



## Review

# Regulation of the cancer cell membrane lipid composition by NaCHoleate<sup>☆</sup>

## Effects on cell signaling and therapeutical relevance in glioma



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## ABSTRACT

This review summarizes the cellular bases of the effects of NaCHoleate (2-hydroxyoleic acid; 2OHOA; Minerval) against glioma and other types of tumors. NaCHoleate, activates sphingomyelin synthase (SGMS) increasing the levels of cell membrane sphingomyelin (SM) and diacylglycerol (DAG) together with reductions of phosphatidylethanolamine (PE) and phosphatidylcholine (PC). The increases in the membrane levels of NaCHoleate itself and of DAG induce a translocation and overexpression of protein kinase C (PKC) and subsequent reductions of Cyclin D, cyclin-dependent kinases 4 and 6 (CDKs 4 and 6), hypophosphorylation of the retinoblastoma protein, inhibition of E2F1 and knockdown of dihydrofolate reductase (DHFR) impairing DNA synthesis. In addition in some cancer cells, the increases in SM are associated with Fas receptor (FasR) capping and ligand-free induction of apoptosis. In glioma cell lines, the increases in SM are associated with the inhibition of the Ras/MAPK and PI3K/Akt pathways, in association with p27Kip1 overexpression. Finally, an analysis of the Repository of Molecular Brain Neoplasia Data (REMBRANDT) database for glioma patient survival shows that the weight of SM-related metabolism gene expression in glioma patients' survival is similar to glioma-related genes. Due to its low toxicity and anti-tumoral effect in cell and animal models its status as an orphan drug for glioma treatment by the European Medicines Agency (EMA) was recently acknowledged and a phase 1/2A open label, non-randomized study was started in patients with advanced solid tumors including malignant glioma. This article is part of a Special Issue entitled: Membrane Structure and Function: Relevance in the Cell's Physiology, Pathology and Therapy.

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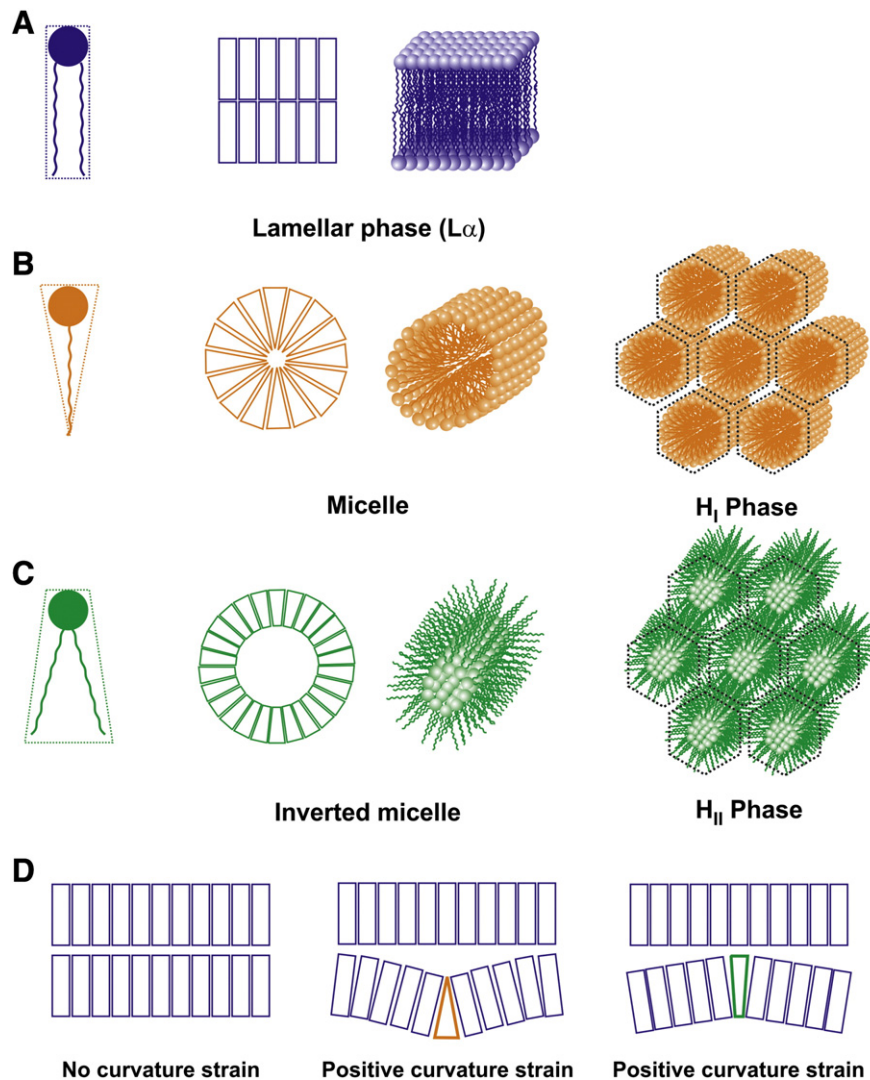
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### 1. Membrane lipid composition and cancer

Normal or pathological cellular functions are generally associated with the activity of specific proteins. As a consequence, most therapeutic molecules are targeted to bind these specific proteins to revert their



**Fig. 1.** Membrane lipid structures. Examples of the relationship between lipid shapes, intrinsic curvatures and lipid phases. A. Lipids with rectangular shapes (e.g. PC, SM) do not confer a curvature strain forming lamellar phases. B. Lipids with a bulky polar head and only one acyl chain (e.g. lysophospholipids) have an inverted cone shape inducing a positive curvature strain in membranes. They favor the formation of micelles or  $H_1$  phases. C. Lipids with a small polar head (e.g. PE, CHO, DAG) have a molecular shape that resembles a truncated cone. They induce a negative curvature strain and favor the formation of inverted micelles and hexagonal  $H_{II}$  phases. D. Examples of phospholipid-induced curvature strains in the membrane bilayer.

malfunction and/or alter their expression. Many of these proteins (channels, receptors and enzymes) interact with lipids of the cell membranes and these protein–lipid interactions are susceptible to be modified, leading to changes in the protein activity [1]. In fact, changes in the levels of lipids have been described in a number of pathologies. For example, modifications in the lipid composition or structure of cell membranes have been associated with the development of cardiovascular pathologies, such as hypertension, atherosclerosis, coronary heart disease and thrombosis [2–5].

Cancer cells show a constitutive activation of the fatty acid biosynthesis to provide enough fatty acids to generate new membranes due to the increased proliferation ratio [6–8]. In this context, it has been demonstrated that in breast tissue the fatty acid and phospholipid profiles are altered not only in the tumor areas but also in the surrounding healthy areas. This provides evidence that changes in phospholipid composition can occur before the morphological tumoral changes [9]. Changes in the lipid composition can also be related to the malignancy of the tumor. For example, the pro-apoptotic ceramide is reduced in tumors and their levels are inversely associated with tumor progression [10]. It is also known that gangliosides, which act as immunosuppressors or as angiogenesis promoters [11], are aberrantly distributed in a number of tumors

[12]. Another important lipid pathway modified in cancer cells is cholesterol (CHO) metabolism. Accumulation of esterified CHO levels are associated with increased cell cycle progression and tumor growth [13].

In the last few years our group has accumulated evidence showing the relationship between the plasma membrane sphingomyelin (SM) levels and tumorigenesis. SM is synthesized by sphingomyelin synthase (SGMS) which catalyzes the transfer of a phosphocholine head group from a phosphatidylcholine (PC) to a ceramide yielding one diacylglycerol (DAG) [14]. This process can occur in the Golgi apparatus mediated by SGMS1 or in plasma membrane by SGMS2 [15]. As SGMSs are able to reverse the SM production forming again SM and DAG, they are considered regulators of proapoptotic ceramides and DAG as a second messenger [16]. In this context, we have described that SM levels are reduced in a variety of cancer cells compared to non-tumoral cells, and the restoration of normal levels of SM by the activation of SGMS inhibits cell cycle proliferation of cancer cells and/or induces cell death [16].

In this revision, we first discuss how the lipids are organized in the cell membrane and how lipids affect the cell signaling events involved in different cancer-related processes. Therefore, lipid molecules could be used as therapeutic molecules for the treatment of cancer. As an example, we describe the mechanism of action of one of these lipid

drugs, NaChOleate, a SGMS activator that has a promising potential in cancer therapy.

## 2. How is the cell membrane organized?

In the 70s, a model for the cell plasma membrane structure where lipids were distributed homogeneously as a double layer was proposed. This structure not only provided shape and protection to the cell but also a fluidic environment surrounding the embedded proteins [18]. Although nowadays the basic structure of this lipid bilayer is still accepted, we know that lipids and proteins are not homogeneously distributed in the plasma membrane [19]. First, there is a heterogeneity or asymmetry in the lipid composition of the membrane: the inner mono-layer contains a higher concentration of phosphatidylserine (PS) and phosphatidylethanolamine (PE) whereas the extracellular mono-layer is enriched in PC and SM [20]. An additional level of complexity and asymmetry is present in lipid rafts, caveolae, receptors/channel clusters and synaptosomes [21,22]. Even more, in a cell membrane there exists an enormous combination of lipid distributions due to the large amount of different lipid species (about 1300–1500 on average), which allows lipophilic or electrostatic interactions among each species permanently interchanging their “partners” through lateral diffusion along the cell membrane [23].

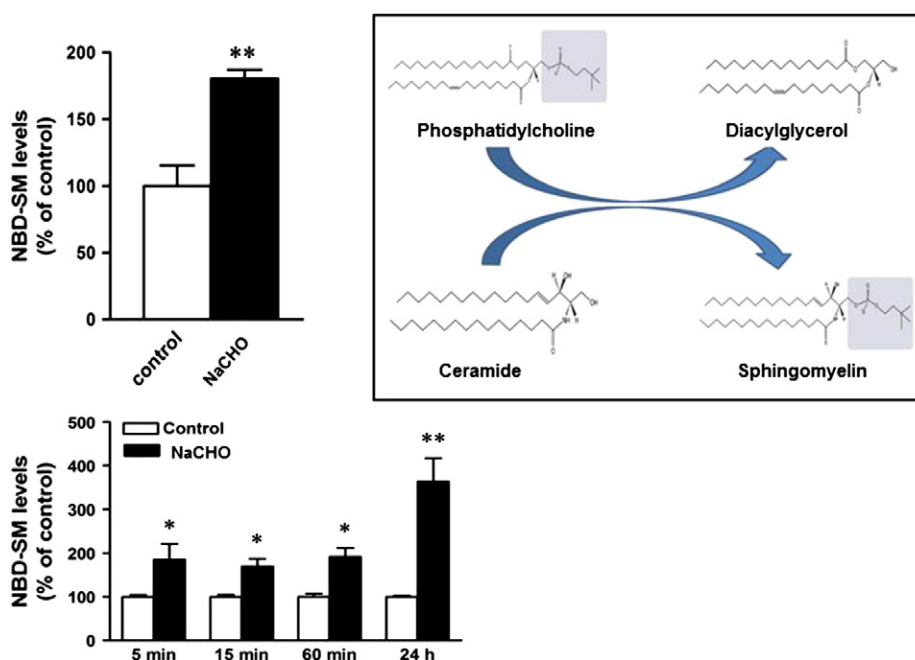
Depending on the lipid composition, the lipids are organized in supra-molecular structures that present characteristic biophysical properties, such as fluidity, electric charge, cross sectional area, lateral pressure profile, surface packing and non-lamellar-phase propensities (Fig. 1). The most common lipid organization in the membrane is the lamellar phase which can be subdivided in several types of lamellar sub-structures. These sub-structures can change from one to another depending of lipid composition, pH, ionic strength, temperature, water concentration or lateral pressure and the temperature that modulates the fluidity [24]. The lamellar  $\alpha$  sub-structure ( $L_\alpha$ ), also known as fluid lamellar phase, liquid crystalline or liquid disordered ( $L_d$ ), is the structure found in most domains and regions of the cell membrane and shows high levels of lipid and protein mobility. This lamellar structure can evolve to a variety of more organized structures, such as the gel phase ( $L_\beta$ ), pseudo-

crystalline ( $L_c$ ), ripped ( $P_\beta$ ) membranes and the solid ordered ( $s_o$  or  $L_o$ ) [25]. The  $L_o$  phase is found in the lipid raft domains. Lipid rafts are enriched in CHO and SM with the acyl chains of the lipids extended and highly packed, but highly dynamic [26]. Essential cellular regulation processes are compartmentalized in these CHO and SM raft domains [22,27]. CHO and other sterols, do not form a bilayer by themselves, but are important functional membrane components, by modifying the fluidity and organizing the  $L_o$  phases [28].

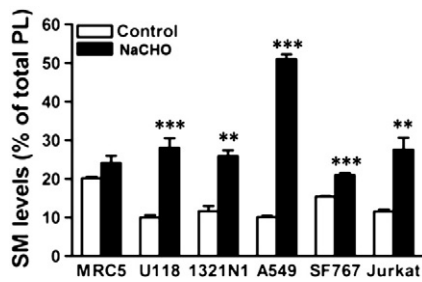
In addition to the lipids that form fluid and organized lamellar phases, the cell membrane includes the so-called non-lamellar-prone lipids such as PE, CHO, DAG and acidic PS. These lipids are structurally characterized by a non-cylinder shape that induces a curvature stress into one of the layers of the cell membrane. If non-lamellar-prone lipids are abundant, the membrane can adopt a conformation in which some lipids adopt an extended shape with one of the acyl chain out of the bilayer allowing a better access to the inner part of the cell membrane [21]. The lipid curvature stress induced by the non-lamellar-prone lipids can be organized into hexagonal and cubic phases among others [29]. For example, the DAG and PE induce negative curvature stress into the cell membrane and can even form inverted hexagonal structure mono-layers ( $H_{II}$ ) or inverted micelles. The formation of  $H_{II}$  phases is critical in the budding, fusion and fission processes [30]. On the other hand, other lipids with big polar heads as lysophospholipids induce positive curvature strains in the membranes, forming normal hexagonal monolayers ( $H_I$ ) or micelles.

The presence of these domains or the prevalence of one type of phase in the membrane can direct the activation state and location of a number of key membrane proteins as enzymes, receptors, channels and effectors of a variety of cell signaling pathways.

G proteins and G protein-coupled receptors (GPCRs) are interesting examples of protein activities modulated by membrane structure. When the GPCRs are activated by their ligand, the signal is transmitted by inducing the activation/dissociation of the heterotrimeric  $G\alpha\beta\gamma$  proteins into the  $G\alpha$  and  $G\beta\gamma$  subunits. Whereas the GPCRs and  $G\alpha\beta\gamma$  have greater affinity for  $L_c$  regions (with  $H_{II}$  propensity), the  $G\alpha$  subunits are recruited in  $L_o$  regions where they interact with their effectors.



**Fig. 2.** NaChOleate directly activates SMS. Upper-left panel, SMS activity is observed immediately after incubation with NaChOleate, in intact U118 cells. This effect was also observed in vitro (bottom-left). Values represent the mean  $\pm$  SEM;  $n = 3$ ; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ . The upper-right panel shows the reaction catalyzed by SMS. This figure was adapted from G. Barceló-Coblijn et al., Proc. Natl. Acad. Sci. U. S. A., 2011 December 6; 108 (49): 19569–19574 [17].

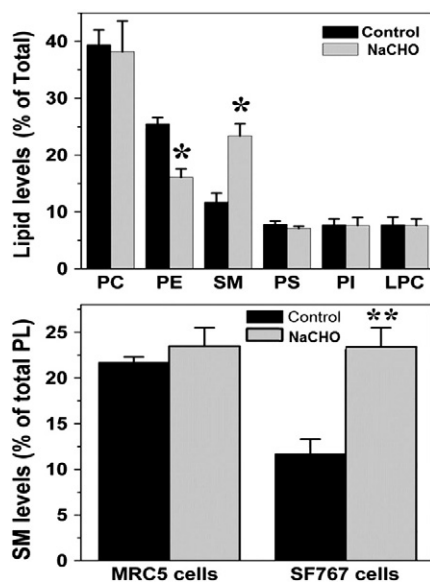


**Fig. 3.** Selective induction of SM levels in cancer cells by NaChOleate. NaChOleate induces a marked increase of SM levels in human glioma (U118, 1321N1, SF767), lung cancer (A549) and leukemia (Jurkat) cells, but not in normal cells. In this context, SM is found at very low levels in all cancer cells studied and its membrane concentration is normalized by treatments. Values represent the mean  $\pm$  SEM;  $n = 3$ ; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . This figure was adapted from G. Barceló-Coblijn et al., Proc. Natl. Acad. Sci. U. S. A., 2011 December 6; 108 (49): 19569–19574 [17].

In fact, the adenylyl cyclase, and the  $G\beta\gamma$  subunits are located in regions where they interact with their own specific effectors and inactivate the GPCRs [31–33]. Similarly, some isoforms of protein kinase C (PKC) are activated by a non-lamellar lipid, such as PE and DAG, and translocate to the membrane regions with propensity to form  $H_{II}$  non-lamellar phases [31]. Even more, inverted phases can induce the activation of PKC by itself [34–36].

Another example is phospholipase C which once activated, converts the phosphatidylinositol (PI) into inositol phosphate and DAG. Recent studies have described how the inositol phosphate effectors, with the FYVE peptide sequence that targets phosphatidyl inositol tri-phosphate are accumulated in the inner layer of the membrane in PI enriched domains [37].

The lipid rafts can also modify the activity of key proteins in cell survival. For example, in conditions of increased synthesis of SM, the FasR can be activated and trigger extrinsic apoptosis due to the



**Fig. 4.** NaChOleate (72 h, 200  $\mu$ M) induces changes in SF767 membrane-lipid composition. Upper panel, levels of the major phospholipid classes before (black bars) and after (gray bars) 20HOA treatment: PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; PS, phosphatidylserine; PI, phosphatidylinositol; LPC, lyso-phosphatidylcholine. Lower panel, effects of NaChOleate (72 h, 200  $\mu$ M) on SM levels in normal (MRC5) and glioma (SF767) cells before (black) and after (gray) treatment ( $n = 6-8$ ). This figure was adapted from S. Teres et al., Proc. Natl. Acad. Sci. U. S. A., 2012 May 29; 109 (22): 8489–8494 [62].

clustering of the receptor in the lipid rafts [38]. In addition, some membrane-acting drugs as edelfosine, mitelfosine and NaChOleate can cluster and activate death receptors without the presence of their ligand as will be discussed below [39,40].

Besides the differential affinity of the intrinsic or interacting proteins with the membrane, the membrane structure is also modulated by the interaction with proteins. Several proteins have posttranscriptional lipid modifications that allow the protein to interact with the membrane [41]. For example, the non-lamellar-phase binding propensity of G $\gamma$  subunit is enhanced by the interaction of the membrane with the isoprenyl moieties and with the transmembrane domain of membrane receptors [42,43].

If we consider that most of the cancer-related pathways are, initially, activated in the membrane and the lipid modifications occurring in cancer cells are associated with the activation of proliferation and tumorigenesis, it is conceivable that lipid modifications can regulate these pathological cell signaling pathways.

### 3. Membrane-lipid therapy

The membrane-lipid therapy is a novel therapeutic approach in which the drugs are designed to target the membrane of diseased cells, modulating its composition and structure and therefore modifying the activity of membrane-interacting proteins. These drugs are lipophilic or amphiphilic molecules with the ability to change the general lipid membrane organization and structure.

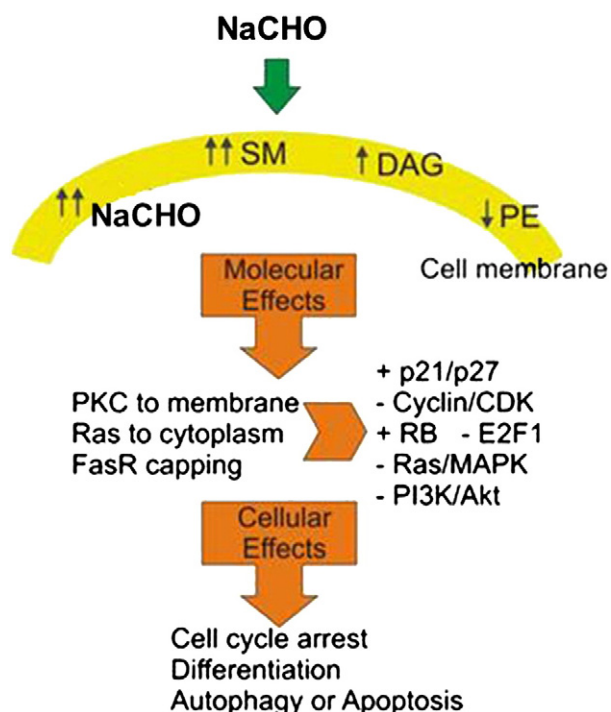
There are a number of natural lipid molecules that precisely do so. For example, oleic acid increases the  $H_{II}$  non-lamellar phase propensity whereas its analogs, the trans-mono-unsaturated elaidic acid and the saturated stearic acid, do not affect to the membrane structure. This difference is because of their differences in the shape of the molecule. While oleic acid presents a distinctive “boomerang” shape, the elaidic and stearic acids have a “rod-shape” that does not modify the membrane structure. Some of the membrane-bound proteins that are known to be modulated by oleic acid are the GPCRs, protein G and their effectors controlling blood pressure [44–46]. In fact, a diet rich in olive oil (presents high content of oleic acid) has been associated with a lower risk of developing cancer [47].

Other natural lipids such as n-3 polyunsaturated fatty acids when introduced in the organism by the diet, are readily inserted in the lipid rafts displacing the CHO and provoking a de-clustering effect in the lipid rafts, modifying the location of some intrinsic membrane proteins, such as for example, the MHC class I proteins [48].

One of the first evidences that antitumor drugs could act through the membranes was the discovery that anthracyclines do not need to be internalized into the cell to exert its anti-tumoral function [49]. In the past decade a number of other molecules have shown to be effective as an anti-cancer drug by targeting the membrane. For example, daunorubicin and hexamethylene bisacetamide modify the nonlamellar  $H_{II}$  phase formation in the membrane [50,51]. In addition, hexamethylene bisacetamide induces differentiation and cell cycle arrest of erythroleukemia cells by reducing the DAG levels [52] and CHO esterification [13].

Alkylphospholipids belong to another class of drugs that target the cell membrane. Among this class edelfosine, mitelfosine and perfosine, induce clustering and activation of Fas death receptor (FasR) through structural changes of the lipid rafts [40,53].

Some anti-tumoral drugs have been recently modified by adding a lipid motive in order to reduce the toxicity and/or increase its effectiveness. For example, NEO6002, a gemcitabine derivate is modified by a cardiolipin motive to reduce the toxicity and overcome the gemcitabine resistance in cancer cells [54]. Another representative example is propofol-DHA; the combination of the anesthetic propofol with the n-3 fatty acid induces apoptosis in breast cancer cells [55,56].



**Fig. 5.** Selective molecular and cellular effects of NaCHOLEate on cancer cells. NaCHOLEate induces marked changes in the membrane levels of lipids that modify the interaction of pivotal signaling proteins with membranes and their corresponding activities. The signaling pathways regulated by these proteins are modified accordingly, resulting in induction of cell cycle arrest, differentiation and autophagy or apoptosis in cancer but not normal cells.

Not only lipid molecules are used to modify the cell membrane structure and function. Bactericidal peptides bind the membrane altering its structure inducing anti-tumoral effects [57,58].

#### 4. Plasma membrane modifications by NaCHOLEate

NaCHOLEate was specifically designed to regulate the lipid composition and structure of the cell membrane (membrane lipid therapy).

Several years ago our group showed that NaCHOLEate modified plasmatic membrane composition and structure, altering the capacity of different proteins to bind to the membrane and as a consequence interfere with its activity [34,59].

NaCHOLEate is incorporated in a variety of cancer cells faster than in non-tumoral control fibroblasts [60]. Moreover, the hydroxylation of the fatty acids present in the phospholipid fraction of cell membranes is increased after the treatment with NaCHOLEate, indicating the incorporation of NaCHOLEate in the glycerophospholipids of the cell [61]. The NaCHOLEate content in the total fatty acid in the glycerophospholipid fraction is raised 15% after NaCHOLEate treatment. Even more, NaCHOLEate is also incorporated in the triacylglyceride and DAG fraction of the neutral lipid, but not into the sphingolipids. The above represent solid evidences that NaCHOLEate targets and it is incorporated into the cell membranes.

NaCHOLEate treatment modifies the composition, fluidity and the membrane lipid structure of cancer cells and other diseased cells [21]. For example, the treatment of hypertensive rats with NaCHOLEate lowers the blood systolic pressure to normal values and increases the propensity of the membranes to form  $H_{II}$ -phases [59].

It has been shown that in NaCHOLEate-treated glioma cells the  $L_d$  domains become more disordered, and conversely, the  $L_o$  domains become more rigid and compact [17,61]. In this context, one of the critical events triggered by NaCHOLEate in cancer cell membrane is the activation of SGMS (Fig. 2), increasing the SM levels in the membrane (Figs. 3 and 4). These changes appear to be essential for the antiproliferative effect of NaCHOLEate in glioma cells [17,62].

In addition to the SGMS activation, it has been shown that NaCHOLEate decreases PE levels (Fig. 4), reduces oleic acid levels and increases saturated fatty acids incorporated in PC and PE in a number of cancer cells but not in non-cancer cells (MRC5 human fibroblasts) [61,62]. The reduction of oleic acid levels can be explained by its substitution by NaCHOLEate and/or the inhibition of fatty acid biosynthesis by the effect of NaCHOLEate in inhibiting stearoyl-CoA desaturase (SCD1) activity, an enzyme that catalyzes a rate-limiting step in the synthesis of unsaturated fatty acids.

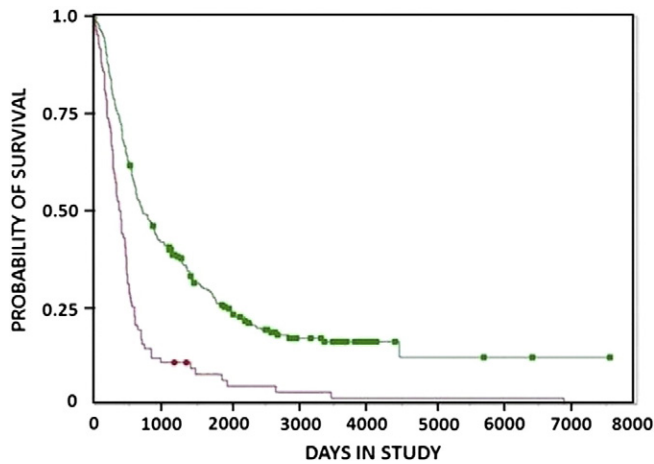
In summary NaCHOLEate treatment induces a variety of changes in membrane composition and in lipid metabolism enzymes modifying the lipid structure and inducing the formation of different domains in the cell membrane. For example, the increase in the order of the  $L_o$  domains could be attributed to the changes in membranes levels of SM and as a consequence the proportion of lipid rafts, with SM and CHO as major components. As discussed, the lipid rafts are important signaling platforms and their structure is sensitive to membrane lipid composition as well as the activity proteins that interact in these domains [63–65]. This decreased order of  $L_d$  domains can be associated with a reduction in the surface lateral pressure of the lipid bilayer that can modify the binding and activity of some enzymes to the membrane, such as PKC [34,66], and also activates SGMS and inhibits SCD1 [60,61]. As will be discussed next, a consequence of the above described changes in the membrane structure and membrane-lipid enzymes is the inhibition of the proliferative signaling pathways in cancer cells.

**Table 1**

Demographic and clinical characteristics of patients in REMBRANDT.

Variable		Astrocytoma	Glioblastoma	Mixed	Oligodendroglioma	Total
<b>N (%)</b>	n = 343	105 (30.6%)	181 (52.8%)	7 (2.0%)	50 (14.6%)	<b>343 (100.0%)</b>
Male, n (%)	n = 249	54 (66.7%)	79 (61.7%)	4 (57.1%)	16 (48.5%)	153 (61.4%)
Age, m ± SD	n = 297	44.6 ± 14.8	55.2 ± 12.7	40.6 ± 6.9	47.1 ± 15.9	50.3 ± 14.6
Grade, n (%)	n = 247					
Mild		0 (0%)	2 (1.9%)	0 (0%)	0 (0%)	2 (0.8%)
Moderate		50 (53.2%)	0 (0%)	2 (40.0%)	19 (47.5%)	71 (28.7%)
Severe		44 (46.8%)	0 (0%)	3 (60.0%)	21 (52.5%)	68 (27.5%)
Very severe		0 (0%)	106 (98.1%)	0 (0%)	0 (0%)	106 (42.9%)
Race, n (%)	n = 224					
White		61 (91.0%)	114 (95.8%)	6 (85.7%)	29 (93.5%)	210 (93.8%)
Black		3 (4.5%)	2 (1.7%)	0 (0%)	1 (3.2%)	6 (2.7%)
Other		3 (4.5%)	3 (2.5%)	1 (14.3%)	1 (3.2%)	8 (3.6%)
Karnofsky baseline, m ± SD	n = 160	87.4 ± 18.2	74.6 ± 22.4	90.0 ± 10.9	80.8 ± 24.1	80.1 ± 21.8
Follow-up in months, m ± SD	n = 343	50.1 ± 14.6	19.6 ± 18.5	52.7 ± 41.5	47.1 ± 55.9	33.6 ± 38.1

REMBRANDT database (Glioma Molecular Diagnostic Initiative) contains gene expression arrays, gene copy number arrays and clinical data of 343 glioma patients (astrocytoma, glioblastoma, mixed and oligodendroglioma).



**Fig. 6.** The levels of gene expression for SMS1 were down-regulated at least two-fold in 84 (24.5%) of 343 glioma samples. The survival on Kaplan–Meier plots of patients with gliomas showed a significant adverse association with the lower expression of SMS1 with  $p$  values  $< 0.0001$ . Green: glioma patients with normal SMS1 expression ( $n = 258$ ); red: glioma patients with low expression of SMS1 ( $n = 84$ ).

## 5. NaChOleate: a treatment against glioma

Glioma is the most common type of primary brain tumor and is associated with very high rates of mortality and a high resistance to chemotherapy and apoptosis induction [67]. The reference drug in glioma treatment is temozolomide, an alkylating agent, which increases the life expectancy of patients to about 2.5 months [68]. Due to the promising anticancer effect of NaChOleate in lung and leukemic cancer cells and its lipid nature we decided to explore it as a treatment against glioma. We have demonstrated that NaChOleate is potentially more effective than temozolomide using xenographic mice models. Moreover, in contrast to temozolomide, there is no apparent relapse in the tumor formation after treatment with NaChOleate [62].

**Table 2**  
Expression of selected lipid and cancer genes in REMBRANDT.

	Gene	Down (n, %)	Intermediate (n, %)	Up (n, %)	
Lipid	ASAH2	115 (33.5%)	224 (65.3%)	4 (1.2%)	
	FASN	101 (29.4%)	234 (68.2%)	8 (2.3%)	
	SMPD2	0 (0%)	288 (84.0%)	55 (16.0%)	
	SMPD3	74 (21.6%)	269 (78.4%)	0 (0%)	
	PHYH	30 (8.7%)	311 (90.7%)	2 (0.6%)	
	SPTL3	15 (4.4%)	197 (57.4%)	131 (38.2%)	
	SGMS1	84 (24.5%)	258 (75.2%)	1 (0.3%)	
	SGMS2	16 (4.7%)	254 (74.1%)	73 (21.3%)	
	SPHK1	6 (1.7%)	283 (82.5%)	54 (15.7%)	
	SPHK2	20 (5.8%)	292 (85.1%)	31 (9.0%)	
	UGCG	21 (6.1%)	283 (82.5%)	39 (11.4%)	
	Cancer	AKT1	0 (0%)	317 (92.4%)	26 (7.6%)
		AKT2	0 (0%)	285 (83.1%)	58 (16.9%)
		AKT3	48 (14.0%)	291 (84.8%)	4 (1.2%)
MYC		4 (1.2%)	111 (32.4%)	228 (66.5%)	
MAPK13		17 (13.7%)	288 (84.0%)	8 (2.3%)	
PIK3CB		26 (7.6%)	317 (92.4%)	0 (0%)	
PIK3CG		16 (4.7%)	235 (68.5%)	92 (26.8%)	
PIK3CD		23 (6.7%)	313 (91.3%)	7 (2.0%)	
NRAS		0 (0%)	236 (68.8%)	107 (31.2%)	
KRAS		20 (5.8%)	322 (93.9%)	1 (0.3%)	
TNF		34 (9.9%)	294 (85.7%)	15 (4.4%)	

Analysis of the expression of eleven representative genes related to lipid metabolism and eleven genes related to glioma from the data of gene expression arrays taken from the REMBRANDT database (Glioma Molecular Diagnostic Initiative). Genes are listed with standard abbreviations. Down: 2-fold survival below average. Intermediate: average survival. Up: 2-fold survival above average.

## 6. Mechanism of action of the antiproliferative effect of NaChOleate

We have demonstrated that NaChOleate is effective in inducing cell cycle arrest in lung cancer cells [69,70], apoptosis in several cancer cell lines, including leukemic cancer cells [69,70], and ER stress, autophagy and sphingomyelinolysis in glioma cell lines [62,71]. NaChOleate modifies the membrane composition, enhancing the propensity to induce the non-lamellar  $H_{II}$  phases in the membrane [34,59]. This correlates with PKC translocation to the membrane with its concomitant activation, inducing the activation/overexpression of  $p21^{Cip1}$  and  $p27^{Kip1}$ , members of CDK1 family [34,62,72]. The NaChOleate-induced overexpression of CDK1s inhibits the formation of cyclin D–CDK4/6 complexes which are associated with the hypophosphorylation of Rb [34,62,71,72]. E2F1 is a transcription factor that induces the expression of proteins related to the cell cycle progression and its regulation is dependent of the Rb-phosphorylation status, being recruited and inactivated when Rb is hypophosphorylated. One of E2F1 target genes is dihydrofolate reductase (DHFR), essential for the synthesis of DNA, which is down-regulated in a variety of NaChOleate-treated cancer cell lines [62,70].

In Jurkat cells, the membrane modifications induced by NaChOleate facilitate the FasR capping without ligand binding, probably due to the induction of lipid raft domains in the membrane. The FasR capping by itself is able to trigger caspase activation [40] that, together with the DHFR down-regulation, induces apoptosis in these cells [39,70].

In addition to the activation of PKC and FasR in the membrane of NaChOleate-treated cancer cells, modifications in the interaction of Ras to the membrane have been identified as well. Ras is a family of peripheral proteins that binds to membrane through its bulky isoprenyl moiety in domains with high levels of PE and is constitutively activated in many types of cancers. The restoration of SM levels and reduction of PE in the membrane of NaChOleate-treated glioma cells, exclude Ras proteins from the plasma membrane [62]. In this context, NaChOleate has a similar effect to that of farnesyl transferase inhibitors, such as tipifranib, which has an anti-cancer effect that is based on the impairment of the binding of Ras to the membrane by inhibiting Ras isoprenylation [73]. This Ras exclusion from the membrane inhibits the activation of the different downstream pathways triggered by Ras, such as MAPK and PI3K/Akt pathways [62]. MAPK as well as PI3K/Akt pathways are related with the loss of cellular differentiation and the survival of cancer cells [74–76]. In fact, NaChOleate treatment in glioma cancer cells enhances the expression of markers of differentiation, such as glial fibrillary acidic protein (GFAP) and glutamine synthase (GS) [62]. The overexpression of these markers in cells exposed to NaChOleate suggests an induction of differentiation after DHFR downregulation, through PKC– $p21$ –CyclinD/CDK4/6–Rb pathway and Ras–MAPK and Ras–PI3K/Akt pathway inhibition (Fig. 5) [18,62]. NaChOleate activates the ceramide synthesis and the recycling pathways of lipid metabolism with accumulation of SM, ceramide and hexosylceramide species, resulting in sphingolipidosis, compromising cell viability [60,62] and inducing autophagy in glioma cells [71]. The autophagy induction in glioma cells is related to the activation of the ER stress pathway in glioma cells and cell cycle arrest in G2/M phase [71]. Due to its anti-tumoral effect in cell and animal models and low toxicity NaChOleate was recently acknowledged the status of orphan drug for glioma treatment by the EMA and a phase 1/2A open label, non-randomized study in patients with advanced solid tumors including malignant glioma was started [77].

## 7. The role of lipid metabolism genes in brain cancer

The alterations in lipid composition and in enzymes involved in lipid metabolism are beginning to be associated with tumorigenesis and cancer patient survival. For example, a recent study demonstrated that some lipid metabolism genes, such as, OLR1, GLRX, and SNAP23, are co-ordinately upregulated in transformed cells and late-stage breast and prostate cancer tissues [78]. In addition, some hepatic lipase

**Table 3**  
Combination distribution of lipid and cancer genes altered in REMBRANDT.

	Number of lipid genes altered	Number of cancer genes altered	N (%)	Median survival (months)	P	Survival (one year)	Survival (three years)	Survival (five years)	Survival (ten years)
1 – Low risk	<2	<2	104 (30.3%)	30.8	Ref.	0.57	0.34	0.20	0.08
2 – Medium risk lipid	>3	<2	42 (12.2%)	14.6	0.068	0.40	0.26	0.17	0.10
3 – Medium risk cancer	<2	>3	83 (24.2%)	22.6	0.144	0.48	0.22	0.13	0.04
4 – High risk	>3	>3	114 (33.2%)	13.2	<0.001	0.20	0.10	0.04	0.01
All			343 (100.0%)	18.5		0.41	0.22	0.13	0.05

Median glioma patient survival analysis from data taken from REMBRANDT database (Glioma Molecular Diagnostic Initiative). Patients were divided in low risk, medium risk and high risk categories depending of the number of genes (lipid and cancer) altered. Ref.: Reference group; the P values were calculated with respect to the Reference group.

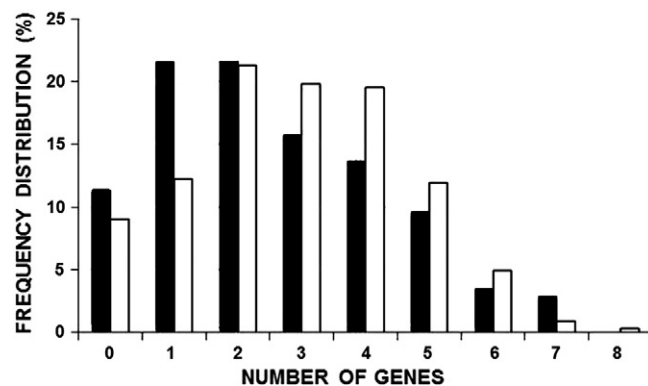
polymorphisms have been associated with colorectal carcinoma risk [79]; and, it has been described that different enzymes related to fatty acid biosynthesis and lipid metabolism regulation, such as, ACACA, FASN, INSIG1, and SREBP1 are highly expressed in breast cancer tumors and associated with low patient survival [80]. In this context, the Cancer Genome Atlas (TCGA) database analysis for 38 ovarian cancer samples demonstrated 39 differentially expressed lipid genes in ovarian cancer tissue compared to normal ovarian tissues [81].

The role of lipid metabolism genes in brain cancer patient survival is largely unknown. The REMBRANDT database is a suitable data source to explore the potential role of genes to modify the prognosis of individuals with brain cancer [82,83]. We have used this database as an additional source to investigate the relevance of lipid metabolism genes in the survival of patients with glioma. It includes demographic, clinical and genotype information of a cohort of glioma patients. To date, REMBRANDT contains data generated through the Glioma Molecular Diagnostic Initiative from glioma specimens comprising approximately 566 gene expression arrays, 834 copy number arrays, and 13,472 clinical phenotype data points. Overall, 343 glioma patients were included in our study (Table 1). As a representative example of a lipid metabolism gene we first studied the expression of SGMS1 (which we have previously demonstrated as being activated by NaCHOLEate) in the clinical outcome of glioma (Fig. 6). Haplotype frequencies were produced for down-regulated ( $n = 84$ ) and normally expressed ( $n = 258$ ) SMGS1. Survival of patients with the up-regulation of SGMS1 expression was not studied given its low proportion ( $n = 1$ ). Frequency distributions were obtained and survival was assessed using standard Kaplan–Meier statistics and survival differences were quantified with Log Rank (Mantel–Cox) tests, and the median follow-up time to death [84]. In all analyses, a  $p$  value lower than 0.05 was considered for statistical significance. We found that at least two-fold down-regulation of SMGS1 lowered very significantly the median of survival of patients with glioma. Moreover, the 10-year survival of patients with low SGMS1 expression was ca. 10-fold lesser than that of patients with normal SGMS1 expression. This result is in concordance with the mechanism of action of NaCHOLEate described above and supports the hypothesis that the administration of NaCHOLEate, a specific activator of SGMS1, may reverse the low survival of glioma patients. We extended this analysis to eleven representative genes related to lipid metabolism and we compared the survival analysis to a set of eleven glioma cancer-related genes (Table 2) [62,85,86]. When grouping samples by the number of lipid metabolism and consensus cancer genes altered, the average number of genes altered among the indicated list was  $2.6 \pm 1.8$  vs.  $2.9 \pm 1.7$  respectively ( $p = 0.432$ ) so that no differences were found. These data highlight the importance of SM and other lipid metabolism genes in the malignant transformation. Moreover, alterations in genes related to lipid metabolism induce a regulation in the median glioma patient survival from 22.6 months to 14.6 months (Table 3), further supporting the relevance of membrane lipids in the cell physiology and glioma etiopathology. According to an arbitrary threshold of 3 genes altered, based on the data shown in Fig. 7, REMBRANDT participants were stratified as those with genetic low risk (each  $\leq 2$  lipid and cancer genes), those with medium risk (only one set of genes  $\geq 3$  altered)

or those with high risk (each  $\geq 3$  lipid and cancer genes). Median survival in those with  $\geq 3$  lipid genes altered was 13.2 months, significantly lesser than those of patients with  $\leq 2$  lipid genes altered with a median survival of 30.8 months ( $p < 0.001$ ). This result was independently associated with death when compared to other glioma patients with an increasing number of cancer genes altered, with a median of 15.0 months ( $\geq 3$  lipid genes) and 13.2 ( $\geq 3$  lipid genes and  $\geq 3$  cancer genes altered). To our knowledge, this is the first assessment of the study of lipid metabolism genes in REMBRANDT and elsewhere. Lipid-related genotypes may help to predict survival in glioma patients, and their effect appears independent of the number of cancer genes altered. In addition, the molecular features of tumors may be relevant for patient stratification and treatment selection. In this context, companies such as Lipopharma Therapeutics and Ability Pharmaceuticals are developing molecules for the regulation of the membrane lipid composition and structure.

#### Abbreviations

CHO	cholesterol
DAG	diacylglycerol
DHFR	dihydrofolate reductase
EMA	European Medicines Agency
ER	endothelial reticulum
FasR	death Fas receptor
GFAP	glial fibrillary acidic protein
GPCRs	G protein-coupled receptors
GS	glutamine synthase
OA	oleic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PKC	protein kinase C
PS	phosphatidylserine
SCD1	stearoyl-CoA desaturase
SGMS	sphingomyelin synthase
SM	sphingomyelin



**Fig. 7.** Diagram graph showing the frequency distribution (%) of lipid metabolism related genes (filled bars) and consensus glioma cancer genes (white bars) whose expression is altered in patients as shown in the REMBRANDT glioma database.

## Conflict of interest

M.I, D.J.L. and M.A. were supported by Torres-Quevedo Research Contracts granted to Lipopharma Therapeutics, S.L. by the Ministerio de Economía y Competividad (Spanish Government). Lipopharma Therapeutics, S.L. is a spin-off, pharmaceutical company from the University of the Balearic Islands.

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