidized PLs, BSs and TAGs showed the presence of two populations, the main one  
 604 with size of 63 nm and a smaller population (< 1 %) with hydrodynamic dimension of 405 nm (Table  
 605 4). Similarly to the previous sample type (native PL/BS/TAG emulsion), the size distribution measured  
 606 based on the object volume was significantly different. The structural aggregations with average  
 607 hydrodynamic dimension of 538 nm were predominant population. Additionally, two other populations  
 608 of particles were observed: one with diameter of 76 nm and the other with 5.5  $\mu\text{m}$  (Fig. A1D).

609 **Table 4. PDI values and hydrodynamic diameters of aggregation structures PL/BS or**  
 610 **PL/BS/TAG systems containing native or oxidized PLs.**

System components	PDI*	The size of aggregation structures determined on the basis of			
		Object volumes		Object number	
		Diameter [nm]*	Content [%]*	Diameter [nm]*	Content [%]*
Native PLs + BSs	0.15 $\pm$ 0.02	93.0 $\pm$ 12.4	100	66.8 $\pm$ 1.9	100
Oxidized PLs + BSs	0.39 $\pm$ 0.03	122 $\pm$ 15	63.2 $\pm$ 15.7	77.0 $\pm$ 5.9	100
		2784 $\pm$ 693	36.8 $\pm$ 9.3	-	-
Native PLs + BSs + TAGs	0.50 $\pm$ 0.08	408 $\pm$ 118	55.3 $\pm$ 4.7	55.9 $\pm$ 9.3	99.6 $\pm$ 0.5
		66.0 $\pm$ 14.0	33.3 $\pm$ 8.6	272 $\pm$ 23	0.78 $\pm$ 0.29
		5579 $\pm$ 216	4.0 $\pm$ 2.0	-	-
Oxidized PLs + BSs + TAGs	0.57 $\pm$ 0.09	538 $\pm$ 160	67.5 $\pm$ 6.9	62.6 $\pm$ 10.9	99.3 $\pm$ 0.2
		75.7 $\pm$ 25.0	31.6 $\pm$ 7.4	405 $\pm$ 82	0.69 $\pm$ 0.20
		5489 $\pm$ 99	2.6 $\pm$ 1.0	-	-

611 \* Results are the means of five independent determinations (n=5)  $\pm$  standard deviation (SD) values.

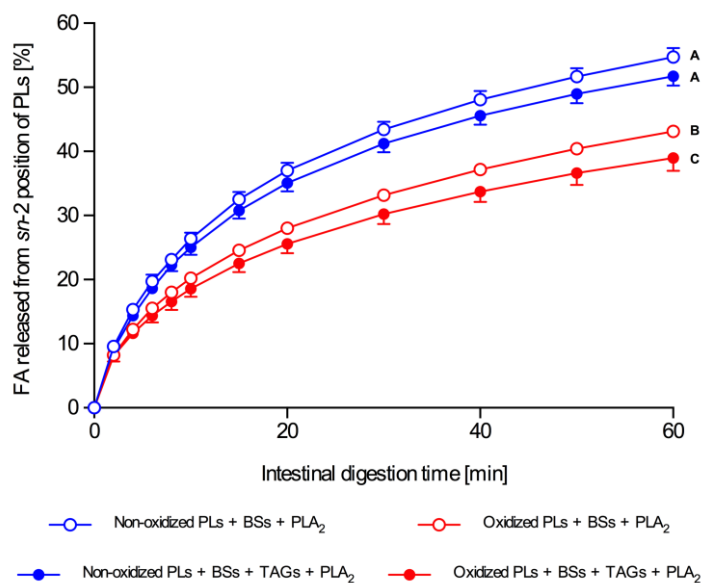
612 Abbreviations: BS – bile salt; PDI – polydispersity index; PL – phospholipid; TAG – triacyloglycerol.

613 **3.3. PLA<sub>2</sub>-catalysed hydrolysis of native and oxidized PLs embedded in mixed micelles and**  
614 **emulsions**

615 Kinetics of PLA<sub>2</sub>-catalysed hydrolysis of native and partially oxidized PLs present in mixed micelles or  
616 emulsions was determined using pH-stat method. This approach enabled monitoring of enzymatic  
617 hydrolysis of lipids based on the volume of NaOH solution used for neutralization of released FAs to  
618 maintain the pH at a constant value (pH=8 in this case) (Beisson, Tiss, Rivière, & Verger, 2000).

619 Among all tested sample types, the native PL/BS mixed micelles were the most liable to hydrolysis  
620 catalyzed by PLA<sub>2</sub>. After 10 min from the initiation of hydrolysis by the addition of PLA<sub>2</sub>, approximately  
621 26.4 mol % of FAs present in *sn*-2 position of PLs were released. In this period, the reaction  
622 proceeded at the highest rate (Fig. 2). The amounts of FAs released after 30 and 60 min were  
623 respectively 43.4 and 54.7 mol %, which clearly showed that the hydrolysis rate decreased with the  
624 reaction time. The addition of olive oil TAGs led to slight decrease of PL hydrolysis catalyzed by PLA<sub>2</sub>.  
625 However, the differences in hydrolysis rate between native PL-containing systems without and with  
626 TAGs were not statically significant over the entire hydrolysis time range.

628 Oxidation of UFAs found in the structure of PLs caused a statistically significant slowdown of  
629 hydrolysis of oxPL/BS mixed micelles catalyzed by PLA<sub>2</sub>. The discrepancy between hydrolysis rate of  
630 native and oxidized PL was growing especially in the range of 2 – 20 min. The amount of FAs  
631 released after 10 min of the reaction amounted to 20.2 mol % of FAs present in the *sn*-2 position of  
632 oxPLs, while this parameter achieved the value of 33.2 and 43.1 mol % after 30 and 60 min,  
633 respectively. For the oxPL/BS/TAG emulsion sample, the lowest digestibility by PLA<sub>2</sub> was observed.  
634 Similarly to the emulsion containing native PLs, the addition of olive oil TAGs slightly decreased the  
635 PL hydrolysis rate in oxPL/BS/TAG system, compared to mixed micelles with oxidized PL fraction.  
636 However, in these cases, the effect of TAG addition made statistically significant difference ( $p \leq 0.05$ )  
637 beginning from 40 min of the reaction. After 10 min of the hydrolysis, approximately of 18.6 mol % of  
638 FAs were released. Longer hydrolysis times (30 and 60 min) resulted in 30.2 and 39.0 mol % of FAs  
639 released from the *sn*-2 position of PLs, respectively.



640

641 **Fig. 2.** PLA<sub>2</sub>-catalysed hydrolysis of native and oxidized PL in the aggregation structures of mixed  
 642 micelles and emulsions. The results are the average of three independent analyses (n=3). For the  
 643 sake of legibility of the drawing, the standard deviation (SD) values in the graph is presented in one  
 644 direction only (plus or minus). In the curve for oxidized PLs + BSs + PLA<sub>2</sub>, the SD values were smaller  
 645 than the symbol size used. Different letters at the endpoint of hydrolysis time indicate significantly  
 646 different values examined using one-way ANOVA with Tukey's post hoc test ( $p \leq 0.05$ ).

647 (Abbreviations: BS – bile salt; FA – fatty acid; PL – phospholipid; PLA<sub>2</sub> – phospholipase A<sub>2</sub>; TAG –  
 648 triacylglycerol)

### 649 3.4. Impact of hexanal and (*E*)-2-nonenal on PLA<sub>2</sub> activity

650 As mentioned earlier, low-molecular weight volatile aldehydes are formed as a result of degradation of  
 651 lipid hydroperoxides by the cleavage of the C-C bond in UFA chains. Due to the frequent occurrence  
 652 of linoleic acid in the structure of hen egg yolk PLs and its relatively high oxidability, hexanal was  
 653 found to be one of the most abundant aldehydes among the volatile compounds identified in the  
 654 headspace of the hen egg yolk PL fraction heated for 20 min at 100 °C (Chen, Balagiannis, & Parker,  
 655 2019). Another type of volatile aldehydes that are formed during lipid oxidation are electrophilic  $\alpha$ - $\beta$ -  
 656 unsaturated aldehydes, which are susceptible to nucleophilic attack at the  $\beta$ -carbon position. The most  
 657 abundant  $\alpha$ - $\beta$ -unsaturated aldehydes identified in the hen egg yolk PL fraction exposed to elevated  
 658 temperature were (*E*)-2-heptenal, (*E*)-2-octenal, (*E*)-2-decenal and (*E*)-2-nonenal (Chen et al., 2019).  
 659 In order to determine the effect of low-molecular volatile aldehydes on PLA<sub>2</sub> activity, two of those





660 aldehydes were used: hexanal and (*E*)-2-nonenal. The IC<sub>10</sub> and IC<sub>30</sub> values for both studied aldehydes  
661 are shown in Table 5.

663 The obtained IC<sub>10</sub> and IC<sub>30</sub> values were lower for (*E*)-2-nonenal compared to the saturated aldehyde –  
664 hexanal (Table 5). Importantly, in the case of both tested aldehydes – hexanal and (*E*)-2-nonenal–  
665 further increase in concentrations (above ~ 20 mM) did not lead to a linear decrease in the PLA<sub>2</sub>  
666 activity. Therefore, a 50 % reduction in enzyme activity was not achieved and consequently the IC<sub>50</sub>  
667 values for neither of these aldehydes could be determined.

668 **Table 5. Inhibitory concentration (IC) values determined for studied aldehydes causing 10 %**  
669 **and 30 % inhibition of porcine pancreatic PLA<sub>2</sub> activity.**

Aldehyde	IC <sub>10</sub> [mM]*	IC <sub>30</sub> [mM]*
Hexanal	5.2 ± 0.6	15.7 ± 1.8
( <i>E</i> )-2-nonenal	3.3 ± 0.4	13.3 ± 1.5

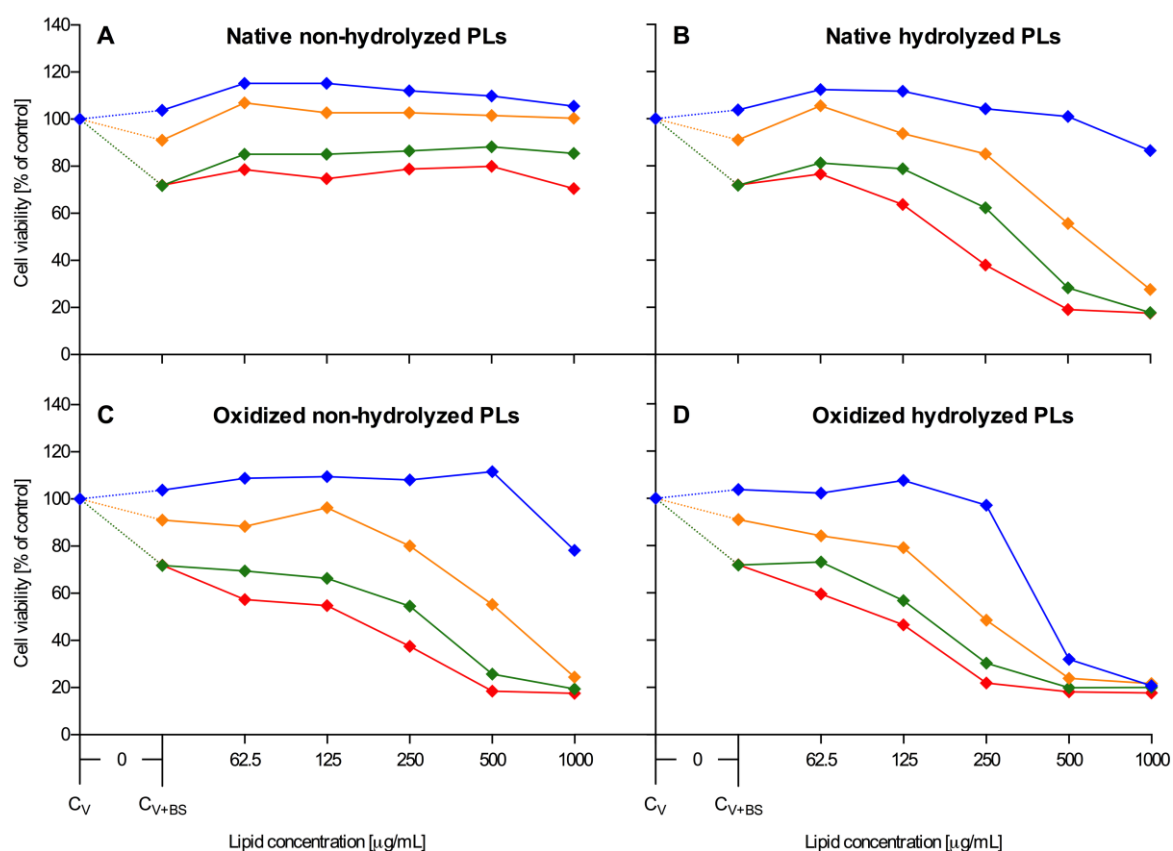
670 \* Results are calculated based on the equation of aldehyde concentration vs. percentage of PLA<sub>2</sub>  
671 inhibition in the linear range. All concentration points (0, 5, 10, 15, 20 mM) were determined in  
672 triplicate (n=3) ± standard deviation (SD) values for each aldehyde.

673 Abbreviation: PLA<sub>2</sub> – phospholipase A<sub>2</sub>.

### 674 3.5. Impact on cell viability

675 The impact of native and oxidized PLs on HT29 cell viability was assessed using MTT test. The HT29  
676 cell line was chosen as a model of the intestinal epithelium tissue that comes into direct contact with  
677 ingested food components. This cell line is recommended for nutritional studies i.a. by the National  
678 Centre for the Replacement, Refinement and Reduction of Animals in Research (UK) (Krishna &  
679 Gopalakrishnan, 2016) and a detailed explanation of the reason for choosing this cell line is presented  
680 in the section 1. Since earlier studies indicated that part of food-delivered PLs could be absorbed by  
681 intestinal cells without their prior hydrolysis (Zierenberg & Grundy, 1982), the cells were exposed to  
682 native and oxidized PLs in the presence of BS, both before and after intestinal hydrolysis. In the  
683 present study, two types of control cell cultures were used. The first one involved HT29 cells treated  
684 with the buffer (150 mM NaCl, 6 mM CaCl<sub>2</sub>, 0.28 mM Tris-base, pH=8). The second type of control  
685 constituted of HT29 cells exposed to SIF that contained BS mixture at the concentration of 500 µg/mL.  
686 BSs are important surface-active compounds responsible for solubilization of food-delivered lipids,  
687 which enables their effective hydrolysis. Therefore, they are essential components of SIF used during  
688 *in vitro* intestinal digestion studies. In our study, BS mixture was used during lipolysis on native and  
689 oxidized PL fractions at the concentration of 5 mg/mL. However, the final concentration of BS mixture

690 in culture medium containing native or oxidized PLs before or after intestinal hydrolysis was 500  
 691  $\mu\text{g/mL}$ . Additionally, the culture medium contained 0.5  $\mu\text{M}$  Varespladib, which inhibits the PLA<sub>2</sub>-  
 692 catalyzed hydrolysis of PLs. The inhibitor was added to all types of cell cultures, including controls and  
 693 cells exposed to non-hydrolyzed PLs, to eliminate its influence on cell viability while comparing  
 694 treatments with different samples. The preliminary studies showed that Varespladib was not cytotoxic  
 695 at the concentration used. Dose-response curves after 6, 24, 48 and 72 h of cell exposure to native  
 696 and oxidized PLs before and after hydrolysis are shown in **Fig. 3**. Results of the statistical analysis are  
 697 available in the Supplementary Materials (Table A3).



698  
 699 **Fig. 3.** Cell viability of HT29 cells determined by MTT test after 6 (blue), 24 (orange), 48 (green) and  
 700 72 h (red) exposure to: native non-hydrolyzed PL/BS mixed micelle solution (A); native hydrolyzed  
 701 PL/BS mixed micelle solution (B); oxidized non-hydrolyzed PL/BS mixed micelle solution (C) and  
 702 oxidized hydrolyzed PL/BS mixed micelle solution (D). Results represent means of three independent  
 703 experiments carried out in quadruplicate (standard deviation (SD) values are lower than 15 %).  
 704 Results of statistical analysis are available in Supplementary Materials (Table A3). (Abbreviations: BS  
 705 – bile salt; C<sub>v</sub> – control cells treated with buffer containing 0.5  $\mu\text{M}$  Varespladib; C<sub>v+BS</sub> – control cells

706 treated with SIF containing 500 µg/mL BSs and 0.5 µM Varespladib; PL – phospholipid; SIF –  
707 simulated intestinal fluid)

708 The exposure of HT29 cells to SIF containing only a BS mixture led to the decrease of their viability  
709 (Fig. 3). This effect was observed especially for prolonged treatment times (48 and 72 h), for which the  
710 decrease of cell viability was around 30 %, while for 24 h the cell viability achieved around 90 %  
711 compared to control cells exposed to buffer only. Importantly, the simultaneous presence of native  
712 non-hydrolyzed PLs, across the range of concentrations used (62.5 – 1000 µg/mL), led to a reversal of  
713 the cytotoxic effect of BS for all tested exposure times (Fig. 3A). Additionally, there was no significant  
714 effect of the increasing PL concentration on cell viability. However, it was observed that the prolonged  
715 exposure times (48 and 72 h) decreased the viability of the cells compared to the control ones treated  
716 with buffer only..

717 PL digestion catalyzed by PLA<sub>2</sub> leads to the formation of 1-LPL and FA. In our experiments, after  
718 releasing 40 % of the FAs present at the *sn*-2 position of PL, the reaction was terminated by the  
719 addition of PLA<sub>2</sub> inhibitor Varespladib. For this sample type, containing native PL and product of their  
720 lipolysis, the reduction of the cytotoxic effect of BS was observed in the case of the lowest lipid  
721 concentration used (62.5 µg/mL) for all tested exposure times (Fig. 3B). However, the protective effect  
722 was not observed for higher concentrations (125 – 1000 µg/mL). For the shortest incubation time (6 h),  
723 only the highest concentration tested (1000 µg/mL) caused a decrease in cell viability by about 15 %  
724 compared to the buffer-treated control cells. While, for exposure times of 24, 48 and 72 h, there was a  
725 significant dose-dependent decrease in HT29 cell viability with an increase in the concentrations of  
726 PLs and products of their hydrolysis (1-LPL and FA) in the tested mixtures containing constant BS  
727 concentration.

728 The oxidative modification of PL significantly contributed to the viability of HT29 cells compared to  
729 their native counterparts (Fig. 3C). In the case of the shortest incubation time (6h), no significant  
730 decline in HT29 cell viability was observed in the concentration range of 62.5 – 500 µg/mL. However,  
731 the cell exposure to the highest concentration of oxidized PL at the same incubation time (6 h) led to a  
732 20 % decrease of cell viability. For longer exposure times in the range of 24 – 72 h, negative  
733 correlation between oxidized PL concentrations and cell viability was observed. A significant decrease  
734 in cell viability , for these treatment times, was observed for all concentrations above 125 µg/mL.

735 Furthermore, for this sample type, no significant protective effect against BS-induced cytotoxicity was  
736 observed.

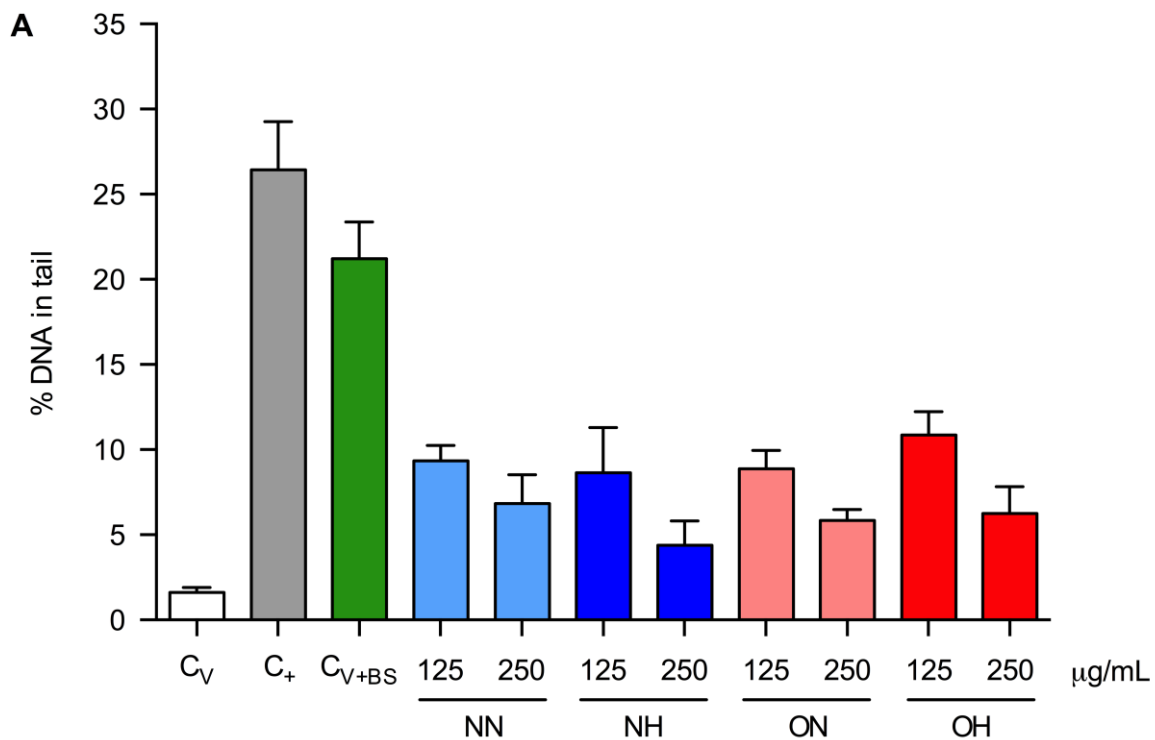
737 The oxidized PLs after their partial hydrolysis (around 40 % of total PL) were characterized by the  
738 highest cytotoxicity among all tested samples (Fig. 3D). For the incubation time of 6 h, no significant  
739 decrease in HT29 cell viability was observed in the concentration range of 62.5 – 250 µg/mL. Above  
740 this range, a sharp decrease in cell survival was observed, reaching around 70 % decline in cell  
741 viability for concentration of 500 µg/mL. For the incubation times of 24, 48 and 72 h, negative  
742 correlation between lipid (PL/1-LPL/FA) concentrations and cell viability was observed. For 24 and 48  
743 h exposure times, maximum reduction of cell viability was observed for concentrations of 500 and  
744 1000 µg/mL, while for 72 h the maximum viability decrease was achieved for concentrations above  
745 250 µg/mL. Similarly to oxidized non-hydrolyzed PLs, the protective effect against cytotoxicity induced  
746 by BS mixture was not observed for oxidized PL fraction after partial hydrolysis.

### 747 **3.6. Genotoxicity**

748 The effect of native and oxidized PL before and after hydrolysis on the DNA integrity of cells treated  
749 for 24 h with tested samples was determined by a comet assay. The test is based on the detection of  
750 DNA strand breaks in single cells embedded in LMP agarose (single-cell gel electrophoresis test). The  
751 lipid concentrations used in the comet assay (125 and 250 µg/mL) were selected on the basis of the  
752 results obtained by MTT test, so that the viability of HT29 cells treated for 24 h with the tested  
753 solutions was not lower than 80 % of control cell viability. The only exception was the cells exposed to  
754 oxidized PL/BS mixed micelles after partial hydrolysis, where the cell viability was inhibited by about  
755 50 % for the concentrations of 250 µg/mL.

756 Among all tested samples, the treatment with BS mixture alone at the concentration of 500 µg/mL  
757 caused the highest genotoxicity (Fig. 4). Interestingly, the BS-induced the genotoxic effect was similar  
758 to DNA damage observed in the case of positive control cells treated with 150 µM H<sub>2</sub>O<sub>2</sub> for 1 h. The  
759 percentage of DNA in tail for these samples was 21.2 and 26.5 %, respectively. On the other hand, the  
760 obtained results indicated that the simultaneous presence of PLs, either native or oxidized before and  
761 after hydrolysis at both tested concentrations (125 and 250 µg/mL), significantly reduced BS-induced  
762 DNA fragmentation. Moreover, for most of the tested sample types, a statistically significant difference  
763 in DNA integrity was observed between the two applied concentrations, with 250 µg/mL offering better  
764 protection. The statistical analysis does not confirm concentration dependence only for native non-

765 hydrolyzed PL, for which the p value was 0.1148. Overall, however, the same effect on BS-induced  
 766 genotoxicity was observed regardless of PL oxidation or hydrolysis.



**B**

vs.	C <sub>+</sub>	C <sub>V+BS</sub>	NN 125	NN 250	NH 125	NH 250	ON 125	ON 250	OH 125	OH 250
C <sub>V</sub>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>0.0001</b>	<b>&lt;0.0001</b>	0.0577	<b>&lt;0.0001</b>	<b>0.0010</b>	<b>&lt;0.0001</b>	<b>0.0002</b>
C <sub>+</sub>		<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>0.0195</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>
C <sub>V+BS</sub>			<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>
NN 125				0.1148	0.9987	<b>&lt;0.0001</b>	>0.9999	<b>0.0040</b>	0.7845	<b>0.0183</b>
NN 250					0.5527	0.1471	0.3725	0.9861	<b>0.0004</b>	0.9998
NH 125						<b>0.0002</b>	>0.9999	0.0541	0.2484	0.1722
NH 250							<b>&lt;0.0001</b>	0.8107	<b>&lt;0.0001</b>	0.5039
ON 125								<b>0.0251</b>	0.4020	0.0912
ON 250									<b>&lt;0.0001</b>	>0.9999
OH 125										<b>&lt;0.0001</b>

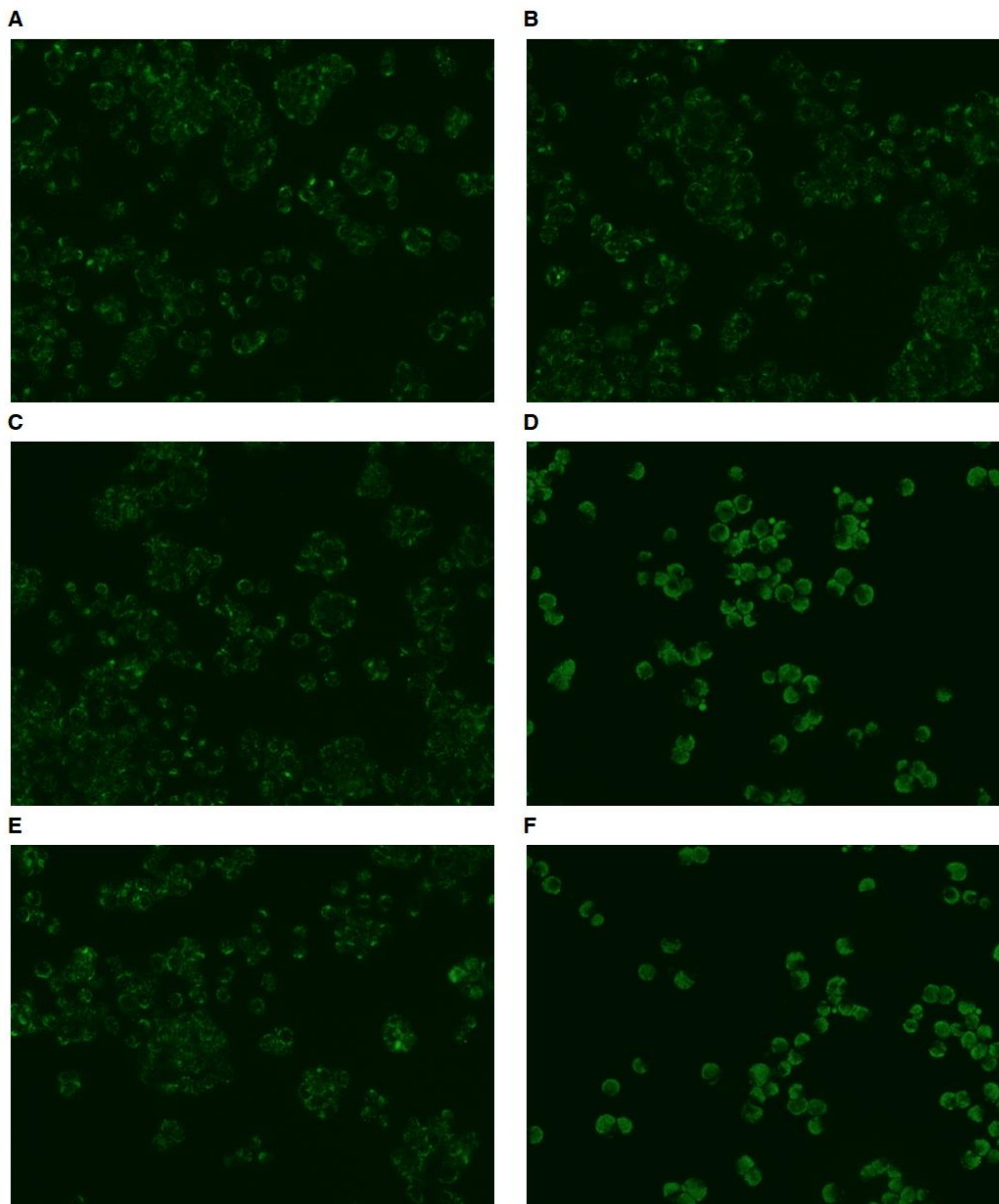
767  
 768 **Fig. 4.** Genotoxic effects of native and oxidized PL/BS mixed micelles before and after hydrolysis  
 769 determined in HT29 cells by comet assay and expressed as % DNA in the comet tail (Panel A).  
 770 Results represent means of three independent experiments and standard deviation (SD) values. In the  
 771 graph, the SD values are presented only in the plus direction. The final concentration of PLA<sub>2</sub> inhibitor  
 772 in cell medium for all tested samples was 0.5 µM. Probability values for individual sample pairs were  
 773 compared using one-way ANOVA with Tukey's post hoc test. Values with statistically important  
 774 difference (p < 0.05) between the two compared samples are marked in bold red, while probability  
 775 values p ≥ 0.05 in normal font (panel B). (Abbreviations: BS – bile salt; C<sub>+</sub> – positive control cells

776 treated with 150  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 1 h;  $C_v$  – control cells treated with buffer containing 0.5  $\mu\text{M}$  Varespladib;  
777  $C_{v+BS}$  – control cells treated with SIF containing 500  $\mu\text{g}/\text{mL}$  BSs and 0.5  $\mu\text{M}$  Varespladib; NH – native  
778 hydrolyzed; NN – native non-hydrolyzed; OH – oxidized hydrolyzed; ON – oxidized non-hydrolyzed;  
779 PL – phospholipid;  $\text{PLA}_2$  – phospholipase  $A_2$ ; SIF – simulated intestinal fluid)

### 780 **3.7. Neutral lipid accumulation**

781 CLDs are intracellular organelles composed of a core containing neutral lipids (mainly TAGs)  
782 surrounded by a PL monolayer with various proteins embedded in it. CLDs can be visualized using  
783 fluorescence microscopy after staining of CLDs with lipophilic dye – Nile Red. In a neutral lipid  
784 environment (e.g., CLD core), Nile Red exhibits green fluorescence with a maximum emission at the  
785 wavelength of 585 nm (excitation wavelength of 510 nm). In contrast, in PL-rich structures (e.g.,  
786 cellular membranes), Nile Red exhibits red fluorescence with emission and excitation wavelengths of  
787 640 and 550 nm, respectively. In the present study, the accumulation of natural lipids in CLDs was  
788 assessed using the green fluorescence of Nile Red (Listenberger & Brown, 2007). Before CLD  
789 imaging, HT29 cells were treated for 24 h with native or oxidized PL/BS mixed micelles (125  $\mu\text{g}/\text{mL}$ )  
790 before and after hydrolysis catalyzed by  $\text{PLA}_2$ .

791 Visual analysis of the fluorescence intensity of CLDs present in HT29 cells suggested that the  
792 presence of free FA increases the accumulation of neutral lipids since it was significantly higher for  
793 native and oxidized PL/1-LPL/FA mixture compared to non-hydrolyzed PLs (Fig. 5). There was  
794 however no effect of PL oxidation on fluorescence intensity, which indicates no increase in the  
795 accumulation of neutral lipids. No differences were observed, between cells exposed to non-  
796 hydrolyzed both native or oxidized PLs and control cells treated with the buffer (150 mM NaCl, 5 mM  
797  $\text{CaCl}_2$ , 0.28 mM Tris) containing 5  $\mu\text{M}$  Varespladib. Additionally, based on the visual analysis, there  
798 was no influence of BS mixture (500  $\mu\text{g}/\text{mL}$ ) on lipid accumulation.



799

800 **Fig. 5.** Visualization of CLDs in HT29 cells after 24 h exposure to PL/BS mixed micelles intact or  
 801 submitted to PLA<sub>2</sub> catalyzed hydrolysis using fluorescent microscopy and Nile Red staining: cells  
 802 treated with buffer containing 0.5 μM Varespladib (A); cells treated with SIF containing 500 μg/mL BSs  
 803 and 0.5 μM Varespladib (B); cells treated with native PL/BS mixed micelle solution (C); cells treated  
 804 with native PL/BS mixed micelle solution after hydrolysis (D); cells treated with oxidized PL/BS mixed  
 805 micelle solution (E) and cells treated with oxidized PL/BS mixed micelle solution after hydrolysis (F).  
 806 The final concentrations of PL and BS in cell medium containing mixed micelles were 125 and 500  
 807 μg/mL, respectively. (Abbreviations: BS – bile salt; CLD – cytosolic lipid droplet; PL – phospholipid;  
 808 PLA<sub>2</sub> – phospholipase A<sub>2</sub>)

809 **4. Discussion**

810 Oxidative modifications of FAs found in the structure of lipids lead to the loss of the nutritional value  
811 and deterioration of sensory properties of food products (Jacobsen, Paiva-Martins, Schwarz, &  
812 Bochkov, 2019). Moreover, a variety of harmful substances can be formed, including lipid  
813 hydroperoxides and products of their decomposition, containing hydroxy, epoxy, keto or aldehyde  
814 groups. It has been estimated that daily intake of LOOHs is around 1.5 mmol (Kanner, 2007). While  
815 the consumption of acrolein ( $\alpha$ - $\beta$  unsaturated aldehyde formed from PUFAs *via* LOOHs) can  
816 significantly exceed 1 mg per day (according to some data the maximum exposure is 0.1 mg/kg/day  
817 (Henning, Johnson, Coyle, & Harbison, 2017; Vieira, Zhang, & Decker, 2017)), which is far above the  
818 Tolerable Daily Intake (TDI) value of 7.5  $\mu$ g/kg/day recommended by the World Health Organization  
819 (WHO) (Jiang et al., 2022). Unfortunately, general health recommendations for the maximum  
820 acceptable human daily intakes (MHDIs) of other LOPs are either extremely limited or completely  
821 unavailable (Grootveld, Percival, Leenders, & Wilson, 2020).

822 An *in vivo* study in a rat model fed with non-oxidized and oxidized (PVs in the range of 100 – 1200  
823 *mEq O<sub>2</sub>/kg*) soybean oils indicated that animal exposure to oils with PVs above 400 *mEq O<sub>2</sub>/kg*  
824 significantly decreased weight gain compared to the control group. A diet containing oxidized oil with a  
825 PV of 1200 *mEq O<sub>2</sub>/kg* caused deaths within the approximately three weeks. Among the symptoms,  
826 weight loss during the experiment and severe diarrhea were observed. Animals fed with oil  
827 characterized by PV of 800 *mEq O<sub>2</sub>/kg* developed moderate to severe diarrhea, but the symptom had  
828 subsided somewhat by the eighth week. Additionally, the histological examinations of the animals fed  
829 with highly oxidized fraction (PV above 3000 *mEq O<sub>2</sub>/kg*) indicates that intestines were one of the most  
830 affected organs and were characterized by the presence of cytoplasmic vacuoles distending mucosal  
831 (Andrews, Griffith, Mead, & Stein, 1960). Cases of food poisoning manifested by acute symptoms  
832 such as diarrheal, nausea, emesis, abdominal pain, fatigue, and headache, were also observed in  
833 humans consuming processed foods containing oils and fats with PVs of at least 100 *mEq O<sub>2</sub>/kg*  
834 (Gotoh et al., 2006). Moreover, chronic consumption of such foodstuffs has been postulated to  
835 increase the risk of disorders such as alimentary tract cancers, cardiovascular diseases and nervous  
836 system disorders (neurotoxicity) (Gotoh et al., 2006; Jackson & Penumetcha, 2019). Therefore, it  
837 seems important to understand the influence of oxidation of dietary lipid on important physiological  
838 processes occurring in the intestinal lumen, namely digestion and up-take as well as the subsequent  
839 biological effects of lipid oxidation and hydrolysis towards IECs.



840 To address these questions, in the present study, we used PL fraction isolated from hen egg yolk that  
841 is the main nutritional source of PLs in typical Western diet (Blesso, 2015). As shown in Table 2, hen  
842 egg yolk PL species contained a significant share of UFAs, including PUFAs such as  $\alpha$ -linolenic or  
843 arachidonic acids. Therefore, PL fraction isolated from egg yolk was selected as a suitable model for  
844 oxidation and further assessment of its impact on IECs. In general, oxidized lipids can be formed in  
845 three ways: by non-enzymatic autoxidation and photo-oxidation or *via* enzyme-catalysed reaction, but  
846 all of these reactions lead to the formation of lipid hydroperoxides as primary oxidation products  
847 (Domínguez et al., 2019). However, to ensure a high yield of PL oxidation needed in our study, the  
848 LOX-catalysed reaction was chosen. The PV determined for oxidized PL fraction (323 *mEq O<sub>2</sub>/kg*)  
849 confirmed that PL species were oxidized with high efficiency and could be successfully used in further  
850 stages of our research (Table 3). Our previous analysis, using two-step high-performance liquid  
851 chromatography (HPLC) coupled with mass spectrometry (MS) technique, confirmed the presence of  
852 not only PL hydroperoxides, but also PL dihydroperoxides in the LOX-catalysed hen egg PL fraction  
853 (Parchem, Kusznierevicz, et al., 2019). The latter are not typical lipid oxidation products, however,  
854 previous studies indicated that they can be formed *in vitro* at high enzyme concentration (Brash,  
855 1999). Additionally, lipid dihydroperoxides were detected in virgin and refined soybean oils exposed to  
856 elevated temperature (70°C) (Martin-Rubio, Sopelana, Ibargoitia, & Guillén, 2021). Our current study  
857 also showed a statistically significant difference in TBARS content between oxidized and native  
858 fraction (Table 3). This indicated at least a partial decomposition of full-chain length PL  
859 (di)hydroperoxides by C-C bond cleavage and formation of low-molecular weight aldehydes, known to  
860 inhibit pancreatic PLA<sub>2</sub> activity, and thereby obstructing PLs hydrolysis (Litvinko et al., 2018).  
861 In the next step of the study, the effect of PL oxidation on the parameters of aggregation structures  
862 present in SIF containing BS mixture was evaluated. In general, PLs can form different aggregation  
863 structures, whose spatial organization depends on the presence and composition of surfactants (e.g.,  
864 BSs), other lipid molecules (e.g., TAGs), non-lipid compounds (e.g., proteins) as well as on  
865 physicochemical properties of aqueous phase (Birru et al., 2014; Meyuhas et al., 1997). Under the  
866 conditions used in our study, native PLs formed the homogeneous population of aggregation  
867 structures. Taking into account their size (67 and 93 nm based on the object number and volume,  
868 respectively), it can be assumed that the PL/BS mixed micelles or/and vesicles were obtained. The  
869 typical size of mixed micelles, based on the literature data, ranges from 50 to 150 nm (Riethorst et al.,

2016), while this parameter for mixed vesicles achieves values from 50 to several hundred nm (Birru et al., 2014), though unequivocal identification of aggregation structure type is not possible only on the basis of their size. In this study, the obtained aggregation structures are referred to as mixed micelles, however, noted that unambiguous identification was not made in this study. The size distribution of native PL/BS sample (Fig. A1A) indicated also the presence of structures with the diameter below 50 nm, which are most likely single-component BS micelles. Their typical size ranges from 10 to 50 nm (Riethorst et al., 2016). The replacement of native PLs by their oxidized counterparts led to slightly increased hydrodynamic diameter of the structures formed (Table 4, Fig. A1B). This can result from the displacement of oxidized FAs present in surface PLs from hydrophobic part of the aggregation structure towards the aqueous environment, which is related to the increased hydrophilicity of FAs due to their oxidative modification. Consequently, the hydrophilic groups of oxidized FAs could interact with water molecules leading to the formation of solvation shell and increase in hydrodynamic diameter. The presence of oxidized FAs on the liposome surface has also been suggested in previous studies, in which a model composed of cholesterol and PC species with hydroperoxy linoleic acid was applied (Kambayashi, Yamamoto, & Nakan, 1998). Moreover, the size distribution of the oxidized PL/BS structures determined by volume revealed not only the major population with the hydrodynamic dimension of 122 nm, but also another fraction with a much larger dimension of 2.8  $\mu\text{m}$ . This could be a result of the aggregation of the primary structures into larger clusters *via* e.g., cross-linking of PL molecules by imine formation. Such reaction is known to occur between the primary amines found in the PE head group (ethanolamine) and the aldehyde groups. The latter can be present in the structure of truncated oxPL formed by C-C bond cleavage along UFA acyl chain. Importantly, oxidation of PLs followed by imine formation was reported as a necessary step in sphingosine-induced vesicles aggregation (Jiménez-Rojo et al., 2014).

The addition of olive oil TAGs to native and oxidized PL/BS systems resulted in significant changes of the size distributions based on object volume compared to TAG-free systems (Fig. A1). For native PLs, a heterogeneous system was obtained with the main population characterised by hydrodynamic diameter of 408 nm. It may be postulated that lipid droplets were formed under the conditions used. Their typical size, according to literature data, ranges from 20 to 500 nm for lipid droplets forming nanoemulsion, while aggregation structures with dimension of 1 – 100  $\mu\text{m}$  are typical for macroemulsions (A. Gupta, Eral, Hatton, & Doyle, 2016). Additionally, the lipid droplets with the size

900 specific for macroemulsions (5.6  $\mu\text{m}$ ) were also observed in our system. However, their share was  
901 small and accounted for about 4% based on particle volume. The second most abundant population in  
902 the analyzed system were aggregation structures with the size below 100 nm, which corresponds to  
903 small lipid droplets present in nanoemulsion (A. Gupta et al., 2016). Importantly, these structures  
904 represented a practically homogeneous population when the size distribution was based on the object  
905 number. Similarly to PL/BS models, the use of oxidized PLs had a slight effect on the size distribution  
906 of the structures composed of PLs, BSs and TAG compared to native ones. Most likely this was  
907 associated with changes in spatial structure and physicochemical properties of PL species containing  
908 oxidized FAs that affected their behaviour during the assembly of the aggregation structures.

909 Although the addition of olive oil TAGs to the system containing native PLs and BS mixture affected  
910 the size distribution of formed aggregation structures, no effect was observed on the PL hydrolysis  
911 catalyzed by pancreatic  $\text{PLA}_2$ . This suggested that in both analyzed systems (without and with TAGs),  
912 the enzyme had an unimpeded access to the substrates located on the surface of aggregation  
913 structures. In contrast, a significant decrease in the hydrolysis rate was observed for the systems  
914 containing oxPLs, especially in the model containing additionally TAGs. As already mentioned, PL  
915 hydroperoxides are preferentially hydrolyzed by  $\text{PLA}_2$  compared to the native counterparts, at least  
916 when standard PC hydroperoxide (PC18:0/18:2(-OOH)) was used (Baba et al., 1993). In contrast,  
917 another study suggested that UV-irradiation of hen egg yolk PL/sodium deoxycholate mixed micelles  
918 decreased porcine pancreatic  $\text{PLA}_2$  activity after 40 min of the reaction. In this case, however, the  
919 authors linked their observation to significant increase of MDA level formed over this time (Litvinko et  
920 al., 2018). Therefore, in our study we have attempted to determine the effect of low-molecular  
921 aldehydes on the porcine pancreatic  $\text{PLA}_2$  activity.

922 The chemical structures of formed aldehydes depend on the structure of precursor UFA (e.g., n-6 or n-  
923 3 family) and the oxidation pathway. In general, saturated and  $\alpha$ - $\beta$ -unsaturated aldehydes, can be  
924 distinguished. In our study, we determined the effect of hexanal and (*E*)-2-nonenal – representatives  
925 of saturated and unsaturated aldehydes, respectively, previously identified in the hen egg yolk PL  
926 fraction exposed to elevated temperature (Chen et al., 2019) – on  $\text{PLA}_2$  activity. The obtained data  
927 indicated that both tested aldehydes showed the ability to inhibit this enzyme with (*E*)-2-nonenal being  
928 more effective inhibitor of  $\text{PLA}_2$  activity (Table 5). Aldehydes can covalently modify enzymes by  
929 electrophilic addition to primary amines of the amino acids, such as lysine and arginine, with the

930 formation of Schiff's bases. Porcine pancreatic PLA<sub>2</sub> (1SFW according to the PDB database) is a  
931 small enzyme composed of 124 amino acids. Its active side includes His-48, Asp-99 and Ca<sup>2+</sup> ion that  
932 is bound by Tyr-28, Gly-30, Gly-32, Asp-49. Additionally, PLA<sub>2</sub> structure is stabilized by seven disulfide  
933 bonds, which makes this enzyme highly resistant to chemically and thermally induced unfolding  
934 (Kölbel, Weininger, Ihling, Mrestani-Klaus, & Ulbrich-Hofmann, 2015). However, there are nine lysine  
935 and four arginine residues in the amino acid sequence of PLA<sub>2</sub> that may be modified by the mentioned  
936 aldehydes, which can cause the change in the enzyme conformation and lower its activity. As  
937 described earlier, for both tested aldehydes, the reduction of enzymatic activity by 50 % could be not  
938 achieved, despite the increases in aldehyde concentration. This explanation could be that after  
939 reaching a certain aldehyde concentration, all possible liable to modification enzyme sites become  
940 modified, leading to a decrease in its activity, but not complete inactivation. Therefore, an increase in  
941 the aldehyde concentration did not cause further reduction of enzymatic activity.

942 Summarizing this part of the work, it can be stated that oxidation of dietary (phospho)lipids, and  
943 consequently the formation of low-molecular weight aldehydes, declines (ox)PL digestibility by  
944 pancreatic PLA<sub>2</sub>. Moreover, it seems that this enzyme is responsible not only for direct PL digestion,  
945 but also may modulate digestion of TAGs catalyzed by pancreatic lipase-colipase complex  
946 (Borgström, 1980). The role of PLA<sub>2</sub> in neutral lipid digestion is to facilitate the access of lipase to  
947 TAGs present in the core of lipid droplets surrounded by dietary and bile PLs. The initiation of PL  
948 hydrolysis by PLA<sub>2</sub> results in binding of lipase to their substrates and rapid hydrolysis of TAGs  
949 (Borgström, 1980). Taking the above into account, it can be hypothesized that PLA<sub>2</sub> activity decline in  
950 the presence of oxidized (phospho)lipids is a physiological protective mechanism against too rapid  
951 release of oxidized FAs, which similarly to their native counterparts may be effectively absorbed by IECs  
952 (Penumetcha, Khan, & Parthasarathy, 2000). Otherwise, in the cells exposed to relatively high  
953 concentration of oxidized lipid hydrolysis products, the cellular redox homeostasis could become  
954 disturbed and IEC death induced (T. Wang, Gotoh, Jennings, & Rhoads, 2000).

955 In order to examine this hypothesis, we evaluated the biological effect of PL oxidation and hydrolysis  
956 towards HT29 cells serving as a model of IECs. However, it should be reminded the cell line used in  
957 this study is characterized by cancerous origin. Therefore, due to some changes in nutrient transport  
958 and metabolism as well as redox status, the tested compounds can affect the cells differentially  
959 compared to healthy intestinal cells. Nevertheless, the aim of our research was to understand the

960 general mechanism of toxicity of oxidized PLs and products and their hydrolysis in the presence of  
961 BSs, therefore we found this model to be appropriate. Based on the data obtained using MTT test, it  
962 was observed that hydrolysis and oxidation of PLs led to significant decrease of cell viability in a time-  
963 and concentration-dependent manner, while oxidized hydrolyzed PLs were characterized by the  
964 highest cytotoxic effect (Fig. 3, Table A3). Additionally, BSs alone were found to decline HT29 cell  
965 viability, especially for prolonged exposure time, compared to control cells treated with the buffer only.  
966 However, this effect was reversed by the simultaneous presence of native non-hydrolyzed PLs across  
967 the range of concentrations used. This observation can be explained by the limited bioavailability of  
968 BSs for cells due to promoting the formation of less cytotoxic PL/BS mixed micelles/vesicles, which  
969 was also confirmed by previous studies using other intestinal epithelium cell line delivered from human  
970 colorectal adenocarcinoma – Caco-2 (Tan et al., 2013). Most probably, such a protective effect also  
971 occurs under physiological conditions, where BSs are released to duodenum in the bile form  
972 containing PLs. Bile is composed of 95 % water and various inorganic and organic compounds. The  
973 latter include *i.a.* bile acids (40 mM), PLs (7 mM), cholesterol (3 mM) (Macierzanka, Torcello-Gómez,  
974 Jungnickel, & Maldonado-Valderrama, 2019).

975 In addition, comet assay results showed that cell exposure to BS mixture caused significant DNA  
976 strand breaks (Fig. 4). The genotoxic mechanism of BSs is not yet fully understood. It has been  
977 suggested that DNA damage can be caused by the increased production of ROS and RNS *via*  
978 stimulatory effect of BSs on the activation of LOX, cyclooxygenase (COX) and NADPH oxidase  
979 (Nguyen, Ung, Kim, & Jung, 2018; Rosignoli et al., 2008). Moreover, BSs due to their surfactant  
980 properties can cause mitochondrial damage, leading to the release of ROS generated in the  
981 mitochondrial electron transport chain (Nguyen et al., 2018). However, the BS-induced genotoxicity  
982 was reversed in the presence of not only native non-hydrolyzed PLs, as observed in the case of  
983 cytotoxic effect of BS, but also in cells treated with oxidized and hydrolysed samples. Moreover, higher  
984 lipid concentration (250 vs. 125 µg/mL) led to statistically significant stronger protection against BS-  
985 induced genotoxicity for all sample types. This observation indicates that cytotoxic effect caused by  
986 prolonged cell exposure to higher concentrations of native hydrolyzed and oxidized, both non-  
987 hydrolyzed and hydrolyzed PLs, was not related to DNA strand breaks, as determined using comet  
988 assay. Most probably, for HT29 cells treated with these samples, an overlap of other toxic effects was  
989 observed. For example, hydrolysis of both native and oxidized PLs significantly affected the cellular

990 accumulation of neutral lipids. The products of PL hydrolysis (LPLs and FAs), after intestinal  
991 absorption, are re-esterified at the ER membrane (Küllenberg de Gaudry et al., 2012). However, the  
992 prolonged cell exposure to high concentration of LPLs and FAs can lead to excessive of cellular PL  
993 levels causing alterations in cellular lipid metabolism. The previous data showed that cell exposure to  
994 exogenous LPCs or LPEs led to increased synthesis of cellular PCs. However, the cells compensated  
995 this elevated PC level by their enzymatic degradation to glycerophosphocholine (GPC) and FA by  
996 lysophospholipase (lysoPLA<sub>1</sub>) activity (Baburina & Jackowski, 1999).

997 Additionally, taking into account the presence of FAs delivered from egg yolk PL hydrolysis in the  
998 culture medium, cellular esterification of these FAs to TAGs would be necessary to limit their lipotoxic  
999 effect (Lipke, Kubis-Kubiak, & Piwowar, 2022). This metabolic pathway most probably led to increased  
1000 accumulation of TAGs in the cytosolic droplets within the cells exposed to hydrolyzed native and  
1001 oxidized PLs. However, long-term cell exposure to high concentration of PL hydrolysis products could  
1002 lead to the intensification of the lipotoxic effect, which was manifested as decreased cell viability.

1003 Importantly, under the lipotoxic conditions, the increased mitochondrial FA oxidation can occur in order  
1004 to metabolize the cellular lipid excess. This can lead to disturbance of cellular redox homeostasis and  
1005 increased lipid peroxidation accompanied by the formation of other toxic compounds such as  
1006 electrophilic  $\alpha,\beta$ -unsaturated 4-hydroxyalkenals, which are capable of covalent modification of cellular  
1007 proteins or DNA. Such type of DNA damage however, may not be detected by comet assay used in  
1008 this study. This method determines DNA strand fragmentation, but not necessarily the stable covalent  
1009 modifications of nucleic acid bases by aldehydes.

1010 HT29 cell exposure to non-hydrolyzed oxidized PLs led to a significant decrease of their viability,  
1011 especially for prolonged incubation times. On the one hand, absorption of (phospho)lipid oxidation  
1012 products containing reactive functional groups, such as hydroperoxide or aldehyde, can lead to  
1013 modification of cellular structures such as proteins, membrane lipids and DNA, as well as may disturb  
1014 the cellular redox homeostasis. The previous research using other cell line – Caco-2 – confirmed our  
1015 observations and conclusions. The exposure of Caco-2 cells to not-hydrolyzed oxidized menhaden oil  
016 lead to significant GSH/GSSG ratio, inducing cellular redox imbalance and initiation of apoptotic cell  
017 death (T. Wang et al., 2000). Moreover, the literature data indicated that oxPLs can trigger numerous  
018 signaling pathways leading also to cellular apoptosis as well as inflammation induction (Fruhwrith et  
019 al., 2007). Interestingly, the biggest differences in the cell viability curves were observed between

1020 native hydrolyzed, oxidized non-hydrolyzed and oxidized hydrolyzed PLs for the cells exposed the  
1021 shortest incubation time (6 h). This indicates that while comparing all, the release of oxidized FAs from  
1022 the *sn*-2 position of PLs in a short time induced the highest cytotoxic effect among all tested samples.  
1023 An *in vitro* studies using Caco-2 cell line indicated that hydroperoxy octadecadienoic acid (HPODE)  
1024 and hydroxy octadecadienoic acid (HODE) were effectively absorbed by cells within the exposure time  
1025 of 30 min. Additionally, the uptake of modified FAs was comparable to non-oxidized FAs such as  
1026 linoleic or oleic (Penumetcha et al., 2000). In contrast, the absorption of these oxidized FAs was  
1027 significantly (about 20 times) lower for smooth muscle cells (Penumetcha et al., 2000). One of the  
1028 most common oxidized FA is 13-hydroperoxy octadecadienoic acid (13-HPODE). In addition to  
1029 inducing oxidative stress and cellular death at higher concentrations (above 100  $\mu$ M) and insufficient  
1030 activity of GPx2 enzyme, an *in vitro* studies using Caco-2 model showed that subtoxic concentration of  
1031 13-HPODE can induce pro-inflammatory gene expression and affect lipid and glucose metabolism,  
1032 energy production and mitochondrial functions (Faizo, Narasimhulu, Forsman, Yooseph, &  
1033 Parthasarathy, 2021; Keewan, Narasimhulu, Rohr, Hamid, & Parthasarathy, 2020).

## 1034 **5. Conclusions**

1035 Oxidation of dietary (phospho)lipids is associated not only with the deterioration of the quality and  
1036 sensory properties of foodstuffs, but also may pose a serious threat to the health of consumers,  
1037 especially leading to some gastrointestinal pathologies. Firstly, our results indicated that oxidation of  
1038 PLs decreased their hydrolysis catalyzed by pancreatic PLA<sub>2</sub> in the presence of BS mixture. We  
1039 suggested that low-molecular weight aldehydes such as hexanal or (*E*)-2-nonenal, which are formed  
1040 as a result of lipid hydroperoxide breakdown by the cleavage of the C-C bond in UFA chains, can  
1041 covalently modify the enzyme leading to inhibition of its activity. Our further research using HT29 cell  
1042 line indicated that both hydrolysis and oxidation of PLs led to decrease of cell viability in a time- and  
1043 concentration-dependent manner, while combination of both these factors resulted in the highest  
1044 cytotoxic effect determined by MTT test. On the other hand, the BS-induced genotoxic effect was  
1045 reversed in the presence PLs regardless of whether they were oxidized or hydrolyzed, while the  
1046 protective effect against the BS-induced cytotoxicity was observed for native non-hydrolyzed PLs, but  
1047 was not clearly visible for other samples. This can be related to an overlap of several toxic effects  
1048 including lipotoxicity or disturbance of cellular redox homeostasis. The obtained data have driven us to  
1049 a non-obvious conclusion that the inhibition of PLA<sub>2</sub> activity in the presence of oxidized

1050 (phospho)lipids, may be a kind of physiological protective mechanism against rapid release of oxidized  
1051 FAs that are characterized by cytotoxic effect towards IECs.

## 1052 **6. Limitation of the study**

1053 It should be noted that the presented work was aimed at a preliminary assessment of the influence of  
1054 oxidative modification of dietary (phospho)lipids on their digestion and biological effects towards  
1055 intestinal epithelium. Therefore, to understand the fundamental mechanisms or their action, the  
1056 proposed models did not fully reflect the physiological conditions (e.g., only the intestinal phase of  
1057 digestion was considered; only one of pancreatic lipolytic enzymes was used; there were no other  
1058 nutrients such as protein included). However, despite simplified experimental design, it was possible  
1059 to observe the important differences between native and oxidized egg yolk PL fractions on PL  
1060 hydrolysis as well as cell physiology and pathophysiology. Nevertheless, further work in this area is  
1061 needed for a deeper understanding of mechanisms occurring under the physiological conditions.

## 1062 **CRedit authorship contribution statement**

1063 **Karol Parchem:** Conceptualization, Data Curation, Formal analysis, Funding Acquisition,  
1064 Investigation, Methodology, Project Administration, Validation, Visualization, Writing –  
1065 original draft, Writing – review & editing. **Monika Baranowska:** Investigation, Methodology,  
1066 Validation. **Anna Kościelak:** Investigation. **Ilona Kłosowska-Chomiczewska:** Investigation,  
1067 Methodology, Validation. **Maria Rosário Domingues:** Funding Acquisition, Supervision,  
1068 Writing – review & editing. **Adam Macierzanka:** Supervision, Writing – review & editing.  
1069 **Agnieszka Bartoszek:** Conceptualization, Formal analysis, Funding Acquisition, Resources,  
1070 Supervision, Visualization, Writing – original draft, Writing – review & editing.

## 1071 **Declaration of Competing Interest**

1072 The authors declare that they have no known competing financial interests or personal  
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## 1081 **Appendix A. Supplementary data**

1082 Supplementary data to this article can be found online.

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