

1 **Ether lipid and sphingolipid expression patterns are G-protein coupled**  
2 **estrogen receptor 1-dependently altered in breast cancer cells**

3 Lisa Hahnefeld<sup>1</sup>, Lisa Gruber<sup>1</sup>, Nina Schömel<sup>1</sup>, Caroline Fischer<sup>1</sup>, Peter Mattjus<sup>2</sup>, Robert  
4 Gurke<sup>1,3</sup>, Martina Beretta<sup>4</sup>, Nerea Ferreirós<sup>1</sup>, Gerd Geisslinger<sup>1,3</sup>, Marthe-Susanna Wegner<sup>1,4</sup>

5 <sup>1</sup>*pharmazentrum frankfurt/ZAFES*, Institute of Clinical Pharmacology, Johann Wolfgang  
6 Goethe University, Theodor Stern-Kai 7, 60590 Frankfurt am Main, Germany.

7 <sup>2</sup>Åbo Akademi University, Biochemistry, Faculty of Science and Engineering Artillerigatan 6A,  
8 III, BioCity FI-20520 Turku, Finland.

9 <sup>3</sup>Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Branch for  
10 Translational Medicine and Pharmacology TMP, Theodor Stern-Kai 7, 60590 Frankfurt am  
11 Main, Germany.

12 <sup>4</sup>School of Biotechnology and Biomolecular Sciences, University of New South Wales,  
13 Sydney, New South Wales 2052, Australia.

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22 Corresponding author:

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24 Marthe-Susanna Wegner

25 School of Biotechnology & Biomolecular Sciences

26 UNSW SYDNEY NSW 2052 AUSTRALIA

27 Phone: +61 (2) 9385 6516

28 E-Mail: [wegner@med.uni-frankfurt.de](mailto:wegner@med.uni-frankfurt.de)

29 **Abstract**

30 Identifying co-expression of lipid species is challenging, but indispensable to identify novel  
31 therapeutic targets for breast cancer treatment. Lipid metabolism is often dysregulated in  
32 cancer cells, and changes in lipid metabolism affect cellular processes such as proliferation,  
33 autophagy, and tumor development. In addition to mRNA analysis of sphingolipid  
34 metabolizing enzymes, we performed liquid chromatography time-of-flight mass  
35 spectrometry analysis in three breast cancer cell lines. These breast cancer cell lines differ in  
36 estrogen receptor and G-protein coupled estrogen receptor 1 status. Our data show that  
37 sphingolipids and non-sphingolipids are strongly increased in SKBr3 cells. SKBr3 cells are  
38 estrogen receptor negative and G-protein coupled estrogen receptor 1 positive. Treatment  
39 with G15, a G-protein coupled estrogen receptor 1 antagonist, abolishes the effect of  
40 increased sphingolipid and non-sphingolipid levels in SKBr3 cells. In particular, ether lipids  
41 are expressed at much higher levels in cancer compared to normal cells and are strongly  
42 increased in SKBr3 cells. Our analysis reveals that this is accompanied by increased  
43 sphingolipid levels such as ceramide, sphingadiene-ceramide and sphingomyelin. This shows  
44 the importance of focusing on more than one lipid class when investigating molecular  
45 mechanisms in breast cancer cells. Our analysis allows unbiased screening for different lipid  
46 classes leading to identification of co-expression patterns of lipids in the context of breast  
47 cancer. Co-expression of different lipid classes could influence tumorigenic potential of  
48 breast cancer cells. Identification of co-regulated lipid species is important to achieve  
49 improved breast cancer treatment outcome.

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## 57 **Keywords**

58 sphingolipids, ether lipids, GPER1, AGMO, AGPS

## 59 **Highlights**

- 60 • LC-HRMS analysis allows identification of co-expression between lipid classes
- 61 • Putative co-expression of sphingolipid and non-sphingolipid classes
- 62 • Ether lipids are strongly upregulated in SKBr3 cells (ER negative, GPER1 positive)

## 63 **1. Introduction**

64 Breast cancer is the most common cancer among females in North America, Europe and  
65 Oceania and shares the lead with cervical cancers in South America, Africa and most of Asia  
66 (reviewed in (Torre et al., 2016)). 70 to 78 % of the breast tumors express the  
67 transcriptionally more active *estrogen receptor* (ER) subtype  $\alpha$ , which is declared as an ER  
68 positive (+) status (Pujol et al., 1994, Chu and Anderson, 2002). ER + tumor proliferation is  
69 hormone driven. Current therapies are based on either lowering patient estrogen levels by  
70 aromatase inhibition or by blocking estrogen-mediated signaling pathways through *selective*  
71 *ER modulator* (SERM) or *selective ER downregulator* (SERD) treatment. Normally, following  
72 diffusion into the cell, estrogen mediates its function by binding on ERs, which leads to  
73 translocation of the receptor into the nucleus. This results in activation of manifold signaling  
74 pathways by gene transcription alteration. Membrane-associated ERs also include *G-protein*  
75 *coupled estrogen receptor 1* (GPER1). GPER1 mediates rapid non-genomic as well as indirect  
76 genomic responses (reviewed in (Hsu et al., 2019)). This membrane-associated ER is involved  
77 in physiological processes such as cell growth and pathophysiological processes such as  
78 tumor development (reviewed in (Olde and Leeb-Lundberg, 2009, Wang et al., 2010)) and is  
79 discussed controversially in the literature. For example, it is still unclear where exactly  
80 GPER1 is subcellularly located and whether this receptor contributes to tumorigenic  
81 potential, or indicates less aggressiveness of breast cancer cells.

82 It has been shown that abnormalities of lipids influence cellular processes resulting in  
83 metabolic disorders or tumor development (reviewed in (Long et al., 2018, Pakiet et al.,  
84 2019)). Thereby, the influence of lipids ranges from promoting cancer to suppressing cancer

85 (reviewed in (Lim, 2018)). It has been observed that ether lipids are expressed at much  
86 higher levels in cancer as compared to normal cells (reviewed in (Dean and Lodhi, 2018)).  
87 Also, plasma ether-linked *phosphocholine* (PC) species, which are elevated in breast cancer  
88 patients, can be used as a biomarker for the diagnosis of breast cancer (Chen et al., 2016).  
89 Furthermore, Benjamin et al. showed elevated ether lipid levels in more aggressive breast  
90 cancer cell lines such as 231 MFP (Benjamin et al., 2013). It has also been speculated that  
91 ether lipids seem to be capable of taking over the role of certain sphingolipids both in cells  
92 and organisms (reviewed in (Jimenez-Rojo and Riezman, 2019)). Furthermore, deregulated  
93 sphingolipid metabolism contributes to tumor development and progression (reviewed in  
94 (Ryland et al., 2011, Wegner et al., 2016)). Sphingolipids such as ceramide and  
95 sphingomyelin are significantly increased in human breast cancer (Nagahashi et al., 2016)  
96 compared to normal tissue (reviewed in (Furuya et al., 2011)). However, there is  
97 contradictory data relating to the involvement of lipid classes in cellular pathophysiological  
98 processes. One example is the finding that inhibition or silencing of *acetyl-CoA carboxylase*  
99 (ACC), which is essential for fatty acid synthesis, is shown to limit cancer cell growth  
100 (reviewed in (Lim, 2018)). Surprisingly, liver-specific ACC knockout leads to increased tumor  
101 incidence in a hepatocellular carcinogen *diethylnitrosamine* (DEN) mouse model (Nelson et  
102 al., 2017). This indicates that lipogenesis is not essential for liver tumorigenesis in the DEN  
103 mouse model. Another example is *adipose triglyceride lipase* (ATGL), which inhibits growth  
104 of several cancer cell types when suppressed, but mice lacking ATGL develop lung tumors  
105 (reviewed in (Chen and Huang, 2019)). Given the complexity of the influence that lipids have  
106 on tumor development, it is important to analyze biological samples by a method which  
107 allows an unbiased investigation of several species of lipids instead of focusing on a single  
108 lipid class.

109 Here, we investigated co-expression of different lipid species in breast cancer cells with  
110 differing ER and GPER1 status using *liquid chromatography time-of-flight mass spectrometry*  
111 (LC-HRMS). Our LC-HRMS analysis show co-regulation of sphingolipid and non-sphingolipid  
112 expression and indicate that tumorigenic potential of breast cancer cells is affected by this.  
113 Strikingly, ether lipids and sphingolipids are strongly increased in SKBr3 cells (ER negative,  
114 GPER1 positive). This GPER1-dependent co-regulation of sphingolipid and non-sphingolipid  
115 expression is a novel finding, which might contribute to the identification of novel  
116 therapeutic targets in breast cancer therapy.

## 117 2. Results

### 118 2.1. *Estrogen receptor (ER) and G-protein coupled estrogen receptor 1 (GPER1) status of* 119 *breast cancer cells*

120 First we analyzed *estrogen receptor (ER)* and *G-protein coupled estrogen receptor 1 (GPER1)*  
121 mRNA expression status of T47D, MCF-7 and SKBr3 cells by *quantitative RealTime (qRT)-PCR*.  
122 T47D cells only express ER $\alpha$  resulting in an ER + and GPER1 – status (**Figure 1A**). MCF-7 cells  
123 are ER $\alpha$ , ER $\beta$  and GPER1 expressing breast cancer cells. Accordingly, MCF-7 cells are  
124 identified to exhibit an ER + and GPER1 + status. GPER1 mRNA expression is the highest in  
125 SKBr3 cells as compared to the other cell lines showing a GPER1 + status, whereas ER $\alpha$  and  
126 ER $\beta$  are rarely detectable. Therefore, SKBr3 cells are an ER - status breast cancer cell line.  
127 Relative mRNA expression below the value of 200 is assumed to be a negative status for the  
128 respective gene. The results displayed in **Figure 1A** are in line with other studies (Mota et al.,  
129 2017, Deng et al., 2020).

## 130 2.2. Basal mRNA expression of sphingolipid metabolizing enzymes

131 Next we determined the basal mRNA expression of several anabolic and catabolic  
132 sphingolipid metabolizing enzymes in T47D, MCF-7 and SKBr3 cells. In general,  
133 *sphingomyelin synthase 2* (SMS2), *ceramide kinase* (CERK), *UDP-glucose ceramide*  
134 *glucosyltransferase* (UGCG), *acid ceramidase* (aCDase) and *neutral sphingomyelinase 1*  
135 (nSMase 1) are the most highly expressed genes in all three breast cancer cell lines (**Figure**  
136 **1B** and **C**). Exceptions are SPHK1 and GalCerS, which are highly expressed in SKBr3, but not  
137 T47D and MCF-7 cells (**Figure 1B**). Compared to MCF-7 and SKBr3 cells, T47D cells express  
138 higher amounts of UGCG (**Figure 1B**). MCF-7 cells as compared to the other cell lines exhibit  
139 a strong mRNA expression of the anabolic enzymes *ceramide synthase 2* and *6* (CerS2 and  
140 CerS6) (**Figure 1B**). Notably, UGCG mRNA expression is comparable to CerS2 and CerS6  
141 mRNA expression in MCF-7 cells and SMS2 is the most highly expressed gene in MCF-7 cells  
142 (**Figure 1B**). The catabolic enzymes aCDase and nSMase 1 are also highly expressed in MCF-7  
143 cells when compared to the other cell lines (**Figure 1C**). Since MCF-7 cells express ER  
144 subtypes  $\alpha$  and  $\beta$  and GPER1 these enzymes could potentially be regulated by ER $\alpha$ ,  $\beta$  and  
145 GPER1. Interestingly, *ceramide synthase 4* (CerS4), *sphingosine kinase 1* (SPHK1), CERK and  
146 *galactosylceramide synthase* (GalCerS) are strongly expressed in GPER1 + SKBr3 cells  
147 compared to the other cell lines (**Figure 1B**). The data reveal differing expression patterns of  
148 sphingolipid metabolizing enzymes in breast cancer cells exhibiting unequal ER and GPER1  
149 status.

## 150 2.3. ER- and GPER1-dependent sphingolipid levels

151 In order to screen for ER-dependent changes on sphingolipid levels we performed LC-HRMS  
152 analysis. ER - SKBr3 cells exhibit significantly reduced *dihydroceramide* (dhCer) levels  
153 compared to MCF-7 cells (**Figure 2A** and **supplemental data 1A**), whereas ceramide levels  
154 are strongly increased (**Figure 2B** and **supplemental data 1A**). Sphingadiene-ceramide  
155 concentration is also strongly increased in SKBr3 cells compared to ER + cells, albeit MCF-7  
156 cells exhibit a higher sphingadiene-ceramide concentration than T47D cells (**Figure 2C** and  
157 **supplemental data 1A**). *Galactosylceramide* (GalCer)/*glucosylceramide* (GlcCer) levels in  
158 SKBr3 cells are increased compared to MCF-7 cells, whereas no significant differences  
159 between MCF-7 and T47D cells were detected (**Figure 2D** and **supplemental data 1B**).  
160 *Lactosylceramide* (LacCer) concentration in T47D cells is the lowest compared to MCF-7 and

161 SKBr3 cells (**Figure 2E** and **supplemental data 1B**). Additionally, *sphingomyelin* (SM)  
162 concentration appears to follow the expression of GPER1-mRNA with highest level in SKBr3  
163 cells and significantly lowest level in T47D cells (**Figure 2F** and **supplemental data 1C**).  
164 Treatment with G15, a GPER1 antagonist, significantly reduced ceramide and sphingadiene-  
165 ceramide level in SKBr3 cells. Dihydroceramide, ceramide, GalCer/GlcCer, LacCer and  
166 sphingomyelin levels are significantly increased in MCF-7 cells (**supplemental data 1D**). No  
167 effect of G15 treatment on the analyzed lipid levels in T47D (GPER1 -) could be detected  
168 (**supplemental data 1D** and **2H**). In summary, sphingolipid analysis revealed that the high  
169 anabolic enzyme mRNA expression in SKBr3 cells is reflected on sphingolipid levels by high  
170 ceramide, sphingadiene-ceramide, GalCer/GlcCer and SM levels. Furthermore, the G15  
171 treatment data indicate that sphingolipid levels are GPER1-dependently regulated.

#### 172 2.4. ER- and GPER1-dependent non-sphingolipid levels

173 LC-HRMS analysis revealed GPER1- and ER-dependent alterations of non-sphingolipid levels.  
174 Cholesterol levels are the lowest in T47D and the highest in SKBr3 cells (**Figure 3A**). Total  
175 sterol ester concentration is the lowest in MCF-7 cells and the highest in SKBr3 cells (**Figure**  
176 **3B** and **supplemental data 2A**). By far the most altered lipid concentration between the  
177 three cell lines is the ether lipid concentration. SKBr3 cells exhibit a 140-fold increased ether  
178 lipid level compared to ER + cell lines (**Figure 3C** and **supplemental data 2B**). *Diglycerides*  
179 (DG) follow the same trend as cholesterol with T47D cells having the lowest and SKBr3 cells  
180 the highest concentration (**Figure 3D** and **supplemental data 2C**). In contrast, *triglyceride*  
181 (TG) content is low in MCF-7 cells as compared to T47D and SKBr3 cells (**Figure 3E** and  
182 **supplemental data 2D**). Glycerophospholipids appear most abundant in MCF-7 cells (**Figure**  
183 **3F** and **supplemental data 2E**), whereas lyso-glycerophospholipids are the lowest in MCF-7  
184 cells (**Figure 3G** and **supplemental data 2F**). Lyso-glycerophospholipids are statistically  
185 increased in SKBr3 cells as compared to T47D cells (**Figure 3G** and **supplemental data 2F**).  
186 Acylcarnitine levels are significantly decreased in SKBr3 cells compared to MCF-7 cells,  
187 whereas no difference compared to T47D cells could be detected (**Figure 3H** and  
188 **supplemental data 2G**). Treatment with the GPER1 antagonist G15 leads to significantly  
189 reduced levels of sterol ester and ether lipids in SKBr3 cells (**supplemental data 2H**). In  
190 contrast, G15 stimulation leads to significantly increased levels of cholesterol, diglyceride,  
191 glycerophospholipids and acylcarnitines (**supplemental data 2H**). SKBr3 cells exhibit

192 increased sphingolipid levels as compared to the other cell lines, as well as increased levels  
193 of several non-sphingolipids such as cholesterol, DGs and ether lipids. This indicates that  
194 sphingolipid and non-sphingolipid pathways might be co-regulated in SKBr3 cells. In addition,  
195 treatment with the GPER1 antagonist G15 indicate GPER1-dependent co-regulation of  
196 sphingolipids and non-sphingolipids in GPER1 + breast cancer cells.

## 197 2.5. Effect of ER and GPER1 on cell proliferation

198 There are differences in morphology between the three cell lines such that T47D cells are  
199 the smallest in size, MCF-7 cells are larger and SKBr3 cells are the largest (**Figure 4A**). The  
200 results of the proliferation assay show that MCF-7 cells proliferate faster than SKBr3 cells,  
201 which in turn proliferate faster than T47D cells (**Figure 4B**). Since the three breast cancer cell  
202 lines differ in their ER and GPER1 status and in their sphingolipid and non-sphingolipid  
203 expression pattern, it is possible that cell size and proliferation is affected by this.

## 204 2.6. GPER1-dependent ether lipid metabolizing enzymes mRNA expression

205 Since a strong GPER1-dependent increase of ether lipid level could be detected, we analyzed  
206 several enzymes involved in the ether lipid metabolism on mRNA level by qRT-PCR.  
207 *Glyceronephosphate O-acyltransferase* (GNPAT) is a crucial enzyme in ether lipid synthesis  
208 and is significantly lowered in MCF-7 cells compared to the other two cell lines (**Figure 4C**).  
209 Another key enzyme in ether lipid synthesis is *alkylglycerone phosphate synthase* (AGPS).  
210 AGPS is highly increased in SKBr3 cells, which fits the finding of strongly increased ether lipid  
211 concentrations in SKBr3 cells (**Figure 4C**). The *alkylglycerol monooxygenase* (AGMO) cleaves  
212 the O-alkyl bond of ether lipids leading to ether lipid degradation. Surprisingly, SKBr3 cells,  
213 which exhibit a high amount of ether lipids, also show strongly increased AGMO mRNA  
214 expression (**Figure 4C**). This might indicate a GPER1-dependent mRNA expression regulation  
215 of AGMO. In addition, *fatty acid desaturase 1* (FADS1) mRNA expression is strongly increased  
216 in SKBr3 cells (**Figure 4C**). *Carnitine palmitoyltransferase 1A* (CPT1A) mRNA expression in  
217 SKBr3 cells is significantly reduced compared to MCF-7 cells, whereas no difference could be  
218 detected as compared to T47D, which verifies the LC-HRMS analysis results for acylcarnitine  
219 levels (**Figure 4C**). When normalizing LC-HRMS analysis results using *median peak ratio*  
220 (MPR) as compared to one *internal standard* (IS) per lipid class, the distribution of the  
221 reading points does not change (**Figure 5** and **supplemental data 3**), whereby effects  
222 ascribable to cell size are excluded. In summary, our data indicate high ether lipid turnover



223 in GPER1 +, but ER - cells (SKBr3) and a putative GPER1-dependent regulation of the  
224 metabolizing enzymes.

### 225 3. Materials and Methods

#### 226 3.1. Cell lines

227 MCF-7, T47D and SKBr3 cells were purchased from the Health Protection Agency (European  
228 Collection of Cell Cultures, ECACC, Salisbury, UK) and were cultured in phenol-red free  
229 *Dulbecco's Modified Eagle's Medium* (DMEM) supplemented with 5 % charcoal *fetal*  
230 *bovine serum* (FBS), 1 % sodium pyruvate and 1 % GlutaMAX. They were maintained in a  
231 humidified, 5 % CO<sub>2</sub> supplied atmosphere incubator at 37 °C.

#### 232 3.2. Quantitative real-time-PCR (qRT-PCR)

233 Total RNA was isolated using RNeasy Mini Kit (QIAGEN, Hilden, Germany). cDNA was  
234 synthesized from 300 ng total RNA using VERSO™ cDNA Synthesis Kit (Thermo Fisher  
235 Scientific, ABgene, Epsom, UK). Gene specific PCR products were assayed using Maxima  
236 EvaGreen qPCR Master Mix on a 7500fast quantitative PCR system (TaqMan®, Life  
237 Technologies, Darmstadt, Germany). Relative gene expression was determined using the  $\Delta\Delta C_T$   
238 method, normalizing relative values to the expression level of *60S ribosomal protein L37a*  
239 (RPL37A) as a housekeeping gene. It is shown that RPL37A is the optimal single reference  
240 gene when normalizing gene expression in meningiomas and control tissue (Pfister et al.,  
241 2011). Furthermore, Maltseva et al. showed that RPL37A has similar high expression stability  
242 values compared to the other genes such as ACTB or RPS23 in breast cancer cells (Maltseva  
243 et al., 2013). Accordingly, RPL37A is a suitable housekeeper gene for our study. The primer  
244 mix for *sphingomyelin synthase 1* und 2 (SMS1 and SMS2) and *galactosylceramide synthase*  
245 (GalCerS) detection were purchased from GeneCopeia (Rockville, USA) and the GPER1-  
246 primermix from Realtimeprimers (Pennsylvania, USA).

247 **Table 1** qRT-PCR primer sequences.

Gene	Sequence (5'→3')	
	Forward primer	Reverse primer
<b>RPL37A</b>	ATT GAA ATC AGC CAG CAC GC	AGG AAC CAC AGT GCC AGA TCC
<b>CerS2</b>	CCA GGT AGA GCG TTG GTT	CCA GGG TTT ATC CAC AAT GAC
<b>CerS4</b>	CTG GTG GTA CCT CTT GGA GC	CGT CGC ACA CTT GCT GAT AC
<b>CerS5</b>	CAA GTA TCA GCG GCT CTG T	ATT ATC TCC CAA CTC TCA AAG A
<b>CerS6</b>	AAG CAA CTG CAG TGG GAT GTT	AAT CTG ACT CCG TAG GTA AAT ACA

<b>ER<math>\alpha</math></b>	GGG AAG TAT GGC TAT GGA ATC TG	CTG GAG AGT AGC GAG TCT CC
<b>ER<math>\beta</math></b>	AGC ACG GCT CCA TAT ACA TAC C	TGG CTG GAT ATT CAT GGT GGC
<b>SPHK1</b>	GTC ACG TGC AGC CCC TTT	CGC GCG TGG TTC CG
<b>UGCG</b>	TGC TCA GTA CAT TGC CGA AGA	TGG ACA TTG CAA ACC TCC AA
<b>CERK</b>	TAA CCC CCA AAG TCA CAA AA	CAT CTC CAC CAA CAC AGA CA
<b>aCDase</b>	TGT GGA TAG GGT TCC TCA CTA GA	TTG TGT ATA CGG TCA GCT TGT TG
<b>aSMase</b>	CCT GGA GAG CCT GTT GAG TG	GTT GGT CCT GAC GAG TCT GG
<b>nSMase 1</b>	TTT GGT GTC CGC ATT GAC TA	TAG AGC TGG GGt TCT GCT GT
<b>nSMase 2</b>	CAA CAA GTG TAA CGA CGA TGC C	CGA TTC TTT GGT CCT GAG GTG T
<b>nSMase 3</b>	CAC CCA GGA TGA GAA TGG AAA	GTC CGT CCT CAC CCA GCA T
<b>AGMO</b>	CTG ACC TTG ACT TCC ATT GGA TT	CAA GCA ACG GAG AGT TTC CAT A
<b>GNPAT</b>	GCT GCT ACG AAT GTC GGG T	TGT CCC TTC GAG GAA AAA TTC AA
<b>AGPS</b>	AGG GAA GGA ATG TTT GAG CGA	GCA GGA CAC ATC AGG CCAT
<b>FADS1</b>	CCA ACT GCT TCC GCA AAG AC	GCT GGT GGT TGT ACG GCA TA
<b>CPT1A</b>	ATC AAT CGG ACT CTG GAA ACG G	TCA GGG AGT AGC GCA TGG T

### 248 3.3. Lipidomics analysis

#### 249 3.3.1. Materials

250 Water, isopropanol, methanol (LC-MS grade) and *methyl-tert-butyl-ether* (MTBE, HPLC-  
 251 grade) were purchased from Carl Roth (Karlsruhe, Germany). Ammonium formate (for mass  
 252 spectrometry,  $\geq 99.0\%$ ) and Sulfinpyrazon (100 %) were purchased from Sigma-Aldrich  
 253 (Munich, Germany) and acetonitrile (ULC-MS grade) from Biosolve B. V. (Valkenswaard,  
 254 Netherlands). APCI positive calibration solutions was obtained from Sciex (Darmstadt,  
 255 Germany) and formic acid (98-100 %) from AppliChem (Darmstadt, Germany). All internal  
 256 standards were purchased from Avanti Polar Lipids (Alabaster, AL, USA).

#### 257 3.3.2. Liquid chromatography time-of-flight mass spectrometry (LC-HRMS)

258 Three experiments with three replicates each were performed. The sample processing and  
 259 LC-HRMS measurement was carried out as described previously (Hahnefeld et al., 2020).  
 260 Approximately  $5 \times 10^5$  cells in 150  $\mu\text{L}$  PBS were thawed in the fridge for 30 min before  
 261 extraction with 150  $\mu\text{L}$  internal standards in methanol and 500  $\mu\text{L}$  MTBE. After centrifugation  
 262 at 20,000 g for 5 min, the upper organic phase was transferred and the aqueous phase was  
 263 reextracted using 200  $\mu\text{L}$  of MTBE: methanol: water (10:3:2.5, v/v/v). The combined organic  
 264 phases were split for measurement in positive and negative ionization mode, dried at 45  $^\circ\text{C}$   
 265 under a nitrogen stream and stored at -20  $^\circ\text{C}$  pending analysis. The samples were dissolved  
 266 in 120  $\mu\text{L}$  methanol before analysis. For quality control, the different cell lines were pooled  
 267 with two injections at the beginning and the end of each run and one after every 10th  
 268 sample. The LC-MS measurement was carried out on a Shimadzu Nexera-X2 (Shimadzu  
 269 Corporation, Kyoto, Japan) with a Zorbax RRHD Eclipse Plus C8 1.8  $\mu\text{m}$  50x2.1 mm ID column

270 (Agilent, Waldbronn, Germany) coupled to TripleTOF 6600 (Sciex, Darmstadt, Germany) with  
271 electrospray ionization operating in positive and negative ionization mode. A mass range  
272 from 100 to 1000 m/z was scanned together with data-dependent acquisition for improved  
273 identification with a mass error of  $\pm 5$  ppm. The data acquisition was performed using  
274 Analyst TF v1.71. Compound identification and semi-targeted analysis was achieved with  
275 MasterView v1.1 and MultiQuant v3.02 software as described previously (Hahnefeld et al.,  
276 2020). The software was obtained from Sciex (Darmstadt, Germany).

### 277 3.3.3. Sample Normalization

278 LC-HRMS analysis results were compared after normalization once with one *internal*  
279 *standard* (IS) per lipid class and once with *median peak ratio* (MPR) as calculated by  
280 MarkerView v1.1 software. For MPR calculation a selected reference sample, usually the first  
281 QC sample, is used to generate a list of all peaks with a minimum peak area of 1 % of the  
282 largest signal. For each peak in the list the peak area is divided by the peak area of the  
283 reference sample and subsequently the median of the area ratios is calculated. If no peak  
284 appears in the analyzed sample, the area ratio is set to the value 1. Accordingly, a  
285 normalization factor for each sample was generated and the values of all analytes were  
286 multiplied by this normalization factor. The results normalized with one internal standard  
287 per lipid class were used for further analysis.

### 288 3.4. Proliferation assay

289 For quantitative proliferation assays, cells were seeded at a density of  $5 \times 10^4$  cells/well of a  
290 6-well plate (cell culture multiwell plate, 6 well, clear, sterile, (Greiner AG, Kremsmünster,  
291 Austria)). Since the cells were not 100 % confluent following 5 days of culture, the media was  
292 not replenished during the 5 days of culture. Cells were harvested at day 1, 2, 3, 4, 5 and  
293 living cell number was counted using a Neubauer counting chamber and trypan blue (labels  
294 dead cells exclusively).

### 295 3.5. Statistical analysis

296 Data are presented as mean  $\pm$  *standard error of the mean* (SEM). Statistical analyses were  
297 performed with GraphPad Prism 7 software. Significant differences ( $p < 0.05$ ) between  
298 groups were assessed by Tukey's multiple comparison test as indicated in the figure  
299 descriptions.

#### 300 4. Discussion

301 With our LC-HRMS analysis approach we were able to show co-expression of sphingolipids  
302 and non-sphingolipids in breast cancer cells. Importantly, GPER1 might be involved in  
303 regulating this co-expression of different lipid species.

304 Our analysis identified a distinct mRNA expression pattern in both anabolic and catabolic  
305 sphingolipid metabolizing enzymes among the three breast cancer cell lines tested. Low  
306 CerS5 and CerS6 mRNA expression (responsible for the production of C16 to C20 ceramides)  
307 in SKBr3 cells (GPER1 +, ER -) is in line with results we published previously showing  
308 decreased CerS5 and CerS6 mRNA (production of C16 ceramides) level in MCF-7 cells  
309 following stable GPER1 plasmid transfection (MCF-7/GPER1) (Wegner et al., 2019). However,  
310 following GPER1 overexpression in MCF-7 cells, CerS2 mRNA (production of C20-26  
311 ceramides) level is increased and CerS4 mRNA level unchanged (Wegner et al., 2019),  
312 whereas SKBr3 cells exhibit lower CerS2 and higher CerS4 mRNA levels compared to MCF-7  
313 wild type cells. This indicates that regulation of CerS2 and CerS4 is regulated by both GPER1  
314 and ER and that the lack of ER $\alpha$  and  $\beta$  in SKBr3 cells contributes to the differences in SKBr3  
315 and MCF-7/GPER1 cells. GPER1-dependent SPHK1 activity is shown (reviewed in (Sukocheva  
316 and Wadham, 2014)) and is in line with our data, which reveal high SPHK1 mRNA levels in  
317 GPER1 + cells (SKBr3). Sukocheva et al. have shown estrogen-dependent SPHK1 activation  
318 that leads to *sphingosine 1-phosphate* (S1P) release, accordingly to Edg-3 activation and  
319 results in *enhanced growth factor receptor* (EGFR) transactivation (Sukocheva et al., 2006).  
320 Our previous data show that GPER1 regulates CerS transcription ligand-independently  
321 (Wegner et al., 2014). Also, since phenol-red is known to mediate estrogen-like mechanisms  
322 (Wesierska-Gadek et al., 2007), we used phenol-red free media and 5 % charcoaled FBS in  
323 our current study. This could indicate that in addition to ligand-dependent mechanisms,  
324 ligand-independent mechanism mediated by ERs could also contribute to SPHK1 regulation.  
325 Since SPHK1 is a marker for poor prognosis in breast cancer patients (reviewed in  
326 (Sukocheva and Wadham, 2014)) it would be intriguing to investigate GPER1-dependent  
327 mechanisms of SPHK1 regulation. No distinct differences on SMS1 and SMS2 mRNA  
328 expression levels between the three cell lines were observed indicating that SMS are  
329 constitutively expressed. However, we detected SMS1 and SMS2 mRNA expression increase  
330 following stable GPER1 overexpression in MCF-7 cells (Wegner et al., 2019). This indicates a  
331 putative interaction of GPER1 and ER $\alpha$  and or  $\beta$ . Moro et al. showed suppression of CERK

332 expression in cancer cells (Moro et al., 2018). The breast cancer cell line SKBr3 exhibits  
333 strongly increased CERK mRNA expression. This indicates that high CERK expression might  
334 not be a prognostic marker for cancer in general, but rather identifies a less aggressive type  
335 of cancer. Interestingly, the product of CERK activity, *ceramide 1-phosphate* (C1P) is a known  
336 inducer of inflammatory processes in cancer cells, which could contribute to the activation  
337 of pro-cancerous signaling pathways (reviewed in (Dei Cas and Ghidoni, 2018, Arana et al.,  
338 2010)). Indeed, we measured increased FADS1 mRNA expression in SKBr3 cells. FADS1  
339 catalyzes the final step in arachidonic acid synthesis. However, Owczarek et al. showed that  
340 GalCerS, which is also highly expressed in SKBr3 cells, functions as a pro-tumorigenic protein  
341 by inhibiting apoptosis (Owczarek et al., 2013). This is contradictory to the finding that SKBr3  
342 cells are less aggressive, however, we have already shown that UGCG overexpression in  
343 MCF-7 cells leads to increased glutamine uptake (Schomel et al., 2019). This results in  
344 reinforced oxidative stress response and fueled *tricarboxylic acid* (TCA) cycle, which is  
345 accompanied by increased cell proliferation (Schomel et al., 2019). Since MCF-7 cells exhibit  
346 a p53 wildtype while T47D (Lim et al., 2009) and SKBr3 cells (Garufi et al., 2016) display a p53  
347 mutant status and a high UGCG mRNA expression, it is likely that UGCG overexpression is  
348 connected to p53 signaling pathway inhibition. In line with this, Liu et al. showed that UGCG  
349 suppression restores apoptosis mediated by p53 in mutant p53 cancer cells (Liu et al., 2011).  
350 However, MCF-7 cells exhibit the most increased aCDase mRNA expression as well as  
351 increased proliferation. This fits the finding of Lucki et al. showing aCDase driven increased  
352 proliferation of MCF-7 cells (Lucki and Sewer, 2011). Usually, aCDase overexpression in  
353 breast cancer patients correlates with a better prognosis (Ruckhaberle et al., 2009, Sanger et  
354 al., 2015).

355 Overall, levels of anabolic enzyme mRNA expression are more distinct than the mRNA levels  
356 of catabolic enzymes in the analyzed cell lines. Increased anabolic sphingolipid enzyme  
357 mRNA expression levels are indicated by an increase in total ceramide, sphingadiene-  
358 ceramide, GalCer/GlcCer and SM levels in SKBr3 cells. Each sample contained the same cell  
359 number, but SKBr3 cells are larger in size than T47D and MCF-7 cells. We assumed that the  
360 total metabolite concentration might correlate with cell size and therefore differences in cell  
361 size are compensated by normalization with MPR (Muschet et al., 2016). Normalization with  
362 one IS per lipid class cannot compensate for unequal cell size, but often improves  
363 measurement deviations. The volcano plots show that the differences between lipid levels

364 are not ascribable to cell size, whereas the proliferation differs between the cell lines.  
365 However, since dhCer levels are decreased and ceramide levels strongly increased in SKBr3  
366 cells, it is likely that the *dihydroceramide desaturase 1* (DES1) activity is elevated.  
367 Interestingly, SKBr3 cells exhibit strongly increased sphingadiene-ceramide concentration.  
368 Information about the biological function of sphingadiene-ceramides is limited.  
369 Sphingadiene-ceramides inhibit the *phosphoinositide 3-kinase* (PI3K)/Akt and Wnt signaling  
370 pathway leading to apoptosis (reviewed in (Hannun, 2015)).

371 Furthermore, we observed similar sphingolipid and non-sphingolipid expression patterns in  
372 SKBr3 cells indicating a co-regulation of these lipid species. Especially, ether lipids are  
373 strongly increased in SKBr3 cells, which is reversible by treatment with G15, a GPER1  
374 antagonist. This is confirmed by strongly increased AGPS (generation of ether lipids) mRNA  
375 expression (**Summary Figure**). Interestingly, it is shown that aggressive cancer cells exhibit  
376 increased AGPS expression and ether lipid metabolism (Benjamin et al., 2013). Furthermore,  
377 Chen et al. postulated that plasma ether-linked *phosphocholine* (PC) species can be used as a  
378 biomarker for the diagnosis of breast cancer (Chen et al., 2016). One limitation of the study  
379 from Chen et al. is the lack of information about the ER and GPER1 status of the breast  
380 cancer. Our results do not confirm a general induction of ether lipid concentration in breast  
381 cancer cells, but indicate a GPER1-dependent regulation. Since SKBr3 cells are a non-  
382 aggressive cell line, GPER1-dependent increase of ether lipid synthesis does not state  
383 aggressiveness of breast cancer cells in general. However, AGPS is located in the peroxisome  
384 and is the rate-limiting enzyme in ether lipid synthesis (reviewed in (Dean and Lodhi, 2018)).  
385 Surprisingly, GNPAT, which is also located in the peroxisome and essential for ether lipid  
386 synthesis is only compared to MCF-7 cells significantly increased in SKBr3 cells. The reason  
387 for this could be the finding that GNPAT enzyme activity does not only require AGPS  
388 presence, but also depends on the integrity of channeling the substrate from GNPAT to  
389 AGPS, which is shown by Itzkovitz et al. (Itzkovitz et al., 2012). Therefore, GNPAT activity  
390 could be increased in SKBr3 cells leading to increased ether lipid synthesis without an  
391 increased GNPAT mRNA level. *Phosphocholine* (PC)-ether species could not be detected in  
392 T47D cells, but T47D cells exhibit a similar GNPAT mRNA expression as SKBr3 cells. GNPAT  
393 does not contribute to ether lipid production in T47D cells. Either GNPAT is less active in  
394 T47D cells or is expressed, because the enzyme also executes other tasks in T47D cells.  
395 AGMO, an ether lipid cleaving enzyme, also exhibits strongly increased mRNA levels in SKBr3

396 cells. This indicates that SKBr3 cells exhibit an accelerated ether lipid metabolism (**Summary**  
397 **Figure**). A crosstalk between ether lipids and sphingolipids has been shown in  
398 pathophysiological processes such as cancer and atopic dermatitis (reviewed in (Jimenez-  
399 Rojo and Riezman, 2019)). GlcCer levels in cancer have been shown to correlate to ether  
400 lipids through mechanisms that are linked to *mammalian target of rapamycin* (mTOR)  
401 signaling, which is activated in most tumors (Guri et al., 2017). Therefore, previous studies  
402 confirm our data showing that high ether lipid levels correlate with GSL levels in SKBr3 cells.  
403 Ether lipids can be covalently attached to proteins as components of  
404 *glycosylphosphatidylinositol* (GPI)-anchors. GPI-anchored proteins are linked to the  
405 membrane in the ER via the hydrophobic part of the glycolipid and are mostly delivered to  
406 the cell surface to execute diverse functions (reviewed in (Jimenez-Rojo and Riezman,  
407 2019)). Typically GPI-anchored proteins are enriched in membrane microdomains (rafts) and  
408 these microdomains exhibit high sphingolipid and cholesterol levels (reviewed in (Kinoshita,  
409 2016)). In addition to increased ether lipid concentration, SKBr3 cells exhibit elevated  
410 cholesterol levels. This indicates either increased raft formation or changed lipid  
411 composition of rafts as compared to the other cell lines. Ether lipids also affect membrane  
412 fluidity and cellular processes such as membrane fusion (reviewed in (Dean and Lodhi,  
413 2018)). However, SKBr3 cells exhibit elevated DG levels. One possible mechanism leading to  
414 increased DG levels is GPER1-dependent phospholipase C activation, which results in DG  
415 production (reviewed in (Newton et al., 2016)). DGs function as second messengers in the  
416 cell by activating protein kinase C (PKC) and PKC function in the cell is manifold (reviewed in  
417 (Newton, 2018)). Interestingly, TG are the lowest in MCF-7 cells. Lofterød et al. showed that  
418 increased TG levels are connected to promoted tumor growth (Lofterød et al., 2018), which  
419 underlines the finding that SKBr3 cells have poor tumorigenic potential. Another interesting  
420 finding is the low CPT1A mRNA level in SKBr3 cells, which follows the same trend as total  
421 acylcarnitine level. Following production by CPT1A acylcarnitine is imported into  
422 mitochondria and used for  $\beta$ -oxidation in form of acyl-CoA (reviewed in (Schooneman et al.,  
423 2013)). The  $\beta$ -oxidation products *nicotinamide adenine dinucleotide* (NADH) and reduced  
424 *flavin adenine dinucleotide* (FADH<sub>2</sub>) are oxidized at the *oxidative phosphorylation* (OXPHOS)  
425 complexes I and II. This generates the mitochondrial membrane potential, which is essential  
426 for proper mitochondrial respiration function. The data indicate that SKBr3 cells execute less  
427  $\beta$ -oxidation, which might lead to reduced mitochondrial respiration. We have shown that

428 GPER1 overexpression in MCF-7 cells leads to decreased basal respiration and reduced  
429 glycolysis rate resulting in reduced cell proliferation (Wegner et al., 2019). However, acyl-  
430 CoA needed for ether lipid synthesis could be generated by increased *de novo* lipogenesis.

## 431 5. Conclusion

432 In conclusion, the results of our semi-targeted analysis show co-regulation of sphingolipids  
433 and non-sphingolipids in breast cancer cells with differing ER and GPER1 status. Especially,  
434 GPER1 seems to influence expression of sphingolipids and ether lipids. Importantly, this co-  
435 regulation might lead to a less tumorigenic potential. This finding might contribute to  
436 identification of novel potential therapeutic targets in breast cancer treatment.

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## 443 7. Competing Interests

444 We declare that the authors have no competing interests that might be perceived to  
445 influence the content of this manuscript.

## 446 8. Author approvals

447 All authors have seen and approved the manuscript. Furthermore, we ensure that the  
448 manuscript hasn't been accepted or published elsewhere.

## 449 Figure descriptions

450 **Figure 1: Estrogen receptor (ER) and G-protein coupled estrogen receptor 1 (GPER1) status**  
451 **and mRNA expression analysis of sphingolipid metabolizing enzymes in breast cancer cells**  
452 **by qRT-PCR. A** ER $\alpha$ , ER $\beta$  and GPER1 mRNA expression related to the housekeeping gene  
453 RPL37A. Data are presented as a mean of  $n=3-8 \pm$  standard error of the mean (SEM). Tukey's  
454 multiple comparison test. \*\*\*\* $p \leq 0.0001$ . **B** Heatmap of anabolic sphingolipid metabolizing  
455 enzyme mRNA expression. mRNA expression is related to the housekeeping gene RPL37A.  
456 High values are represented as *black*, middle range values are *red* and low values are shown



457 in *white*. Data are presented as a mean of  $n=3$ . *Ceramide synthase X* (CerS X), *sphingosine*  
458 *kinase 1* (SPHK1), *sphingomyelin synthase 1 and 2* (SMS1 and 2), *ceramide kinase 1* (CERK),  
459 *galactosylceramide synthase* (GalCerS), *UDP-glucose ceramide glucosyltransferase* (UGCG). **C**  
460 Heatmap of catabolic sphingolipid metabolizing enzyme mRNA expression. mRNA expression  
461 is related to the housekeeping gene RPL37A. High values are represented as *black*, middle  
462 range values are *red* and low values are shown in *white*. Data are presented as a mean of  
463  $n=3$ . *Acid ceramidase* (aCDase), *acid sphingomyelinase* (aSMase), *neutral sphingomyelinase*  
464 *1, 2 and 3* (nSMase 1, 2 and 3).

465 **Figure 2: Sphingolipid species in breast cancer cells identified by LC-HRMS analysis. A** Total  
466 *dihydroceramide* (dhCer) levels. Total of the following analytes: Cer d18:0/22:0, Cer  
467 d18:0/24:0, Cer d18:0/24:1. **B** Total ceramide levels. Total of the following analytes: Cer  
468 d18:1/16:0, Cer d18:1/18:0, Cer d18:1/22:0, Cer d18:1/22:1, Cer d18:1/23:0, Cer d18:1/24:0,  
469 Cer d18:1/24:1. **C** Total sphingadiene-ceramide levels. Total of the following analytes: Cer  
470 d18:2/22:0, Cer d18:2/24:0. **D** Total *galactosylceramide* (GalCer)/*glucosylceramide* (GlcCer)  
471 levels. Total of the following analytes: HexCer d18:1/16:0, 24:0, 24:1. **E** Total  
472 *lactosylceramide* (LacCer) levels. Total of the following analytes: Hex2Cer d18:1/16:0, 24:1. **F**  
473 Total *sphingomyelin* (SM) levels. Total of the following analytes: SM 30:1, 32:1, 32:2, 33:1,  
474 34:0, 34:1, 34:2, 36:1, 36:2, 36:3, 37:1, 38:1, 38:2, 40:1, 40:2, 40:3, 41:1, 41:2, 42:1, 42:2,  
475 42:3, 43:1, 43:2. The identified single analytes are displayed in a heatmap in **supplemental**  
476 **data 1**. Data are presented as a mean of  $n=3 \pm$  SEM. Tukey's multiple comparison test.  
477  $*p \leq 0.05$ ,  $**p \leq 0.01$ ,  $***p \leq 0.001$ ,  $****p \leq 0.0001$ . Area under curve (AUC), internal standard  
478 (IS), not significant (ns).

479 **Figure 3: Non-sphingolipid species in breast cancer cells identified by LC-HRMS analysis. A**  
480 Cholesterol (ST 27:1\_OH). **B** Total sterol ester levels. Total of the following analytes: SE  
481 27:1/14:1, 16:1, 17:1, 18:1, 18:2, 20:3, 20:4, 22:6, 24:1. **C** Total ether lipid levels. Total of the  
482 following analytes: LPC O-16:0, 16:1, 18:0, 18:1, PC O-16:0\_16:0, 18:2, 20:4, PC O-16:1\_16:0,  
483 18:1, 18:2, 20:4, PC O-18:1\_20:4, PC O-34:1, PE O-16:1\_18:2, 20:4, PE O-18:1\_18:1, 18:2,  
484 20:4, LPE O-16:1, 18:1. **D** Total *diglyceride* (DG) levels. Total of the following analytes: DG  
485 32:1, 32:2, 34:1, 34:2, 34:3, 36:1, 36:2, 36:3, 38:2, 38:5. **E** Total *triglyceride* (TG) levels. Total  
486 of the following analytes: TG 42:1, 42:2, 43:0, 44:0, 44:1, 44:2, 46:0, 46:1, 46:2, 46:3, 48:0,  
487 48:1, 48:2, 48:3, 48:4, 50:1, 50:2, 50:3, 50:4, 52:1, 52:2, 52:3, 52:4, 52:5, 54:1, 54:2, 54:3,

488 54:4, 54:5, 54:6, 56:1, 56:2, 56:3, 56:4, 56:6, 58:1, 58:2, 58:3, 58:4, 58:6. **F** Total  
489 glycerophospholipids levels. Total of the following analytes: PE 32:1, 34:1, 34:2, 34:3, 36:1,  
490 36:2, 36:3, 36:4, 36:5, 38:4, 38:5, 38:6, 40:6, 40:7, PG 34:1, 36:2, PI 32:1, 34:1, 34:2, 36:1,  
491 36:2, 36:4, 38:4, 38:6, 40:5, 40:6, PS 32:1, 34:1, 34:2, 36:1, 36:2, 38:2, 38:3, PC 30:0, 30:1,  
492 30:2, 32:0, 32:1, 32:2, 33:2, 34:0, 34:1, 34:2, 34:3, 34:4, 34:5, 36:1, 36:2, 36:3, 36:4, 36:5,  
493 38:2, 38:3, 38:4, 38:6, 38:7, 40:6, 40:7. **G** Total lyso-glycerophospholipid levels. Total of the  
494 following analytes: LPE 16:0, 18:0, LPG 16:0, 18:1, 18:2, LPI 16:0, 18:0, 18:2, 20:3, 20:4, LPS  
495 18:0, 18:1, LPC 14:0, 15:0, 16:0, 17:0, 18:0, 18:3, 20:0, 20:1, 20:3, 22:0, 24:0. **H** Total  
496 acylcarnitine levels. Total of the following analytes: acylcarnitine 14:1, 16:0, 18:0, 18:1. The  
497 identified single analytes are displayed in heatmaps in **supplemental data 2**. Data are  
498 presented as a mean of  $n=3 \pm$  SEM. Tukey's multiple comparison test.  $*p \leq 0.05$ ,  $**p \leq 0.01$ ,  
499  $***p \leq 0.001$ ,  $****p \leq 0.0001$ . Area under curve (AUC), internal standard (IS), not significant  
500 (ns).

501 **Figure 4: Morphology, proliferation and ether lipid metabolizing enzyme mRNA expression**  
502 **analysis by qRT-PCR of MCF-7, T47D and SKBr3 cells.** **A** Light microscopy images of breast  
503 cancer cells with differing ER and GPER1 status. A representative image of each breast  
504 cancer cell line is displayed. **B** Living cell number on day 1, 2, 3, 4 and 5. Data are presented  
505 as a mean of  $n=3 \pm$  SEM. Tukey's multiple comparison test. **C** *Glyceronephosphate O-*  
506 *acyltransferase* (GNPAT), *alkylglycerone phosphate synthase* (AGPS), *alkylglycerol*  
507 *monooxygenase* (AGMO), *fatty acid desaturase 1* (FADS1) and *carnitine palmitoyltransferase*  
508 *1A* (CPT1A) mRNA expression related to the housekeeping gene RPL37A. Data are presented  
509 as a mean of  $n=3 \pm$  SEM. Tukey's multiple comparison test.  $*p \leq 0.05$ ,  $**p \leq 0.01$ ,  $***p \leq 0.001$ ,  
510  $****p \leq 0.0001$ .

511 **Figure 5: Internal Standard (IS) normalized LC-HRMS data (upper row) and median peak**  
512 **ratio (MPR) normalized LC-HRMS data (lower row).** **A** SKBr3/MCF-7. **B** T47D/MCF-7. **C**  
513 SKBr3/T47D.

514 **Summary Figure: Summary of ether lipid metabolism alterations in GPER1 +, but ER - cells.**  
515 The acyl CoA required for ether lipid synthesis in SKBr3 cells might be generated by *de novo*  
516 lipogenesis rather than  $\beta$ -oxidation. Both key enzymes of ether lipid synthesis  
517 (*glyceronephosphate O-acyltransferase* (GNPAT) and *alkylglycerone phosphate synthase*  
518 (AGPS)) are strongly increased in SKBr3 cells. Since ether lipid degrading *alkylglycerol*

519 *monoxygenase* (AGMO) is also increased, accelerated ether lipid metabolism in GPER1 +  
520 (ER-) cells is assumed (red arrow = displays changes in mRNA expression level).

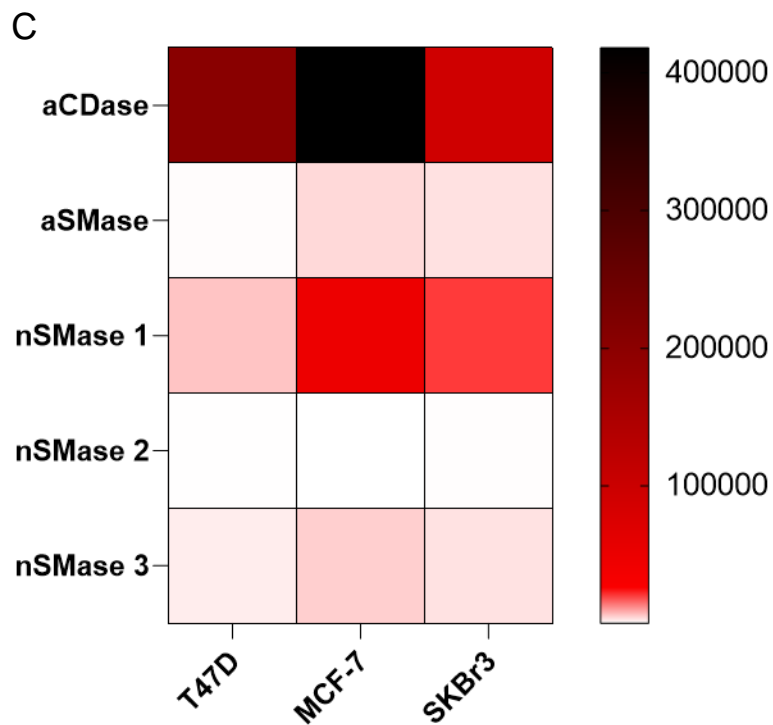
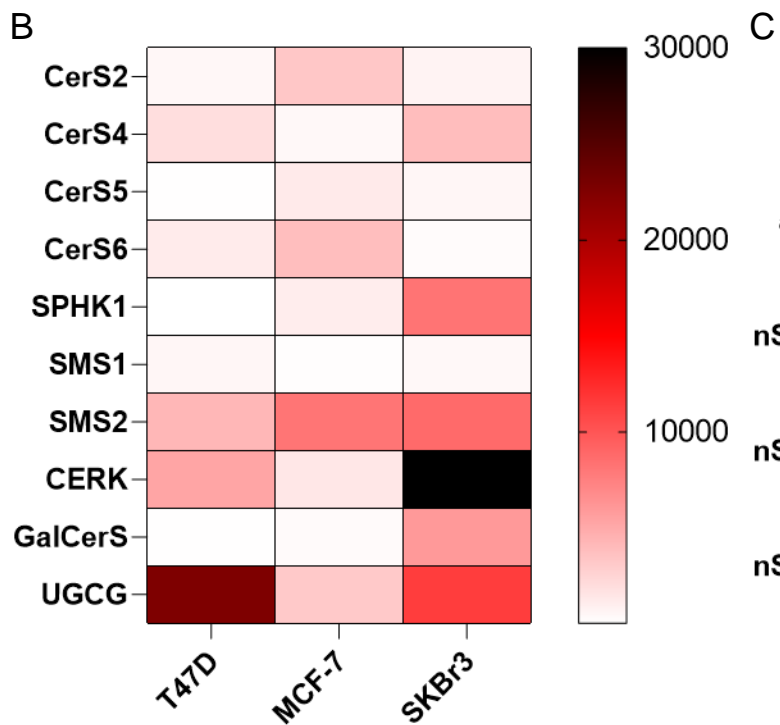
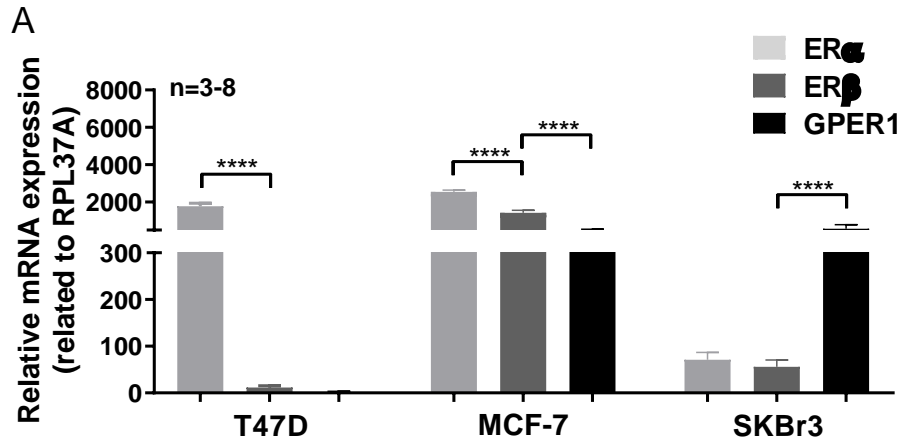
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Figure 1



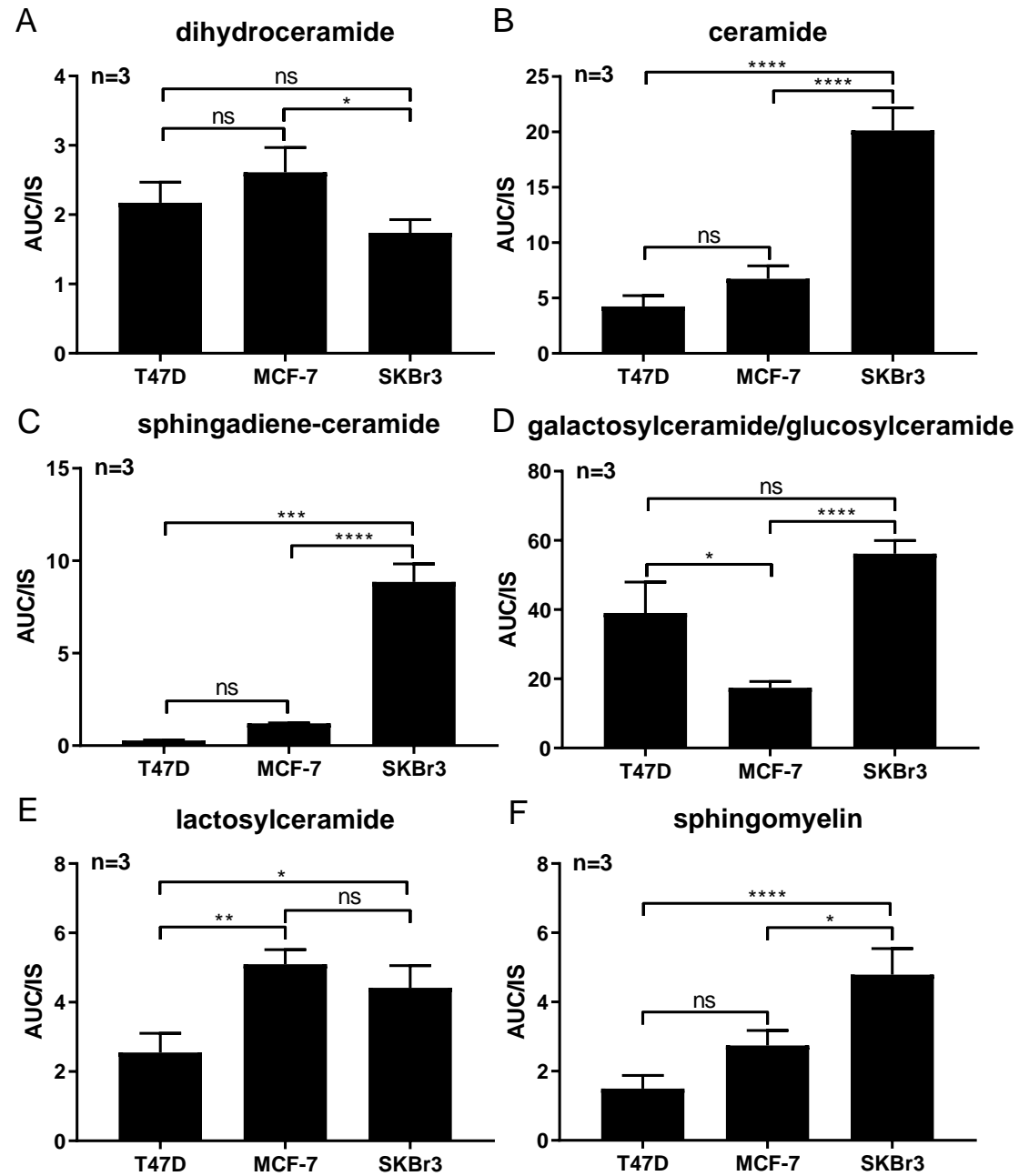


Figure 2

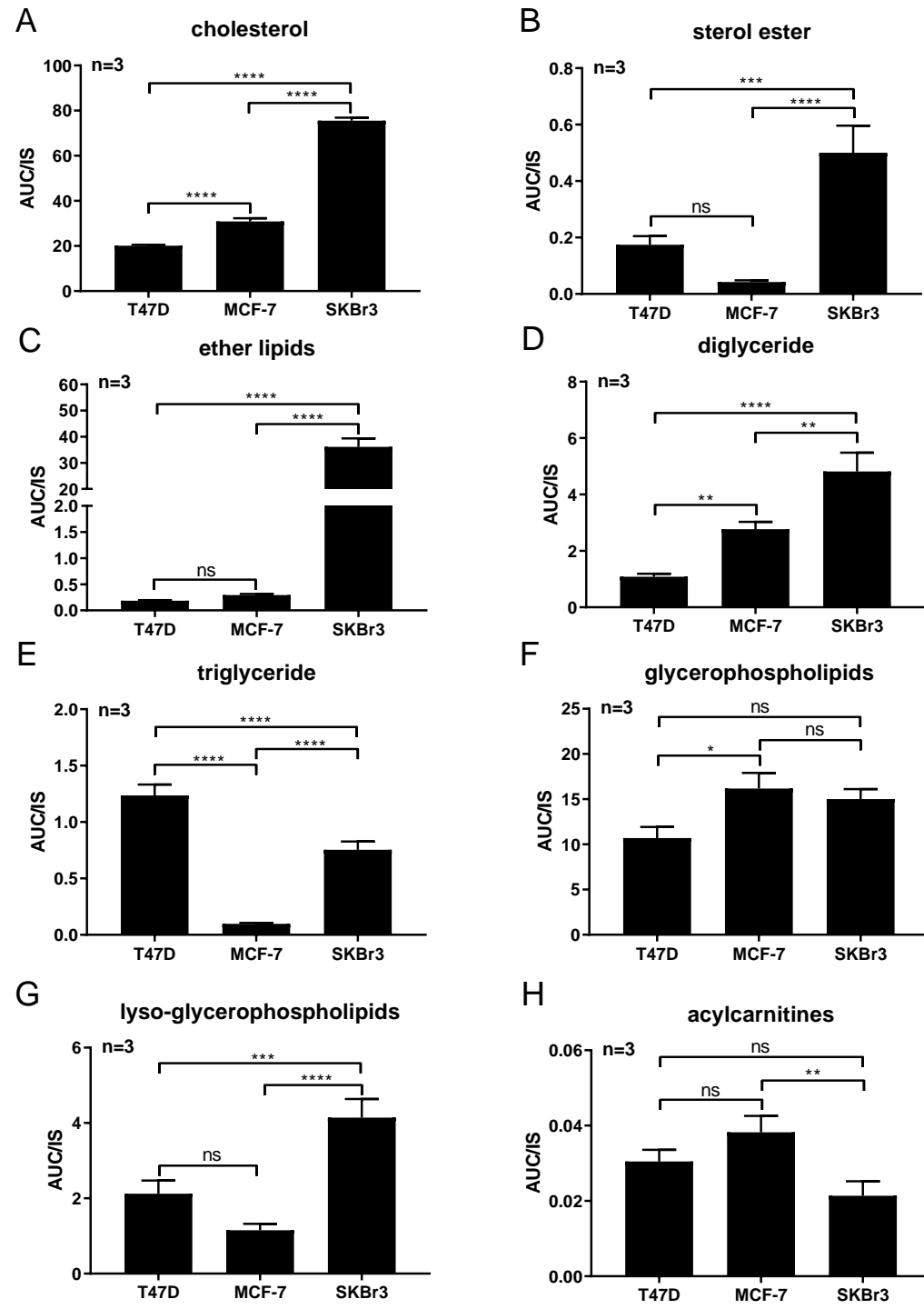


Figure 3



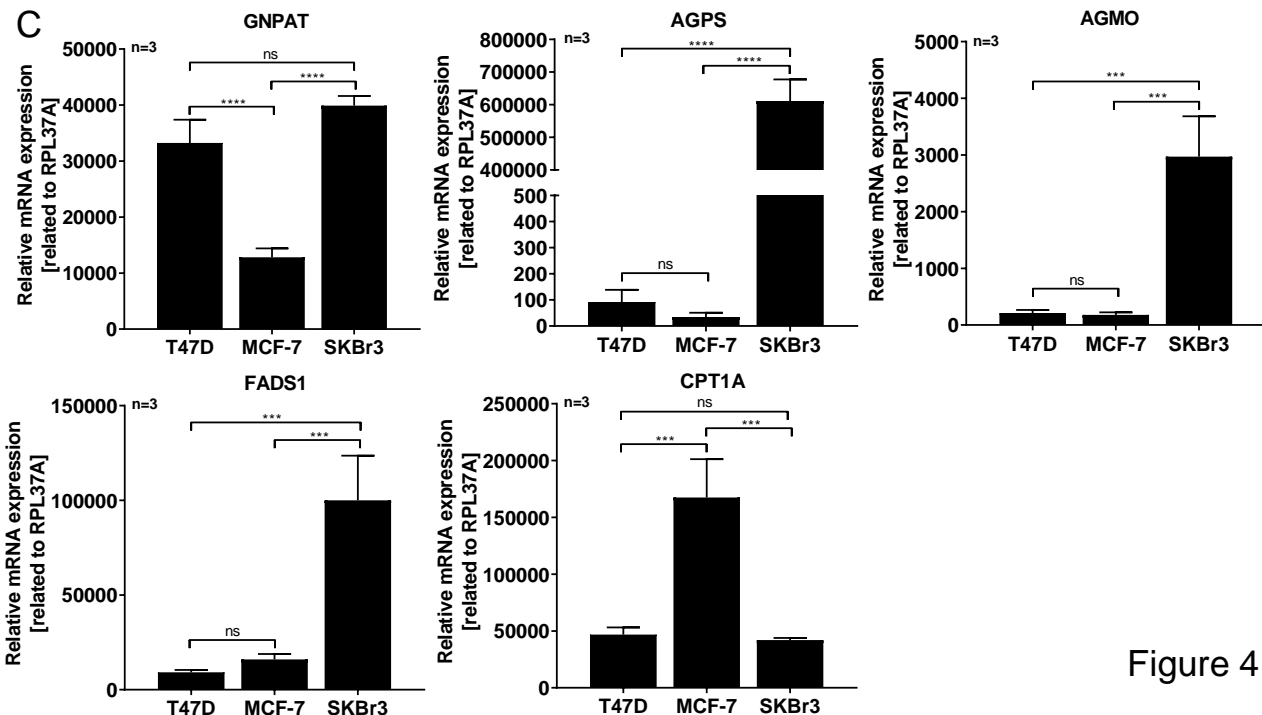
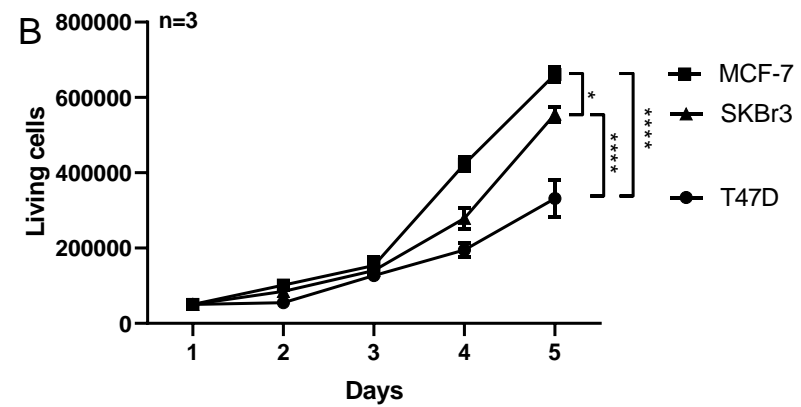
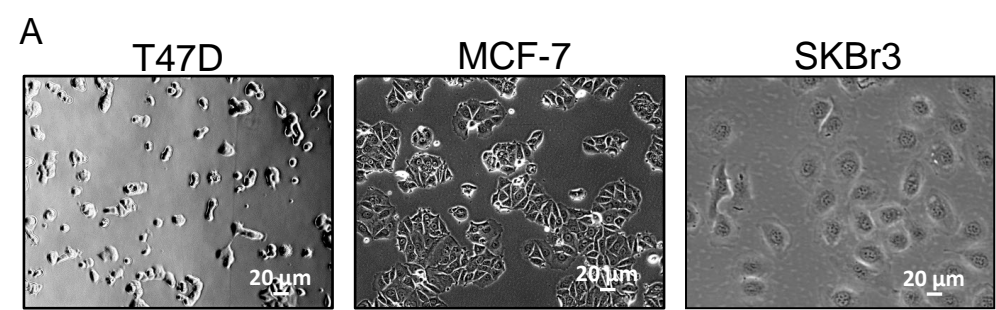


Figure 4

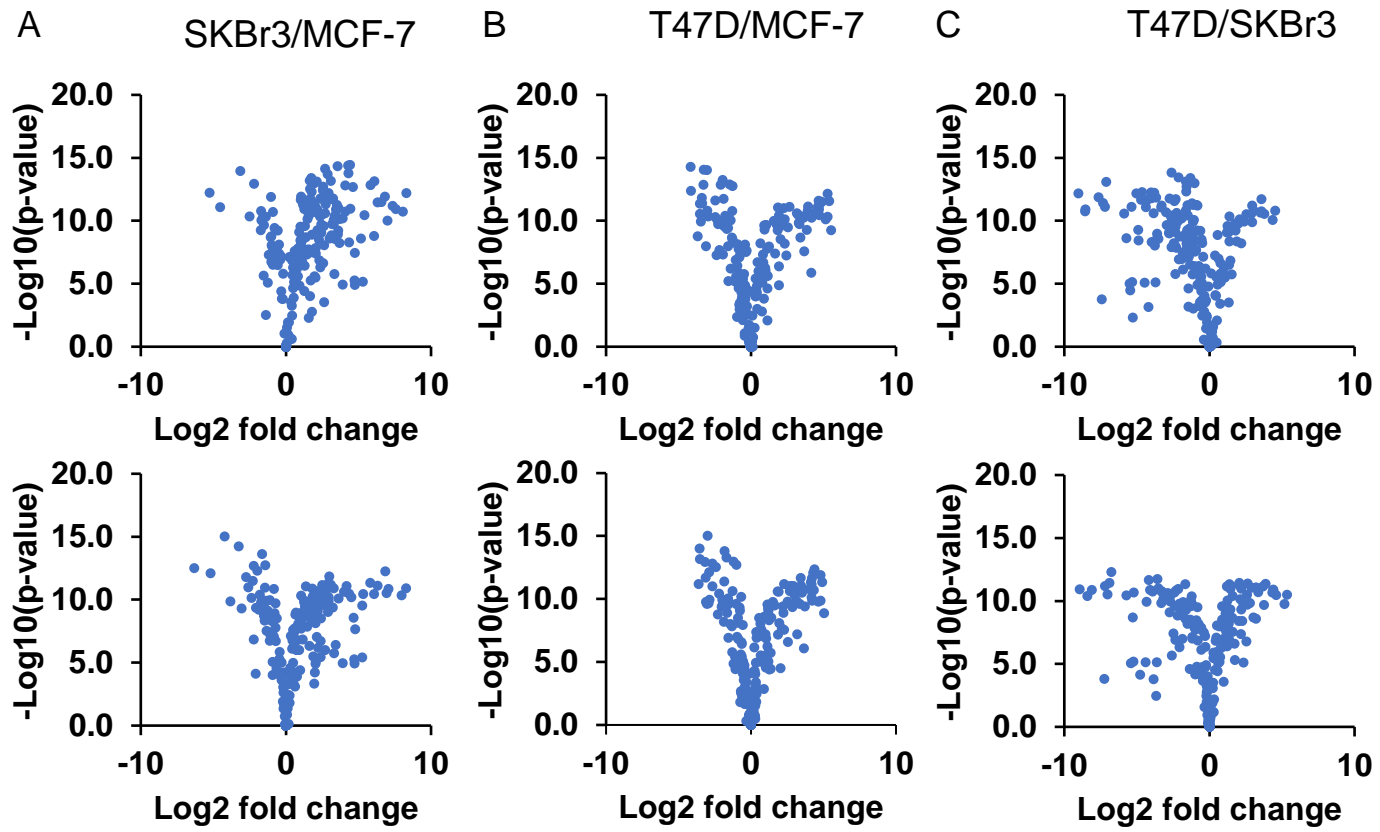
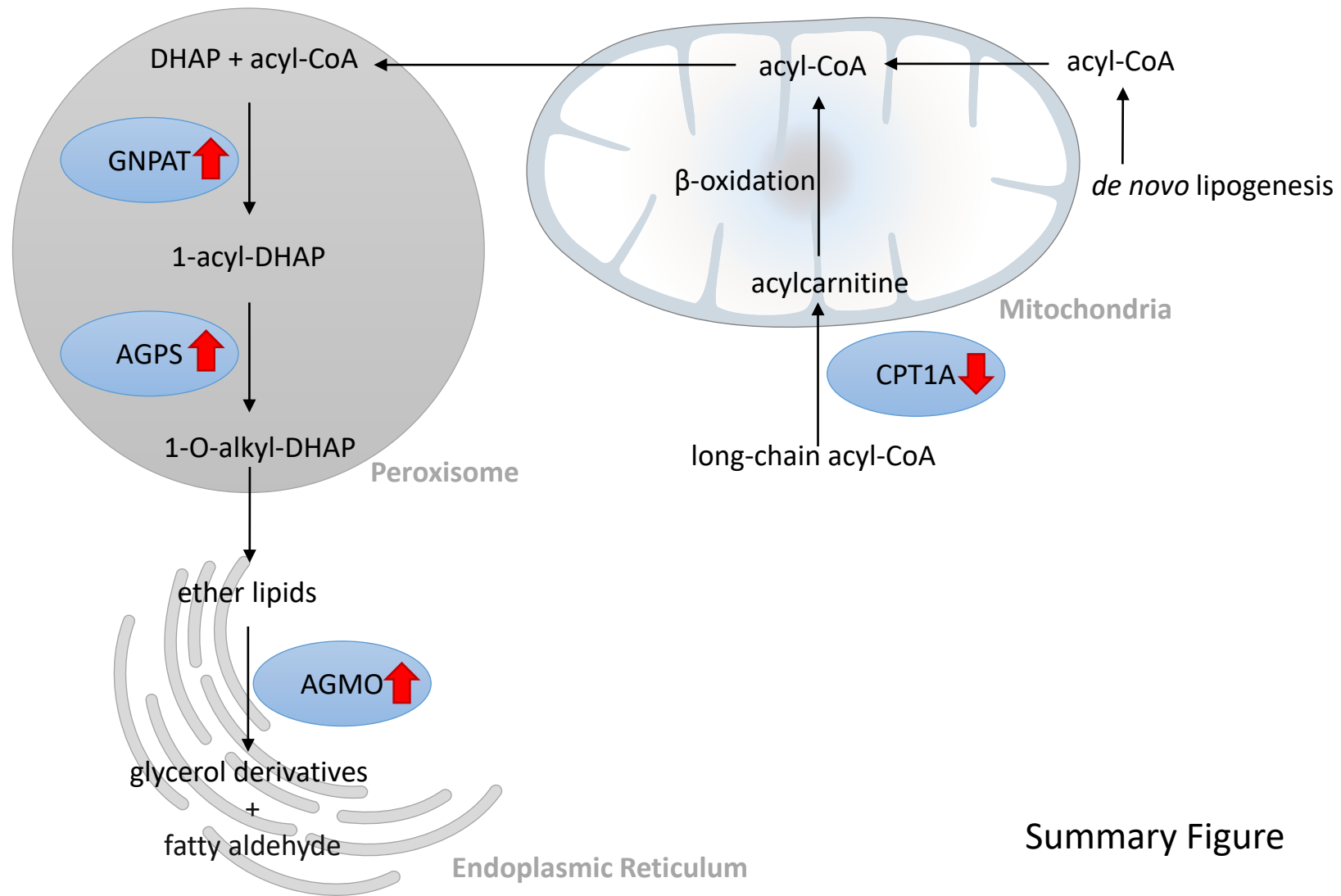


Figure 5



Summary Figure