Università degli Studi di Milano

Scuola di Dottorato di Ricerca in Scienze Biochimiche, Nutrizionali e Metaboliche

Dipartimento di Biotecnologie Mediche e Medicina Translazionale

DOTTORATO DI RICERCA IN BIOCHIMICA
CICLO XXV - BIO/10

Sphingolipids as signaling molecules: their involvement in health and disease.

Elena CHIRICOZZI
Matricola n. R08820

Docente guida:
Prof. Sandro SONNINO

Coordinatore del Dottorato:
Prof. Franco BONOMI

Anno Accademico 2011-2012

A Paola, Mamma e Papa'

(per il Nobel ci vorra' ancora un po'…)

Abstract

Sphingolipids (SLs) are minor cell membrane amphyphilic components, residing in the external layer of the plasma membrane (PM), with the hydrophobic moiety, the ceramide (Cer), inserted into the membrane layer and the hydrophilic head group protruding toward the extracellular environment. They are a family of several compounds with different structural properties: the phospholipid, sphingomyelin (SM), the glycosphingolipids (GSLs) characterized for containing a complex oligosaccharide chain as hydrophilic moiety and gangliosides, GSLs containing sialic acid. As membrane components, SLs participate to modulate several cell processes, such as cell growth, differentiation, morphogenesis, cell to matrix interaction and cell to cell communication. From this, it follows that a defect in SL metabolism can obviously lead to a great number of dysfunctions, ranging from neurodegeneration to cancer.

Along my Ph.D. course I considered the different faces of SLs roles: from their involvement in physiology to that in pathology.

i.Sphingolipids and health.

SLs cluster to form SL-enriched domains on cellular PM (lipid rafts, caveolae, and glycosynapses) providing a microenvironment within the PM for reciprocal interaction between lipids and proteins. In particular biochemical analyses have demonstrated that GSLs-enriched microdomains contain several transducer molecules, especially membrane-anchored signal transduction molecules, such as tyrosine kinases belonging to the Src family. Although it has been speculated that GSLs are involved in cell differentiation, proliferation and functions such as phagocytosing, there are quite few evidence that GSLs, by themselves, directly mediate signal transduction, which lead to these cell functions.

Lactosylceramide-enriched microdomain in neutrophils Lactosylceramide (LacCer), a neutral GSL, is abundantly expressed on human neutrophils, and specifically recognizes several pathogenic microorganisms. It has been previously demonstrated that LacCer forms PM lipid domains, that can be separated by detergent treatment of cells followed by ultracentrifugation, and that these lipid domains are coupled with Lyn, a Src family kinase. Ligand binding to LacCer activates Lyn, resulting in neutrophils functions, such as superoxide generation, phagocytosis and migration. The presence of LacCer molecular species with Cer containing a very long fatty acid chain is necessary for the association of Lyn with LacCer-enriched PM domains and LacCer-mediated functions. Lyn is associated by a palmitoyl anchor to the cytoplasmic leaflet, while LacCer is inserted into the

outer layer of membrane bilayer. So the question is: how does LacCer interact with signal transducer molecules?

The GSL-protein interactions in neutrophilis have been investigated by photoactivable GSLs. These molecules have been administered and taken up by the cell PM. When cells are illuminated, the photoactivable group, linked to the terminal portion of Cer, yields a very reactive intermediate, that covalently binds the molecules in the close environment.

For the first time, at the best of our knowledge, we show a direct connection, across the PM, between GSLs and palmitoylated proteins: these results suggest that LacCer with a long fatty acid chain in Cer moiety could be the key-player of the transduction of information across the PM, modulating membrane interdigitation, through the long acyl chain, and forming specific PM microdomains.

ii. Sphingolipids and diseases.

Implication in neurodegenerative disorders

SLs are particularly abundant in the nervous system where they play crucial roles regulating signaling events. Severe neurodegeneration is the prominent pathological hallmark of the most sphingolipidoses, inherited metabolic diseases characterized by a defect in the lysosomal GSL catabolism. Most of sphingolipidosis are caused by deficiencies of a specific lysosomal hydrolases, resulting in the accumulation of undegraded lipid metabolite in many cells.

Secondary accumulations of gangliosides: the case of Niemann-Pick type A

Niemann-Pick Disease (NPD) is a rare, autosomal recessive, lysosomal storage disease resulting from a deficiency in acid sphingomyelinase (ASM) activity. If ASM is absent or not functioning properly, SM cannot be metabolized properly and is accumulated within the cell, eventually causing cell death and the malfunction of major organ systems. In NPD type A, residual enzyme activity is very low (<5%) and in NPD-A patients, storage pathology is severe within neurons and glial cells throughout the nervous system resulting in progressive, global deterioration of neurological function and death by 3 years of age.

To better understand the secondary biochemical mechanism underling the pathogenesis and the importance of these secondary alterations of SL metabolism on lysosomal diseases, we analyzed lipid composition of CNS and extraneural tissues from the acid sphingomyelinase-deficient (ASMKO) mouse, the animal model of NPD type A, that has been developed using gene targeting and embryo transfer techniques. Our data show an unexpected tissue specific selection of the accumulated molecular species of SM, and an accumulation of GM3 and GM2 gangliosides in both neural and extraneural tissues, that

cannot be solely explained by the lack of ASM. In ASMKO mice, we observed the preferential accumulation of SM molecular species with shorter acyl chains in the nervous system, but not in extraneural tissues. The unbalance toward C18/C16-fatty acid containing SM species was detectable as early as SM accumulation started, and monosialoganglioside accumulation followed immediately afterwards. These changes in SL patterns should thus represent the effect of secondary biochemical pathways altered as a consequence of a non-related primary cause. The mechanism underlying these changes still remains to be elucidated and is probably the result of changes in the expression and/or activity of more than one single enzyme, and of anomalies in the traffic of the substrate/product concentrations in multiple cellular compartments. Several pieces of evidence suggest that altered SL metabolism results in a non-physiological PM composition and organization, leading to altered PM-originated signalling pathways that could be relevant to the onset of cellular damage and of tissue pathology.

Chaperone therapy for GM2 gangliosidosis: Pyrimethamine and Sandhoff Disease Sandhoff disease is an autosomal recessive neurodegenerative disease characterized by the intralysosomal accumulation of GM2 ganglioside. This is due to mutations in the β-hexosaminidases β-chain gene, resulting in a β-hexosaminidases A (αβ) and B (ββ) deficiency. Lysosomal enzymes are synthesized in the endoplasmic reticulum (ER), and most of them are transported to the Golgi apparatus for glycosylation and taggeting for lysosomes, by the addition of mannose-6-phosphate. The ER contains a highly conserved degradation pathway, to protect cells from misfolding and potentially toxic proteins. Thus, the protein turnover ensures integrity and biological functions of cells. Treatment of sphingolipidoses, such as Sandhoff disease, have received increasing interest despite the low incidence. However, the replacing enzyme therapy meets the problem to be ineffective over the blood brain barrier and thus new approaches are now under investigation. One of these is the use of pharmacological chaperones. Pyrimethamine, a drug used to treat or prevent serious parasite infections, such as toxoplasmosis and malaria, has been described to act as chaperone for the β-hexosaminidase. Fibroblasts from two cases of juvenile Sandhoff disease, displaying the new mutations C1082+5G>A (#1) and C446-13A>G (#2) in the HEX B gene, were treated with pyrimethamine. The two examined cell lines, after treatment with pyrimethamine, showed an increased total β-hexosaminidase activity, so that the residual value was around 10%. This value should be enough to limit the accumulation of GM2. In contrast to this, cells treated with pyrimethamine and fed with isotopically tritium labeled ganglioside GM1 did not show any GM2 lysosomal catabolism. This negative result was explained after fractionation of the

total cell proteins by ion exchange DEAE column chromatography. Clearly, we found that the increased β-hexosaminidase activity on the artificial substrate was due to the increase of the activity of β-hexosaminidase isoform S. Unlikely, the isoform S, even if it hydrolyzes the artificial compound, does not recognize the natural substrate GM2 and, therefore, the pyrimethamine is unable to modify cell lysosomal activity in the two cell lines showing the new mutations C1082+5G>A and C446-13A>G, and, moreover, cannot be used as a therapeutic drug for the these two patients. Our results are in agreement with a previous information on the selective activity of pyrimethamine depending on the mutation and confirm the advantages deriving from the use of patients' fibroblasts for the decision on the use of pyrimethamine as therapeutic drug.

Implication in cancer

Tumor cells are characterized by aberrant glycosylation processes responsible to increase proliferation and motility. A precise content and molar ratio between SLs is necessary in cells to have the correct membrane organization and the correct interaction processes between membrane components and membranes of adjacent cells.

Cell surface glycohydrolase modulation during tumor irradiation. Radiotherapy has actually been used clinically for a number of years, with very positive results on tumor reduction. Irradiation of cancer cells leads to cell death by different mechanism. DNA damage, mitochondrial damage and oxidative stress bring mainly to necrosis, whereas the PM production of ceramide leads to apoptosis. The ceramide mediated apoptosis has been reported to depend on activation of cell surface sphingomyelinase and the following activation of the ceramide dependent phosphorylation cascade. Other enzymes of the SL metabolism have been recently found associated to the external leaflet of the PMs, like the α-sialidase, the β-hexosaminidase, the β-galactosidase and the β-glucosidase. These glycohydrolases lead to ceramide from complex GSLs and suggest a new way to trigger the ceramide-induced apoptosis. The PM enzymatic activities display optimal pH under mild acidic condition. This is found in specific membrane domains, known as lipid rafts, where GSLs are highly enriched together with ion exchanger proteins such as proton pumps.

Human breast cancer cell line T47D was studied in detail. In these cells the increase of activity of β-glucosidase and β-galactosidase was parallel to the increase of irradiation dose up to 60 Gy and continued with time, at least up to 72 hr from irradiation. β-glucosidase increased up to 17 times and β-galactosidase up to 40 times with respect to control. Sialidase Neu3 and sphingomyelinase increased about 2 times at a dose of 20 Gy but no further significant differences were observed with increase of radiation dose and time. After irradiation, we observed a reduction of cell proliferation, an increase of apoptotic cell

death and an increase of PM ceramide up to 3 times, with respect to control cells. Tritiated GM3 ganglioside has been administered to T47D cells under conditions that prevented the lysosomal catabolism. GM3 became component of the PMs and was transformed into LacCer, GlcCer and ceramide. The quantity of ceramide produced in irradiated cells was about two times that of control cells.

We are characterizing the role of PM glycohydrolases in the process of apoptosis activated by cell irradiation and developing new protocols for radiation therapy combined with pharmacological treatments capable to exert enhanced antitumor activity through synergic action. Increase of the activities of the PM glycohydrolases, and of the apoptotic process, cold be obtained by i) activating with specific drugs the PM proton pumps to decrease the local extracellular pH; ii) the use of recombinant glycohydrolases; iii) using drugs or chaperones capable to modulate directly the glycohydrolase activities. Concerning this last opportunity, molecules acting as chaperones able to up-regulate the activity of lysosomal enzymes are known. Also if no information is available, considering the nature of PM enzymes, we believe that these regulators should be effective also on PM enzymes.

Index

ABBREVIATIONS *pag. 10*

STATE of THE ART *pag. 13*
REFERENCES pag. 22

Theme I:

"Role of Lactosylceramide eriched lipid domains in activation processes of neutrophils" *pag. 27*
INTRODUCTION pag. 28
AIM pag. 37
MATHERIALS and METHODS pag. 42
RESULTS pag. 46
DISCUSSION pag. 53
REFERENCES pag. 56

Theme II

"Are secondary accumulation of gangliosides the triggering factor for Sphingolipidosis? The case of Niemman Pick type A" *pag. 61*
INTRODUCTION pag. 62
AIM pag. 70
MATHERIALS and METHODS pag. 72
RESULTS pag. 75
DISCUSSION pag. 86
REFERENCES pag. 90

Theme III

"Chaperone therapy for the treatment of GM2 gangliosidosis: Pyrimethamine and Sandhoff Disease" *pag. 97*
INTRODUCTION pag. 98
AIM pag. 107
MATHERIALS and METHODS pag. 109
RESULTS pag. 113
DISCUSSION pag. 120
REFERENCES pag. 123

Theme IV:

"Cell surface sphingolipid glycohydrolases modulation during tumor irradiation" *pag. 128*
INTRODUCTION pag. 129
AIM pag. 141
MATHERIALS and METHODS pag. 143
RESULTS pag. 149
DISCUSSION pag. 156
REFERENCES pag. 160

Abbreviations

AMP-DNM	*N*-(5-adamantane-1-yl-methoxy)pentyldeoxynojirimycin
BME	Basal Modified Eagle's Mediumù
CBE	Conduritol B Epoxide
Cer	Ceramide
CNS	Central Nervous System
CTRL	Control
DMEM	Dulbecco Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
D-HL-60	DMSO-treated neutrophilic differentiated human promyelocytic leukemia
DRM	Detergent Resistant Membrane
EIPA	5-(N-Ethyl-N-isopropyl)amiloride
ER	Endoplasmatic Reticulum
ERAD	Endoplasmatic Reticulum Associated Degradation
ERT	Enzyme Replacement Terapy
ETT	Enzyme Target Terapy
FBS	Fetal bovine serum
GalCer	Galactosyl ceramide
GBA2	Non-lysosomal β-glucosylceramidase
GD	Gaucher Diseases
GlcCer	Glucosyl ceramide
GSL	Glycosphingolipid
HD	High Density
HL-60	Human promyelocytic leukemia
HPTLC	High performance thin layer chromatography
HPLC	High performance liquid chromatography
KO	Knock out
LacCer	Lactosyl ceramide
LSD	Lysosomal Storage Disease
MEM	Minimum Essential Medium with Earle's Salt
MUB	4-Methylumbelliferone
MUB-Gal	4-Methylumbelliferyl-□-D-galactopyranoside
MUB-Glc	4-Methylumbelliferyl-□-D-glucopyranoside
MUG	4-Methylumbelliferyl-□-N-acetylglucosaminide
MUGS	4- Methylumbelliferyl-□-N-acetylglucosaminide-6-sulphate
Neu3	PM associated sialidase

NPD	Niemann Pick Disease
PAGE	Polyacrylamide gel electrophoresis
PAMPS	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PC	Pharmacological Chaperone
PM	Plasma membrane
PNS	Post Nuclear Supernatant
PRR	Pattern recognition receptor
PVDF	Polyvinylidene di fluoride
SD	Sandhoff Disease
SL	Sphingolipid
SM	Sphingomyelin
SMase	Sphingomyelinase
SRT	Substrate Reduction Terapy
ST-1	GM3 synthase, CMP-NeuAc: LacCer α2-3 sialyltransferase
ST-II	GD3 synthase, CMP-NeuAc: GM3 α2-8 sialyltransferase
ST-IV	CMPNeuAc: GA1/GM1/GD1b/GT1c α2-3 sialyltransferase
WT	Wild-type
β-Gal	β-galactosidase
β-GlC	β-glucosidase
β-Hex	β-hexosaminidase

Ganglioside nomenclature is in accordance with IUPAC-IUBMB recommendations (IUPAC-IUBMB 1998)

1. State of the Art

SPHINGOLIPIDS

Sphingolipids (SLs) are minor cell components, residing in the external layer of the plasma membrane (PM) [1] consisting in a hydrophobic moiety, ceramide, and in a hydrophilic headgroup. The ceramide (Cer) is responsible for the insertion into the outer layer of the PM, with the hydrophilic headgroup protruding in the extracellular environment.

Cer itself is also a SL, the most simple SL, and its structure is that of a long chain amino alcohol like the 2-amino-1,3-dihydroxyoctadec-4-ene (sphingosine), connected to a fatty acid by an amide linkage. Of the four possible configuration of sphingosine, only the 2*S*,3*R* is present in nature [2,3]. Cer is the common precursor of complex SLs, which are synthesized by addition of polar molecules to hydroxyl group in position 1 of the sphingoid base [4].

Hundreds of SL molecular species, differing in their polar head group, sphingoid base and fatty acyl moiety, have been described. Referring to the polar head group there is the phosphocholine, component of sphingomyelin (SM), and a very wide group of oligosaccharide chains, components of glycosphingolipids (GSLs) [5]. GSLs containing one or more sialic acid residues in the carbohydrate chain are referred to as gangliosides. Based on the sequences of the core carbohydrate residues, GSLs are classified into a number of series (including gala-, ganglio-, isoganglio-, lacto-, neolacto-, lactoganglio-, globo-, isoglobo-, and muco-series). Structural diversity in their carbohydrate chains is a hallmark of GSLs. At present, 172 neutral GSLs, 24 sulfated GSLs, and 188 gangliosides with variations in the carbohydrate chain have been reported in a variety of vertebrate tissues and organs [6]. This complexity is increased many fold when heterogeneity in the lipophilic components is taken into consideration.

The glycolipids are clearly significant contributors to the structure of the outer leaflet of most eukaryotic cell membranes. The molar ratio of these molecules relative to the other major membrane lipids (phospholipids, cholesterol and glycerolipids) varies from being <0.5% in erythrocytes to about 6% in neurons. Thus, in neurons SL are about 12% of total lipids of external PM layer.

Variations in type, number, linkage and further modification of sugar and sialic acid residues within the oligosaccharide chain but also the different composition of the lipid moiety give rise to a combinatorial variety of naturally occurring GSLs.

BIOSYNTHESIS

Glycolipid biosynthesis in animals requires the intracellular formation of the membrane anchor [7] and the subsequent addition of single carbohydrate residues [8]. Both events are coupled to intracellular movement of metabolic intermediates and final association of products to the PM

[9]. The combinatorial variety of naturally occurring GSLs can be largely attributed the combination of glycosyltransferase activities found in different species and cell types.

Ceramide

The *de novo* biosynthetic pathway of SLs starts at the cytosolic face of the endoplasmic reticulum (ER), where enzyme activities responsible for the reaction sequence leading to the formation of Cer are localized. Cer synthesis (Figure 1) is due to the condensation, catalysed by the enzyme serine palmitoyl-transferase, of the amino acid L-serine with a fatty acyl coenzyme A, usually palmitoyl coenzyme A and in less extent stearoyl coenzyme A, to give 3-ketosphinganine [*10-12*]. In the following NADPH-dependent reaction, 3-ketosphinganine is reduced to D-*erythro*-sphinganine by 3-ketosphinganine reductase [*13*]. Sphinganine is subsequently acylated to dihydroceramide by a *N*-acyltransferase [*14-16*]. The major part of the dihydroceramide pool is desaturated to Cer in the dihydroceramide desaturase reaction [*17-19*].

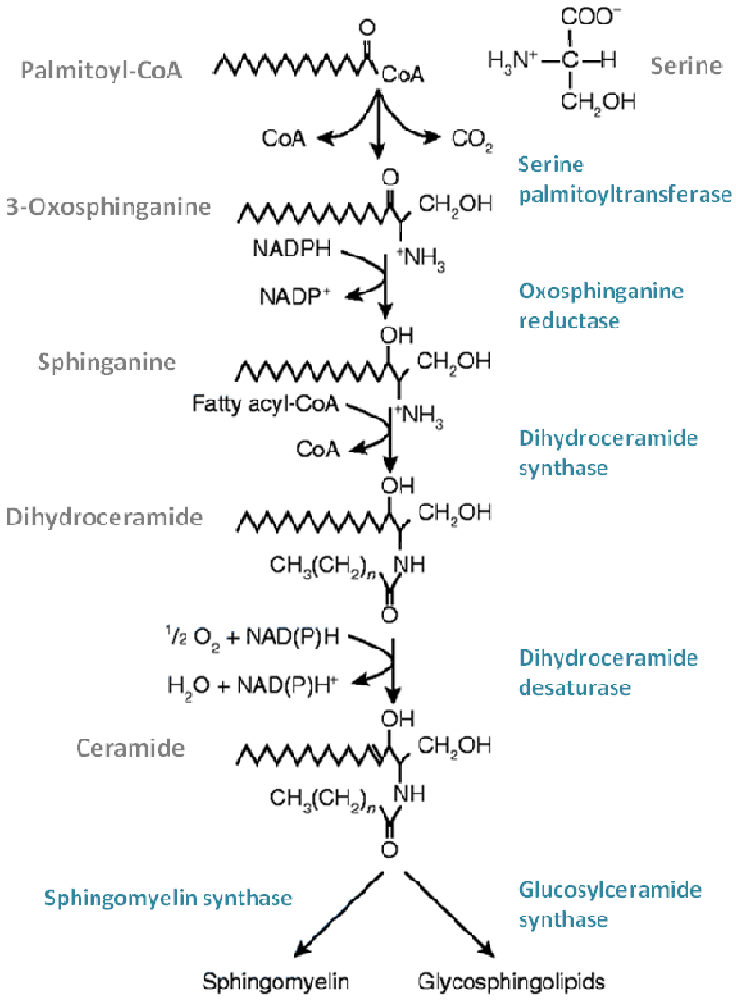


Fig. 1 De novo ceramide biosynthesis.

Cer is also generated during the catabolism of SLs that occurs in lysosomes, and rapidly converted to sphingosine which is largely recycled. Cer synthase is able to use as substrate both sphingosine and sphinganine with similar efficiency [20-22], so Cer can be also formed by *N*-acylation of sphingosine produced by the catabolism of complex SLs (Figure 2).

Cer can be also produced by the hydrolysis of SM and GSL catalyzed by enzymes associated to the PM [23,24].

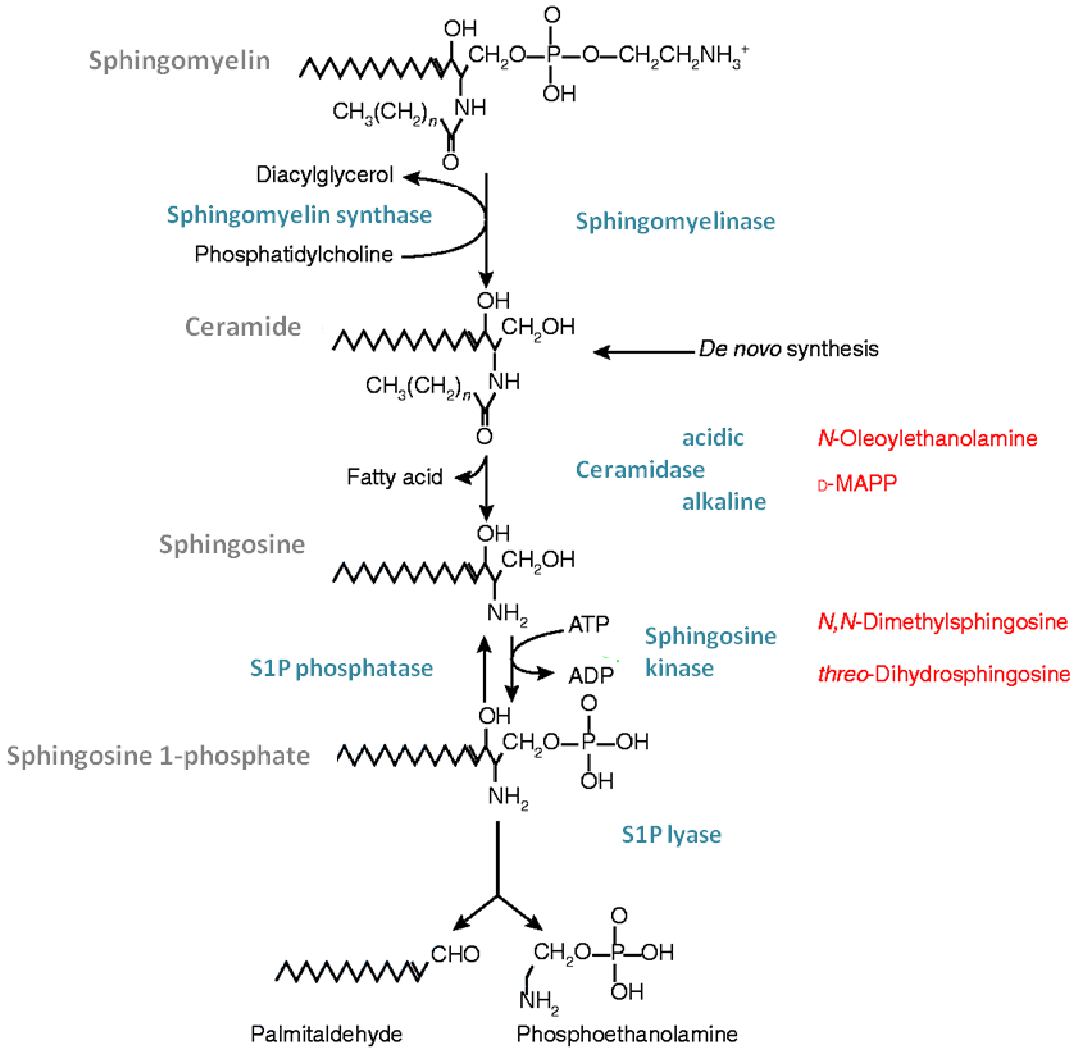


Fig. 2 Ceramide from catabolism.

Sphingomyelin and Sphingolipids

The neo-synthesized Cer reaches directly the PM or it is used as common precursor in the GSLs and SM biosynthesis. In both cases the Cer reaches the Golgi apparatus by a not well defined mechanism [9], capable to address the synthesis of SM or GSL. Synthesis of SM occurs via addition of phosphocholine group to the hydroxyl group in position 1 of the sphingoid base of the Cer. In the case of the biosynthesis of GSLs, different membrane-bound glycosyltransferases are responsible for the sequential addition of sugar residues to the hydroxyl group in position 1 of the Cer, leading to the growth of the oligosaccharide chain.

Glucosylceramide (GlcCer) is the first glycosylated product, formed by a Cer glucosyltransferase activity localized at the cytosolic side of the early Golgi membrane [25]. GlcCer can either directly reach the PM [26], presumably transported in a non-vesicular way [26], or be translocated to the luminal side of the Golgi, where it is further glycosylated by other glycosyltransferases located in this cellular district to generate more complex GSLs. Lactosylceramide(LacCer), the common precursor for the GSL series found in vertebrates, is formed by the addition of a galactose moiety from UDP-Gal to GlcCer catalysed by galactosyltransferase. The enzyme has been purified and cloned from rat brain [27]. LacCer formation and also the reactions leading to higher glycosylated lipids occur on the luminal leaflet of Golgi membranes [28]. Neo-synthesized GSLs move through the Golgi apparatus to the PM following the mainstream exocytotic vesicular traffic.

Complex gangliosides

A GSL series that is especially abundant on neuronal cells is the ganglio series. The biosynthesis of sialic acid-containing GSLs of this series, the gangliosides, is catalysed by glycosyltransferases in the lumen of the Golgi apparatus [8, 29, 30]. Gangliosides are structurally and biosynthetically derived from LacCer. LacCer and the gangliosides GM3, GD3 and GT3, serve as precursors for complex gangliosides of the 0-, a-, b- and c-series (Figure 3). In adult human tissues, gangliosides from the 0- and c-series are found only in trace amounts. The transferases that catalyse the first steps in ganglioside biosynthesis show high specificity towards their glycolipid substrates, i.e. for the formation of LacCer, GM3 and GD3. The relative amount of these GSLs in the steady state seems to determine the amount of 0-series GSLs, which are derived only from LacCer, a-series gangliosides which are only derived from ganglioside GM3, and b-series gangliosides which are only derived from ganglioside GD3. Sialyltransferases I and II are much more specific for their glycolipid substrates than sialyltransferases IV and V, or than galactosyltransferase-II and GalNAc transferase. It was assumed that different transferases catalyse the formation of homologous gangliosides of different series.

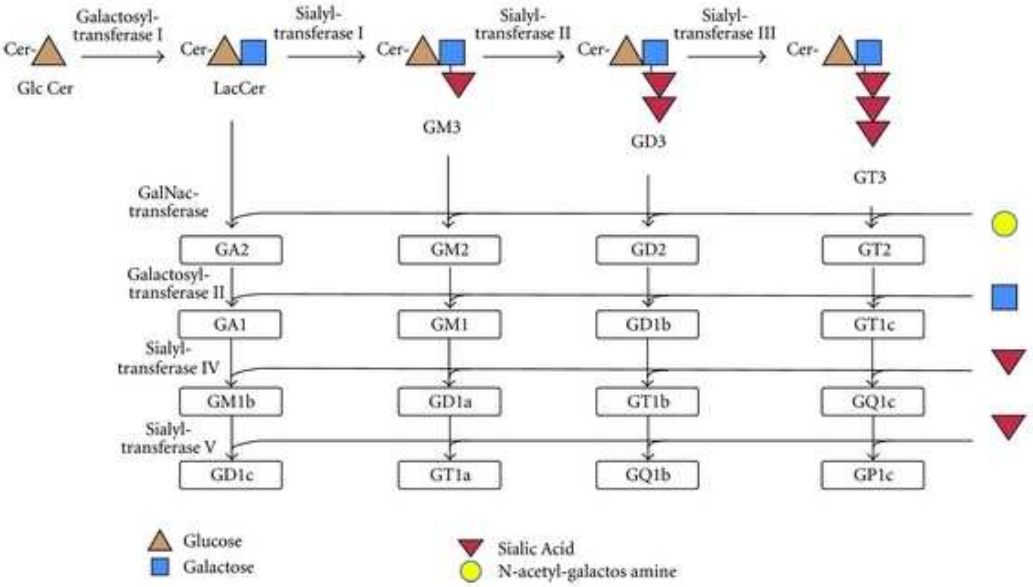


Fig. 3 Scheme of Ganglioside biosynthesis. "G" denotes "ganglioside;" "A" denotes "asialo" or lacking sialic acid; "M" denotes "monosicalo," "D" denotes "disialo;" numbers denote carbohydrate sequence. Cer: ceramide; GlcCer: glucosylceramide; LacCer: lactosylceramide; GalNac: N-acetylgalactosamine. (Adapted from [31]).

CATABOLISM

Another important point of regulation of PM SLs composition is the lipidic degradation that occurs in the acidic compartments of the cells, the lysosomes, where SLs are transported by the endocytic vesicular flow through the early and late endosomal compartment to be catabolyzed.

Lysosomal glycosidases sequentially cleave off the sugar residues from the non-reducing end of their glycolipid substrates. The resulting monosaccharides, sialic acids, fatty acids and sphingoid bases can leave the lysosome and be used within recovery processes or can be further degraded. The intralysosomal degradation of most, if not all, GSLs requires, besides exoglycohydrolases, effector protein molecules named "sphingolipid activator proteins (SAPs, or saposines)" [32] .

The sequence of sugar removal from gangliosides within the lysosomes is as follows: from GM1, galactose is then removed by a β-galactosidase, working in the presence of either the GM2-AP or SAP-B [33], to produce GM2. The resulting ganglioside GM2 is cleaved to ganglioside GM3 and *N*-acetyl-galactosamine only by the β-hexosaminidase isoenzymes Hex A which requires the GM2-AP, an activator that is essential for the *in vivo* degradation of the GM2 gangliosides.

In some cells and animals, sialic acid is removed from GM1 and GM2 by a specific sialidase (GM1- and GM2- sialidase) producing the corresponding a-sialo derivatives GA1 and GA2, that, by the

action of β-galactosidase and β-hexosaminidase or only β-hexosaminidase respectively, are converted to LacCer.

LacCer is produced also directly from GM3 by the action of a sialidase which cleaves this ganglioside into LacCer and sialic acid in a reaction stimulated by SAP-B [34]. LacCer is then degraded to Cer by the sequential action of a β-galactosidase (in the presence of either SAP-B or –C) and β-glucosidase [35] (which is a glucosidase enzyme which acts upon □-1,4 bonds linking two glucose).

Cer is cleaved by acid ceramidase in the presence of SAP-D [36] into sphingosine and a fatty acid. Together with the other cleavage products, these two metabolites are able to leave the lysosome. An alternative pathway for ganglioside/GSLs degradation is the splitting of the β-glucosidic linkage between glucose and Cer, with the formation of Cer and the oligosaccharide. The enzymes catalysing this reaction, named "endoglycoceramidases" or "Cer glycanases" [37-42] appear to require, or to be markedly activated, by specific activator proteins whose action would be essential under in vivo conditions [37]. Endoglycoceramidases have been found to occur in some bacteria [37] and leeches [38]. Although described to occur in lactating mammary glands of rodents [40], the presence of this enzyme in vertebrate and, particularly, mammalian tissues is yet to be definitely assessed.

For the non-glycosylated SLs, like Cer and SM, non-lysosomal degradation steps are known which apparently do not need the assistance of an activator protein. SM is cleaved to Cer and phosphorylcholine. Cer later on is degraded into sphingosine and a fatty acid by ceramidases. Sphingosine moves into the cytosol where it can be phosphorylated to sphingosine-1-phosphate and further degraded to phosphoethanolamine and long chain aldehyde. But at the same time sphingosine-1-phosphate acts as metabolic regulator [32,43]

Enzymes that are capable to remove the fatty acid moiety from several SLs (SM, gangliosides and some neutral GSLs) producing the corresponding lyso-derivatives were also described [43,44]. These enzymes, known as SL Cer *N*-deacylases, were detected in bacteria. No evidence was yet provided for their occurrence in vertebrates. However, it is known that in the brain of patients suffering from some sphingolipidoses where there is accumulation of lyso-GSLs [45], these accumulated compounds seem~~s~~ to be the products of proteins having this kind of enzymatic activity. These products can leave the organelle and re-enter the biosynthetic pathway or serve as energy sources and be further degraded.

BIOCHEMICAL ROLES OF SPHINGOLIPIDS

SLs are a family of membrane lipids with important roles in the regulation of the membrane fluidity and membrane organization [1].

SL are amphiphilic molecules: they are characterized by a hydrophobic portion, embedded in the lipid core of biological membranes, and by a hydrophilic portion protruding in the extracellular milieu. The Cer backbone confers some physical-chemical properties to the SLs that differs from those of other membrane lipids. In fact, the amidic linkage, with the contemporary presence in the same molecule of a hydrogen bond donor and an acceptor group (the carbonyl oxygen and the amidic hydrogen), allows the formation of a network of hydrogen bonds. The presence of hydrogen linkages considerably stabilizes the SL segregation in specific membrane areas, which appear enriched with this lipid family and for this reason they have been defined "SL-rich membrane domains" [46,47]. In addition, the numerous hydrogen bonds in these domains confer rigidity and resistance to the membrane allowing their differentiation by physical-chemical properties from the remaining membrane.

Recent results suggest new aspects of lipid function: not only structural component of cell membrane but also fundamental actors of signalling and regulatory pathways.

Unexpectedly, advances in biochemical and molecular studies of SL metabolism and function during the past 2 decades revealed Cer, Cer-1-phosphate, GlcCer, LacCer, galactosylceramide (GalCer), sphingosine, sphingosine-1-phosphate (S1P) are not only precursors or catabolytes, but are biological active having key roles in the regulation of several fundamental biological processes (Figure 4) [46,47].

These molecules forming cell type specific profiles, have essential roles in several aspect of cell biology:

- Cell growth
- Differentiation
- Morphogenesis
- Cell to matrix interaction
- Cell to cell communication

Since SLs have many putative biological roles, it's easy to think that a defect in their metabolism lead to a great number of biochemical dysfunctions, that consequently result in a several pathological manifestation from neurodegeneration to cancer.

In this Thesis, I report different aspects of SLs biochemistry, with particular interest in both the physiological and pathological SL processes, with the aim to understand how perturbation of SLs metabolism can alter intracellular signalling pathways.

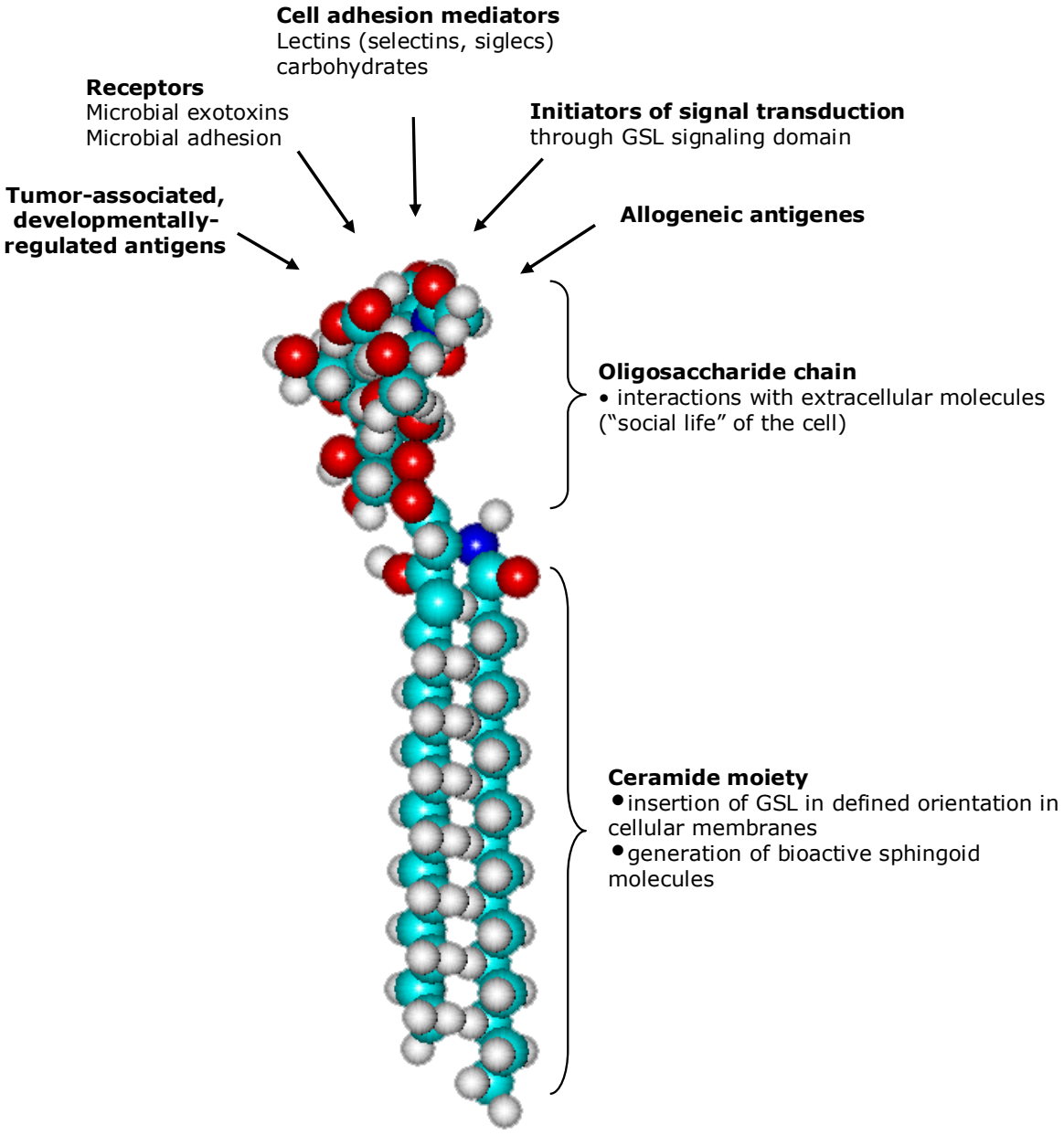


Fig. 3 Sphingolipid functions.

References

1. Feizi T. (1985) Demonstration by monoclonal antibodies that carbohydrate structures of glycoproteins and glycolipids are onco-developmental antigens. *Nature 314*:53-57.
2. Carter H. E., Glick F. J., Norris W. P., and Phillips G. E. (1947) Biochemisrty of sphingolipides. III. Structure of sphingosine. *J. Biol. Chem. 170*:285-294.
3. Carter H. E., Rothfus J. A., Gigg R. (1961) Biochemistry of the sphingolipids: XII. conversion of cerebrosides to ceramides and sphingosine; structure of Gaucher cerebroside. *J Lipid Res* 2:228-234.
4. Kolesnick R. N. (1991) Sphingomyelin and derivatives as cellular signals. *Prog Lipid Res 30*:1-38.
5. Hakomori S. (1990) Bifunctional role of glycosphingolipids. Modulators for transmembrane signaling and mediators for cellular interactions. *J Biol Chem* 265:18713-18716.
6. Yu R. K., Yanagisawa M., and Ariga T. (2007) Glycosphingolipid Structures, In *Comprehensive Glycoscience* (Johannis, P. K., Ed.), pp 73-122, Elsevier, Oxford.
7. Merrill A. H. (2002) De novo sphingolipid biosynthesis: a necessary, but dangerous, pathway, *J Biol Chem* 277:25843-25846.
8. Kolter T., Proia R. L., and Sandhoff K. (2002) Combinatorial ganglioside biosynthesis, *J Biol Chem* 277:25859-25862.
9. van Meer G., and Lisman Q. (2002) Sphingolipid transport: rafts and translocators, *J Biol Chem* 277:25855-25858.
10. Mandon E. C., van Echten G., Birk R., Schmidt R. R., and Sandhoff K. (1991) Sphingolipid biosynthesis in cultured neurons. Down-regulation of serine palmitoyltransferase by sphingoid bases, *Eur J Biochem 198*:667-674.
11. Nagiec M. M., Baltisberger J. A., Wells G. B., Lester R. L., and Dickson R. C. (1994) The LCB2 gene of Saccharomyces and the related LCB1 gene encode subunits of serine palmitoyltransferase, the initial enzyme in sphingolipid synthesis, *Proc Natl Acad Sci U S A* 91:7899-7902.
12. Weiss B., and Stoffel W. (1997) Human and murine serine-palmitoyl-CoA transferase--cloning, expression and characterization of the key enzyme in sphingolipid synthesis, *Eur J Biochem* 249:239-247.
13. Stoffel W., LeKim D. and Sticht G. (1968) Metabolism of sphingosine bases. 8. Distribution, isolation and properties of D-3-oxosphinganine reductase. Stereospecificity of the NADPH-dependent reaction of 3-oxodihydrospingosine (2-amino-1-hydroxyoctadecane-3-one), *Hoppe Seylers Z Physiol Chem* 349:1637-1644.
14. Rother J., van Echten G., Schwarzmann G., and Sandhoff K. (1992) Biosynthesis of sphingolipids: dihydroceramide and not sphinganine is desaturated by cultured cells, *Biochem Biophys Res Commun 189*:14-20.
15. Merrill A. H. and Wang, E. (1986) Biosynthesis of long-chain (sphingoid) bases from serine by LM cells. Evidence for introduction of the 4-trans-double bond after de novo biosynthesis of N-acylsphinganine(s) *J Biol Chem* 261:3764-3769.

16. Shimeno H., Soeda S., Sakamoto M., Kouchi T., Kowakame T. and Kihara, T. (1998) Partial purification and characterization of sphingosine N-acyltransferase (ceramide synthase) from bovine liver mitochondrion-rich fraction *Lipids 33*:601-605.
17. Geeraert, L. Mannaerts, G. P. and van Veldhoven P. P. (1997) Conversion of dihydroceramide into ceramide: involvement of a desaturase *Biochem J 327*:125-132.
18. Michel C., van Echten-Deckert G., Rother J., Sandhoff K., Wang E., and Merrill A. H., Jr. (1997) Characterization of ceramide synthesis. A dihydroceramide desaturase introduces the 4,5-trans-double bond of sphingosine at the level of dihydroceramide. *J Biol Chem 272*:22432-22437.
19. Mikami T., Kashiwagi M., Tsuchihashi, K. Akino and Gasa, S. (1998) Substrate specificity and some other enzymatic properties of dihydroceramide desaturase (ceramide synthase) in fetal rat skin, *J Biochem (Tokyo) 123*:906-911.
20. Merrill A. H., Jr. (1983) Characterization of serine palmitoyltransferase activity in Chinese hamster ovary cells, *Biochim Biophys Acta 754*:284-291.
21. Merrill A. H. Jr., and Jones D. D. (1990) An update of the enzymology and regulation of sphingomyelin metabolism *Biochim Biophys Acta 1044*:1-12.
22. Spence, M. W. (1993) Sphingomyelinases, *Adv Lipid Res 26*, 3-23.
23. Aureli M., Bassi R., Prinetti A., Chiricozzi E., Pappalardi B., Chigorno V., Di Muzio N., Loberto N., Sonnino S. (2012) Ionizing radiations increase the activity of the cell surface glycohydrolases and the plasma membrane ceramide content *Glycoconj.* 29:585-597
24. Valaperta R., Chigorno V., Basso L., Prinetti A., Bresciani R., Preti A., Miyagi T., Sonnino S. (2006) Plasma membrane production of ceramide from ganglioside GM3 in human fibroblasts. *FASEB J.* 20:1227–1229.
25. Jeckel D., Karrenbauer A., Burger K. N., van Meer G., and Wieland F. (1992) Glucosylceramide is synthesized at the cytosolic surface of various Golgi subfractions, *J Cell Biol 117*:259-267.
26. Warnock D. E., Lutz M. S., Blackburn W. A., Young W. W., Jr and Baenziger J. U. (1994) Transport of newly synthesized glucosylceramide to the plasma membrane by a non-Golgi pathway, *Proc Natl Acad Sci U S A 91*:2708-2712.
27. Nomura T., Takizawa M., Aoki J., Arai H., Inoue K., Wakisaka E., Yoshizuka N., Imokawa G., Dohmae N., Takio K., Hattori M., and Matsuo N. (1998) Purification, cDNA cloning, and expression of UDP-Gal: glucosylceramide beta-1,4-galactosyltransferase from rat brain, *J Biol Chem 273*:13570-13577.
28. Lannert H., Gorgas K., Meissner I., Wieland F. T., and Jeckel D. (1998) Functional organization of the Golgi apparatus in glycosphingolipid biosynthesis. Lactosylceramide and subsequent glycosphingolipids are formed in the lumen of the late Golgi, *J Biol Chem 273*:2939-2946.
29. Kaufman B., Basu S., and Roseman S. (1968) Enzymatic synthesis of disialogangliosides from monosialogangliosides by sialyltransferases from embryonic chicken brain, *J Biol Chem 243*:5804-5807.
30. Yip M. C., and Dain J. A. (1969) The enzymic synthesis of ganglioside. 1. Brain uridine diphosphate D-galactose: N-acetyl-galactosaminyl-galactosyl-glucosyl-ceramide galactosyl transferase, *Lipids 4*:270-277.

31. Miklavcic J.J., Schnabl K.L., Mazurak V.C., Thomson A.B.R., and Clandinin M.T., (2012) Dietary Ganglioside Reduces Proinflammatory Signaling in the Intestine Journal of Nutrition and Metabolism doi: 10.11552012280286
32. Huwiler A., Kolter T., Pfeilschifter J., and Sandhoff K. (2000) Physiology and pathophysiology of sphingolipid metabolism and signaling, *Biochim Biophys Acta 1485*:63-99.
33. Wilkening G., Linke T., Uhlhorn-Dierks G., and Sandhoff K. (2000) Degradation of membrane-bound ganglioside GM1. Stimulation by bis(monoacylglycero)phosphate and the activator proteins SAP-B and GM2-AP, *J Biol Chem 275*:35814-35819.
34. Fingerhut R., van der Horst G. T., Verheijen F. W., and Conzelmann E. (1992) Degradation of gangliosides by the lysosomal sialidase requires an activator protein, *Eur J Biochem 208*:623-629.
35. Zschoche A., Furst W., Schwarzmann G., and Sanhoff K. (1994) Hydrolysis of lactosylceramide by human galactosylceramidase and GM1-beta-galactosidase in a detergent-free system and its stimulation by sphingolipid activator proteins, sap-B and sap-C. Activator proteins stimulate lactosylceramide hydrolysis, *Eur J Biochem 222*:83-90.
36. Linke T., Wilkening G., Sadeghlar F., Mozcall H., Bernardo K., Schuchman E., and Sandhoff K. (2001) Interfacial regulation of acid ceramidase activity. Stimulation of ceramide degradation by lysosomal lipids and sphingolipid activator proteins, *J Biol Chem 276*:5760-5768.
37. Ito M., and Yamagata T. (1986) A novel glycosphingolipid-degrading enzyme cleaves the linkage between the oligosaccharide and ceramide of neutral and acidic glycosphingolipids, *J Biol Chem 261*:14278-14282.
38. Zhou B., Li S. C., Laine R. A., Huang R. T., and Li Y. T. (1989) Isolation and characterization of ceramide glycanase from the leech, Macrobdella decora, *J Biol Chem 264*:12272-12277.
39. Ito M., Ikegami Y., and Yamagata T. (1991) Activator proteins for glycosphingolipid hydrolysis by endoglycoceramidases. Elucidation of biological functions of cell-surface glycosphingolipids in situ by endoglycoceramidases made possible using these activator proteins, *J Biol Chem 266*:7919-7926.
40. Basu M., Girzadas M., Dastgheib S., Baker J., Rossi F., Radin N. S., and Basu S. (1997) Ceramide glycanase from rat mammary tissues: inhibition by PPMP(D-/L-) and its probable role in signal transduction, *Indian J Biochem Biophys 34*:142-149.
41. Basu M., Kelly P., Girzadas M., Li Z., and Basu S. (2000) Properties of animal ceramide glycanases, *Methods Enzymol 311*:287-297.
42. Dastgheib S., Basu S. S., Li Z., Basu M., and Basu S. (2000) Analyses of glycosphingolipids using clam, Mercenaria mercenaria, ceramide glycanase, *Methods Enzymol 312*:196-205.
43. Hirabayashi Y., Kimura M., Matsumoto M., Yamamoto K., Kadowaki S., and Tochikura T. (1988) A novel glycosphingolipid hydrolyzing enzyme, glycosphingolipid ceramide deacylase, which cleaves the linkage between the fatty acid and sphingosine base in glycosphingolipids, *J Biochem 103*:1-4.
44. Furusato M., Sueyoshi N., Mitsutake S., Sakaguchi K., Kita K., Okino N., Ichinose S., Omori A., and Ito M. (2002) Molecular cloning and characterization of sphingolipid ceramide N-deacylase from a marine bacterium, Shewanella alga G8, *J Biol Chem 277*:17300-17307.

45. Rodriguez-Lafrasse C., and Vanier M. T. (1999) Sphingosylphosphorylcholine in Niemann-Pick disease brain: accumulation in type A but not in type B, *Neurochem Res* 24:199-205.
46. Sonnino S, Prinetti A. (2012) Membrane domains and the "lipid raft" concept. *Curr Med Chem*. Nov 8. PMID:23150999
47. Lahiri S. and Futerman A.H., (2007) The metabolism and function of sphingolipids and glycosphingolipids *Cell. Mol. Life Sci.* 64.2270-2284

2. Theme I

Role of Lactosylceramide enriched lipid domains in activation processes of neutrophils

Introduction

THE LIPID RAFT CONCEPTS

In the late 1980s, a diverse series of experimental findings collectively gave rise to the lipid domain hypothesis. Lipid domains were originally defined as membrane zones, *i.e.*, ordered structures created as a consequence of the lateral segregation of sphingolipids (SLs) and differing from the surrounding membrane in their molecular composition and properties. This definition was subsequently modified to introduce the notion that membrane domains correspond to membrane areas stabilized by the presence of cholesterol within a liquid-ordered phase. During the past two decades, the concept of membrane domains has become extremely popular among cell biologists, and these structures have been suggested to be involved in a great variety of cellular functions and biological events [1,2,3].

The notion that different levels of order exist in biological membranes is deeply rooted in cellular biology. Cellular membranes are composed mainly of phospholipids, SLs, cholesterol, and several types of membrane associated protein. The first level of ordered organization is the creation of the lipid bilayer, a consequence of the aggregational properties of complex amphipathic membrane lipids. The lipid bilayer provides a physical boundary between the cellular and extracellular environments, and a scaffold for molecules and molecular complexes that physically and functionally link these two environments. The lipid bilayer is characterized by several basic properties that are relevant to its biological functions: the bilayer as a whole is a very stable structure, however it allows its components a certain degree of lateral motility. As a consequence of this fluidity, components of biological membranes can be arranged following a non-homogenous lateral distribution, leading to the creation of membrane areas ("domains") with a highly differentiated molecular composition and supermolecular architecture. The non-homogeneous lateral distribution of membrane components is made possible by the existence of lateral interactions stabilizing different membrane domains, and creating a second level of order in the organization of biological membranes. Membrane microdomains are historically proposed to be lateral assemblies of specific lipids, in particular, cholesterol and SLs, involved in the sorting of proteins to the apical surface of polarized epithelial cells, and with which specific proteins interact [3-5].

Today different terms are used to define the membrane domains, as in the case of "caveolae", membrane invaginations containing the protein caveolin [6] or "Lipid rafts" membrane domain that sort or transport proteins inside the cells. Others define membrane portions on the basis of chemico-physical or compositional features: DIM (detergent insoluble material) [7], DISAM (detergent-insoluble substrate attachment matrix) [7], DIG (detergent insoluble glycolipid-enriched material) [8], DRM (detergent resistant membranes) [9], SEMF (SL enriched membrane

fraction) [10], GEM, (glycolipid enriched membrane) [1]. Among all these definitions, the one that probably makes a better idea is "DRM", because it relies on the biochemical characteristics that are used for the purification of lipid domains: they are relatively insolubly in nonionic detergent under specific experimental conditions and this is one of the most important characteristic of these domains from an experimental point of view (Figure 1), that allows us to separate the lipid rafts from the other part of cellular membrane.

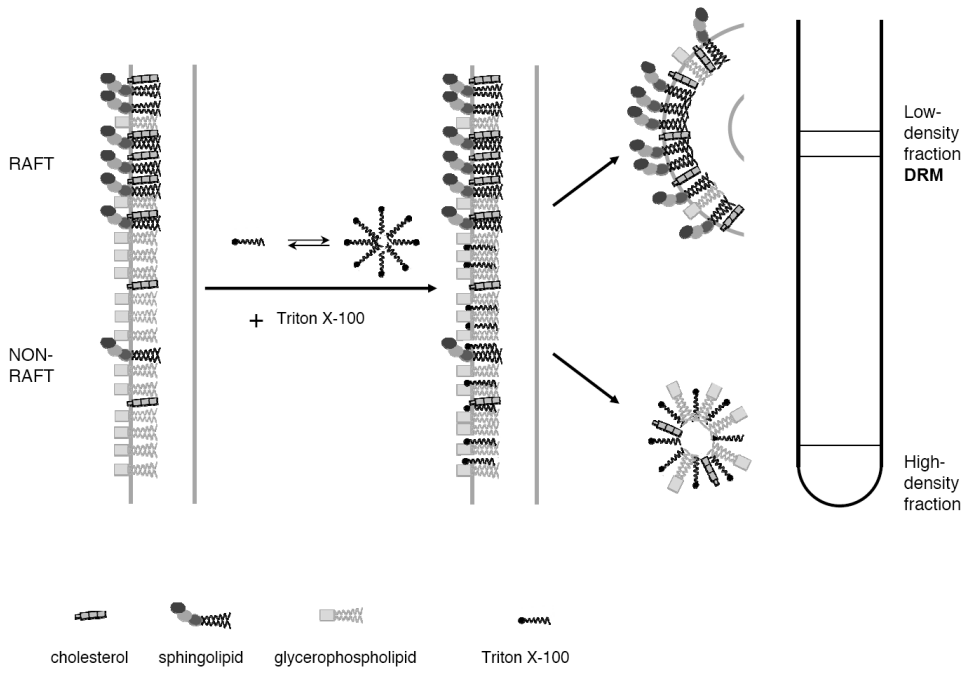


Fig. 1 Insolubility of lipid raft components in the presence of Triton X-100 and preparation of detergent-resistant membranes (DRMs). Detergents in water solutions aggregate as small micelles over a critical micellar concentration (c.m.c.). The c.m.c. for Triton X-100 is 0.31 mM; at a 1% concentration many detergent monomers are therefore present in solution and enter into the fluid ("non-raft") portions of the membrane. Fluid membranes containing Triton X-100 are dissolved and form small mixed micelles that are enriched in detergent, GPLs, and proteins. The detergent does not enter into the less fluid ("raft") portions of membrane, which contain few proteins but are enriched in SLs and cholesterol. The membrane in these areas therefore maintains the bilayer structure. Detergent-resistant and detergent-soluble components can be separated by gradient centrifugation. The figure schematically depicts only the external membrane layer. Proteins are not illustrated, and the relative proportions of the membrane components as shown are not accurate. (Adapted from [3]).

The great interest for lipid-rich membrane domains, that gave rise to the complex and controversial discipline of raftology in the last two decades, derived from the observation that some membrane-associated proteins are highly concentrated in these domains, even if the overall

protein content of these membrane areas in very low. It has been assumed that the trapping of certain proteins in lipid rafts might be somehow functional to their biological role [11]. Studies on model membranes, on detergent-resistant membrane fractions and in intact cells indicated, without doubt, that several classes of membrane-associated proteins display a strong preference for the association of lipid-rich membrane domains [12].

Among most common raft-targeting motifs are the presence of GPI anchor or of a lipid modification (NH_2 terminal myristoylation/palmitoylation and double palmitoylation), also transmembrane proteins are sometimes concentrated in lipid ratfs. Thus the presence of a lipid modifications or of a specific targeting sequence within the hydrophobic or the extracellular/cytoplasmatic domains is required. Finally peripheral proteins can be associated or recruited to lipid rafts, possibly indirectly via interaction with raft-resident proteins.

Anyway it has been suggested and in some cases proven that the association with lipid-rich membrane domains, or with lipids within lipid domains, that can affect the functional properties of a membrane protein.

Glycosphingolipids-ENRICHED MICRODOMAINS

Glycosphingolipids (GSLs) cluster to forms GSL-enriched microdomains on cellular plasma membrane (PM), provide a microenvironment within the PM for reciprocal interactions between lipids and protein molecules participating in the control of signal transduction (Figure 2) [13].

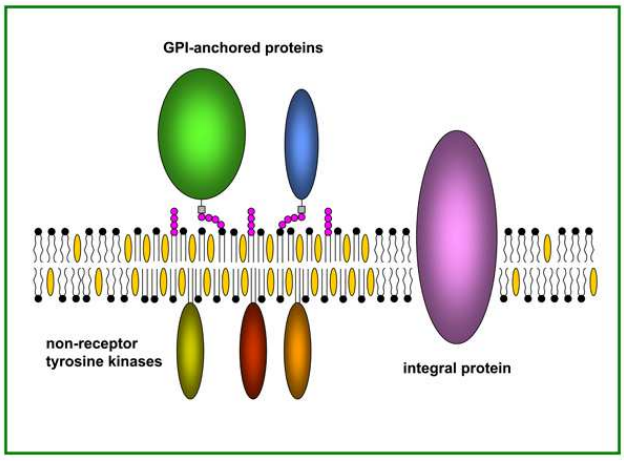


Fig. 2 Proposed structure and organization of a lipid raft microdomain in the PM. SLs, which include both SM and GSLs, associate with cholesterol to form a more tightly packed domain. The regions rich in PC and other glycerol-based phospholipids are less densely packed, and form fluid regions outside the raft microdomains. Lipid rafts are enriched in GPI-anchored proteins and enzymes at their external surface, and acylated proteins, such as tyrosine kinases of the Src family at the cytoplasmic surface. Transmembrane

integral proteins are generally excluded from rafts, and are found in the more fluid phospholipid-rich regions of the membrane [From http://www.uoguelph.ca/~fsharom/research/lipid.shtml].

The chemical structure of GSLs molecules makes them ideal in play the role of mediators of information transfer across the PM: the hydrophilic component is able to provide recognition sites and of interaction, while the hydrophobic portion allows it to interact with other components of the PM.

There are a lot of evidence on the ability of GSLs to interact and modulate the activity of PM proteins, in some cases in a highly specific manner, such as receptor tyrosine kinase. This can be consider the starting step in the process of transferring the information across the membrane. But for some interaction between GSLs and proteins there is no information on how the process continues after the initial interaction: still remain to elucidate the molecular mechanism of such GSLs-proteins interactions [14-17].

The oligosaccharide chain of GSLs could interact with a membrane protein via i) aminoacid residues belonging to the intracellular loops of the protein, if the conformation of the polypeptide chian allows them to be sufficiently close to the membrane surface; ii) sugar residues in the glycans of a glycosylated protein, if the dynamics of the protein oligosaccharide chain allows the correct orientation toward the cell surface; iii) the hydrophilic portion to the anchor in the case of GPI-anchored proteins [18-21].

Some evidence suggest that ceramide (Cer), the portion of the molecule that is insert into the outer layer and makes the GSLs to be a component of the membrane, could be involved in the intermediate steps of signalling process.

The amide group of Cer is a rigid group, with a perpendicular orientation towards the two hydrocarbonyl chains, which parallel orientation stabilized by the presence of an unsaturation at position 4-5 of sphingosine. The hydroxyl group at position 2 of sphingosine, the amidic proton and the carbonyl oxygen, enable the Cer moiety to form hydrogen bonds, acting as hydrogen donors and acceptors. The orientation of the hydrogen bond donor and acceptor groups of SLs, is optimal to form lateral interactions and to considerably increase the stability in lipid-lipid interaction. This, together with a reduced GSL dynamics due to GSL-protein interactions, are very good requisites to promote the formation of rigid zones within the membrane. The Cer moiety contains a long chain base linked to a fatty acid chain. Sphingosine [(2S,3R,4E)-2-amino-1,3-dihydroxy-octadecene] is largely the main long chain base in mammals. However, the structure with 20 carbons is relatively abundant in neurons, where its content progressively increases along aging. This introduced the hypothesis of an involvement of C20-sphingosine containing GSLs in

functional processes. On the other hand, GSL's Cer is heterogeneous concerning its fatty acid content [17, 22-26].

The thickness of a cellular membrane is related to the length of the hydrocarbonyl chains present in the membrane lipids. The maximum length of is determinate by the length of sphingosine belong to the water-lipid interface, so that, with the exception of less abundant $Cer_{(s)}$ containing very short fatty acids, it is the fatty acid structure that define the length of the whole Cer into the outer membrane layer [27,28].

Lactosylceramide-ENRICHED MICRODOMAINS IN NEUTROPHILIS

Although we are constantly exposed to infectious agents, we can generally resist these infections using the two immune systems: the innate and the adaptive immune systems. Even if the innate and adaptive immune systems both function to protect against microorganisms, they differ in a number of ways. The innate immune system is the inborn defense mechanism and the first line of defense against pathogenic organisms, such as bacteria, fungi, viruses, etc. Professional phagocytes, such as neutrophils and macrophages, are essential for the innate immunity system. Phagocytes express several specific receptors and adhesion molecules (pattern recognition receptors or PRRs) on their cell surface, including Toll-like receptors and β2 integrins, to recognize pathogen-associated molecular patterns (PAMPs) on microorganisms [29-32].

Lactosylceramide (LacCer) is a neutral GSL, and is associated with a number of key cellular processes. It has been demonstrated that LacCer activates NADPH oxidase to modulate intercellular adhesion molecule-1 expression on human umbilical vein endothelial cells, and to induce the proliferation of human aortic smooth muscle cells. Therefore, it is possible that LacCer activates NADPH oxidase, and thereby affects the functions of superoxide producing cells. LacCer is also a receptor activator of NF-κB ligand and is essential for osteoclastogenesis mediated by macrophage colony stimulation factor. Recently, LacCer has been shown to recruit PCKα/ε and phospholipase A2 to stimulate PECAM-1 expression in human monocytes and adhesion to endothelial cells and to regulate β1-integrin clustering and endocytosis on cell surfaces. LacCer has been shown to bind specifically to several types of pathogenic microorganism, including Escherichia coli, Bordetella pertussis, Bacillus dysenteriae, Propionibacterium freudenreichii, and Candida albicans, suggesting that LacCer plays roles in the interactions between these microorganisms and host cells [13,28,32-35].

Neutrophils play important roles in innate immunity. After infection immediately migrate toward the infecting pathogen and phagocytose it via PRRs such as beta(2) integrin Mac-1(CD11b/CD18, CR3), CD14 and Dectin-1. In the second step, neutrophils kill the microorganisms with microbicidal molecules and through superoxide generation.

LacCer is the most abundant GSL in human neutrophils where accounts for about 70% of the GSL and it has been shown to bind specifically to several types of pathogenic microorganism. In addition, chemotactic and superoxide-generating activities are induced by addition of anti-LacCer monoclonal antibodies to cells. These immune cells migrate toward C. Albicans-derived β-glucan (CSBG), and generate superoxide anions via LacCer. Therefore, LacCer is thought to be involved in neutrophil microbicidal functions [33, 36].

We know that on neutrophil PM, LacCer forms lipid microdomanins with the Src family kinase Lyn, and serve as PRR responsible for several biological functions including chemotaxis, phagocitosis and superoxid generation. Anti-LacCer monoclonal antibody activates Lyn, which becomes phosphorylated, and it immunoprecipitates Lyn-associated domains from a detergent resistant membrane fraction prepared from cell membranes (Figure 3)[13,28,32-35].

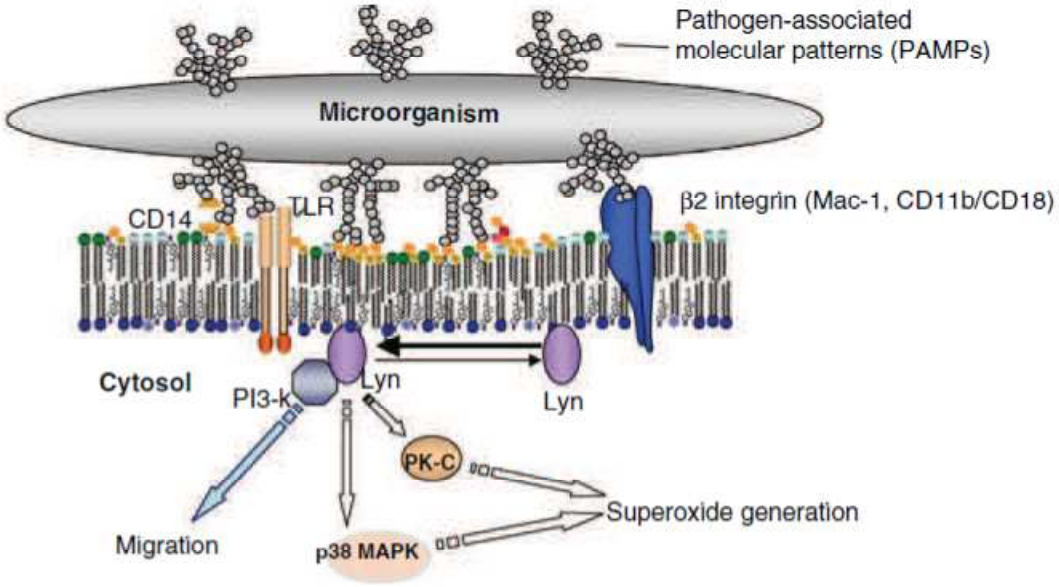


Fig. 3 Role of neutrophils in innate immunity. The binding of microorganisms to LacCer induces clustering of LacCer enriched lipid domains, leading to superoxide generation and migration through Lyn-, PI3-k-, p38 MAPK-, and protein kinase C-dependent signal transduction pathways (Adapted from [28]).

Dimethyl sulfoxide (DMSO)-treated neutrophilic differentiated human promyelocytic leukemia HL-60 cells (D-HL-60 cells) possess chemotactic and superoxide-generating activities induced by formyl peptide fMLP. Interestingly, D-HL-60 cells do not show superoxide-generating or chemotactic activity induced by anti-LacCer antibodies, although these cells express almost the same amount of LacCer on the plasma membrane as neutrophils (Figure 4a and b). Moreover most of LacCer and Lyn, in both neutrophils and D-HL-60 cells, was recovered in DRM fraction.

Interestingly Lyn was co immunoprecipitate from DRM of neutrophilis but not of DHL-60 cells (Figure 4c). These data suggested that some essential molecule(s) which are indispensable for linking LacCer with Lyn are absent from the LacCer microdomains of DHL60[13].

The HPLC analysis of molecular species of LacCer has revealed that in neutrophils PM the very long fatty acid C24:0 and C24:1 chains are the main component of LacCer, whereas PM of DHL60 cells mainly includes C16 LacCer species (Figure 4d) [33].

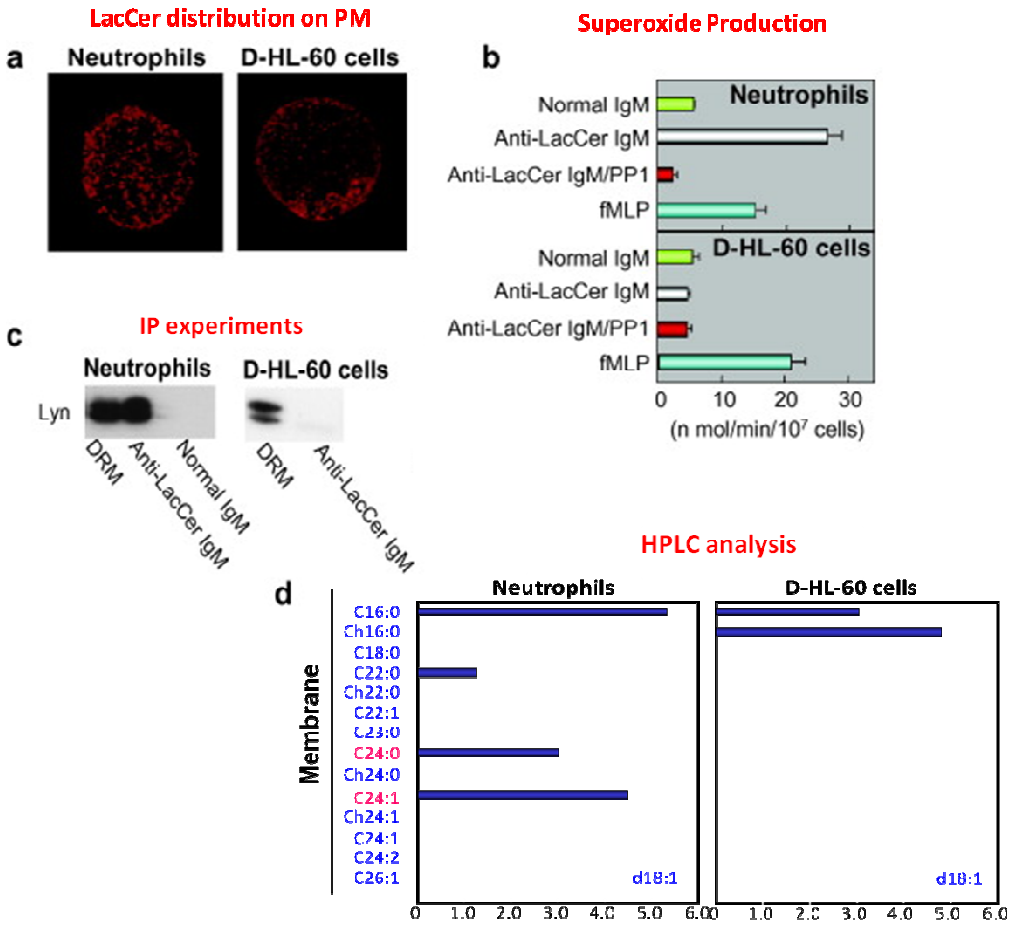


Fig. 4 (a) Three-dimensional reconstructed images of LacCer on the PM of LacCer-loaded D-HL-60 cells. (b) Anti-LacCer antibody-induced superoxide generation in neutrophils but not D-HL-60 cells. (c) Association of Lyn with LacCer in neutrophils but not D-HL-60 cells. (d) HPLC analysis of molecular species of LacCer from PM of neutrophils and D-HL-60 cells (Adapted from [33,35]).

These information suggest that C24 fatty acid chains LacCer, but not C16 fatty acid chains LacCer, play an important role in the formation of LacCer enriched lipid raft coupled with Lyn as functional lipid domains responsible for neutrophilic functions.

In support of this information, when the D-HL-60, which as mentioned before are not able to fagocitate the non-opzonized bacteria, are incubated with LacCer C24, are able to replace the

LacCer present in the membrane and reconstruct the domains between LacCer and Lyn and show phagocytosis, chemotaxis and superoxide anion production mediated by LacCer (Figure 5) [35].

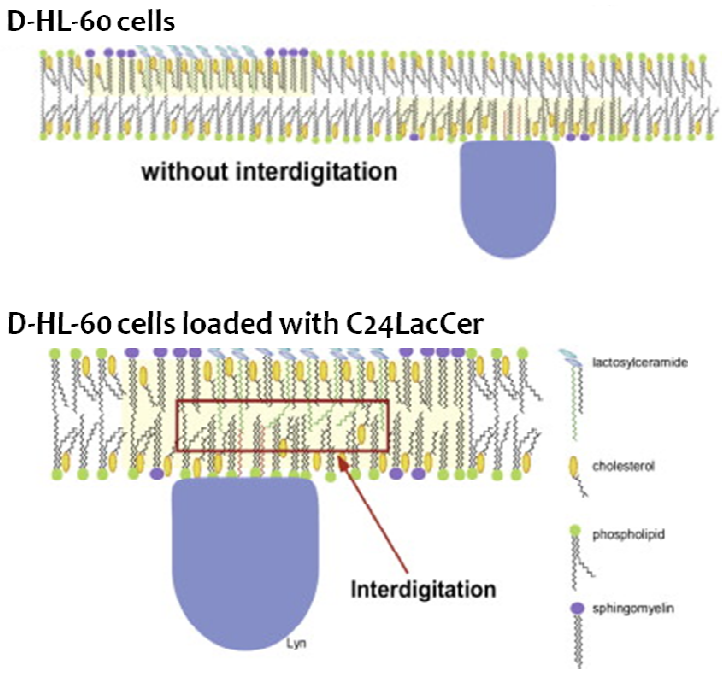


Fig. 5 Proposed model of signaling through LacCer-enriched microdomains. SL-enriched microdomains are more tightly packed than the surrounding non-raft phase of the bilayer, containing complex lipid species carrying 16–18 carbon atom acyl chains. Due to their high transition temperatures, these lipids participate in efforts to reduce membrane fluidity and favor cholesterol segregation within the same membrane areas. The assembly of cholesterol could induce vacant pockets in the central part of the membrane, allowing interdigitation with longer alkyl chains. Due to the mismatch in lengths between cholesterol and the alkyl chains, the assembly of cholesterol could induce packing defects in their central parts. Interdigitation of C24:0- and C24:1-LacCer hydrophobic chains could occur in lipid rafts of neutrophils, causing an association of lipid rafts of external and cytoplasmic leaflets and the interaction of alkyl chains of LacCer and Lyn. In contrast, neutrophilic lineage D-HL-60 cells have quite a small amount of C24 fatty acid chain-contained LacCer in their plasma membranes. Thus LacCer cannot form Lyn-coupled membrane microdomains on the plasma membrane of D-HL-60 cells. When D-HL-60 cells where loaded with C24 fatty acid chain-containing LacCer are able to reconstruct the PM domains allowing interdigidation phenomena (adapted from [35]).

Aim

The aims of this project was understand the real organization of LacCer enriched domains with the objective to identificate the proteins interacting with LacCer in the innate response of human neutrophilis.

In particular the goal was to find essential molecule(s) linking LacCer with Lyn in the LacCer-enriched lipid rafts of D-HL-60 cells in order to elucidate the role of very long fatty acid chains in GSL-enriched membrane microdomains, especially the significance of C24 fatty acid chains of LacCer in LacCer-mediated neutrophil functions.

Lyn is a myristoylated protein, associated with the membrane inner lipid layer and LacCer is not located in the cytoplasmic leaflet of membrane bilayer. Our question is how LacCer is able to modulate a signal transducer molecule? Is it a direct interaction or is mediated by other molecules?

On the basis of a series of data, our hypoteis is that GSLs, with a very long fatty acid chain of Cer mojety, have a specific role in modulating membrane interdigitation, reducing the membrane thickness and forming a specific PM microdomanins. This would allow contact between the outer leaflet and cytoplasmic proteins as well as among the GSLs in the outer leaflet of the cytoplasmic side and components (Figure 6) [28].

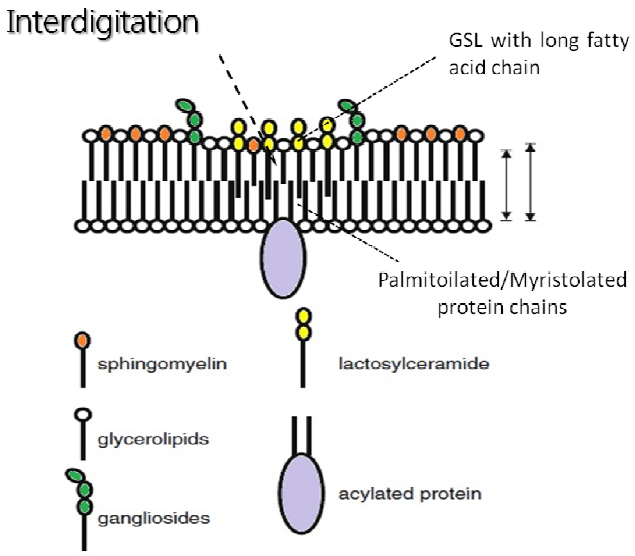


Fig. 6 Schematic representation of the membrane with partially interdigitated layers. The presence of components with very long fatty acid, here represented by LacCer, requires a partial interdigitation to allow the insertion of the long hydrocarbonyl chain reducing the membrane thickness (Adapted from [28]).

Several techniques are available for the detection of lipid rafts or organized domains, but the study of the PM lipid protein interactions is not easy because the biochemical assays available are very limiting. In particular the difficulty lies in the possibility to study the lipid-protein interaction directly at the level of PM of living cells and not in vitro studies.

A methodology useful for the study of GSLs-proteins interaction is the **photoaffinity labelling**, which consist in the administration of photoactivable GSLs molecules (Figure 7), that are able to become components of the cell membrane, followed by illumination [37-46]. As radioactive tracer we used tritium, chosen because it does not change the physic-chemical properties of the molecules, it is not dangerous, whereas as photoactivable group we use a reactive azide.

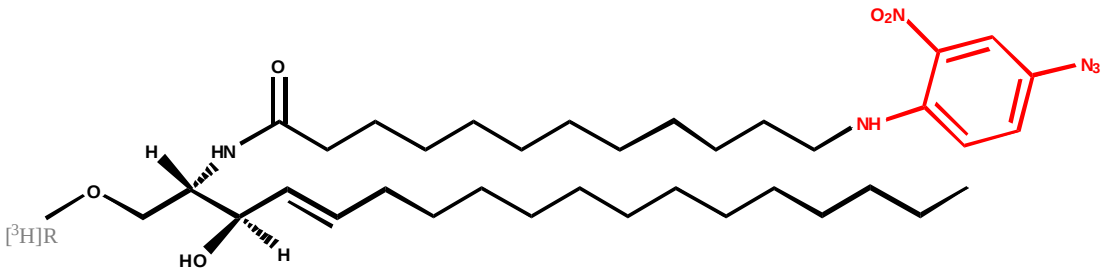


Fig. 7 General structure of a radioactive and photoactivable GSL derivate containing a nytrophenylazide. [^{3}H]R is the tritium labeled ganglioside oligosaccharide chain.

Linked to nitrophenyl, the azide becomes very sensitive to light when solubilized and in this condition it must handled with care and under dark conditions (red safelight). When illuminated, yield a very reactive nitrene intermediate that, by reactions of addition, insertion and nucleophilc attack, covalently binds to the molecules in the environment and in part link to the proteins (Figure 8).

Thus, after the probe is taken up by the cells and becomes membrane component, the azide group will be deeply inserted into the membrane lipid bilayer. According to the scheme reported in Figure 7, when the photoactivable GSLs, inserted in the membrane level, is illuminated, the major part of it cross-links to membrane lipid molecules which are more abundant and close to the photoactivable group. A minor part of the nitrene-containing GSLs links to proteins and by a radioactive tracer, present in the molecule, is possible to detect the proteins cross-linked to photoactivable probe.

Figure 9 shows the three possible types of GSLs-protein cross-linkage. Given the final azide position inside the PM, a cross linkage might occur with the lipid moiety of a GPI-anchored protein, with a hydrophobic aminoacidic stretch of a transmembrane protein, or with the fatty acid anchoring a protein to the cytoplasmatic side of the membrane.

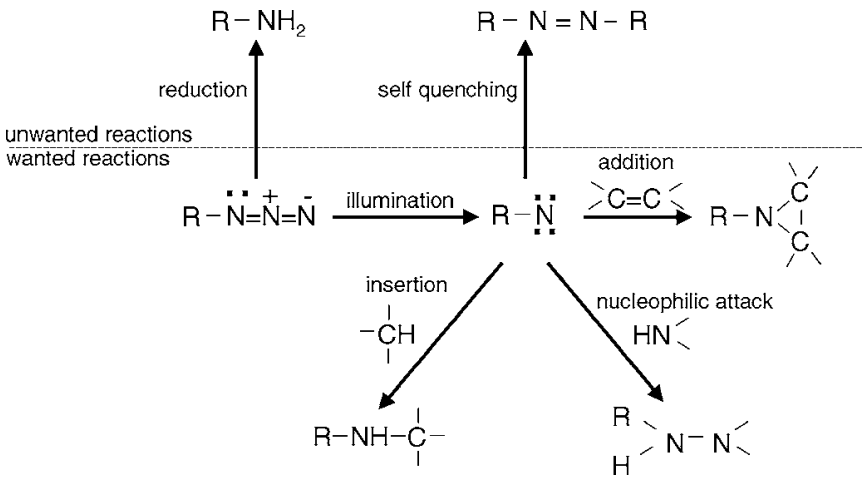


Fig. 8 Scheme of reactions involving the azide before and after illumination. The reduction of azide is a rapid reaction inside the cells in the presence of reducing proteins such as glutathione or SH enriched proteins (from [43]).

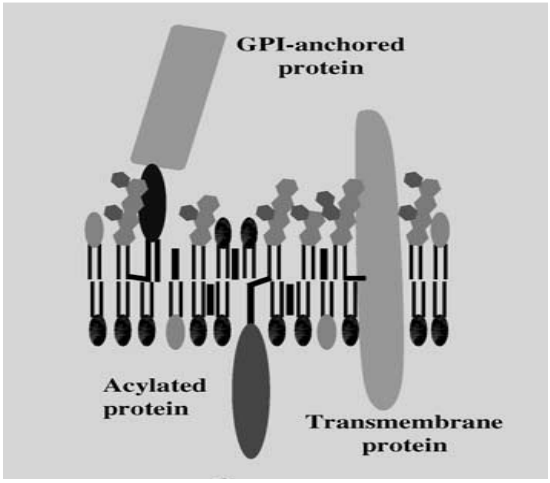
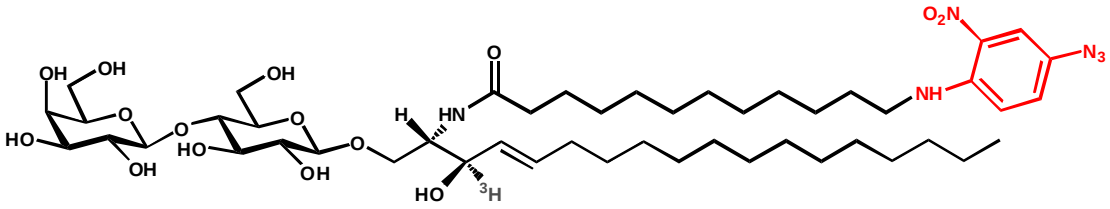
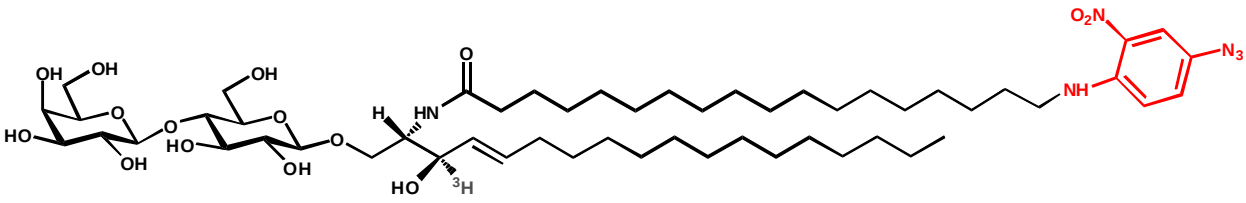


Fig. 9 Cartoon representing the protein-GSLs cross linkages that can be obtained after illumination of the photoactivable GSLs inserted into the PM (adapted from [43]).

To study the LacCer/Lyn interaction, the chemical group of Prof. Sonnino developed a radio-photo labeled analogue of C24-LacCer ([^{3}H]-C24-(N_3)-LacCer), with long acyl chain and C16-LacCer ([^{3}H]-C16-(N_3)-LacCer), with short acyl chain (Figure 10).



[^{3}H]-C16-(N_3)-LacCer



[^{3}H]-C24-(N_3)-LacCer

Fig. 10 Structure of a radioactive and photoactivable [^{3}H]-C24-(N_3)-LacCer, with long acyl chain and [^{3}H]-C16-(N_3)-LacCer, with short acyl chain. The photoactivable group, the nitrophenylazide, is located at the end of the acyl chain, while the tritium group is located in position 3 of shingosine.

Materials and Methods

MATERIALS

Mouse anti-LacCer IgM T5A7 was established as described previously [47]. Mouse anti-LacCer IgM Huly-m13 was purchased from Ancell (Bayport, MN, USA). Rabbit anti-Lyn IgG, and mouse anti-Gαi were purchased Cell Signaling Technology (Beverley, MA, USA) and from Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. All the other monoclonal antibody were purchased from BD Biosciences (San Jose, CA, USA). PE-conjugated anti Human CD11b and mouse IgGIK were from eBioscience (San Diego, CA, USA).

Synthesized GSLs, C16:0 (palmitic acid)- and C24:0 (lignoceric acid)-LacCer were synthesized as described previously [48]

Culture dishes IWAKI were purchased from Bibby Scientific (Tokyo, Japan). RPMI-1640, DMEM/F12 and FBS were purchased from Nissui (Tokyo, Japan). PBS, BSA, DMSO, DFP, PMSF, TRIZMA, EDTA, NaCl, NaF, Triton X-100, Na_3VO_4 and sucrose were from Sigma-Aldrich (St. Louise, MO). Complete protease inhibitor cocktail (Complete) was obtained from Roche (Indianapolis, IN, USA).

METHODS

Cell Culture

HL-60 cells were maintained in culture in RPMI-1640 medium supplemented with 10% FBS. To induce differentiation into neutrophilic lineage cells (D-HL-60 cells), HL-60 cells were cultured with 1.3% DMSO for 8 days. Differentiation was confirmed by CD11b expression on D-HL-60 cells using flow cytometry analysis [49].

Flow cytometric analysis

DMSO-treated and untreated HL-60 cell suspensions were labeled with phycoerythrin-conjugated antihuman CD11b antibody (eBioscience, San Diego, CA) or with phycoerythrin-conjugated mouse IgG1, kappa isotype control (eBioscience) for 30 min on ice, and then the expression of CD11b was analyzed by flow cytometry (FACScalibur; BD Biosciences,Mansfield, MA) [53].

Treatment of cell cultures with radioactive photoactivable LacCers.

For loading of D-HL-60 cells with LacCer, aliquots of 10 ml D-HL-60 cells ($2x10^7$ cells/ml) in Dulbecco's PBS were incubated with a mixture of 0.25ug of [^{3}H]--(N_3)LacCer and 0.25 µg of LacCer (final concentration 0.5 µg/ml) for 30 min at 20°C. After incubation, cells were washed once with 10 ml PBS + 0.1% BSA in DMSO to remove non incorporated or attached LacCer [48]. The cells were washed twice with 10 ml of cold PBS, and then 15 ml of cold PBS were added and the cells

were illuminated for 45 min under UV light (λ= 360 nm) on ice. All procedure before exposure to UV light were performed under red safelight [37-46]. Cells were collected and treated with 5mM DFP in PBS for 10 minutes on ice. After centrifugation at 270 g for 10 minutes, cell pellet ($2x10^8$ cells) was lysed in the 1 ml of lysis buffer (1% Triton X-100, 10 mM Tris-HCl (pH7.5), 150 mM NaCl, 5 mM EDTA, 1 mM DFP, 1mM Na3VO4, 1 mM PMSF and 1/20 (vol/vol) Complete Roche at 4°C for 20 minutes. After Dounce homogenized (70 strokes), the lysate was centrifugated (1300g for 5 min) to remove nuclei and cellular debris, and the Post Nuclear Supernatant (PNS) was removed and transferred into new tubes.

Determination of cell viability

At the end of loading with radioactive and photoactivable LacCer cell viability was assessed by Tripan Blue exclusion methods [50,51].

Preparation of SLs-enriched membrane domains by sucrose gradient centrifugation.

The PNS fraction was mixed with an equal volume of 85% of sucrose (w/v) in 10 mM Tris Buffer HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na3VO4, placed at the bottom of a discountinuos sucrose concentration gradient (30-5%) in the same buffer and centrifugate (17h at 200,000g) at 4°C. After ultracentrifugation, eleven fraction were collected starting from the top of the tube. The light-scattering band, corresponding to the DRM fraction, was located at the interface between 5% and 30% sucrose and correspond to fraction 5. The entire procedure was performed at 0 to 4°C in ice immersion [33,52].

Analysis of protein patterns

PNS and sucrose gradient fraction obtained after cell photolabeling with [^{3}H]-(N_3)-LacCer were analyzed with SDS-PAGE. After separation, proteins were transferred to PVDF membranes. Proteins cross-linked with [^{3}H]-(N_3)-LacCer were analyzed by digital autoradiography. The presence of Lyn and G protein was assessed by immunoblotting with specific antidoby, followed by reaction with secondary horseradish peroxidase-conjucated antibody and enhanced chemioluminescence detection (Super Signal Pierce) [36-42]

Immunoprecipitation experiments

The lysates ($1.2x10^7$ cell equivalent) or sucrose gradient fraction were diluted 10-fold volumes of immunoprecipitation buffer (50mM HEPES, pH 7.5, 1% Triton X-100, 150 mM NACl, 2mM Na3VO4, 10 mM NaF, with 1/20 Complete) and then precleared by incubation with 30 µl rat anti-mouse IgM/IgG IgG-bound Dynabeads (Invitrogen) for 1 h at 4°C. After pre-cleaning, the supernatants were incubated with 5 µg anti-LacCer IgM Huly-m13 or normal mouse IgM overnight at 4°C for LacCer immunoprecipitation or with 5 µg anti-Lyn IgG or normal mouse IgG overnight at 4°C for Lyn immunoprecipitation, followed by incubation with 30 µl rat anti-mouse IgM/IgG IgG-bound Dynabeads for 4 h at 4°C. The immunoprecipitated beads were then washed three times with immunoprecipitation buffer and were denatured under nonreducing conditions and then separated on 7.5% polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes. The Lyn and αGi were detected using SuperSignal™ reagent (Pierce Chemical Co., Rockford, IL, USA).

In some experiment IP samples were treated with 1% SDS in lysis buffer at 100°C for 5 min, then diluted 10 fold with lysis buffer (0.1% SDS final concentration) and immunoprecipitated as above. These condition are known to breack up the membrane organization and to allow the disaggregation of DRM domains [54]

Phagocytosis assays

LacCer-loaded D-HL-60 cells were incubated with Alexa 647-conjugated NOZs at a concentration of 10 particles per cell for 45 min at 37°C in DMEM/F12. After incubation, cells were washed with ice-cold PBS and fixed with 2% paraformaldehyde in PBS for 20 min on ice. At least 200 cells per sample were counted using a Leica TCS-SP2 confocal microscope equipped with a Plan-Apochromat ×100 oil differential interference contrast (DIC) objective (Leica Microsystems, Wetzlar, Germany), and phagocytic index was defined as percent-positive ingestion multiplied by the average number of phagocytosed particles per cell [35,55]

Other experimental procedures

The radioactivity associated with cells and with cell fractions, was determinate by liquid scintillation counting. Digital autoradiography of the PVDF membranes was performed with a Beta-Imager 2000 instrument (Biospace, Paris)

Results

i. Effect of [^{3}H]-C16/C24-(N_3)-LacCer on D-HL-60 cells

On the bases of the previously experiment [33], we fixed the concentration of administrated [^{3}H]-C16/C24-(N_3)-LacCer around 0.5 µg/ml of cells. In this condition D-HL-60 cells shown an increased production of superoxide.

Like First step we performed experiment to verify cell toxicity due to the administration of photo-LacCer at this concentration, because these compounds are not natural compound but analogues. Cell viability was evaluated by Tripan Blue assay. As show in Figure 11, D-HL-60 cells do not present alteration in cell proliferation and death in the presence of both [^{3}H]-C16-(N_3)-LacCer and [^{3}H]-C24-(N_3)-LacCer, compared with untreated cells.

Cell Viability (%)	
No treated cells	93
+ [^{3}H]-C16-(N_3)-LacCer	99
+ [^{3}H]-C24-(N_3)-LacCer	90

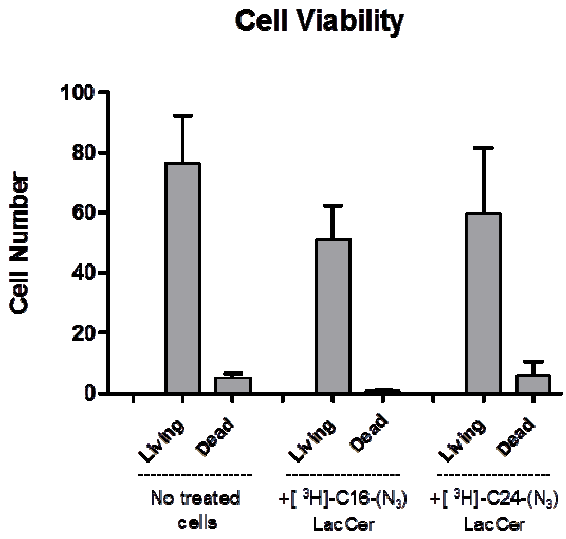


Fig. 11 Photoactivable LacCer's effect on D-HL-60 cells's viability.

ii. Radio-photo analog LacCer are "phisiologically" insert in the PM

The use of these derivates implies to demonstrate that they mimics as close as possible that of natural endogenous compounds. From the previously experiment we know that LacCer is essentially recovered in Triton-X-100 insoluble DRM fraction, of neutrophilis as well as D-HL-60 cells [13].

After the feeding of the cells with the photoactivable compound, cells were illuminated under UV light, harvested, lysed in lysis buffer containg Triton X-100 and in order to remove nuclei cell homogenate were centrifugate at 1300xg for 5 minutes to obtain the PNS. PNS were loaded on a discontinuos sucrose gradient and ultracentrifugated at 200000xg for 17h and eleven fractions were collect from the top of the tube.

The radioactivity associated to each fraction was determinate by liquid scintillation counting.

As show in Figure 12 in both treatments with [^{3}H]-C16-(N_3)-LacCer and [^{3}H]-C24-(N_3)-LacCer, cell radoactivity, then LacCer, was largely associated with fraction 9-11, corresponding to the high density fraction (HD fraction) that contain membrane solubized by the detergent and the higher quantity of cell proteins. Moreover some radioactivity was also associated with fraction 5-6 corresponding to the low density fraction (DRM) that are resistent to the detergent solubilization. The radioactivity associated in the bottom fraction is mainly due to the cross linking with lipid, that are the more abundant and close to the photoactivable group. We obtained a different distribution of radioactivity on gradient fraction from cell labelled with [^{3}H]-C16-(N_3)-LacCer and from the cells labelled with [^{3}H]-C24-(N_3)-LacCer. These probably is due to the different physical property of these 2 compound, and need to be explore.

To understand if the radioactivity distribution was an artefact due to the illumination process, we also prepare the gradient fraction from no illuminating cells. Like show in the graph (Figure 12) no significant different in the distribution of radioactivity was obtained.

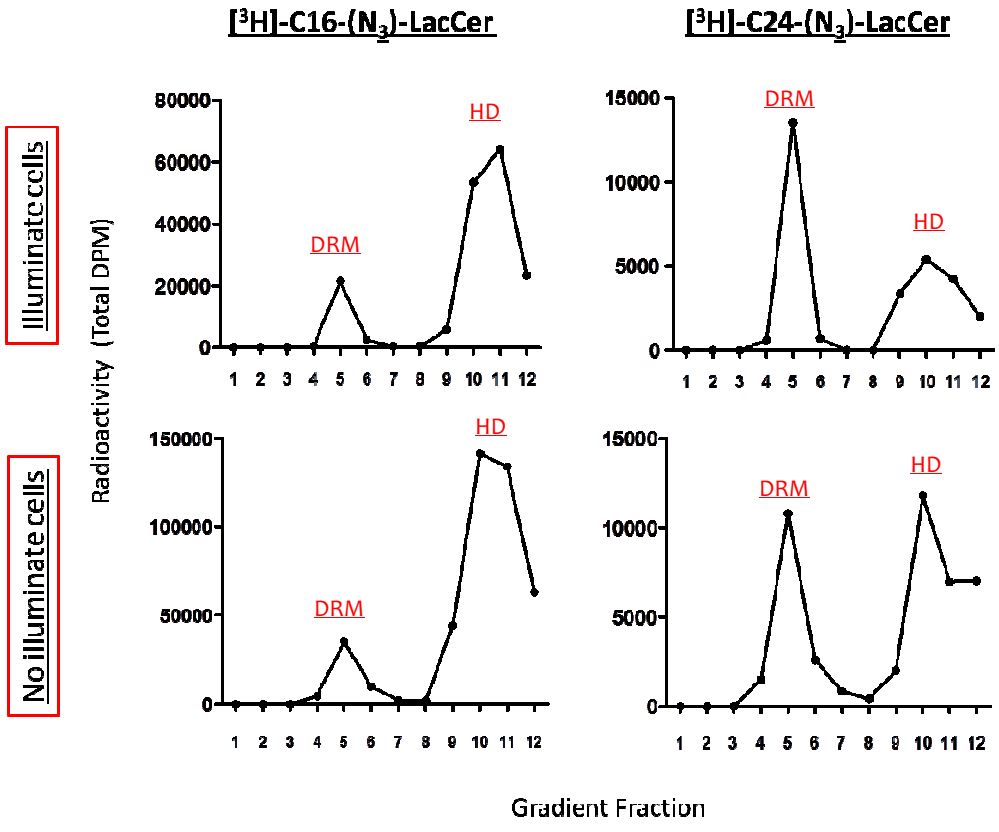


Fig. 12 Gradient fraction analysis. Distribution of total radioactivity ([^{3}H]-C16/C24-(N_3)-LacCer) in sucrose density fraction from illuminate and no illuminate cells

To verify if these compound are biologically active, we assessed the phagocytosis activity of D-HL-60 cells after the loading with radio and photo activable compound. The phagocytosis assay without illumination indicated that and [^{3}H]-C24-(N_3)-LacCer-loaded D-HL-60 cells phagocytosed microorganisms, whereas [^{3}H]-C16-(N_3)-LacCer--loaded D-HL-60 cells failed. These results suggest that both the radio-photo-labeled analog of C24-LacCer and C16-LacCer, are "physiologically" inserted into the PM.

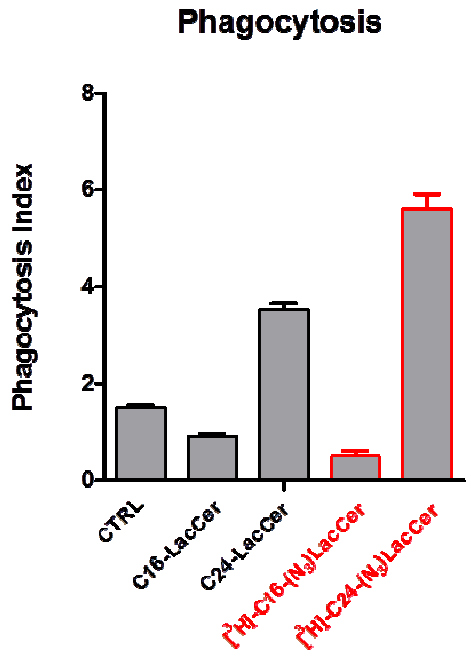


Fig. 13 Phagocytosis index

iii. Radio-photo-labeled analog of C24-LacCer but not C16-LacCer is directly associated with Lyn

To examine interactions among LacCer and Lyn, we performed co-immunoprecipitations assay. We initially performed immunoprecipitation experiments of PNS from cells loaded with [^{3}H]-C16-(N_3)-LacCer and [^{3}H]-C24-(N_3)-LacCer. The immunoprecipitates were submitted to SDS-PAGE and immunoblotted for Lyn . Figure 14a show the WB image: Lyn was recovered in anti-LacCer antibody-immunoprecipitants from and [^{3}H]-C24-(N_3)LacCer but not [^{3}H]-C16-(N_3)LacCer -loaed cells.

In figure 14b we reported the radio-immuno assay using anti LacCer antibody indicating that the immunoprecipitants from [^{3}H]-C24-(N_3)-LacCer but not [^{3}H]-C16-(N_3)-LacCer contained radioactivity.

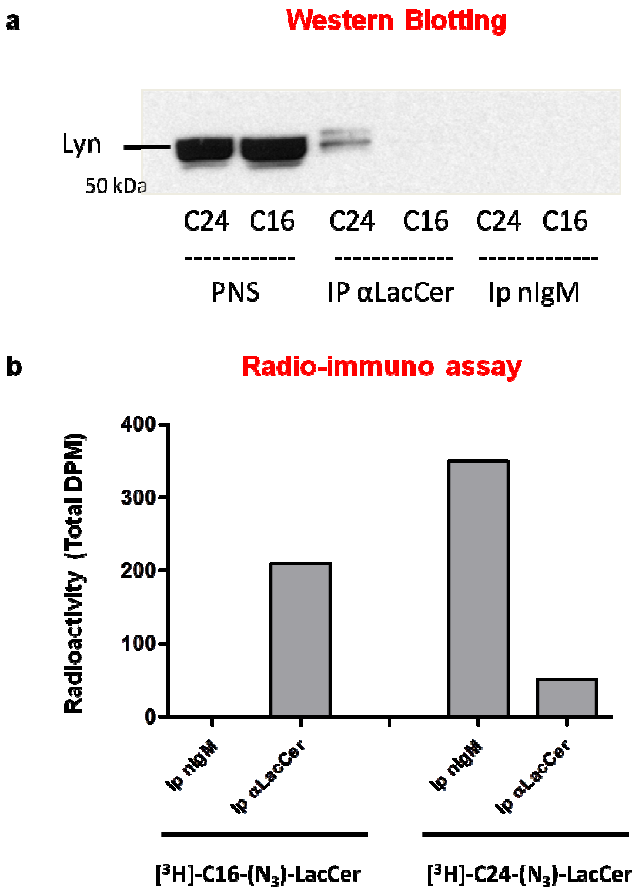


Fig. 14 Imumunoprecipitation of LacCer from D-HL-60 cells loaded with [^{3}H]-C24-(N_3)-LacCer and [^{3}H]-C16-(N_3)-LacCer. a) Western Blotting analysis b) Radio-immuno assay

To determine if the interaction between Lyn and LacCer could be a direct interaction, we performed an experiment of immunoprecipitation for Lyn in disgregant conditions from DRM fraction isolated from the D-HL-60 cells loaded with [^{3}H]-C16-(N_3)-LacCer and [^{3}H]-C24-(N_3)-LacCer. After SDS-PAGE separation, Lyn was recovered from both loaded cells by immunoblotting (Figure 15a). However, [^{3}H]-LacCer (radioactivity) was only recovered in the immunoprecipitant from the cells loaded with analog of [^{3}H]-C24-(N_3)LacCer (Figure 14b). **These results suggest that Lyn is directly connected to LacCer, when cells are loading with C24 LacCer, but not with C16 LacCer.**

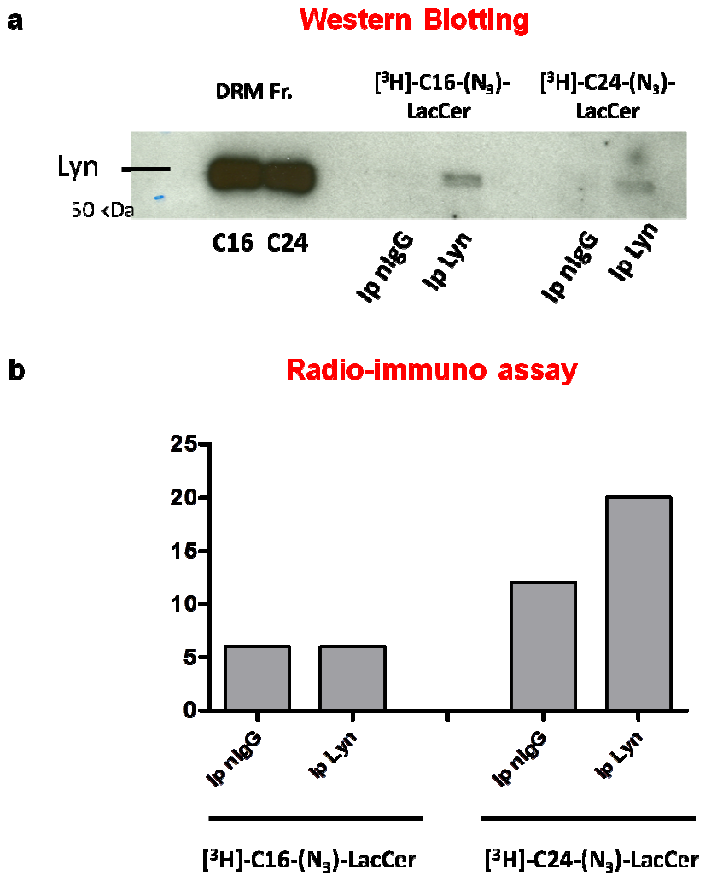


Fig. 14 Imumunoprecipitation of Lyn from D-HL-60 cells loaded with [^{3}H]-C24-(N_3)-LacCer and [^{3}H]-C16-(N_3)-LacCer in disgregant conditions. a) Western Blotting analysis b) Radio-immuno assay

iv. Identification of cross-linkage proteins

We next examined LacCer-protein interactions occurring within the DRM and HD fractions of living cells performing a cross-linking assay using the photoactivable radioactive derivates of LacCer(s).

Target proteins were separated by SDS/PAGE, blotted on PVDF membrane and visualized by autoradiografy for 96 hr (Figure 16a). A broad range of radioactivity reflecting C24-LacCer-protein complexes but not C16-LacCer-protein complexes could be detected from 75 kDa to 20 kDa, suggesting a specific association between LacCer and different DRM proteins.

Moreover the same PVDF was immunostaning both for Lyn and Gαi: a specific radioactive band corresponding to the Lyn protein (56 kDa) and one corresponding to Gαi (41 kDa) were detected, confirming a direct association of LacCer with Lyn and suggesting a possible interaction with Gαi protein.

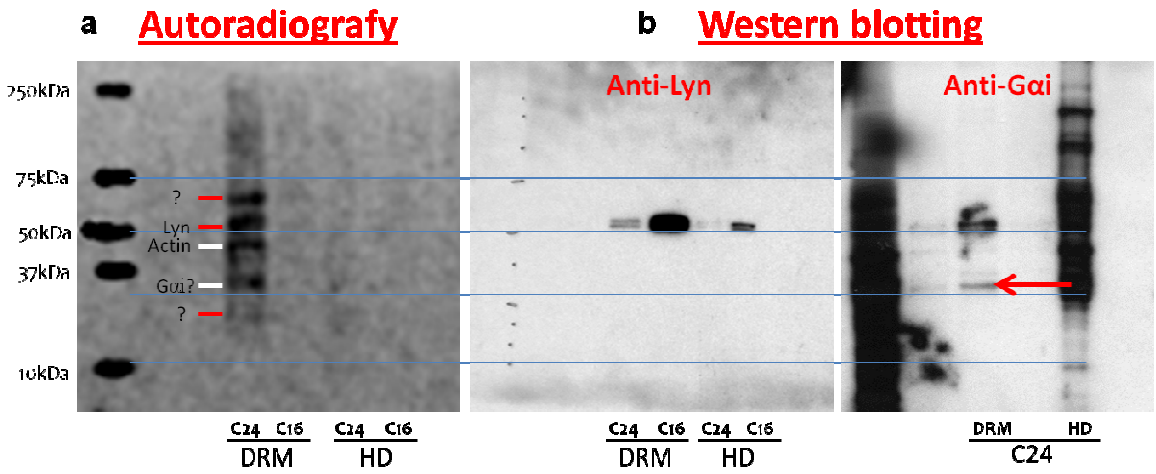


Fig. 16 Protein cross-linked with [^{3}H]-(N_3)-LacCer from DRM and HD fractions prepared from D-HL-60 cells loaded with [^{3}H]-C16-(N_3)-LacCer and [^{3}H]-C24-(N_3)-LacCer. a) Autoradiografy image b) Western Blotting images.

Discussion

The innate immune system is the first line of defense against pathogenic microorganisms, such as bacteria, fungi, and viruses. Phagocytes, such as neutrophils and macrophages, play an important role in the innate immune system by recognizing, engulfing, and eliminating pathogens [29-32]. It has been suggested that lipid membrane microdomains/rafts of phagocytes are involved in these innate immune responses, including superoxide generation, cell migration, and phagocytosis. GSLs are highly enriched in specialized membrane microdomains ("lipid rafts", caveolar domains and glycosynapses), and they participate to the process of transduction of information across the membrane. Recent proteomic analyses of microdomains from phagocytes have provided insight into membrane microdomain-mediated functions in the processes of phagocytosis [13-17,22-26].

LacCer, a neutral GSL, is abundantly expressed on human neutrophils, and specifically recognizes several pathogenic microorganisms. LacCer forms membrane microdomains coupled with the Src family kinase Lyn on the PM, and ligand binding to LacCer activates Lyn, resulting in neutrophil functions, such as superoxide generation, phagocytosis and migration. In contrast, neutrophilic differentiated HL-60 cells do not have Lyn associated LacCer-enriched microdomains and lack LacCer-mediated functions. The presence of a LacCer molecular species with Cer containing a very long fatty acid chain is also required. In neutrophil PMs, the very long fatty acid C24:0 and C24:1 chains are the main components of LacCer, whereas PM of D-HL-60 cells mainly includes C16-LacCer species. LacCer species containing very long fatty acid chains are indispensable for the association of Lyn with LacCer-enriched microdomains and LacCer-mediated functions [13,28-35].

With this project we want to study the role of very long fatty acid-LacCer species in the physical and functional coupling with Lyn and in microdomain-mediated functions in the processes of phagocytosis to determine the molecular mechanisms underlying these functions. For this pourpose, the chemical group of Prof. Sonnino developed the tritium-labeled and photoactivale LacCer derivatives with the aim to give a name to this proteins.

This project was carried out in the Laboratory of Professor Kazuisa Iwabuchi, at the Juntendo University of Tokyo, where I spend 7 months.

First step was to set up the correct condition for the use of the radio-photo labeled derivates. We started founding out the best conditions for the administration of these compound in cell in culture, and like reported in "Materials and Methods" and "Results" sections we fixed the concentration at 0.5 µg/ml of cell sunspecion for 30 minuts at 20°C. In these experimental condition we obtained a sufficient incorporation of radiophoto LacCer into the PM. Indeed this conditions allows us to be sure that LacCer stop at the level of PM and do not enter in the metabolic *via*.

We also demonstrated the physiological activity of these derivated compound: they were incorporated into the PM and showed phagocitosis activity, mimics the natural compound.

To understand what's happen at the level of PM microdomain we prepared the DRM to analyzed and to recognize the protein associated to the LacCer environment.

By means of cross linking and immunoprecipitation experiments with photoactivable and radioactive [^{3}H]-C16/C24-(N_3)-LacCer we showed that Lyn and LacCer with long acyl chain have a direct interaction in LacCer-enriched membrane domain from neutrophilic cells. First biological results with analogues are encouraging. In fact, they seem to confirm the initial hypothesis related to the requirement of long chains LacCer for the innate responses in human neutrophils. These results suggest that GSLs with a long fatty acid chain of Cer moiety, in our specific case LacCer, could be the key- player on the signal transduction across the cellular PM, modulating membrane interdigitation, reducing the membrane thickness and forming a specific PM microdomanins. We know that for LacCer mediated Lyn activation is necessary: LacCer molecular species with Cer containing very long fatty acid chain, but also the presence of β-Gal(1-4)-β-Glc disaccharide structure of LacCer.This means that the oligosaccharide chain is able to recognize some specific protein. Chemical group of Prof. Sonnino is now working on the synthesis of the tritiated and doubly photoactivable LacCer containing fatty acid with long chain, that allow us to understand also which molecules are constituent of these domains in the external PM, like CD11b or CD14.

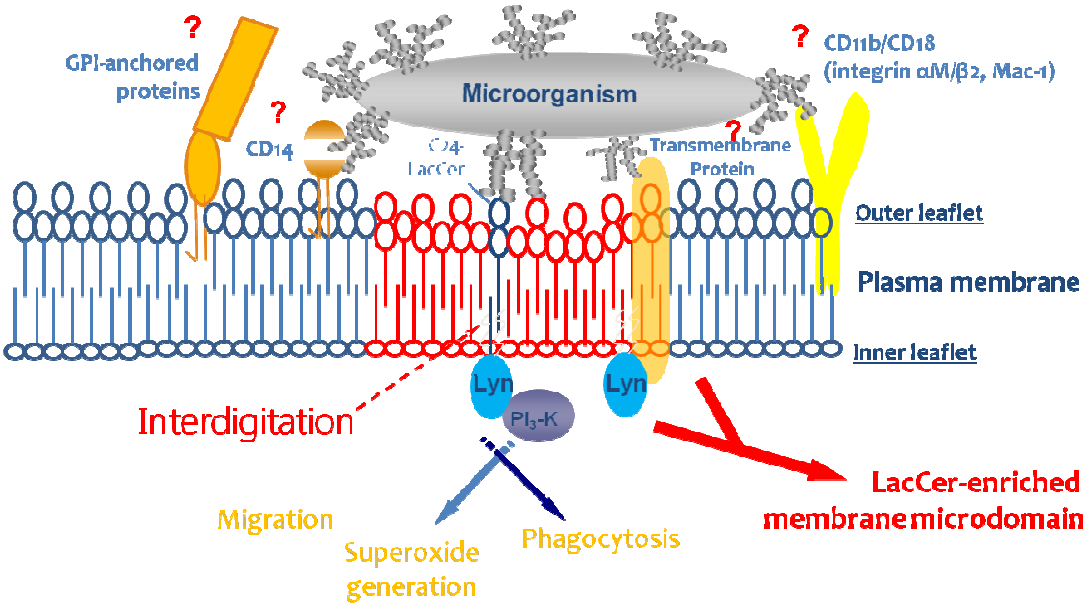


Fig. 17 Cartoon of the possible organization of Lyn coupled LacCer-enriched lipid rafts in neutrophilis.

References

1. Simons K. and Ikoen E. (1997) Functional rafts in cell membranes *Nature* 387:569-572
2. Brown D.A. and Rose J.K. (1992) Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface *Cell* 68:533-544
3. Sonnino S. and Prinetti A. (2012) Membrane Domains and the "Lipid Raft" Concept *Curr Med Chem*
4. Lindner R., Naim H.Y. (2009) Domains in biological membrane *Exp Cell Res* 315:2871-2878
5. Jacobson K., Sheets E.D., Simson R., (1995) Revisiting the fluid mosaic model of membrane *Science* 268:1441-1442
6. Kurzchalia T.V. and Parton R.G. (1999) Membrane microdomanis and caveolae *Curr Opin Cell Biol* 11:424-431
7. Okada Y., Mugnai G., Bremer E.G., and Hakomori S. (1984) Glicosphingolipids in detergent-insoluble substrate attachment (DISAM) prepared from substrate attachment material (SAM). Their possible role in regulating cell adhesion. *Exp Cell Res* 155:448-456
8. Harder T. and Simons K. (1997) Caveolae, DIGS and the dynamics of sphingolipid-cholesterol microdomains. *Curr Opin Cell Biol 9:534-542*
9. Gkantiragas I., Brugger B., Stuven E., Kaloyanova D., Li X.Y., Lohr K., Lottspeich F., Wieland F.T. and Helms J.B. (2001) Sphingomyelin-enriched microdomanins at the Golgi complex *Mol Biol Cell* 12:1819-1833
10. Prinetti A., Chigorno V. Tettamanti G. and Sonnino S. (2000) Sphingolipid-enriched membrane domains from rat cerebellar granule cells differentiated in culture. A compositional study *J Biol Chem* 275:11658-11665
11. Jacobson K., Mouritsen O.G., and Anderson R.G. (2007) Lipid Rafts: at a crossroad between cell biology and physic *Nat Cell Biol* 9:7-14
12. Brown D.A. (2006) Lipid Rafts, detergent-resistant membranes, and Raft targeting signals *Phisiology (Bethesda)* 21:430-439
13. Iwabuchi K. and Nagaoka I. (2011) Lactosylceramide-enriched glycosphingolipid signaling domain mediates superoxide generation from human neutrophilis *Blood* 100:1454-1464
14. Yates A.J. and Rampersaud A. (1998) Sphingolipids as receptor modulators. An overview. *Ann N Y ACAD SCI* 845:57-71
15. Hakomori S. and Igarashi Y. (1995) Functional role of glycosphingolipids in cell recognition and signaling *J Biochem (Tokyo)* 118:1091-1103
16. Hakomori S. (2003) Structure, organization and function of glycosphingolipids in membrane *Curr Opin Hematol* 10:16-24
17. Hakomori S. Handa K Iwabuchi K Yamamura S. Prinetti A. (1998) New insight in glycosphingolipid function: "glycosignaling domain" a cell surface assembly of glycosphingolipids with signal transducer molecole: involved in cell adhesion coupled with signaling *Glycobiology* 8:xi-xix
18. Bremer E.G., Hakomori S., Bowen-Pope D.F., Raines E. and Ross R. (1984) Ganglioside mediated modulation of cell growth, growth factor binding and receptor phosphorilation *J Biol Chem* 259:6818-6825

19. Chan K.F. (1989) Ganglioside-modulate protein phosphorilationin muscle. Activation of phosphorilase B kinase by gangliosides. *J Biol Chem* 264:18632-18637
20. Tsuji S., Nakajima J., Sasaki T., and Nagai Y. (1985) Bioactive ganglioside. IV ganglioside GQ1B/CA2+ dependent protein kinase activity exist in the plasma membrane fraction of neuroblastoma cell line, *J BIOCHEM* 97:969-972
21. Bassi R. Chigorno V., Fiorilli A., Sonnino S., and Tettamanti G. (1991) Exogenous gangliosides GD1B and GD1B-Lactone, stably associated to rat brain P2 subcellular fraction, modulate differently the process od protein phosphorilation *J Neurochem* 57:1207-1211
22. Sonnino, S., Prinetti, A., Mauri, L., Chigorno, V., Tettamanti, G. (2006)Dynamic and structural properties of sphingolipids as driving forces for the formation of membrane domains. *Chem. Rev.* 106,2111–2125
23. Sonnino, S., Mauri, L., Chigorno, V., Prinetti, A. (2007) Gangliosides as components of lipid membrane domains. *Glycobiology* 17, 1R–13R
24. Palestini, P., Masserini, M., Sonnino, S., Giuliani, A., Tettamanti, G. (1990) Changes in the ceramide composition of rat forebrain gangliosides with age. J. Neurochem. 54, 230–235
25. Prinetti, A., Chigorno, V., Prioni, S., Loberto, N., Marano, N., Tettamanti, G., Sonnino, S. (2001) Changes in the lipid turnover, composition, and organization, as sphingolipid-enriched membrane domains, in rat cerebellar granule cells developing in vitro. *J. Biol. Chem*. 276, 21136–21145
26. Pitto, M., Parenti, M., Guzzi, F., Magni, F., Palestini, P., Ravasi, D., Masserini, M.(2002) Palmitic is the main fatty acid carried by lipids of detergent-resistant membrane fractions from neural and non neural cells. *Neurochem. Res*. 27, 729–734
27. Tanford C. (1973) The hydrophobic effect: formation of micelles and biological membranes *Wiley New York*
28. Sonnino S., Prinetti A., Nakayama H., Yangida M., Ogawa H., and Iwabuchi K. (2009) Role of very long fatty acid-containing glycosphingolipids in membrane organization and cell signaling: the model of lactosylceramide in neutrophils *Glycoconj J.* 26:615-621
29. Kabelitz D., Medzhitov (2007) Innate immunity--cross-talk with adaptive immunity through pattern recognition receptors and cytokines. *Curr Opin Immunol*. 19:1-3
30. Gay NJ., Gangloff M. (2007) Structure and function of Toll receptors and their ligands. *Annu Rev Biochem* 76:141-165
31. Mayadas T.N. and Cullere X. (2005) Neutrophil beta2 integrins: moderators of life or death decisions.*Trends Immunol* 26:388-395
32. Yoshizaki F., Nakayama H., Iwahara C., Takamori K., Ogawa H. and Iwabuchi K. (2008) Role of glycosphingolipid-enriched microdomains in innate immunity: mcrodomain-dependent phagocytic cell functions *Bioch Biophy Acta* 1780:383-392
33. Iwabuchi K., Prinetti A., Sonnino S., Mauri L., Kobayashi T., Ishii K., Kaga N., Murayama K., Kurihara H., Nakayama H., Yoshizaki F., Takamori K., Ogawa H., and Nagaoka I. (2008) Involvement of very long fatty acid-containing lactosylceramide in lactosylceramide-mediated superoxide generation and migration in neutrophilis *Glycoconj J.* 25:357-374

34. Nakayama H., Iwahara C., Takamori K., Ogawa H. and Iwabuchi K. (2008) Lactosylceramide is a pattern recognition receptor that forms Lyn-coupled membrane microdomains on neutrophils *Immun Endoc & Metab Agents in Med Chem* 8:327-335
35. Iwabuchi K., Nakayama H., Iwahara C., Takamori K (2010) Significance of glycosphingolipid fatty acid chain lenght membrane micro domain-mediated signal transduction *FEBS Letters* 584:1462-1652
36. Greenberg S., Grinstein S. (2002) Phagocytosis and innate immunity *Curr Opin Immunol* 14:136-145
37. Chigorno V., Valsecchi M., Acquotti D., Sonnino S. and Tettamanti G. (1990) Formation of a cytosolic ganglioside-protein complex following administration of photoreactive ganglioside GM1 to human fibroblasts in culture. *FEBS Lett*. 263, 329–331.
38. Chigorno V., Palestini P., Sciannamblo M. T., Dolo V., Pavan A., Tettamanti G. and Sonnino S. (2000) Evidence that ganglioside enriched domains are distinct from caveolae in MDCK II and human fibroblast cells in culture. *Eur. J. Biochem*. 267:4187–4197.
39. Prinetti A., Marano N., Prioni S., Chigorno V., Mauri L., Casellato R., Tettamanti G. and Sonnino S. (2000b) Association of Srcfamily protein tyrosine kinases with sphingolipids in rat cerebellar granule cells differentiated in culture. *Glycoconjugate J.* 17:223–232.
40. Sonnino S., Chigorno V., Acquotti D., Pitto M., Kirschner G. and Tettamanti G. (1989) A photoreactive derivative of radiolabeled GM1 ganglioside: preparation and use to establish the involvement of specific proteins in GM1 uptake by human fibroblasts in culture. *Biochemistry* 28:77–84.
41. Sonnino S., Chigorno V., Valsecchi M., Pitto M. and Tettamanti G. (1992) Specific ganglioside–cell protein interactions: a study performed with GM1 ganglioside derivative containing photoactivable azide and rat cerebellar granule cells in culture. *Neurochem. Int*. 20:315–321.
42. Loberto N., Prioni S., Prinetti A., Ottico E., Chigorno V., Karagogeos D. and Sonnino S. (2003) The adhesion protein TAG-1 has a ganglioside environment in the sphingolipid-enriched membrane domains of neuronal cells in culture *J Neurochem* 85:224-233
43. Mauri L. Prioni S., Loberto N., Chigorno V., Prinetti A., and Sonnino S. (2004) Synthesis of radioactive and photoactivable ganglioside derivates for the study of ganglioside-protein interactions Glyconj J 20:11-23
44. Prioni S., Mauri L., Loberto N., Casellato R., Chigorno V., Karagogeos D., Prinetti A. and Sonnino S. (2004) Interaction between gangliosides and proteins in the exoplasmic leaflet of neuronal plasma membranes: a study *Glycoconj J* 21:461-470
45. Kabayama K., Sato T., Saito K., Loberto N., Prinetti A., Sonnino S., Kinjo M., Igarashi Y. And Inokuchi J. (2007) Dissociation of the insuline receptor and caveolin-1 complex by ganglioside GM3 in the state of insuline resistence *PNAS* 104:13678-13683
46. Aureli M., Prioni S., Mauri L., Loberto N., Casellato R., Ciampa M.G. Chigorno V., Prinetti A. and Sonnino S. (2010) Photoactivable sphingosine as a tool to study membrane microenvironments in cultured cells *Journal of Lipid Research* 51:798-808

47. Symington, F. W., Bernstein, I. D., Hakomori, S. (1984) Monoclonal antibody specific for lactosylceramide *J. Biol. Chem*. 259,6008-6012
48. Iwabuchi, K., Prinetti, A., Sonnino, S., Mauri, L., Kobayashi, T., Ishii, K., Kaga, N., Murayama, K., Kurihara, H., Nakayama, H., Yoshizaki, F., Takamori, K., Ogawa, H., Nagaoka, I. (2007) Involvement of very long fatty acid-containing lactosylceramide in lactosylceramide-mediated superoxide generation and migration in neutrophils *Glycoconj. J.* EPub ahead of print.
49. Iwabuchi K., Seino K., Kayagaki N. et al (1998) Chemotatic activity of soluble Fas ligand against phagocytes *J.Immunol* 161:4484-4488
50. Aureli M., Loberto N., Lanteri P., Chigorno V., Prinetti A., Sonnino S. (2011) Cell surface sphingolipid glycohydrolases in neuronal differentiation and aging in culture. *J. Neurochem*. 116, 891–899
51. Mehlen P., Rabizadeh S., Snipas S.J., Assa-Munt N., Salvesen G.S., Bredesen D.E. (1998) The DCC gene product induces apoptosis by a mechanism requiring receptor proteolysis. *Nature* 395, 801–804
52. Loberto N., Prioni S., Bettiga A., Chigorno V., Prinetti A. and Sonnino S. (2005) The membrane environment of endogenous cellular prion protein in primary rat cerebellar neurons *J. Neurochem* 95,771-783
53. Yanagida M.,Nakayama H.,Yoshizaki F., Fujimura T., Takamori K., Ogawa H. and Iwabuchi K. (2007) Proteomic analysis of plasma membrane lipid rafts of HL-60 cells Proteomics 7,2398-2409
54. Kasahara K., Watanabe Y., Yamamoto T., and Sanai Y (1997) Association of src family tyrosine kinase LYN with ganglioside GD3 in rat brain. Possible regulation of LYN by glycosphingolipid in caveolae-like domains *J Biol Chem* 272,29947-29953
55. Hua, J., Sakamoto, K., Nagaoka, I. (2002) Inhibitory actions of glucosamine, a therapeutic agent for osteoarthritis, on the functions of neutrophils *J. Leukoc. Biol.* 71,632-640

3. Theme II

Are secondary accumulations of gangliosides the triggering factor for Shingolipidosis?

The case of Niemman-Pick type A

Introduction

SPHINGOLIPIDS in NERVOUS SYSTEM

Among the tissues of the body, nervous tissue is one of the richest in lipid content. Apart from their quantitative importance, the lipids of the nervous tissue show a great deal of structural diversity. They are actively synthesized and increase substantially in quantity during the early phases of development of the nervous system. Lipids in brain tissue have an important role because they modify the structure, fluidity and functions of brain membranes. Lipid deposition and metabolism are connected with the biogenesis of some specialized membranes, such as myelin, clearly indicating a role for lipids in neural function. Lipids account for about 10% of the fresh weight and half the dry matter of the brain.

Sphingolipids (SLs) are particularly abundant in the nervous system and gangliosides, sialic acid content glycosphingolipids (GSLs), are high enriched in neurons. A physiological regulatory role of SLs, and in particular of gangliosides, has been documented for biological events of great relevance for neural cell biology, such as neurotrophic factor signaling [1-4], neural cell adhesion and migration [2,5,6], axon guidance, synaptic transmission [2,7], neuron-glia interactions [8,9] and myelin genesis [10].

It is possible to observe drastic and consistent changes in ganglioside pattern during neuronal development, particularly during the earliest stages. Many studies have shown that the qualitative and quantitative modifications in SLs expression in the nervous system during all phases of life. For this reasons the understanding of the metabolic regulation and the roles of SLs is a major challenge, and particular attention has been paid to determining the roles of SLs in neuronal development and in the central nervous system (CNS).

The crucial role of GSLs in the development and maintenance of the proper functions of nervous system has been demonstrated by different evidences:

i. GSL pattern undergoes deep qualitative and quantitative modifications during the development of the nervous system: the total ganglioside content increases a lot in the postnatal life in vertebrate brains and along in vitro differentiation of cultured neurons of different origin and in mouse neural precursor cells. These increases were accompanied by a dramatic shift from simple gangliosides (GM3 and GD3) to more complex species (GM1, GD1a, GD1b, GT1b). Sphingomyelin (SM) content also dramatically increased during in vitro differentiation in rat cerebellar neurons. Along the adult life, a progressive loss of gangliosides with aging has been reported in human and mouse brain. The most pronounced ganglioside changes associated with aging were an increase in the simpler gangliosides (GM3 and GD3) and a reduction of the complex gangliosides of the a-series (GD1a and GT1a) [12,13]. In addition to development- and age-associated changes in the oligosaccharide portion of gangliosides, deep remodeling of their

hydrophobic portion, involving changes in both the long chain base and fatty acid composition of the ceramide (Cer) moiety, has been described in human [14,15] and rat brains [16-18], rat cerebellum [19] and cultured rat cerebellar neurons [11,20]. Before birth, only C18:0- and C18:1-containing long chain bases were detectable in brain gangliosides [17]. C20-long chain bases in rat brain appeared after birth at a lower amount and gradually increased along aging in all brain gangliosides, reaching 27-55% of the total at the age of 2 years [16]. A similar switch from C18- to C20-long chain bases, both free and ganglioside-associated, was observed in neuronal cells during differentiation and aging in culture [11,10]. The increase in C20-long chain bases was generalized, but followed different trends for different gangliosides, indicating that the final molecular species composition for a certain ganglioside does not simply reflect the availability of different long chain bases as substrates for the biosynthetic pathway. In the ganglioside mixture from the brains of humans and different animals, 18:0 fatty acids generally comprise more than 80% of total fatty acid content, however complex changes in the fatty acid composition of different ganglioside classes have been reported along age. As example, the stearic acid content in C18-long-chain bases-containing gangliosides decreased with age in rat brain, with a concomitant increase in C16:0 and C18:1 fatty acids, while the fatty acid content of C20-long-chain bases-containing gangliosides was age-independent [16]. A greater short-chain fatty acid content in SM has been observed in young children and in a few cases of non-specific brain damage associated with demyelination [21,22].

ii. Experimental manipulations allowing modification of the concentration or pattern of GSLs in the plasma membrane (PM) profoundly affect the behavior of neural cells. The addition of exogenous gangliosides exerts neuritogenic, neurotrophic, and neuroprotective effects in cultured neurons and neural cell lines and in animal models of neural lesions [23,23-27]. In particular, selective depletion of cell surface SM and GSLs, achieved by treating living cells with bacterial sphingomyelinases or with endoglycoceramidase respectively, affected survival in neurotumoral cell lines and oligodendrocytes, axonal transport and sorting, and neuronal signaling transduction.

iii. Many pieces of evidence indicated that SLs biosynthesis is necessary for nervous system development. Blockade of GSLs biosynthesis by pharmacological inhibition of GlcCer synthase or Cer synthase reduced axonal elongation, branching synapses formation and activity [28] in cultured hippocampal and neocortical neurons [29,30]. Neural cell-specific deletion of GlcCer synthase in mice led to severe neurological defects in the early postnatal life and death within 3 weeks [31], demonstrating the importance of GSL for the maturation of the nervous system. On the other hand, pharmacologically induced stimulation of GSL biosynthesis stimulated neurite

outgrowth, formation of functional synapses and synaptic activity in cultured cortical neurons [28,32], and the induced expression of GD3 synthase was able to switch neuroblastoma cells to a differentiated phenotype [33]. NGF- and forskolin-induced neuronal differentiation in PC12 cells was accompanied by the up-regulation of several glycosyltransferase activities (GalGb3-, GM3-, GD1a- and GM2 synthases) [34], and bFGF-stimulated axonal growth in cultured hippocampal neurons resulted in the activation of Cer glucosyltranferase [35]. It has been recently suggested that the activity of the plasma membrane-associated ganglioside sialidase Neu3 might have a role in modifying the cell surface ganglioside composition, causing a decrease of GM3 and shift from polysialylated ganglioside species to GM1, with deep consequences on very important cellular events, including neuronal differentiation. In neuroblastoma cell lines, Neu3 expression increased during pharmacologically induced neuronal differentiation, and Neu3 gene transfection induced neurite outgrowth [36] and enhanced the effect of differentiating agents on the extension or branching of neuritis [37]. Conversely, inhibition of PM sialidase activity resulted in the loss of neuronal differentiation markers [38-40]. In cultured hippocampal neurons, Neu3 activity regulated the local GM1 concentration, determining the neurite axonal fate by a local increase in TrkA activity [41] and affecting axonal regeneration after axotomy [42].

It was recently reported that the sialidase Neu4 was dominantly expressed in the mouse brain [43-45] and its expression was relatively low in the embryonic stage and then rapidly increased at 3-14 days after birth. Moreover during retinoic acid-induced differentiation, Neu4 expression was down-regulated in Neuro2a cells and the overexpression of Neu4 resulted in suppression of neurite formation; on the contrary Neu4 inhibition increases neurite formation [36,37,41,42]. Total β- Galactosidase activity was increased during the aging of the rat hippocampus in vitro and in vitro experiments [46]. These results suggest that a shift of ganglioside expression during development is regulated by changes of the expression level and activity of these key glycosyltransferases. Not only gangliosides but also other GSLs, such as GalCer and sulfatide, present drastic pattern shifts during development.

SPHINGOLIPIDOSIS

Several pieces of evidence indicate a bi-univocal relationship between nervous system dysfunction and altered SLs metabolism [47] : i) inherited disorders caused by defects in SLs metabolism are characterized by acute brain involvement [48]; this is the case of several lysosomal storage diseases, due to defects in SLs catabolism. Recently, the first example of a neurological human disease associated with the genetic disruption of ganglioside biosynthesis has been reported [49]; ii) even in the absence of genetic defects of SLs metabolism underlying

the pathology, SLs metabolism is altered with important consequences in many neurological diseases.

A wide group of inherited lysosomal storage disease is caused by defects in SLs degradation (sphingolipidoses) (Figure 1) (reviewed in [48,50]). Lysosomal storage diseases are caused by the reduced (≤ 10% of normal levels) or absent activity of lysosomal proteins (hydrolytic enzymes, cofactors or lysosomal transporters). As consequence the corresponding lipid substrate accumulates and is stored in lysosomal compartment. Obviously, this accumulation occurs mainly in those cell type and tissues in which the lipids are pronominally synthesized.

For sphingolipidoses, the defective gene encodes for either a hydrolase involved in SLs catabolism, or an activator protein required for the proper activity of a SLs hydrolase. Most sphingolipidoses are characterized by severe neurological involvement. In particular, the most severe infantile forms are characterized by an acute brain involvement, usually leading to death in the early years of life. The enzymatic, genetic and molecular bases underlying the metabolic deficiency have been extensively studied and basically elucidated for most of these diseases.

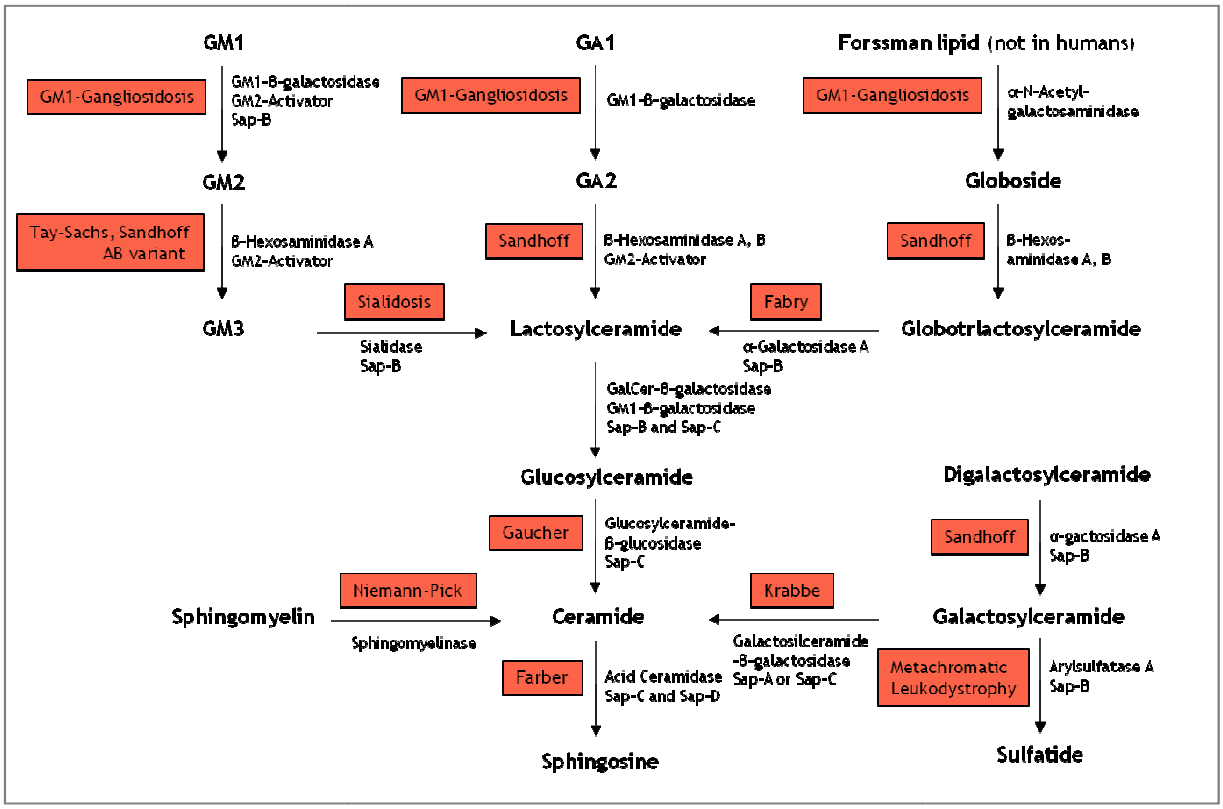


Fig. 1: Sphingolipidosis (Adapted from [50]).

Unexpected alterations of SLs metabolism in Sphingolipidosis

On the other hand, the molecular mechanisms linking the intralysosomal accumulation of unmetabolized substrates to the onset of the pathology are basically unknown. However, it has been suggested that the primary defect might affects multiple secondary biochemical and cellular mechanisms that could be indeed the main cause of tissue damage and death in sphingolipidosis (Figure 2) [48]. Indeed, it has been shown that, in a mouse model of GM1 gangliosidosis, GM1 accumulation in lysosomes resulted in an increase of the ganglioside in the endoplasmic reticulum (ER) and in lipid rafts from mitochondria-associated ER membranes, leading to disruption of Ca^{2+} homeostasis in ER and mitochondria, and to ER stress-induced and mitochondria-mediated apoptosis [51,52]. SLs metabolism and traffic is a complex network of interdependent events, and the recycle of catabolic fragments originated in the lysosomes for biosynthetic purposes is quantitatively relevant. Thus, it can be expected that the blockade of proper SLs catabolism at the lysosomal level would lead to the jamming of the overall flow of metabolites, with consequences on the SLs composition in all cellular regions, including the PM. In addition, it is becoming clear that the mechanisms for the in vivo regulation of SLs levels are multiple, encompassing the de novo synthetic flow, the extent of recycling of catabolic fragments and the enzyme activities of both the biosynthetic and catabolic pathways are likely subjected to a very complex regulation in vivo.

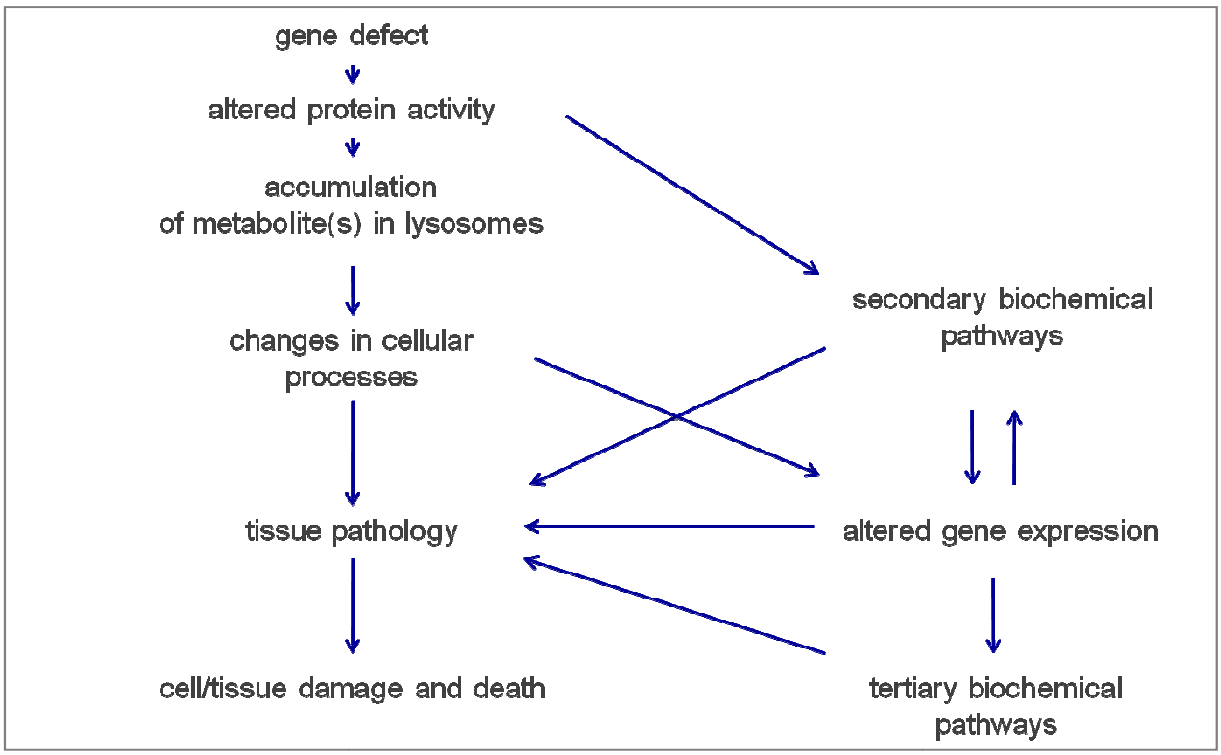


Fig. 2 Secondary biochemical and cellular pathways in sphingolipidosis.

As a consequence, the loss of a certain enzyme activity within SLs metabolism can lead to highly unexpected consequences. A recent example is represented by the Cer synthase 2 knock-out (KO) mice [53,54]. In these mice, in addition to the expected reduction of the synthesis of Cers (and downstream complex SL) containing very long fatty acids (C22-C24), unpredicted changes were observed in the SL composition (including changes in the fatty acyl chain composition of SL that cannot be simply ascribed to the lack of Cer synthase 2 activity, and elevation in sphinganine levels) and in the activity of other enzymes of SL metabolism, including elevated GlcCer synthase and neutral SMase activity (due to the increased expression of neutral SMase II).

Indeed, secondary accumulation of gangliosides was observed in several lysosomal storage diseases, including sphingolipidosis with (GD, galactosialidosis, GM1 gangliosidosis) or without primary defects in ganglioside catabolism (Niemann-Pick disease (NPD) type A and C), as well as lysosomal storage diseases not affecting SL degradation (α-mannosidosis and different types of mucopolysaccharidosis) (reviewed in [55]). Most commonly, accumulated gangliosides were the monosialogangliosides GM3 and GM2, that are relatively minor components in healthy CNS tissues. GM3 and GM2 accumulated in neurons from human patients and animal models [56] of NPD type C (due to a defect in the cholesterol-binding protein NPC1) as well as in the brain of a typical human severe case of NPD type A (caused by defective acid sphingomyelinase (ASM))[57]. It has been suggested that these unexpected changes might be due to a jamming of the intracellular traffic of GSLs, with a consequent lack of feedback regulation of SLs synthesis within the Golgi/trans-Golgi-network [55,58]. However, secondary gangliosides accumulation and, more in general, secondary alterations of other lipid metabolism in sphingolipidosis has never been systematically investigated.

NIEMANN-PICK DISEASE TYPE A

NPD types A and B are caused by mutations in the *SMPD1* gene encoding for ASM [59,60], the lysosomal enzyme required for the hydrolysis of SM into Cer and phosphocholine (Figure 3) [61].

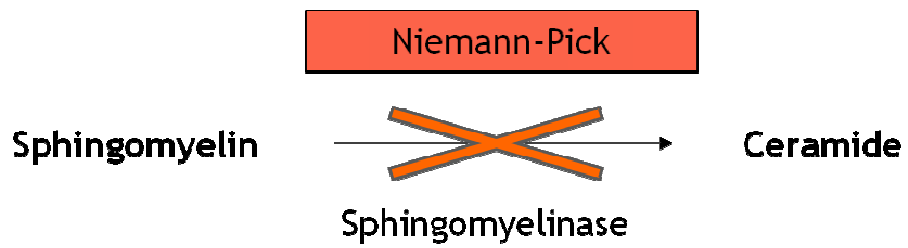


Fig.3 Nieman-Pick disease.

As the consequence of this, SM is accumulated in lysosomes of NPD patient cells, and both NPD type A and B are classified as lysosomal storage diseases [62]. Both forms of the disorder are characterized by progressive visceral organ abnormalities [63]. However, NPD type B, characterized by a higher residual ASM activity, is a late-onset form with no or limited neurological involvement, while NPD type A, with less enzyme residual activity is a severe, neurodegenerative disorder that leads to death by three years of age [64].

To better understand the pathogenesis of this disease and to evaluate the role of ASM, an ASM-deficient mouse line (ASMKO) has been developed by gene targeting using homologous recombination in embryonic stem cells. The wild-type *asm* gene has been destroyed in exon II by the insertion of neomycin (*neo*) resistance gene (Figure 4). The resulting phenotype of these KO mice mimics all the symptoms of the lethal, neurovisceral form of human NPD type A: mice accumulate SM in the reticuloendothelial system of liver, spleen, bone marrow and lung and in the brain. They exhibit hepatosplenomegaly and during the progression of the disease, these mice show a drastic degeneration of Purkinje cell layer in the cerebellum that is associated to the appearance of tremors, ataxia and impairment of motor coordination. The life span of ASMKO is limited to about eight months [65,66].

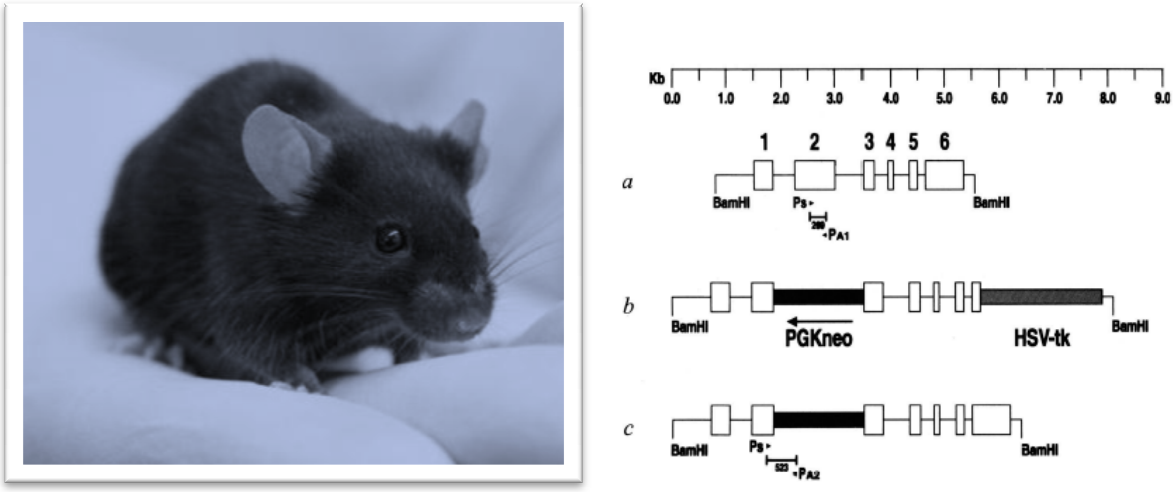


Fig. 4. Schematic representation of the ASM replacement vector. *a*. The mouse ASM gene, consisting in six exons spanning about 4.5kb. The location of the sense and antisense PCR primers (PS and PA1) used for detection of the endogenous mouse gene are shown. *b*. the ASM replacement vector. Note that the PGKneo expression cassette was inserted into exon 2 of the ASM gene in the reverse orientation relative to the ASM coding sequence. An HSV-tk expression cassette was inserted into exon 6, downstream from the TAG termination codon. *c*. The disrupted ASM. The location of the neo-specific antisense PCR primer (PA2) used.

Aim

To better understand the secondary biochemical mechanism underling the pathogenesis and the importance of the secondary alterations of SL metabolism on lysosomal diseases, we decided to analyze the lipid composition of CNS and extraneural tissues from the ASMKO mouse, the animal model of NPD type A.

Matherials and Methods

MATERIALS

Commercial chemicals were of the highest purity available, common solvents were distilled before use and water was doubly distillated in a glass apparatus. Lipids to be used as standard were extracted from rat brain, purified [67], and structurally characterized [68]. High performance thin layer chromatography (HPTLC) plates were from Merck (Darmstadt, Germany). RC DC™ protein assay kit was from Bio-Rad (Hercules,CA,USA). Bovin serum albumin was from Sigma-Aldrich (St. Louis, MO).

METHODS

Animals

The ASMKO mouse model [65] was backcrossed onto the C57BL/6N strain at the Charles River Laboratory, Milan, Italy. Starting from heterozygous mice, homozygous mice colonies were established: wild type (WT) mice (C57BL/6N; ASM+/+) used as control and mutant (ASMKO) mice (C57BL/6N; ASM-/-). Mice were bred according to the NIH Guide for the Care and Use of Laboratory Animals. Genotypes were checked by PCR [65]. Mutants and control mice were killed with CO_2.

Tissue lipid analysis

Tissues from ASMKO and WT mice were weighed and homogenized in iced Millipore water (500 mg of fresh tissue/mL); For lipid analysis we extracted the whole tissue from a single animal. The homogenates were sonicated at ice temperature, snap-frozen and lyophilized; lipids were extracted with chloroform/methanol/water 20 : 10 : 1 (v/v/v) three times. Total lipid extracts were subjected to a two-phase partitioning leading to the separation of an aqueous phase containing gangliosides and an organic phase containing all the other lipids [69]. The ganglioside content of each total tissue was determined in the aqueous phases as lipid-bound sialic acid using the resorcinol method [70] while the phospholipid content was determined as phosphate in the organic phases following perchloric acid digestion using the method of Bartlett [71]. The SM content was determined in the organic phase after alkaline treatment.

Lipids were separated by monodimensional HPTLC carried out using the following solvent systems: chloroform/methanol/0.2% calcium chloride 60:35:8 (v/v/v) for phospholipids and SM, hexane/ethyl-acetate 3:2 (v/v) for cholesterol, and chloroform/methanol/0.2% calcium chloride 50:42:11 (v/v/v) for gangliosides.

Separated lipids were identified on the basis of co-migration with lipid standards [72].

Phospholipids were detected by spraying the HPTLC with a molybdate reagent [73]. Cholesterol was visualized by spraying the HPTLC with anisaldehyde and quantified by densitometry and comparison with 0.1-0.2 µg of a standard compound [72]. SM was recognized using 15% concentrated sulfuric acid in 1-butanol [72]. Gangliosides were visualized after separation on HPTLC by specific detection with the p-dimethylaminobenzaldehyde reagent [74].

The relative amounts of lipids associated with each band after HPTLC separation were determined by densitometry using the Molecular Analyst program (Bio-Rad Laboratories, Hercules, CA, USA). The number of sialyl residues was taken into account for analysis of ganglioside content. The mass content of each phospholipid, or ganglioside, was calculated on the basis of the percentage distribution of total phospholipid or ganglioside content, determined as described above [75,72]. The protein content was determined in all samples using the RC DC™ protein assay and BSA as the reference standard.

Statistical analysis

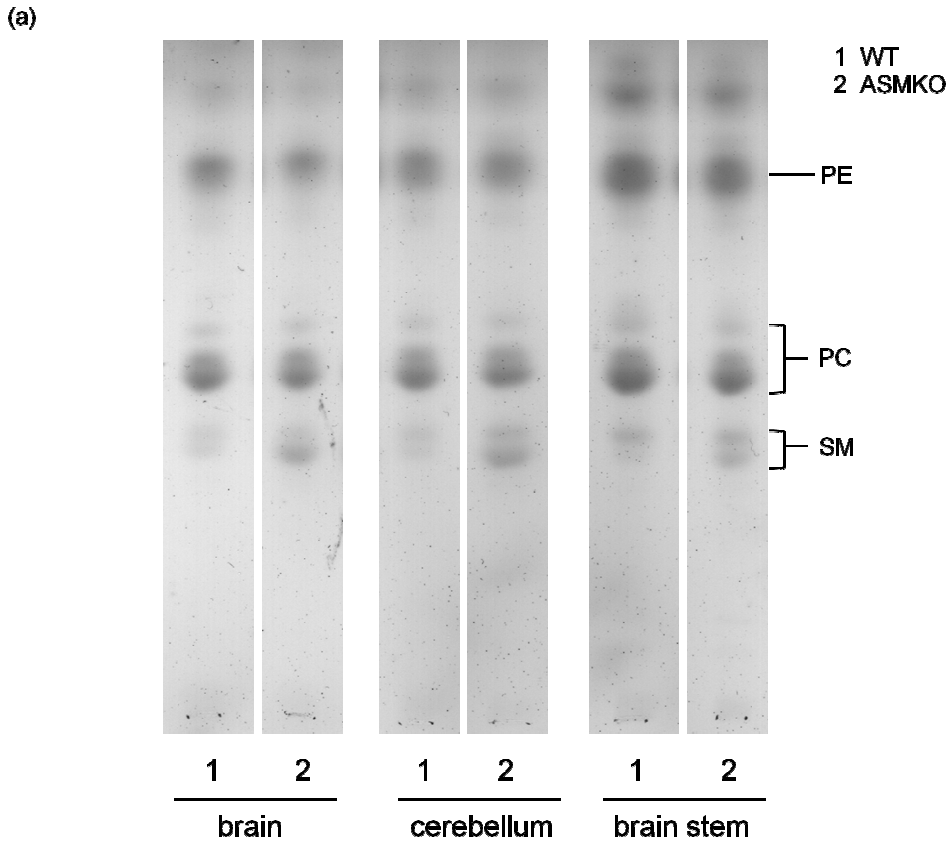
Experiments were performed on three different tissue samples from different animals for each genotype and age group. The results are expressed as mean value ± S.D. Statistical analysis of the data was performed by one-way ANOVA followed by the Student-Newman-Keuls' test. $p<0.05$ was considered significant (compared with WT) and p values are indicated in the legend of each figure and/or table.

Results

We analyzed the lipid composition of CNS and extraneural tissues from the ASMKO mouse, the animal model for NPD type A [90]. Lipids were extracted from nervous (brain, cerebellum, brain stem) and extranervous (spleen, kidney, pancreas, liver) tissues with chloroform/methanol/water, and total lipid extracts were submitted to a two-phase partitioning. The resulting organic phases were analyzed for total phospholipid and cholesterol content and phospholipid pattern, aqueous phases were analyzed for total ganglioside content and ganglioside patterns.

i. Total phospholipid content

Total and individual phospholipid contents are reported in Tables 1, 2, and 3. Figure 5 shows the HPTLC analysis. SM accumulation was evident in 5-month-old ASMKO respect to wild-type (WT) mice in all nervous (Table I) and extranervous (Table II) tissues analyzed, and it was much higher in extranervous (from 30- to 40-fold higher in ASMKO than in WT) than in nervous tissues (4- to 8-fold higher). The total phospholipid phosphorous content in ASMKO vs. WT mice was unchanged in nervous system tissues (Tables I and III), while it was significantly increased in spleen, kidney, liver and, at a lesser extent, pancreas (Table II). The distribution among different glycerophospholipid species, evaluated by HPTLC, was substantially unchanged in nervous and extranervous tissues from both ASMKO and WT mice (Tables I and II).



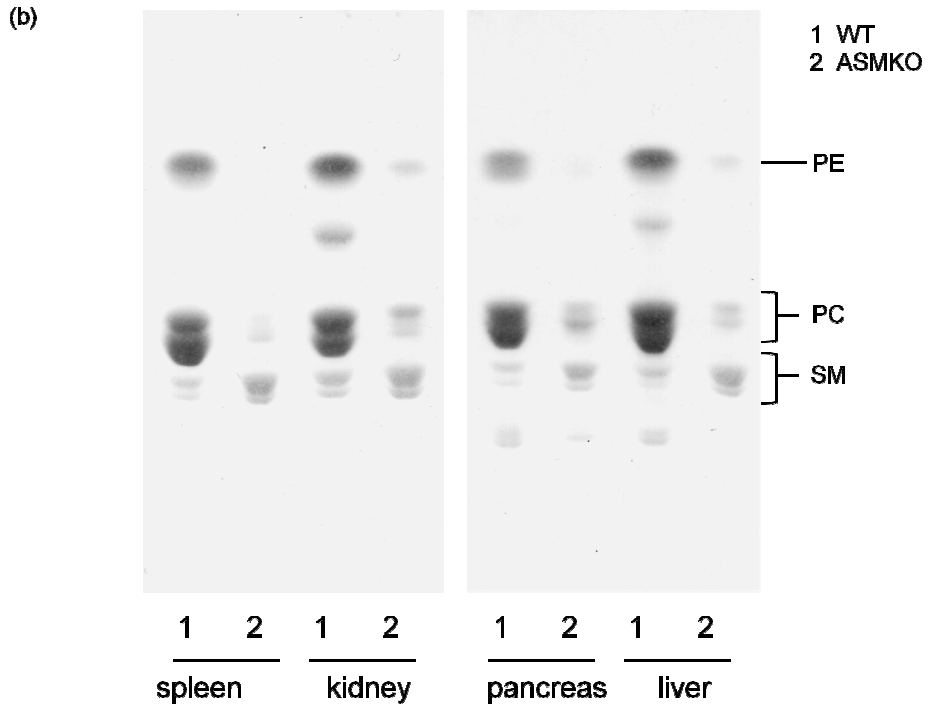


Fig. 5 HPTLC phospholipid patterns from 5 month old WT and ASMKO mouse tissues. Lipids were extracted with chloroform/methanol/water 20:10:1 (v/v/v). Total lipid extracts were subjected to a two phase partitioning [76]. (a) The endogenous phospholipids from WT and ASMKO mouse brain, cerebellum and brain stem were separated from the organic phase, corresponding to 100 µg of tissue proteins, and (b) endogenous phospholipids from WT and ASMKO mouse spleen, kidney, pancreas and liver were separated from the organic phase, corresponding to 300 µg or 30 µg of tissue proteins for WT and ASMKO tissue, respectively, by mono-dimensional HPTLC. The solvent system was chloroform/methanol/0.2% calcium chloride 60:35:8 (v/v/v) and visualization was carried out with a molibdate reagent [73]. Patterns are representative of those obtained in three different experiments. (PE, phosphatidylethanolamine; PC, phosphatidylcholine).

Table I Phospholipid content in 5 month old WT and ASMKO mouse CNS tissue.

	Phospholipids		PC		PE		PI+PS		SM		Others Phospholipids	
	WT	ASMKO	WT	ASMKO	WT	ASMKO	WT	ASMKO	WT	ASMKO	WT	ASMKO
CNS Tissues												
BRAIN	**57.41 ± 2.84**	**54.62 ± 2.41**	22.39 ± 1.11	19.51 ± 1.07	21.17 ± 0.99	18.34 ± 0.83	11.81 ± 0.57	9.94 ± 0.53	**1.33 ± 0.07**	**6.42 ± 0.32****	0.71 ± 0.04	0.41 ± 0.01
CEREBELLUM	**51.96 ± 2.54**	**58.03 ± 2.12**	21.84 ± 0.98	19.76 ± 0.92	21.17 ± 1.06	21.24 ± 1.01	7.98 ± 0.32	9.05 ± 0.39	**0.92 ± 0.04**	**6.81 ± 0.34****	0.05 ± 0.01	1.17 ± 0.06
BRAIN STEM	**69.75 ± 3.42**	**71.85 ± 2.96**	19.99 ± 0.89	21.02 ± 1.02	37.07 ± 1.85	35.04 ± 1.44	11.34 ± 0.05	10.51 ± 0.51	**1.35 ± 0.06**	**5.23 ± 0.21****	n.d.	0.05 ± 0.01

*Data are expressed as nmoles/mg of tissue wet weight (**, p<0.001 versus WT). The results are expressed as means values ± S.D. for three different experiments. WT, wild-type; ASMKO, acid sphingomyelinase knockout; PE, phosphatidyethanolamine; PC, phosphatidylcholine; PI, phosphatidylinosiltol; PS, phosphatidylserine; SM, sphingomyeline.*

Table II Phospholipid content in 5 month old WT and ASMKO mouse extra-neural tissue.

	Phospholipids		PC		PE		PI+PS		SM		Others Phospholipids	
	WT	ASMKO	WT	ASMKO	WT	ASMKO	WT	ASMKO	WT	ASMKO	WT	ASMKO
Extra-neural Tissues												
SPLEEN	**18.76 ± 0.75**	**81.55 ± 1.16****	14.44 ± 0.66	22.49 ± 0.85**	2.62 ± 0.12	5.85 ± 0.25	0.44 ± 0.02	n.d.	1.19 ± 0.06	53.16 ± 2.51**	0.07 ± 0.02	0.05 ± 0.01
KIDNEY	**36.83 ± 1.06**	**69.57 ± 1.41****	18.07 ± 0.79	26.17 ± 1.36*	15.23 ± 0.64	14.60 ± 0.71	0.36 ± 0.01	0.77 ±0.031	0.20 ± 0.09	25.14 ± 1.09**	2.97 ± 0.17	2.89 ± 0.15
PANCREAS	**20.41 ± 1.62**	**29.27 ± 1.33***	15.46 ± 0.67	13.59 ± 0.03	1.42 ± 0.07	0.99 ± 0.04	3.17 ± 0.15	3.96 ± 0.16	0.20 ± 0.03	7.78 ± 0.74*	0.16 ± 0.01	2.95 ± 0.13
LIVER	**30.05 ± 1.37**	**86.81±2.79****	18.77 ± 0.91	34.53 ± 1.57**	6.62 ± 0.27	14.15 ± 0.66*	3.31 ± 0.16	5.11 ± 0.26	1.10 ± 0.05	32.97 ± 0.32**	0.25 ± 0.02	0.05 ± 0.01

Data are expressed as nmoles/mg of tissue wet weight .(, p<0.005 versus WT; **, p<0.001 versus WT). The results are expressed as means values ± S.D. for three different experiments. WT, wild-type; ASMKO, acid sphingomyelinase knockout; PE, phosphatidyethanolamine; PC, phosphatidylcholine; PI, phosphatidylinosiltol; PS, phosphatidylserine; SM, sphingomyeline.*

Table III Sphingomyelin content in WT and ASMKO mouse tissue

	Phospholipids		Total SM		Lower Band (C16+C18)		Upper Band (C24)	
	WT	ASMKO	WT	ASMKO	WT	ASMKO	WT	ASMKO
CNS Tissues								
BRAIN								
0 day	25.82 ± 2,84	30.09 ± 1,78	0.30 ± 0.02	0.38 ± 0.02$^{\delta}$	0.23 ± 0.01	0.29 ± 0.01$^{\delta}$	0.07 ± 0.01	0.09 ± 0.01
15 days	42.57 ± 2,56	42.65 ± 3,81	0.81 ± 0.03	1.10 ± 0.05*	0.65 ± 0.02	0.89 ± 0.04*	0.16 ± 0.01	0.21 ± 0.01*
1 month	46.81 ± 2,04	39.47 ± 3,86	1.30 ± 0.06	2.49 ± 0.12**	1.11 ± 0.05	2.24 ± 0.11**	0.19 ± 0.01	0.25 ± 0.01*
2.5 months	51.67 ± 1,71	49.23 ± 1,67	1.34 ± 0.05	7.29 ± 0.31**	1.11 ± 0.04	6.69 ± 0.29**	0.23 ± 0.01	0.60 ± 0.02**
5 months	57.41 ± 2.84	54.62 ± 2.41	1.33 ± 0.07	6.42 ± 0.32**	1.16 ± 0.06	6.05 ± 0.30**	0.17 ± 0.01	0.37 ± 0.02**
Extra-neural Tissues (5 months)								
SPLEEN	18.76 ± 0.75	81.55 ± 1.16**	1.19 ± 0.06	53.16 ± 2.51*	0.98 ± 0.05	43.68 ± 2.06**	0.21 ± 0.01	9.48 ± 0.45**
KIDNEY	36.83 ± 1.06	69.57 ± 1.41**	0.20 ± 0.09	25.14 ± 1.09**	0.15 ± 0.07	19.22 ± 0.83**	0.05 ± 0.02	5.92 ± 0.26**
PANCREAS	20.41 ± 1.62	29.27 ± 1.33*	0.20 ± 0.03	7.78 ± 0.74*	0.16 ± 0.02	6.32 ± 0.59*	0.04 ± 0.01	1.46 ± 0.15*
LIVER	30.05 ± 1.37	86.81 ± 2.79**	1.10 ± 0.05	32.97 ± 0.32**	0.96 ± 0.04	28.84 ± 0.28**	0.14 ± 0.01	4.13 ± 0.04**

*Data are expressed as nmoles/mg of tissue wet weight ($^{\delta}$, p<0.01 versus WT; *, p<0.005 versus WT; **, p<0.001 versus WT). The results are expressed as means values ± S.D. for three different experiments. WT, wild-type; ASMKO, acid sphingomyelinase knockout; SM, sphingomyeline.*

Table IV Ganglioside content in WT and ASMKO mouse tissue

	Gangliosides		GM3		GM2		GM1	
	WT	ASMKO	WT	ASMKO	WT	ASMKO	WT	ASMKO
CNS Tissues								
BRAIN								
1 month	0.653 ± 0.053	0.725 ± 0.036	0.010 ± 0.002	0.018 ± 0.007	0.007 ± 0.003	0.045 ± 0.026	0.133 ± 0.019	0.136 ± 0.011
2.5 months	0.682 ± 0.032	0.770 ± 0.027†	0.013 ± 0.001	0.037 ± 0.007†	0.013 ± 0.002	0.053 ± 0.007†	0.132 ± 0.012	0.140 ± 0.015
5 months	0.741 ± 0.022	0.887 ± 0.037†	0.017 ± 0.005	0.064 ± 0.011*	0.007 ± 0.001	0.097 ± 0.012†	0.169 ± 0.021	0.162 ± 0.017
CEREBELLUM								
1 month	0.591 ± 0.043	0.586 ± 0.073	0.012 ± 0.002	0.018 ± 0.008	0.007 ± 0.001	0.031 ± 0.018	0.035 ± 0.003	0.028 ± 0.007
2.5 months	0.588 ± 0.031	0.678 ± 0.024†	0.022 ± 0.001	0.027 ± 0.002†	0.011 ± 0.001	0.065 ± 0.014†	0.045 ± 0.012	0.062 ± 0.015
5 months	0.631 ± 0.051	0.831 ± 0.092†	0.018 ± 0.001	0.039 ± 0.007†	0.010 ± 0.002	0.089 ± 0.015†	0.052 ± 0.005	0.066 ± 0.017
BRAIN STEM								
1 month	0.591 ± 0.065	0.635 ± 0.073	0.008 ± 0.002	0.011 ± 0.005	0.007 ± 0.001	0.017 ± 0.003†	0.049 ± 0.007	0.047 ± 0.09
2.5 months	0.507 ± 0.036	0.631 ± 0.035†	0.007 ± 0.001	0.014 ± 0.002†	0.007 ± 0.003	0.019 ± 0.005†	0.070 ± 0.021	0.059 ± 0.014
5 months	0.511 ± 0.047	0.650 ± 0.045†	0.005 ± 0.001	0.029 ± 0.005†	0.006 ± 0.002	0.037 ± 0.009†	0.099 ± 0.006	0.097 ± 0.009
Extra-neural Tissues								
LIVER	0.062 ± 0.052	0.346 ± 0.046*	0.009 ± 0.001	0.124 ± 0.013*	0.053 ± 0.007	0.222 ± 0.024*	n.d.	n.d

	GD3		GD1a		GD1b		GT1b		Others ganglioside	
	WT	ASMKO	WT	ASMKO	WT	ASMKO	WT	KO	WT	ASMKO
CNS Tissues										
BRAIN										
1 month	0.034 ± 0.004	0.043 ± 0.005	0.300 ± 0.057	0.278 ± 0.029	0.062 ± 0.012	0.071 ± 0.014	0.086 ± 0.007	0.100 ± 0.013	0.021 ± 0.009	0.034 ± 0.012
2.5 months	0.042 ± 0.003	0.065 ± 0.007	0.295 ± 0.035	0.270 ± 0.061	0.068 ± 0.013	0.077 ± 0.007	0.097 ± 0.009	0.101 ± 0.007	0.022 ± 0.011	0.027 ± 0.006
5 months	0.042 ± 0.012	0.067 ± 0.014	0.306 ± 0.027	0.279 ± 0.028	0.066 ± 0.007	0.071 ± 0.003	0.101 ± 0.017	0.104 ± 0.010	0.033 ± 0.007	0.043 ± 0.005
CEREBELLUM										
1 month	0.069 ± 0.007	0.073 ± 0.017	0.101 ± 0.015	0.118 ± 0.019	0.040 ± 0.005	0.060 ± 0.012	0.239 ± 0.031	0.205 ± 0.023	0.088 ± 0.007	0.053 ± 0.017
2.5 months	0.070 ± 0.016	0.074 ± 0.009	0.094 ± 0.021	0.096 ± 0.012	0.053 ± 0.009	0.062 ± 0.010	0.241 ± 0.052	0.235 ± 0.013	0.052 ± 0.009	0.057 ± 0.003
5 months	0.063 ± 0.019	0.065 ± 0.015	0.106 ± 0.017	0.146 ± 0.020	0.077 ± 0.012	0.061 ± 0.005	0.244 ± 0.047	0.256 ± 0.023	0.061 ± 0.016	0.109 ± 0.027
BRAIN STEM										
1 month	0.027 ± 0.003	0.030 ± 0.006	0.108 ± 0.027	0.119 ± 0.027	0.088 ± 0.013	0.097 ± 0.023	0.185 ± 0.032	0.181 ± 0.016	0.119 ± 0.024	0.133 ± 0.021
2.5 months	0.023 ± 0.012	0.050 ± 0.016	0.075 ± 0.007	0.094 ± 0.011	0.072 ± 0.003	0.104 ± 0.034	0.133 ± 0.009	0.153 ± 0.013	0.120 ± 0.018	0.138 ± 0.007
5 months	0.029 ± 0.007	0.034 ± 0.007	0.058 ± 0.009	0.065 ± 0.012	0.077 ± 0.015	0.097 ± 0.012	0.127 ± 0.043	0.145 ± 0.024	0.110 ± 0.021	0.146 ± 0.019
Extra-neural Tissues										
LIVER	n.d	n.d	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

ii. Cholesterol content

Cholesterol content was not significantly different in nervous tissues from 5-month-old WT and ASMKO mice (Figure 6a), while a significant increase in cholesterol content was detected in spleen, kidney and liver from ASMKO mice (Figure 6b).

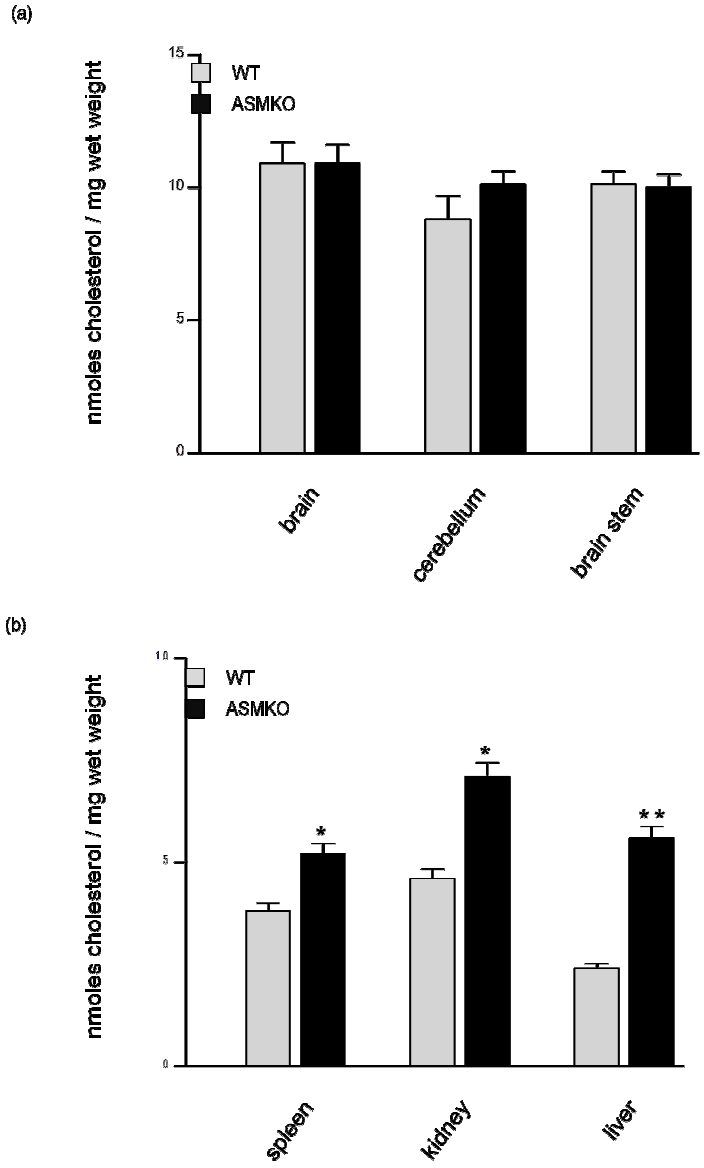


Fig. 6 Cholesterol content in 5 month old WT and ASMKO mouse (a) CNS and (b) extraneural tissue. Lipids were extracted and partitioned as described in the legend of Fig. 1. Cholesterol in the organic phases was quantitatively analyzed as described previously [72]. Data are expressed as nmoles cholesterol per mg of tissue wet weight (*, $p < 0.005$ versus WT; **, $p < 0.001$ versus WT). The results are expressed as means ± S.D. values for three different experiments.

iii. SM pattern

SM accumulation was followed in brains from ASMKO and WT mice along age from birth until the age of 5 months (Table III). SM accumulation occurred very soon in the postnatal life and was progressive along time; it was already significant at 0 days and it reached a plateau around 2.5 months of age, when SM level in ASMKO brain was about 6-fold higher than in WT brain, remaining basically unchanged up to the age of 5 months, in agreement with previously published results [76,77]. Total phospholipid content in brain was similar in ASMKO and WT mice at all ages (Table III), indicating a significant reduction of glycerophospholipids in ASMKO brain along time. As other SL, SM is present in all tissues as a mixture of molecular species differing for their fatty acid composition [78]. HPTLC analysis of alkaline-treated organic phase lipids under the experimental conditions used allowed to separate 2 bands for SM: the lower migrating band corresponds to SM molecular species containing C18 and C16 fatty acids (mainly stearic acid), while the upper migrating band corresponds to SM molecular species with longer chain fatty acids, (mainly C24) [79]. In extranervous tissues, both SM bands accumulated in ASMKO mice at a similar extent (Table III ad Figure 7b). As example, in liver the ratio between lower and upper migrating bands was 6.9 in both ASMKO and WT mice (Figure 7b). On the other hand, in ASMKO brain the lower SM band was preferentially accumulated (Table 3). At 15 days of post-natal age, when SM accumulation was already evident, the ratio between lower and upper SM band was identical in WT and ASMKO brain, while at 1 month of age this ratio was already 1.4 fold higher in ASMKO than in WT and at 5 months it reached 2.5-fold in ASMKO vs WT (Figure 7a), clearly indicating that, in ASMKO brain, SM molecular species containing shorter fatty acyl chains (C18 and C16) accumulate at a higher level that SM species with longer fatty acids (C24), in agreement with preliminary observations in purified myelin from ASMKO mice [79]

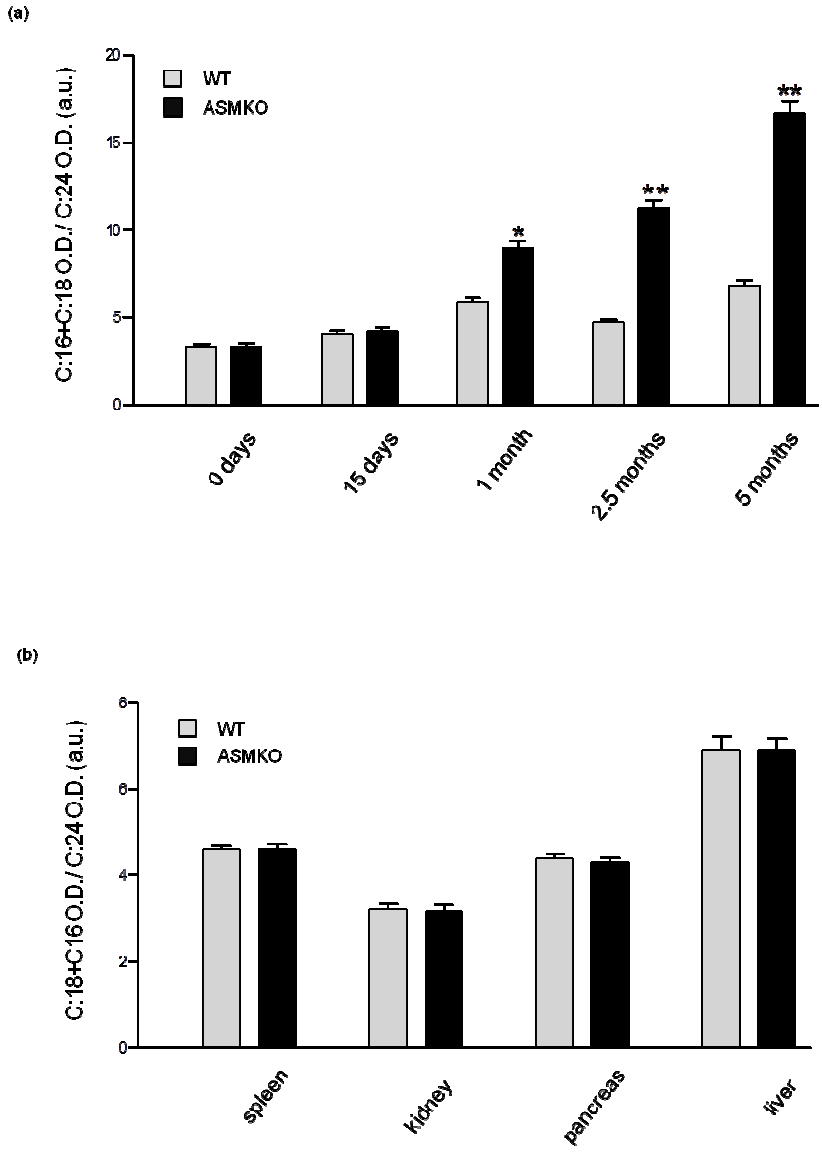
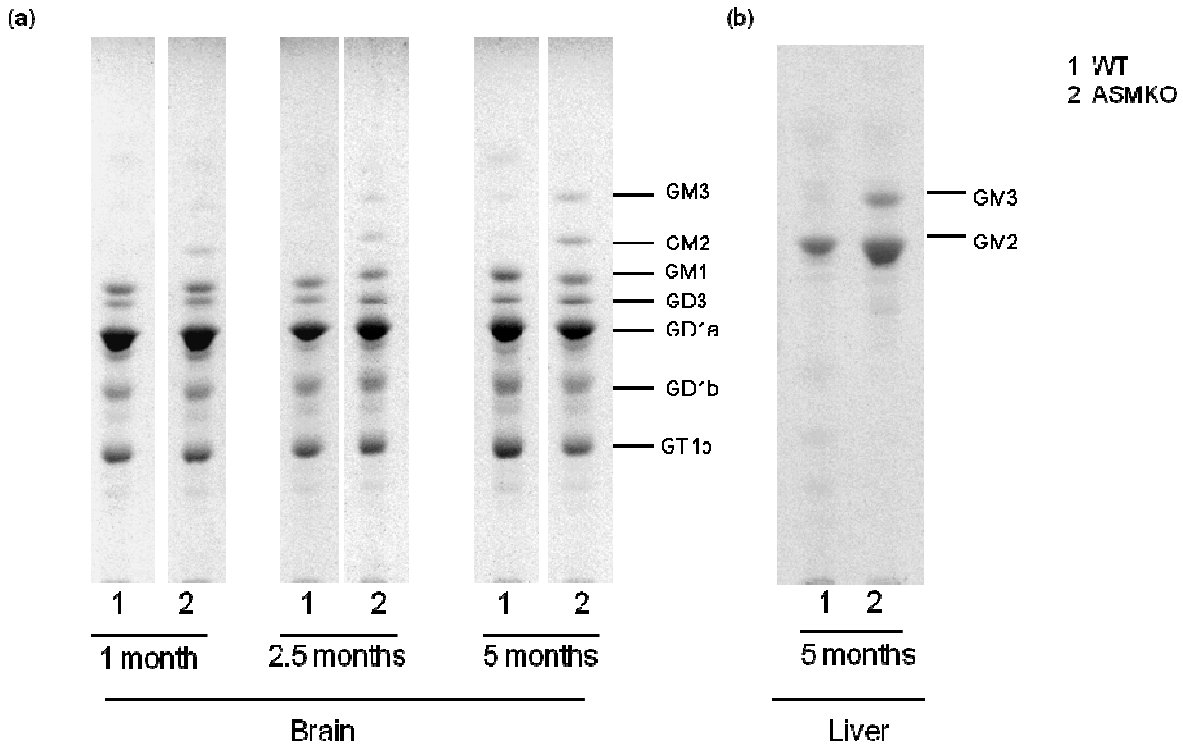


Fig. 7 Ratios between SM in the lower and upper migrating bands in WT (light grey) and ASMKO (black) mouse tissues. Lipids were extracted as described in the legend of Fig. 1. The organic phase obtained was submitted to alkaline treatment followed by partitioning to remove glycerophospholipids. The alkali-treated organic phase was analyzed by mono-dimensional HPTLC, allowing to resolve SM in two separate bands. The relative amounts of SM associated with the upper and lower migrating bands were determined by densitometry. (a) CNS tissues (0 days, 15 days, 1 month, 2.5 months and 5 months) (b) Extra-neural tissues (5 months). Data are expressed as the ratio of arbitrary optical density units between C18/C16 SM and C24 SM (*, $p < 0.005$ versus WT; **, $p < 0.001$ versus WT). The results are expressed as means ± S.D. values for three different experiments.

iv. Ganglioside pattern

In addition to the expected SM accumulation, we found an increase in the gangliosides content in all ASMKO mouse tissues investigated, with the exception of spleen (Table IV). The total ganglioside content was moderately but significantly higher in nervous tissues from ASMKO respect to WT mice already at 2.5 month of age (Table IV). The maximal accumulation was observed in the cerebellum at 5 months (+43% respect to WT). Ganglioside accumulation was much more pronounced in liver (Table IV): at 5 months of age, ganglioside content in liver was 6-fold higher in ASMKO mice respect to controls. The chromatographic analysis of ganglioside patterns revealed that only the monosialoganglioside GM3 and GM2 were accumulated in ASMKO tissues (Figure 8 and Table IV). Since GM3 and GM2 are normally minor components of the ganglioside mixture from nervous tissues, while they are among major gangliosides from extraneural tissues, their preferential accumulation clearly explains the quantitative differences observed between neural and extraneural tissues. In ASMKO brain, GM3 and GM2 accumulation was already significant at the age of 1 month. Both gangliosides accumulated with time, but with a different trend, and at 5 months of age GM2 levels were about 10-fold higher in ASMKO brain respect to WT, while GM3 levels were about 3-fold higher (Figure 8).



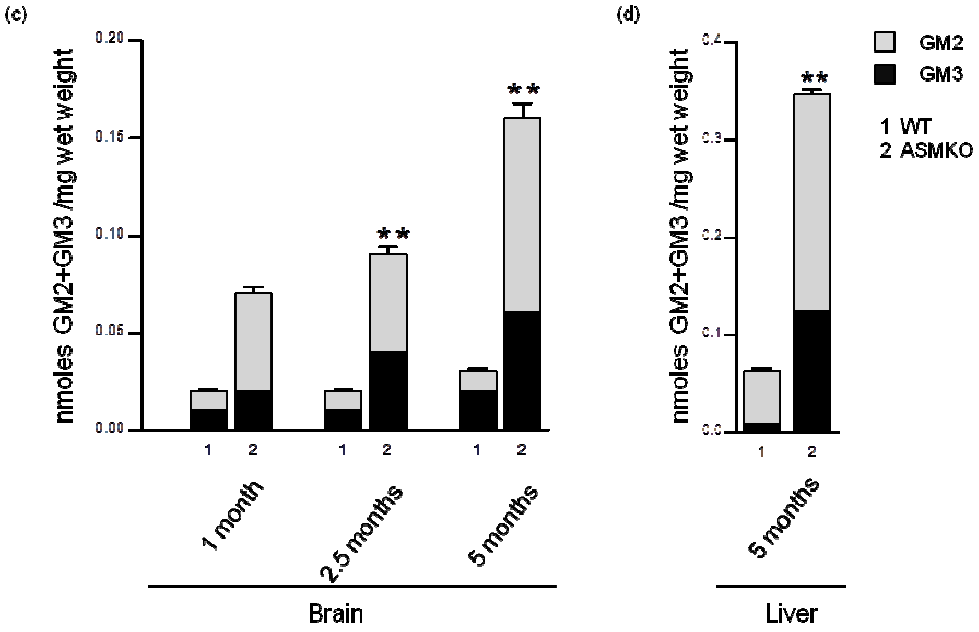


Fig. 8 Ganglioside patterns in WT and ASMKO mouse brain (panel a, 1, 2.5 and 5 months) and liver (panel b, 5 months). Lipids were extracted and partitioned as described in the legend of Fig. 1. The endogenous gangliosides from WT and ASMKO tissues were separated from dialyzed aqueous phases (corresponding to 300 µg of tissue proteins for brain and to 2.5 mg and 1 mg of tissue proteins for WT and ASMKO liver, resepctively) by mono-dimensional HPTLC, using the solvent system chloroform/methanol/0.2% calcium chloride 50:42:11 (v/v/v) and were recognized by specific detection with the p-dimethylaminobenzaldehyde [74]. Patterns are representative of those obtained in three different experiments. The relative amounts of each ganglioside were determined by densitometry. The ganglioside content in each sample was determined as lipid-bound sialic acid using the resorcinol method [70] and the number of sialic residues was taken into account for analysis of gangliosides content. The mass contents of GM3 and GM2 (panels c and d) were calculated on the basis of the ganglioside percentage distribution. Data in panels c and d are expressed as nmoles gangliosides/ mg of tissue wet weight. The results are expressed as means ± S.D. values for three different experiments (**, $p<0.001$ versus WT).

Discussion

Lysosomal storage disorders are caused by the defective activity of lysosomal proteins, which results in the intra-lysosomal accumulation of non-degraded metabolites. For a wide group of disorders, the defective enzyme is a hydrolase involved in SL catabolism. Most sphingolipidoses are characterized by prominent neurological involvement. In particular, the most severe, infantile forms presents with acute brain involvement leading to death within the first year of life. Juvenile and adult forms present both neurological and peripheral symptoms.

The enzymatic, genetic and molecular bases underlying the metabolic deficiency have been extensively studied and basically determined for most of these diseases. However, little is known about the secondary biochemical and cellular pathways that are affected in these diseases, and that eventually result in cell and tissue dysfunction. In other word, we don't know why does the lysosomal accumulation of a particular un-metabolized substrate causes the pathology: it is clear that the intralysosomal accumulation of non-metabolized substrates is the primary cause of the disease, but the wide range of disease symptoms indicates that many secondary biochemical and cellular pathways are affected. Any of these events could be indeed the main cause of tissue damage and death in sphingolipidosis.

It is clearly emerging that, in several sphingolipidosis, complex changes in SL metabolism are observed, that cannot be explained solely on the basis of the genetic and/or enzymatic defect that represents the primary cause of the diseases, or of an impairment of the function of lysosomes, the primary cellular target in these pathologies. Secondary accumulation of gangliosides has been reported in several sphingolipidosis, even if this aspect has never been investigated.

From this point of view, our findings in the ASMKO mice, the animal model of NPD type A, are particularly interesting. In ASMKO mice, we observed the preferential accumulation of SM molecular species with shorter acyl chains in the nervous system, but not in extranervous tissues, and the accumulation of GM3 and GM2 gangliosides in both neural and extraneural tissues. The unbalance toward C18/C16-containing SM species was detectable as early as SM accumulation started, and monosialoganglioside accumulation followed immediately afterwards. At least one report indicates that monosialoganglioside accumulation also occurs in the human pathology [57], while no information is available on the molecular composition of the accumulated SM. Fatty acid composition of SM from human cerebral white matter was reported to be abnormal in patients with juvenile and adult Huntington's disease, with a shift toward shorter chain fatty acid [80].

Clearly, the lack of ASM by itself is not sufficient to explain these unexpected changes in lipid composition. Remarkably, in many neurological disorders, including Alzheimer's, Parkinson's and Creutzfeldt-Jakob diseases, apparently not related to alterations of SL metabolism or of

lysosomal function, a general decrease of ganglioside levels is accompanied by a marked increase in the levels of simpler gangliosides [47]. These changes in SL patterns should thus represent the effect of secondary biochemical pathways altered as a consequence of a non-related primary cause. The mechanisms underlying these changes still remain to be elucidated and are probably the result of changes in the expression and/or activity of more than one single enzyme, of anomalies in the traffic of the substrates and of complex changes in the substrate/product concentrations in multiple cellular compartments. Thus, therapeutic strategies focused on a single enzyme activity might not necessarily represent a choice that can correct all of the metabolic abnormalities. Several pieces of evidence also suggest that altered SL metabolism might result in a non-physiological PM composition and organization, leading to altered plasma membrane-originated signaling pathways that could be relevant to the onset of the cellular damage and of tissue pathology [62]. This hypothesis has been recently confirmed by several observations. In a cell model of Gaucher disease, impaired lysosomal catabolism of GlcCer led to the accumulation of GlcCer at the PM level in lipid rafts, possibly explaining the altered lipid and protein sorting observed in this pathological condition [81]. Psychosine (galactosylsphingosine), one of the galactoslylsphingolipids that accumulates in the brain of Krabbe disease (human globoid cell leukodystrophy) patients due to the deficient activity of □-galactosylceramidase, accumulates in lipid rafts from brain and sciatic nerve from twitcher mice (the animal model for the infantile variant of the disease) and from human Krabbe patients, leading to an altered distribution of lipid raft proteins and to inhibition of protein kinase C [82]. Brain pathology in ASMKO mice is probably at least in part due to a PM involvement [83], possibly associated with a non-conventional lipid raft organization [77,78].

For SM, the shift toward molecular species with shorter fatty acids has been associated in some cases with myelination defects [21,22], and might be a consequence of the myelin deficiency observed in ASMKO mice [77]. On the other hand, it is clear that the composition of the hydrophobic moiety of a certain SL represents one of the factors that most deeply influence its ability to undergo lateral separation within a cellular membrane [84], thus affecting membrane lateral organization. Remarkably, this aspect of SL-mediated biology has been quite neglected in the past, and only recently the metabolic and cellular mechanisms responsible for the creation of the structural heterogeneity of the Cer moiety of cellular SL has been partially elucidated [85,86]. On one side, it resides in the specificity of Cer synthesis with regard of the fatty acid composition: at least six different Cer synthases contribute to the fatty acid heterogeneity of Cer in SL [85]. On the other hand, different transport mechanisms are responsible for the structure-selective delivery of Cer from the sites of synthesis to the sites of its metabolic utilization [86]. Thus, it can be expected that the influence of the structure of the hydrophobic moiety might be for a SL as

important as that of the hydrophilic headgroup. Indeed, it has been recently suggested that clustering of SL with C24 fatty acids might lead to the creation of quasi-crystalline structures in a liquid-ordered membrane matrix, thus providing an additional level of order within biological membranes [87]. On the other hand, it has been suggested that interdigitation of long fatty acid chains in SL might provide a physical and functional link between the inner and outer leaflet of the PM [88,89]. Thus, the significance of the alterations in SM composition hereby reported in ASMKO mice deserves further clarification

References

1. Paratcha G, Ibanez CF (2002) Lipid rafts and the control of neurotrophic factor signaling in the nervous system: variations on a theme. *Curr Opin Neurobiol* 12: 542-549.
2. Tsui-Pierchala BA, Encinas M, Milbrandt J et al (2002) Lipid rafts in neuronal signaling and function. *Trends Neurosci* 25: 412-417.
3. Nagappan G, Lu B (2005) Activity-dependent modulation of the BDNF receptor TrkB: mechanisms and implications. *Trends Neurosci* 28: 464-471.
4. Saarma M (2001) GDNF recruits the signaling crew into lipid rafts. *Trends Neurosci* 24: 427-429.
5. Decker L, Baron W, Ffrench-Constant C (2004) Lipid rafts: microenvironments for integrin-growth factor interactions in neural development. *Biochem Soc Trans* 32: 426-430.
6. Santuccione A, Sytnyk V, Leshchyns'ka I et al (2005) Prion protein recruits its neuronal receptor NCAM to lipid rafts to activate p59fyn and to enhance neurite outgrowth. *J Cell Biol* 169: 341-354.
7. Tooze SA, Martens GJ, Huttner WB (2001) Secretory granule biogenesis: rafting to the SNARE. *Trends Cell Biol* 11: 116-122.
8. McKerracher L (2002) Ganglioside rafts as MAG receptors that mediate blockade of axon growth. *Proc Natl Acad Sci U S A* 99: 7811-7813.
9. Vyas AA, Patel HV, Fromholt SE et al (2002) Gangliosides are functional nerve cell ligands for myelin-associated glycoprotein (MAG), an inhibitor of nerve regeneration. *Proc Natl Acad Sci U S A* 99: 8412-8417.
10. Boggs JM, Wang H, Gao W et al (2004) A glycosynapse in myelin? *Glycoconj J* 21: 97-110.
11. Valsecchi M, Chigorno V, Nicolini M et al (1996) Changes of free long-chain bases in neuronal cells during differentiation and aging in culture. *J Neurochem* 67: 1866-1871.
12. Svennerholm L, Bostrom K, Jungbjer B et al (1994) Membrane lipids of adult human brain: lipid composition of frontal and temporal lobe in subjects of age 20 to 100 years. *J Neurochem* 63: 1802-1811.
13. Barrier L, Ingrand S, Damjanac M et al (2007) Genotype-related changes of ganglioside composition in brain regions of transgenic mouse models of Alzheimer's disease. *Neurobiol Aging* 28: 1863-1872.
14. Mansson JE, Vanier MT, Svennerholm L (1978) Changes in the fatty acid and sphingosine composition of the major gangliosides of human brain with age. *J Neurochem* 30: 273-275.
15. Ando S, Yu RK (1984) Fatty acid and long-chain base composition of gangliosides isolated from adult human brain. *J Neurosci Res* 12: 205-211.
16. Palestini P, Masserini M, Sonnino S et al (1990) Changes in the ceramide composition of rat forebrain gangliosides with age. *J Neurochem* 54: 230-235.
17. Palestini P, Sonnino S, Tettamanti G (1991) Lack of the ganglioside molecular species containing the C20-long-chain bases in human, rat, mouse, rabbit, cat, dog, and chicken brains during prenatal life. *J Neurochem* 56: 2048-2050.
18. Suzuki K (1965) The pattern of mammalian brain gangliosides. II. Evaluation of the extraction procedures, postmortem changes and the effect of formalin preservation. *J Neurochem* 12: 629-638.

19. Valsecchi M, Palestini P, Chigorno V et al (1996) Age-related changes of the ganglioside long-chain base composition in rat cerebellum. *Neurochem Int* 28: 183-187.
20. Valsecchi M, Palestini P, Chigorno V et al (1993) Changes in the ganglioside long-chain base composition of rat cerebellar granule cells during differentiation and aging in culture. *J Neurochem* 60(1): 193-196.
21. Heipertz R, Pilz H, Scholz W (1977) The fatty acid composition of sphingomyelin from adult human cerebral white matter and changes in childhood, senium and unspecific brain damage. *J Neurol* 216: 57-65.
22. Goebel HH, Heipertz R, Scholz W et al (1978) Juvenile Huntington chorea: clinical, ultrastructural, and biochemical studies. *Neurology* 28: 23-31.
23. Facci, L., Leon, A., Toffano, G., Sonnino, S., Ghidoni, R., and Tettamanti, G. (1984) Promotion of neuritogenesis in mouse neuroblastoma cells by exogenous gangliosides. Relationship between the effect and the cell association of ganglioside GM1, *J Neurochem 42*, 299-305.
24. Byrne, M. C., Ledeen, R. W., Roisen, F. J., Yorke, G., and Sclafani, J. R. (1983) Ganglioside-induced neuritogenesis: verification that gangliosides are the active agents, and comparison of molecular species, *J Neurochem 41*, 1214-1222.
25. Kadowaki, H., Evans, J. E., Rys-Sikora, K. E., and Koff, R. S. (1990) Effect of differentiation and cell density on glycosphingolipid class and molecular species composition of mouse neuroblastoma NB2a cells, *J Neurochem 54*, 2125-2137.
26. Tettamanti, G., and Riboni, L. (1994) Gangliosides turnover and neural cells function: a new perspective, *Prog Brain Res 101*, 77-100.
27. Kolesnick, R. N. (1991) Sphingomyelin and derivatives as cellular signals, *Prog Lipid Res 30*, 1-38.
28. Inokuchi, J., Mizutani, A., Jimbo, M., Usuki, S., Yamagishi, K., Mochizuki, H., Muramoto, K., Kobayashi, K., Kuroda, Y., Iwasaki, K., Ohgami, Y., and Fujiwara, M. (1997) Up-regulation of ganglioside biosynthesis, functional synapse formation, and memory retention by a synthetic ceramide analog (L-PDMP), *Biochem Biophys Res Commun 237*, 595-600.
29. Rosner, D. L. (1998) Sleep disordered breathing: an overview, *J S C Med Assoc 94*, 542-545.
30. Mutoh, T., Rudkin, B. B., Koizumi, S., and Guroff, G. (1988) Nerve growth factor, a differentiating agent, and epidermal growth factor, a mitogen, increase the activities of different S6 kinases in PC12 cells, *J Biol Chem 263*, 15853-15856.
31. Jennemann, R., Sandhoff, R., Wang, S., Kiss, E., Gretz, N., Zuliani, C., Martin-Villalba, A., Jager, R., Schorle, H., Kenzelmann, M., Bonrouhi, M., Wiegandt, H., and Grone, H. J. (2005) Cell-specific deletion of glucosylceramide synthase in brain leads to severe neural defects after birth, *Proc Natl Acad Sci U S A 102*, 12459-12464.
32. Usuki, S., Hamanoue, M., Kohsaka, S., and Inokuchi, J. (1996) Induction of ganglioside biosynthesis and neurite outgrowth of primary cultured neurons by L-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol, *J Neurochem 67*, 1821-1830.

33. Kojima, N., Kurosawa, N., Nishi, T., Hanai, N., and Tsuji, S. (1994) Induction of cholinergic differentiation with neurite sprouting by de novo biosynthesis and expression of GD3 and b-series gangliosides in Neuro2a cells, *J Biol Chem* 269, 30451-30456.
34. Kanda, T., Ariga, T., Yamawaki, M., Pal, S., Katoh-Semba, R., and Yu, R. K. (1995) Effect of nerve growth factor and forskolin on glycosyltransferase activities and expression of a globo-series glycosphingolipid in PC12D pheochromocytoma cells, *J Neurochem* 64, 810-817.
35. Boldin, S. A., and Futerman, A. H. (2000) Up-regulation of glucosylceramide synthesis upon stimulation of axonal growth by basic fibroblast growth factor. Evidence for post-translational modification of glucosylceramide synthase, *J Biol Chem* 275, 9905-9909.
36. Proshin, S., Yamaguchi, K., Wada, T., and Miyagi, T. (2002) Modulation of neuritogenesis by ganglioside-specific sialidase (Neu 3) in human neuroblastoma NB-1 cells, *Neurochem Res* 27, 841-846.
37. Hasegawa, T., Yamaguchi, K., Wada, T., Takeda, A., Itoyama, Y., and Miyagi, T. (2000) Molecular cloning of mouse ganglioside sialidase and its increased expression in neuro2a cell differentiation, *J Biol Chem* 275, 14778.
38. Kopitz, J., Muhl, C., Ehemann, V., Lehmann, C., and Cantz, M. (1997) Effects of cell surface ganglioside sialidase inhibition on growth control and differentiation of human neuroblastoma cells, *Eur J Cell Biol* 73, 1-9.
39. Kopitz, J., Sinz, K., Brossmer, R., and Cantz, M. (1997) Partial characterization and enrichment of a membrane-bound sialidase specific for gangliosides from human brain tissue, *Eur J Biochem* 248, 527-534.
40. von Reitzenstein, C., Kopitz, J., Schuhmann, V., and Cantz, M. (2001) Differential functional relevance of a plasma membrane ganglioside sialidase in cholinergic and adrenergic neuroblastoma cell lines, *Eur J Biochem* 268, 326-333.
41. Da Silva, J. S., Hasegawa, T., Miyagi, T., Dotti, C. G., and Abad-Rodriguez, J. (2005) Asymmetric membrane ganglioside sialidase activity specifies axonal fate, *Nat Neurosci* 8, 606-615.
42. Rodriguez, J. A., Piddini, E., Hasegawa, T., Miyagi, T., and Dotti, C. G. (2001) Plasma membrane ganglioside sialidase regulates axonal growth and regeneration in hippocampal neurons in culture, *J Neurosci* 21, 8387-8395.
43. Monti, E., Bassi, M. T., Bresciani, R., Civini, S., Croci, G. L., Papini, N., Riboni, M., Zanchetti, G., Ballabio, A., Preti, A., Tettamanti, G., Venerando, B., and Borsani, G. (2004) Molecular cloning and characterization of NEU4, the fourth member of the human sialidase gene family, *Genomics* 83, 445-453.
44. Seyrantepe, V., Landry, K., Trudel, S., Hassan, J. A., Morales, C. R., and Pshezhetsky, A. V. (2004) Neu4, a novel human lysosomal lumen sialidase, confers normal phenotype to sialidosis and galactosialidosis cells, *J Biol Chem* 279, 37021-37029.
45. Yamaguchi, K., Hata, K., Koseki, K., Shiozaki, K., Akita, H., Wada, T., Moriya, S., and Miyagi, T. (2005) Evidence for mitochondrial localization of a novel human sialidase (NEU4), *Biochem J* 390, 85-93.

46. Geng, Y. Q., Guan, J. T., Xu, X. H., and Fu, Y. C. (2010) Senescence-associated beta-galactosidase activity expression in aging hippocampal neurons, *Biochem Biophys Res Commun* 396, 866-869.
47. Piccinini M, Scandroglio F, Prioni S et al (2010) Deregulated Sphingolipid Metabolism and Membrane Organization in Neurodegenerative Disorders. *Mol Neurobiol.*
48. Futerman AH, van Meer G (2004) The cell biology of lysosomal storage disorders. *Nat Rev Mol Cell Biol* 5: 554-565.
49. Simpson MA, Cross H, Proukakis C et al (2004) Infantile-onset symptomatic epilepsy syndrome caused by a homozygous loss-of-function mutation of GM3 synthase. *Nat Genet* 36: 1225-1229.
50. Kolter T, Sandhoff K (2006) Sphingolipid metabolism diseases. *Biochim Biophys Acta* 1758: 2057-2079.
51. Tessitore A, del PMM, Sano R et al (2004) GM1-ganglioside-mediated activation of the unfolded protein response causes neuronal death in a neurodegenerative gangliosidosis. *Mol Cell* 15: 753-766.
52. Sano R, Annunziata I, Patterson A et al (2009) GM1-ganglioside accumulation at the mitochondria-associated ER membranes links ER stress to Ca(2+)-dependent mitochondrial apoptosis. *Mol Cell* 36: 500-511.
53. Imgrund S, Hartmann D, Farwanah H et al (2009) Adult ceramide synthase 2 (CERS2)-deficient mice exhibit myelin sheath defects, cerebellar degeneration, and hepatocarcinomas. *J Biol Chem* 284: 33549-33560.
54. Pewzner-Jung Y, Park H, Laviad EL et al (2010) A critical role for ceramide synthase 2 in liver homeostasis: I. alterations in lipid metabolic pathways. *J Biol Chem* 285: 10902-10910.
55. Walkley SU (2004) Secondary accumulation of gangliosides in lysosomal storage disorders. *Semin Cell Dev Biol* 15: 433-444.
56. Zervas M, Dobrenis K, Walkley SU (2001) Neurons in Niemann-Pick disease type C accumulate gangliosides as well as unesterified cholesterol and undergo dendritic and axonal alterations. *J Neuropathol Exp Neurol* 60: 49-64.
57. Rodriguez-Lafrasse C, Vanier MT (1999) Sphingosylphosphorylcholine in Niemann-Pick disease brain: accumulation in type A but not in type B. *Neurochem Res* 24: 199-205.
58. Lingwood D, Simons K (2010) Lipid rafts as a membrane-organizing principle. *Science* 327: 46-50.
59. Brady RO, Kanfer JN, Mock MB et al (1966) The metabolism of sphingomyelin. II. Evidence of an enzymatic deficiency in Niemann-Pick diseae. *Proc Natl Acad Sci U S A* 55: 366-369.
60. Schneider PB, Kennedy EP (1967) Sphingomyelinase in normal human spleens and in spleens from subjects with Niemann-Pick disease. *J Lipid Res* 8: 202-209.
61. Kanfer JN, Young OM, Shapiro D et al (1966) The metabolism of sphingomyelin. I. Purification and properties of a sphingomyelin-cleaving enzyme from rat liver tissue. *J Biol Chem* 241: 1081-1084.
62. Schuchman EH Acid sphingomyelinase, cell membranes and human disease: lessons from Niemann-Pick disease. *FEBS Lett* 584: 1895-1900.
63. Schuchman EH (2007) The pathogenesis and treatment of acid sphingomyelinase-deficient Niemann-Pick disease. *J Inherit Metab Dis* 30: 654-663.

64. Graber D, Salvayre R, Levade T (1994) Accurate differentiation of neuronopathic and nonneuronopathic forms of Niemann-Pick disease by evaluation of the effective residual lysosomal sphingomyelinase activity in intact cells. *J Neurochem* 63: 1060-1068.
65. Horinouchi, K., et al. (1995) Acid sphingomyelinase deficient mice: a model of types A and B Niemann-Pick disease *Nat.Gener*. 10:288-293
66. Otterbach, B., and Stoffel, W. (1995) Acid sphingomyelinase deficient mice mimic the neurovisceral form of human lysosomal storage disease (Niemann-pick disease) *Cell* 81:1053-61
67. Tettamanti G, Bonali F, Marchesini S et al (1973) A new procedure for the extraction, purification and fractionation of brain gangliosides. *Biochim Biophys Acta*: 296(291):160-270.
68. Acquotti D, Sonnino S (2000) Use of nuclear magnetic resonance spectroscopy in evaluation of ganglioside structure, conformation, and dynamics. *Methods Enzymol* 312: 247-272.
69. Prinetti A, Basso L, Appierto V et al (2003) Altered Sphingolipid Metabolism in N-(4-Hydroxyphenyl)- retinamide-resistant A2780 Human Ovarian Carcinoma Cells. *J Biol Chem* 278: 5574-5583.
70. Svennerholm L (1957) Quantitative estimation of sialic acids. II. A colorimetric resorcinol-hydrochloric acid method. *Biochim Biophys Acta* 24: 604-611.
71. Bartlett GR (1959) Phosphorus assay in column chromatography. J Biol Chem 234: 466-468.
72. Prinetti A, Chigorno V, Tettamanti G et al (2000) Sphingolipid-enriched membrane domains from rat cerebellar granule cells differentiated in culture. A compositional study. *J Biol Chem* 275: 11658-11665.
73. Vaskovsky VE, Kostetsky EY (1968) Modified spray for the detection of phospholipids on thin-layer chromatograms. *J Lipid Res* 9: 396.
74. Partridge SM (1948) Filter-paper partition chromatography of sugars: 1. General description and application to the qualitative analysis of sugars in apple juice, egg white and foetal blood of sheep. with a note by R. G. Westall. *Biochem J* 42: 238-250.
75. Prinetti A, Chigorno V, Prioni S et al (2001) Changes in the lipid turnover, composition, and organization, as sphingolipid-enriched membrane domains, in rat cerebellar granule cells developing in vitro. *J Biol Chem* 276: 21136-21145.
76. Scandroglio F, Venkata JK, Loberto N et al (2008) Lipid content of brain, brain membrane lipid domains, and neurons from acid sphingomyelinase deficient mice. *J Neurochem* 107: 329-338.
77. Buccinna B, Piccinini M, Prinetti A et al (2009) Alterations of Myelin-Specific Proteins and Sphingolipids Characterize the Brains of Acid Sphingomyelinase-Deficient Mice, an Animal Model of Niemann-Pick Disease Type A. *J Neurochem*.
78. Valsecchi M, Mauri L, Casellato R et al (2007) Ceramide and sphingomyelin species of fibroblasts and neurons in culture. *J Lipid Res* 48: 417-424.
79. Buccinna B, Piccinini M, Prinetti A et al (2009) Alterations of myelin-specific proteins and sphingolipids characterize the brains of acid sphingomyelinase-deficient mice, an animal model of Niemann-Pick disease type A. *J Neurochem* 109: 105-115.

80. Dunbar GL, Sandstrom MI, Rossignol J et al (2006) Neurotrophic enhancers as therapy for behavioral deficits in rodent models of Huntington's disease: use of gangliosides, substituted pyrimidines, and mesenchymal stem cells. *Behav Cogn Neurosci Rev* 5: 63-79.
81. Hein LK, Duplock S, Hopwood JJ et al (2008) Lipid composition of microdomains is altered in a cell model of Gaucher disease. *J Lipid Res* 49: 1725-1734.
82. White AB, Givogri MI, Lopez-Rosas A et al (2009) Psychosine accumulates in membrane microdomains in the brain of krabbe patients, disrupting the raft architecture. *J Neurosci* 29: 6068-6077.
83. Ledesma MD, Prinetti A, Sonnino S et al (2011) Brain pathology in Niemann Pick disease type A: Insights from the acid sphingomyelinase knockout mice. *J Neurochem* 116:779-88.
84. Sonnino S, Prinetti A, Mauri L et al (2006) Dynamic and structural properties of sphingolipids as driving forces for the formation of membrane domains. *Chem Rev* 106: 2111-2125.
85. Pewzner-Jung Y, Ben-Dor S, Futerman AH (2006) When do Lasses (longevity assurance genes) become CerS (ceramide synthases)?: Insights into the regulation of ceramide synthesis. *J Biol Chem* 281: 25001-25005.
86. Bartke N, Hannun YA (2009) Bioactive sphingolipids: metabolism and function. *J Lipid Res* 50 Suppl: S91-96.
87. Quinn PJ (2010) A lipid matrix model of membrane raft structure. Prog Lipid Res 49: 390-406.
88. Iwabuchi K, Prinetti A, Sonnino S et al (2008) Involvement of very long fatty acid-containing lactosylceramide in lactosylceramide-mediated superoxide generation and migration in neutrophils. *Glycoconj J* 25: 357-374.
89. Yoshizaki F, Nakayama H, Iwahara C et al (2008) Role of glycosphingolipid-enriched microdomains in innate immunity: microdomain-dependent phagocytic cell functions. *Biochim Biophys Acta* 1780: 383-392.
90. Prinetti, A., Prioni, S., Chiricozzi, E., Schuchman, E.H., Chigorno, V., and Sonnino, S. (2011) Secondary alterations of sphingolipid metabolism in lysosomal storage disease. *Neurochem. Res.* (doi: 10.1007/s11064-010-0380-3)

4. Theme III

Chaperone therapy, for the treatment of GM2 gangliosidosis: Pyrimethamine and Sandhoff Disease

Introduction

LYSOSOMAL STORAGE DISEASE and THERAPEUTIC TREATMENTS

Biological macromolecule's degradation, such as glycoproteins and glycolipids, takes places in lysosomes. Lysosomal enzymes, such as secretory and plasma membrane (PM) proteins, are synthesized in the endoplasmatic reticulum (ER), and most of them are transported to the Golgi apparatus and tagged for lysosome by the addiction of mannose-6-phosphate. The ER contains a highly retained mechanism to protect the cell from miss-folded and potentially toxic proteins, which is called endoplasmatic reticulum associated degradation (ERAD) pathway [1,2].

The glycosphingolipidosis are a group of inherit disease that are caused by mutation in catabolic enzymes or their cofactors that reduce the efficiency of glycosphingolipid (GSLs) hydrolysis. This leads to a massive accumulation of GSLs in the lysosomes of affected cells. Lysosomal disorders differ from storage product, cells/tissue types affected, and severity. Pathology's degree depends on the mutational impairment of catalytic activity. Most lysosomal storage disorders (LSDs) exist in infantile, juvenile and adult forms. The most severe LSDs, the infantile forms, show acute brain involvement and short lifespan. In adult forms, symptoms develop slower and disability often arises mainly from peripheral symptoms. Juvenile forms are intermediate between infantile and brainstem dysfunctions. In some diseases, phenotypic variability is explained by different levels of residual enzyme activity, but in others there is no obvious correlation between phenotype and genotype. Generally low levels of enzyme activity (<10%) predict that symptom onset occurs more rapidly, leading to infantile or juvenile disease.

When the major site of accumulation is neural tissue, as in gangliosidosis (Sandhoff and Tay-sachs diseases), neurodegeneration is acute and often leads to premature death; whereas others have peripheral dysfunction without brain involvement (Fabry disease) [3-7].

Treatment of rare orphan diseases has received increasing interest despite their very low incidence. There are two fundamental approaches to reducing lysosomal storage of SLs in these disease: Enzyme Target Therapies (ETT), increasing the function of the lysosomes by restoring the deficient enzyme activity and Substrate Targeted Therapies (STT), reducing the substrate burden by inhibiting the synthesis of the SLs that cannot be degraded. The restoration of the defective lysosomal protein could be, in principle, achieved by different ways. i) Enzyme replacement therapy (ERT) that implies the direct delivery of the recombinant enzyme to the defective cells [8]. This approach presents problems related to the efficient targeting of the enzyme to the defective cells (especially within the nervous system), and to the stability of the delivered enzymes. However, it is currently used very effectively for the treatment of non-neurological (type 1) Gaucher and Fabry disease [9,10], and probably represents a valuable option for a non-neurological (type B) Niemann Pick Disease (NPD) [11]. One of the major limitation of ERT

represents the blood-brain barrier that prevents the access of the intravenously administrated enzyme to the Central Nervous System (CNS). ii) Another possible approach is represented by the *ex vivo* Gene Terapy. Hematopoietic cells are able to cross the blood brain barrier and can, therefore, used as vehicles for drugs and genes. After hematopoietic stem cell transplantation, donor derived cells migrate into the brain and differentiate to form microglia. An important principle of gene therapy for lysosomal diseases is the phenomenon of cross-correction, where a genetically corrected cell release enzymes that could be taken from neighbors cells. Nevertheless, at present, somatic gene therapy has led to variable results in animal models and faces difficulties in order to be approved for safety concerns [12-14]. iii) The reactivation of the defective enzyme (e.g., β-glucosidase in an animal model of Gaucher disease (GD)) has been also obtained by the use of "Pharmacological chaperones" (PC), small molecules directly interacting with the enzyme [15], that will be discuss in details in the next section.

Alternatively to the restoration of the defective enzyme activity, it has further been suggested that the accumulation of undegraded substrates might be improved by inhibiting their synthesis (substrate reduction therapy). *N*-butyl-deoxynojirimycin, an inhibitor of glucosylceramide (GlcCer) synthase [16,17], has been approved for the treatment of GD patients who cannot be treated with ERT [18]. It also has being used in patients with type C NPD (due to genetic abnormalities in cholesterol metabolism) and some clinical neurological improvement has been noted [19]. Regarding Tay-Sachs disease model mice, PC therapy is currently under clinical trial (NCT00672022) [20,21].

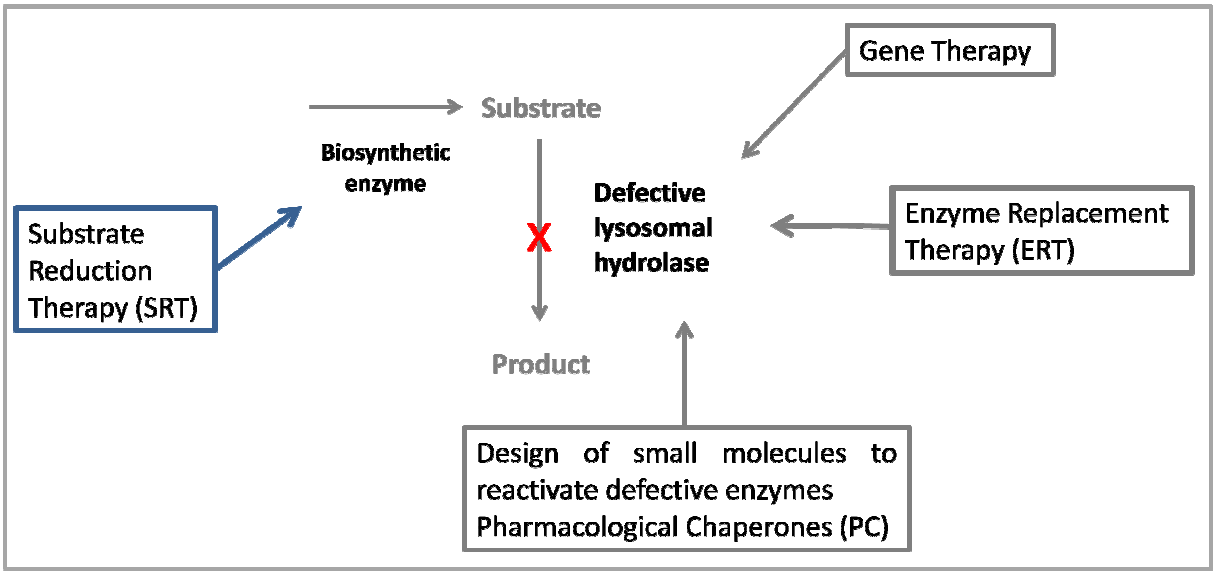


Fig. 1 Therapies for lysosomal storage disorders. LSDs treatments can be divided into two fundamental approaches: one that directly modify the defective enzyme (grey) and other one that reduces the levels of biosynthesis of the accumulating substrate (blue) (Adapted from [4]).

PHARMACOLOGICAL CHAPERONES

Among all the causes that can lead to LSDs, one of them is represented by a genetic defect, a missense mutation, that often proves a destabilizing effect on the enzyme synthesis. These alterations lead to a decrease of termostability and not-correctly folded protein that is not able to retain the native fold and pass the ERAD.

Enzymologists have long know that enzymes in the presence of a substrate or inhibitor are protected from thermo-denaturations.

PCs are small molecules that can stabilize the conformation of mutant proteins, allowing them to pass the quality control system of ER (Figure 2). In this way the property folded mutant enzyme can be transported to the lysosome, increasing the residual enzyme activity of the cells [22-25].

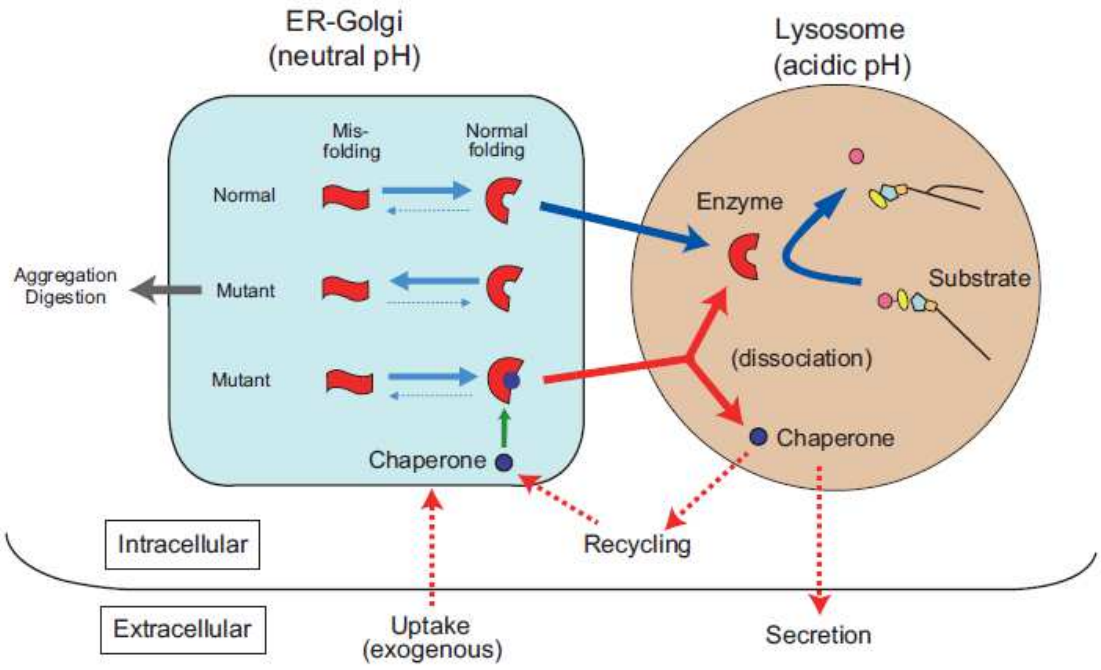


Fig. 2 Postulated molecular events between mutant enzyme molecules and chaperone compounds. Mutant enzyme protein is unstable in the ER-Golgi compartment at neutral pH, and rapidly degraded or aggregated possibly to cause ER stress. An appropriate substrate analogue inhibitor binds to misfolded mutant protein as pharmacological chaperone at the ER-Golgi compartment in somatic cells, resulting in

normal folding and formation of a stable complex at neutral pH. The protein-chaperone complex is safely transported to the lysosome. The complex is dissociated under the acidic condition and in the presence of excessive storage of the substrate. The mutant enzyme remains stabilized, and express catalytic function. The released chaperone is either secreted from the cell or recycled to interact with another mutant protein molecule. These molecular events have been partially clarified by analytical and morphological analyses, and computer-assisted prediction of molecular interactions (Adapted from [22]).

It has been known since many years that the binding of an inhibitor to an enzyme stabilizes the enzyme: all successful PCs must be competitive inhibitors. Of course, we are talking about a reversible inhibitions: once the complex PC-enzyme reaches the lysosome, the stored substrates, that has a higher affinity with the enzyme then with PC, are able to displace the PC and take over the stabilization of the mutant enzymes [26,27].

Furthermore the chaperone affinity for the target protein should be regulated by the pH: the acid pH of lysosome reduces the affinity between the pharmacological chaperone and the enzyme [28,29].

Respect to the ERT, the PCs have the advantages to be orally administrated and to cross the blood brain barrier [28].

PCs represent a very tractable therapeutic approach for a large proportion of genetic diseases where the point mutation do not totally prevent the formation of some functional enzyme/receptor (<2-5% residual activity) [29].

Given the general interest to apply the restoration of the defective lysosomal protein for a variety of late-onset of LSDs, the possibility to find suitable PCs could be a good approach. However, up to now, only five out of more than 40 LSDs have been targeted for this approach.

For example one PC (AT1001, migalastat hydrochloride) is now being evaluated in phase II clinical trials for Fabry disease [30,31]. A further complication, based on the recent experiments with glucocerebrosidase (Gaucher disease), is that not all mutants with residual activity will be equally chaperoned by a single PC, and some were not enhanced at all [32]. This may be a significant issue for the LSDs that are not associated with a single or a few high frequency mutations.

GM2 GANGLIOSIDOSIS

Human β-hexosaminidase (β-hex) isoenzymes are glycosyl hydrolases that remove β-liked non reducing terminal GalNac or GlcNac from a number of macromolecules. There are two different subunits for β-hex, α (528 residues) and β (556 residues), that are encoded by two unlinked but evolutionary related genes, HEXA and HEXB. Each subunit has its own active site. However dimerization is necessary in order to became fully functional [1,33].

These subunits can form 3 different β-hex isoenzymes (Figure 3):

Hex A (αβ),

Hex B (ββ),

Hex S (αα).

The Hex S isoform is unstable and it is only detectable in Sandhoff patients.

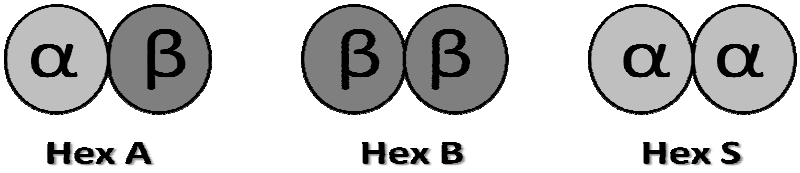


Fig. 3 β-hexosaminidase isoforms.

The active site of the β-subunit hydrolyzes uncharged substrates, whereas the α-subunits in addiction cleaves the negatively charged substrates. Only the αβ heterodimer HexA is able to degrade ganglioside GM2 producing GM3 ganglioside, in presence of GM2 activator protein, a specific co-factor for Hex A [34].

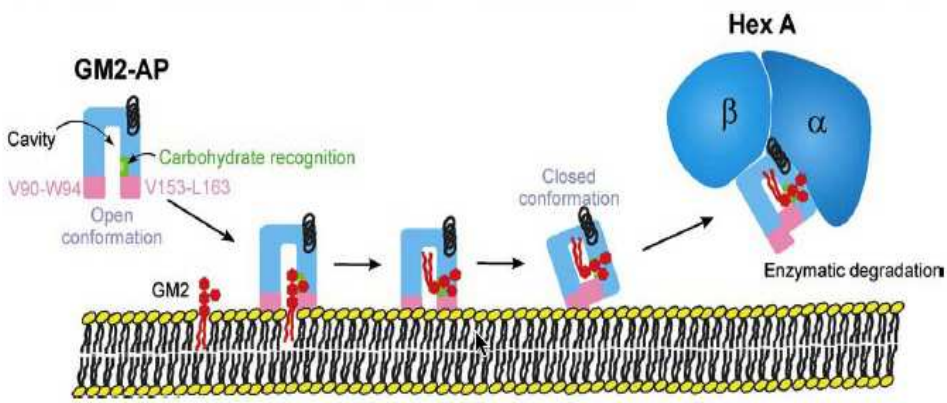


Fig. 4 Model of GM2-activator-stimulated hydrolysis of ganglioside GM2 by human β-hex A. The glycolipid binding site is lined by two hydrophobic (V90-W94 and V153-L163) surface loops and a single short helix. The most flexible of the loops (V153-L163) controls the entrance to the hydrophobic cavity allowing both, an open and closed conformation. GM2AP = GM2-activator protein, Hex A = β-hex A (Adapted from [34]).

Genetic defect in either the gene encoding the subunits of Hex A (αβ) can result in accumulation of GM2 in neural tissue leading at two of the three forms of GM2-gangliosidosis: Tay-sachs disease (defect in α subunits) and Sandhoff disease (defect in β subunits). The third form of the disease, the AB variant, is caused by a deficiency of the GM2 activator protein.

Mutations that cause a complete enzyme deficiency are extremely severe, and children affected with these forms of the disease usually die before they reach the age of four. Mutation that affects folding or dimerization, but compatible with the formation of some functional enzyme, resulting in residual Hex A activity (1-5% of normal) are less severe and results in a "late onset" clinical phenotype. It has been estimated by Sandoff and colleagues that a residual activity between 5-10% of normal is enough to prevent the GM2 accumulation [3, 29].

Use of pharmacological chaperones: the PYRIMETHAMINE

To the best of our knowledge, to date, there is no cure for the treatment of GM2 gangliosidosis.

Several molecules that act as inhibitors of Hex A and Hex B have been identified as a potential PC for late-onset GM2 gangliosidosis, including known inhibitors of Hex, like the N-acetylglucosamine thiazoline (NGT), a sulfur analogue of the oxazolinium ion intermediate, formed during substrate turnover (K_i 300 nM) [1,35] .

Recently, the group of Mauran has identified pyrimethamine [PYR, 5-(4-chlorophenyl)-6-ethyl-2,4-pyrimidinediamine] as PC for the treatment of the late onset form of Tay-Sach disease [28,29] (Figure 5).

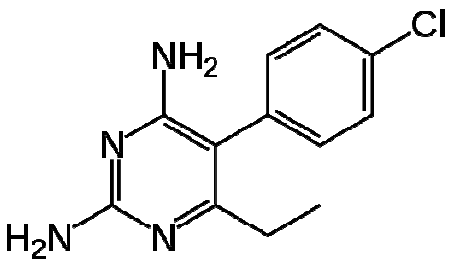


Fig. 5 Pyrimethamine's structure.

PYR is an anti-malarial drug already approved from the FDA, that targets the folic acid synthesis. Specifically, it binds the active site of dihydrofolate reductase (DHFR), preventing substrate access [1,36].

In the structure of Plasmodium vivax DHFR, PYR is located in a deep pocket containing the catalytic residues. The p-chlorophenyl ring makes van der Waals contact with the DHFR cofactor NADPH [36].

Bateman and collaborators [1] showed that PYR also binds the active site in the structure of HexB. Although the active site of HexB appears shallower than DHFR, there are many similarities to DHFR in binding the drug. The pyrimidine ring stacks against an aromatic residue and forms similar hydrogen bonding interactions with residues belonging to the active site.

Even thought, the crystal structure of HexA was difficult to obtain in comparison of HexB, so Bateman's group made a superimposition of the active site residues in the structure of HexB:PYR, suggesting that a HexA:PYR interaction could be closely resemble that of HexB:PYR [1]

The binding of PYR to the active site of HexB introduces five hydrogen bonds per active site and several favorable van der Waals contacts. Potentially PYR increases the stability of the protein fold and develops the correct conformation for the active site [1].

One important characteristic of the PYR is the pH sensibility. NGT inhibits Hex A activity at pH 4.5, whereas PYR has an optimal inhibitory profile at pH of 6.5. Thus, PYR inhibits best at the internal pH of the lumen of ER where a chaperone is needed to assist mutant Hex subunits in their folding, to permit to escape the ERAD control. Once in the lysosome, due to the lowest pH, the PYR affinity for the enzyme decrease and the enzyme could be available for substrate and thus stabilized [29,37].

Mauran and colleagues showed that the PYR is more effective than NGF to increase the activity of β-hex. They also show that PYR is able to increase the hydrolysis of both natural and artificial substrate for β-hex [1,29,37,38,40].

All the data published until now said that PYR is able to increase the folding stability of both subunit α and β, stabilizing the mutant enzymes sufficiently to allow more β-Hex to arrive to the lysosomes [1,29,37-,40]. Mauran and collegues showed that PYR is able to increase the activity of Hex A, the only isoform ables to hydrolyze GM2 [1,29,37,38,40].

One of the limits in the use of this treatment is that the mutations of β-hex gene should not related to the active site of Hex A and Hex B, the binding site of PYR (Table I). If so PYR is not able to sit in the active site and to stabilize the miss-folded protein. Since most of the destabilizing mutations are not in the immediate vicinity of the active site, they will not interfere with PYR binding. *In vitro* studies have shown that many mutations in either the α and β subunit of Hex A can be partially recovered, enhancing the levels of Hex A activity in lysosomes, following the growth of the patients cells in the presence of the PYR (Table I) [1,37,38].

Only last year has been started a clinical trial for the treatment of patients affected by cronic GM2 gangliosidosis, with the aim to establish the efficacy of the treatment with PYR [37].

It's important to note that one of the other reason for the success of PYR, as an antimalarial and potential PC candidate, is that PYR does not seriously harm the function of human DHFR [1].
We also have to report that the treatment with PYR have several adverse effects, that must be considered in an overall perspective of the treatment with PYR as PC [39]

Table I Fibroblast cell lines with relative increase of residual Hex A activity in the presence of PYR (adapted from [38])

Patient cell line number	Mutation I	Mutation 2	Residual Hex A	Hex A with PYR
SD or β Mutants				
32045	R505Q	IVs11+5G>A	4.2	15.5
36986	R505Q	Δ16kb	5.3	41.9
1303	C137Y	C137Y	1.3	6.1
30037	G353R	IVS12-26G>A	3.5	5.2
32429	T150P	P147L	2.6	7.0
3585	Δ16kb	P147L	3.6	5.8
2400	P504S	Δ16kb	12.7	23.1

Aim

Several approaches are available to assay the activity of Hex A: i) The classic one is the use of natural or radiolabeled substrate [41,42] ii) The use of synthetic substrates. MUG is the simplest assay in use. It is hydrolyzed by both the α and β active sites and therefore is used to measure total Hex activity and MUGS, a recent more specific substrate, negatively charged, that reacts only with α subunits. MUGS is activator independent and it is used to measure activity from Hex A and Hex S [40,42].

We perfectly know that assaying Hex A enzymatic activity towards its natural/or radio-labelled substrate is today quite impractical due to the high amount of Hex A activity (purified enzyme), to the difficulty to obtain and use the radio compound as natural compound and to the high cost of these type of research.

Artificial substrates are used to diagnose GM2 gangliosidosis and to determine the efficacy of therapy with different approach, as routine [40].

With this project we want to demonstrate the need to continue to use the classical biochemical approach, especially in the early stages of validation of the new therapy.

Matherials and Methods

MATERIALS

Commercial chemicals were of the highst purity available, common solvents were distilled before use and water was doubly distilled in a glass apparatus. Trypsin, reagent for cell culture , Condutirol B Epoxide (CBE), 4-Methylumbelliferyl-β-N-acetylglucosaminide (MUG) and Pyrimethamine were from Sigma-Aldrich (St. Louise, MO, USA). Minimum Essential Medium with Earle's Salt (MEM) and fetal calf serum were purchased from EuroClone (Leeds, UK).

GM1 and GM2 was prepared by sialidase digestion of a bovine brain ganglioside mixture and purified (Acquotti *et al.* 1994). [3-^{3}H(*sphingosine*)]GM1 (specific radioactivity, 2.3 Ci/mmole) used as substrates of enzymatic assays and as chromatographic standards were prepared as described (228). Highperformance silica gel thin-layer plates (HPTLC Kieselgel 60, 10 x10 cm) were purchased from Merck GmbH (Milan, Italy). AM-calcein was from Invitrogen (Carlsbad, CA, USA). Triton X-100 were from Merck (Darmstadt, Germany). 4-Methylumbelliferyl-β-N-D-galactopyranoside (MUB-Gal). 4- Methylumbelliferyl-β-N-acetylglucosaminide-6-sulphate (MUGS) was from Toronto Research Chemicals Inc. (North York, Ontario, CDN).

METHODS

Cell Cultured

Primary skin fibroblast from unaffected and affected patients were obtained by the punch technique, cultured and propagated as described [43], using Minimum Essential Medium with Earle's Salt (MEM) supplemented with 10% FBS, 1% Glutamine and 1% Penicilin/Streptomicin. The cells were cultivated as monolayer in a humidified atmosphere at 37°C and 5% CO_2.

Cell Viability

Cell viability was assessed by calcein staining [44]. Cells were washed with PBS, the plate was then put on ice and 200 µl of calcein-Am solution (6.25 µg/ml in PBS) were added to esch well. Cells were incubated for 15 min at 37°C, 5% CO_2. Calcein-AM was then removed and 100 µl of PBS and 50 µl of 1% Triton-X100 were added to esch well. Plate was strirred at RT for 15 min and then the fluorescence was detected by a microplate reader (Victor, Perkin-Elmer).

Treatment of cell cultures with PYR

SD and control fibroblast were treated with PYR (dissolved in Ethanol, final concentration 1µg/mL) or with Ethanol (as control), for 72h [38].

Treatment of cell cultures with [3-^{3}H(sphingosine)] GM1

[^{3}H] GM1, dissolved in propan-1-ol/water, 7:3 (v/v), was transferred into a sterile glass tube, and dried under nitrogen stream; the residue was then solubilized in an appropriate volume of pre-warmed cell medium (serum free) to obtain the desired final concentration (….M). After 18h incubation (pulse) in presence of GM2 (5µg/ml) and CBE (50 µM), cells were washed and incubated 2h (chase) with cell medium not containing radioactive ganglioside [40,41].

Radioactive lipid analyses

Cells were lyophilized and subjected to lipid extraction and SLs analysis. Total lipids from lyophilised cells were extracted with chloroform/methanol/water 20:10:1 by vol. followed by chloroform/methanol 2:1 by vol. The total lipid extracts were subjected to a two-phase partitioning by adding 20 % water to the lipid extract; the total lipid extract, the aqueous and organic phases were analyzed by HPTLC (1000 dpm/lane). [^{3}H]SLs of total extracts and organic phases were separated using the solvent system chloroform-methanol–water 110:40:6 by vol, and those of aqueous phases with chloroform-methanol-0.2 % aqueous $CaCl_2$, 50:42:11 by vol.

[^{3}H]SLs were identified by referring to the position of standards in the chromatogram and quantified by radioimaging after 48–96h of acquisition (β-Imager 2000, Biospace, Paris, France) .

Enzymatic activities of cell lysates

The enzymatic activities associated with total cell lysates were determined using fluorigenic substrates as described previously with some modifications [45,46]. Briefly, cells were washed twice with PBS, harvested, and resuspended in water in the presence of a protease inhibitor cocktail (Roche). Aliquots of the cell homogenates were transferred to a 96-well microplate and enzymatic assays performed with five-fold replication. MUB substrates were solubilized in McIlvaine buffer at the opportune final concentration and opportune pH for each enzyme. The reaction mixtures were incubated at 37°C with gentle shaking. The developed fluorescence was detected at various times by a Victor microplate reader (Perkin Elmer) after transferring 10 µl of the reaction mixtures to a microplate and adding 190 µl of 0.25 M glycine, pH 10.7. The data were expressed as nmoles converted substrate/ 106 cells/ hr.

β-hexosaminidase activity

β-hex activities were determinate using the 4-methylumbelliferyl derivates of either β-*N*-acetylglucosaminide (MUG) or β-*N*-acetylglucosamine-6-sulfate (MUGS). MUG was solubilized at

final concentration of 3mM in McIlvaine Buffer (0.1M Citrate/0.2 Phosphate) pH 5.0. MUGS was solubilized at final concentration of 1mM in McIlvaine Buffer (0.1M Citrate/0.2 Phosphate) pH 5.0

β-galactosidase activity

β-Gal activity was determinate using MUB-Gal, solubilized at the concentration of 0.25mM in McIlavine Buffer (0.1M Citrate/0.2 Phosphate) pH 4.0.

β-hexosaminidase isoenzymes analysis (DEAE Column)

Cell lysates were analyzed by the ion-exchange chromatography on DEAE-cellulose [41]. The chromatography was performed by using 1-ml column equilibrated with 10 mM sodium phosphate buffer, pH 6.0 (buffer A). the flow rate was 0.5ml/min. proteins retained by the column were eluted using a linear gradient of NaCl (0.0-0.5 M in 40ml of buffer A) and then eluted with 1.0 M NaCl in the same buffer. Fraction (1ml) were collected and assayed for the Hex activity with the two substrates, MUG and MUGS.

Other analytical methods

The radioactivity associated with lipid extracts was determined by liquid scintillation counting. Digital autoradiografy of HPLC plates was performed with a Beta-Imager 2000 Instrument (Biospace, Paris). The radioactivity associated with individual lipids was determinate with the specific B-Vision software provided by Biospace.

The protein assays were carried out using the DC protein assay in according to manufactures instructions (Biorad).

Results

Here we report the treatment with PYR of two cell lines (fibroblast) derived from two patient, displacing two new mutations for Juvenile Sandhoff disease, #1 and #2.

Complete sequencing of the *HEXB* gene showed that patient #1 carried the common 16 kb deletion (including promoter and exons 1-5) on one allele and a novel splice mutation in intron 2 (c.446-13A>G) on the other allele [48]. Patient #2 was homozygous for a previously reported mutation (c.1082+5G>A) present in intron 8 [49].

Their clinical phenotype was characterized by progressive hypotonia, seizures, exaggerated reaction to sounds and presence of a cherry-red spot at the ophthalmoscopic examination. Biological diagnosis was performed on leukocytes and serum by using enzymatic tests based on the 4-methylumbelliferyl derivatives of β-N-acetylglucosaminide (MUG) or β-N-acetylglucosamine-6-sulfate (MUGS), permitting to evaluate the activity of total hexosaminidases or specific hexosaminidase A, respectively. Both patients had an hexosaminidases A and B deficiency confirming the diagnosis of Sandhoff disease or GM2 gangliosidosis variant 0.

Cultured fibroblasts were obtained from both patients and β-hexosaminidase activities were measured with the artificial substrates MUG and MUGS [28,39]. Both patients showed a residual β-hexosaminidase A activity around 2% with the MUGS substrate, leading the possibility for them to be candidate for an enhancement enzyme therapy [3,29].

i. Preliminary experiments

We have designed a set of preliminary experiments focused to test the concentration of PYR to use and the PYR toxicity.

As control cells we use fibroblast form healthy subjects. The cells not treated with PYR where incubated always in the presence of ethanol, the solvent of PYR.

PYR concentrations

As primary step we tried different concentration of PYR to see the effect on Hex-activity (0.5-10 μg/ml of cell cultured medium). We observed a maximal effect at concentration of 1 μg/ml of cell cultured medium and we noticed that the increase of activity is not dose-dependent (Table II).

Cell Viability

To investigate the toxicity of PYR on cell viability we performed the Calcein analysis. The treatment with PYR for 24, 48, 72 and 96 h do not alter cell viability and death, compared with control cell. For further experiments we fix at 72h the period of incubation (Figure 6).

Table II PYR concentration.

#1 cells	No treated cells	PYR 0,5	PYR 1	PYR 2	PYR 3	PYR 4	PYR 5	PYR 6	PYR 7	PYR 10	CTRL
MUG	202	460	572	580	465	415	314	375	288	255	2778
% Activity	6%	17%	21%	21%	17%	15%	11%	13%	10%	9%	100%
MUGS	67	231	240	230	241	235	202	224	198	170	361
% Activity	19%	64%	67%	60%	67%	65%	56%	62%	55%	47%	100%

PYR concentration is expressed as µg of PYR/ ml of cultured media. MUG and MUGS are representative of the substrate hydrolysis (expressed as arbitrary units of fluorescence). % of activity is respect to the CTRL cells (fibroblast from health donor).

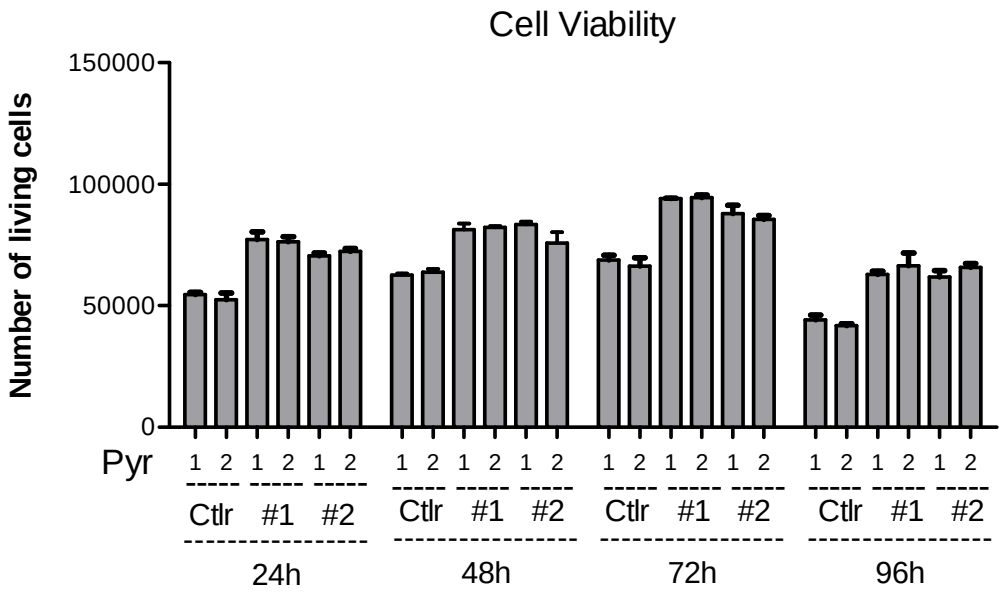


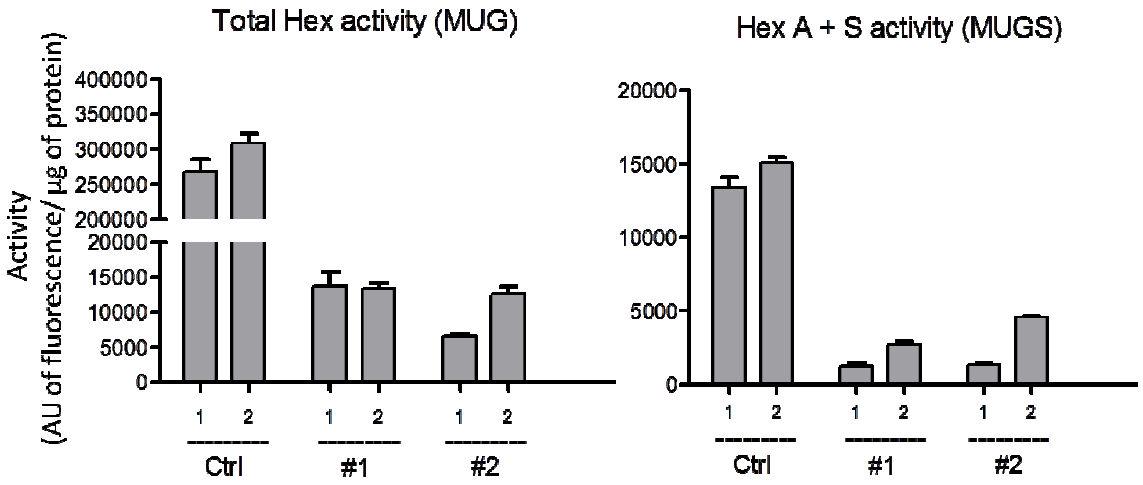
Fig. 6 Cell viability (1, untreated cells; 2, treated cells) Ctrl= control cells incubated with Ethanol; #1: cell line #1; #2: cell line 2.

ii. PYR increase the β-Hex activity using the MUG and MUGS substrates.

To understand if the two fibroblast cell lines from SD patient could be responsive to the PYR treatment, we assesed the β-Hex activity, using artificial substrate MUG and MUGS, from cell lysate after 72h of treatment with PYR (1 µg/ml).

The total Hex activity (MUG) showed no significant increases in treated #1 cells, but, when MUGS was used like substrate, it showed a significant increase in subunit α activity. On the contrary treated #2 cells showed a significant increase in both MUG and MUGS substrates.

Both #1 and #2 fibroblasts, after the treatment with PYR showed an increase up to 10% when measured with artificial substrate MUGS (Figure 7).



	EtOH	Pyr	Increase activity
Ctrl	266700	307466	1x
#1	13653	13272	0
#2	6587	12554	2x

	EtOH	Pyr	Increase activity
Ctrl	13355	15071	1x
#1	1212	2672	2x
#2	1290	4511	3,5x

Fig. 7 β-Hex activity (1, untreated cells; 2, treated cells) Ctrl: control cells incubated with Ethanol; #1: cell line #1; #2: cell line 2.

iii. PYR does not increase others glycohydrolases activities.

We also evaluated the PYR's effect on other glycohydrolases in particular on the β-galactosidase (β-gal). PYR had no effect on the activity of β-gal, after 24, 48, 72 and 96 h of treatment (Figure 8).

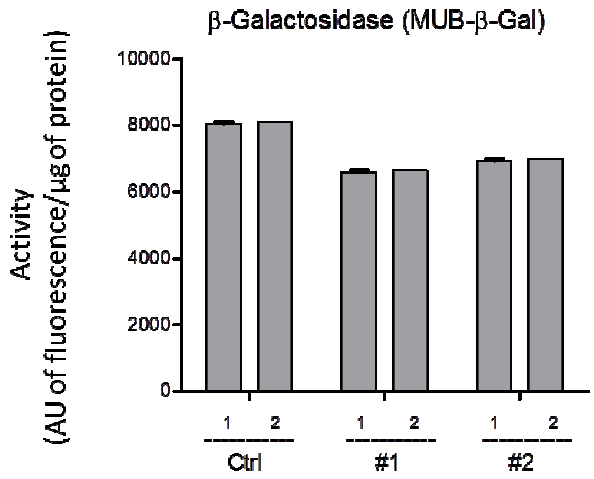


Fig. 8 β-Gal Activity (1, untreated cells; 2, treated cells) Ctrl: control cells incubated with Ethanol; #1: cell line #1; #2: cell line 2.

iv. PYR does not increase the β-Hex activity using the natural substrate.

To confirm the capacity of PYR to increase the activity of β-Hex activity, we fed cell with isotopically tritium labeled GM1 ganglioside, which is the lysosomal precursor of GM2. After 72h of treatment with PYR, GM1 was added to FBS-free media, incubated 18h (pulse), washed and chased for 2h with fresh FBS-contain media.

To reach the same gangliosides concentrations of neurons, we also performed an experiment in the presence of natural GM2 (50 µM) and in the presence of CBE, a non reversible inhibitor of glucocerebrosidase 2, to stop the degradation pathway of GM2 at GlcCer step.

The cells were then subjected to lipid extraction. The total radioactive lipid extracts were analyzed by HPTLC. In both control and PYR treated cells, ganglioside GM1 was transformed into GM2, indicating that GM1 administered to cells was taken up by the cells and reached the lysosomes. In control cells GM2 was transformed into GM3 as indication of the correct lysosomal SL catabolism. As expected, in Sandhoff cells no GM3 was produced due to β-hex chain β. Surprisingly, and in contrast with the increased activity of β-hex in PYR treated Sandhoff cells, we could not see any transformation of GM2 to GM3 (and reduction of GM2) in PYR treated Sandhoff cells (Figure 9).

v. PYR increase the activity of Hex S: DEAE separation of β-Hex isoforms

Then we tried to understand if the observed increase of the activity consequent to artificial substrates administration could be an artifact or not.

To better investigate the β-hex activity after the treatment with PYR, we separated the three isoforms (B, A and S) of β-hex, by DEAE column chromatography (Figure 10).

Separation of β-hex isoforms [41] and determination of their activity showed that the residual activity of Sandhoff fibroblasts is due essentially to the S isoform and that PYR increased β-hex S activity (Fig. 2). Hex S enzyme recognizes the artificial substrates but not ganglioside GM2. No effect on the β-hex A was observed.

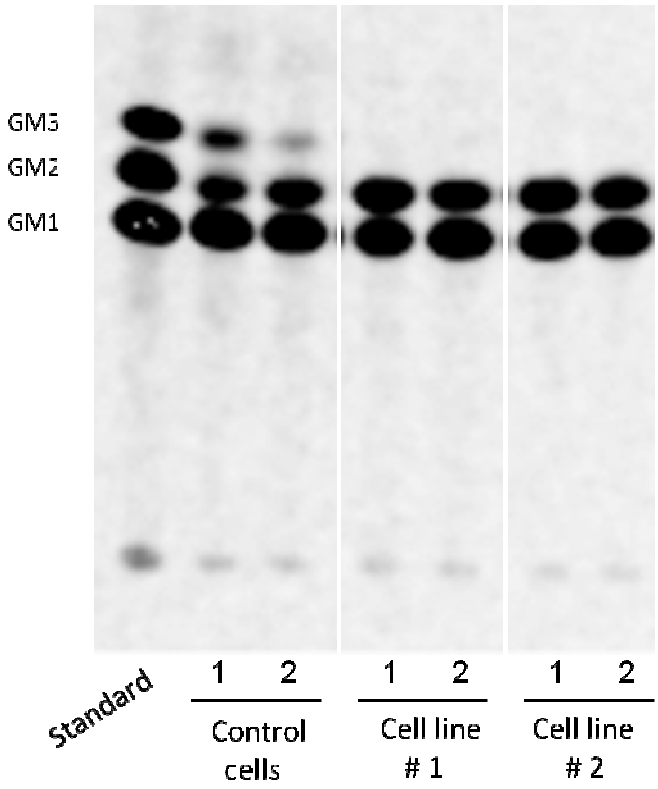


Fig. 9 Cultures of control fibroblasts and Sandhoff fibroblasts were metabolically labeled with [3-^{3}H-*Sph*]GM1 [40] and treated with PYR (1, untreated cells; 2, treated cells). Total lipid extracts were partitionated and gangliosides were separated on HPTLC using the solvent system chloroform/methanol/0.2% calcium chloride 50:42:11 (v/v/v). After separation, the radioactive lipids were visualized by digital autoradiography. In all cells the lysosomal catabolism is clearly recognizable. GM1 administered to cells was taken up by the cells, reached the lysosoms where the β-galactosidase transformed GM1 into GM2, by detachment of the external galactose. In control cells, fibroblasts from healthy donors, the formed ganglioside GM2 was transformed into GM3 by β-hex. In Sandhoff cells and PYR treated Sandhoff cells (both cell lines 1 and 2) no GM3 was formed due to the mutation of β-hex and ineffectiveness of PYR. We confirmed the inhibitory activity of PYR on β-hex A, in the absence of an accumulation of GM2 [28], as suggested by the reduced production of GM3 in control cells.

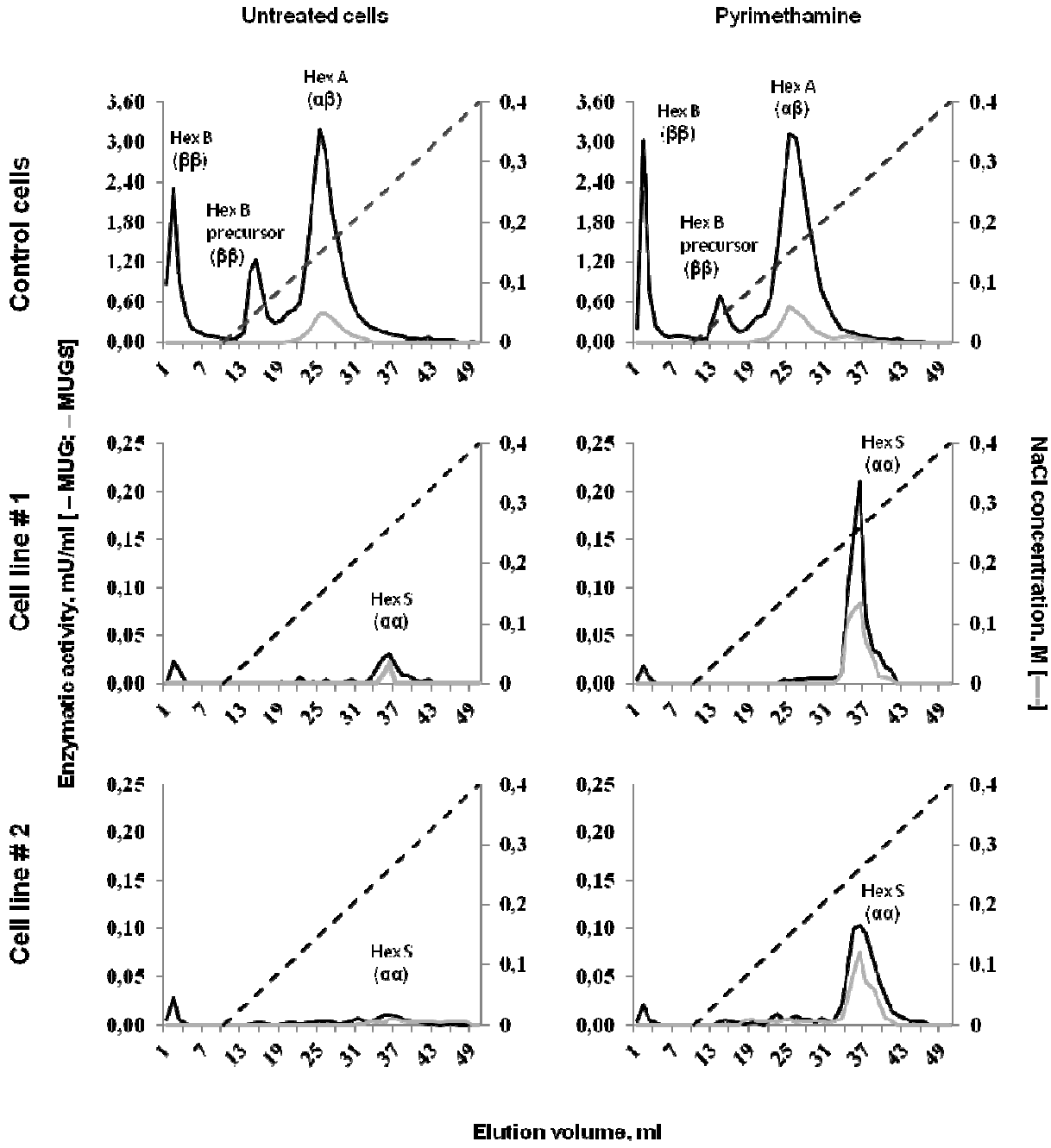


Fig. 10 Analysis of β-hex isoform pattern. β-hex isoenzyme separation was performed by ion exchange DEAE-cellulose column chromatography using a NaCl gradient as eluting system. β-hex B (ββ-dimer) was unretained by the column and eluted with the void volume, whereas β-hex A (αβ-dimer) and other β-hex isoforms (Hex S, αα-dimer) were eluted by the linear saline gradient as previously described [41]. Fractions of 1 ml were collected and assayed for β-hex activity using the fluorescent substrates MUG, which is hydrolyzed by β-hex isoforms A, B and S, and MUGS, which is hydrolyzed by the isoforms A and and S. The same amount of proteins for each sample was loaded on the colum.

Discussion

Sandhoff disease is an autosomal recessive neurodegenerative disease characterized by the intralysosomal accumulation of GM2 ganglioside. It is due to mutations in the β-hexs β-chain gene, resulting in a β-hexs A (αβ) and B (ββ) deficiency. Several β-chain gene mutations have been described. This disease predominantly affects the central nervous system and has a dramatic evolution [47].

Lysosomal enzymes are synthesized in the endoplasmic reticulum, and most of them are transported to the Golgi apparatus for glycosylation and tagged for lysosomes, by the addition of mannose-6-phosphate. The ER contains a highly conserved degradation pathway to protect cells from misfoldingand potentially toxic proteins. Thus, the protein turnover ensures integrity and biological functions of cells [29,40].

Treatment of sphingolipidoses, such as Sandhoff disease, have received increasing interest despite the low incidence. Replacing enzyme therapy meets the problem to be ineffective over the blood brain barrier and new approaches are now under investigation. One of the possible approach is the use of pharmacological chaperones. Pyrimethamine, a drug used to treat or prevent serious parasite infections such as toxoplasmosis and malaria, has been described to act as chaperone for the β-hex [29,39,47]. As chaperone PYR stabilizes the conformation of mutated protein, it allows the missfolded proteins to pass the quality control system of ER. Thereby, the stable folded protein can be transported in higher quantity to lysosomes, increasing the residual enzyme activity.

Pharmacological chaperones that give better results are also competitive inhibitors: inhibition must be reversible by the large amount of substrate accumulated in lysosomes. Furthermore the PYR affinity for the β-hex is regulated by the pH: the acid pH of lysosome reduces the affinity between the pharmacological chaperone and the enzyme [28,29]. Nevertheless, the effectiveness as chaperone of PYR occurs as a function of the conformation of the mutated protein interacting with the chaperone, and not all the mutations on the β-chain allow the protein to interact with PYR.

Here we reported the treatment of two cases of juvenile Sandhoff disease [47], displaying the new mutations C1082+5G>A (#1) and C446-13A>G (#2) in the HEX B gene with PYR. The drug, when administered to Sandhoff patients, is believed to increase β-hex expression and activity and to reduce GM2 lysosomal accumulation. The effects of PYR have been tested on cells carrying several different mutations, but not all responded

as expected. The drug causes also serious adverse effects [39] and should be used only after verifying its effectiveness.

The two examined cell lines, after treatment with PYR showed an increased total β-hex activity, so that the residual value was around 10%. This value should be enough to limit the accumulation of GM2 [41]. In contrast to this, cells treated with PYR and fed with isotopically tritium labeled ganglioside GM1 did not show any GM2 lysosomal catabolism. This negative result was explained after fractionation of the total cell proteins by ion exchange DEAE column chromatography. Clearly, we found that the increased β-hex activity on the artificial substrate was due to the increase of the activity of β-hex isoform S. Unlikely, the isoform S, even if it hydrolyzes the artificial compound, does not recognize the natural substrate GM2 and therefore the PYR is unable to modify cell lysosomal activity in the two cell lines showing the new mutations C1082+5G>A and C446-13A>G and moreover cannot be used as a therapeutic drug for the two patients.

These results suggest that analysis of the enzymatic features of the β-hex isoforms of patient's fibroblasts and the feeding experiments with tritium labeled gangliosides are very useful to determine the effectiveness of the chaperone PYR. These clinical chemistry investigations give a valuable information to decide on the therapeutic use of PYR: our results are in agreement with a previous information on the selective activity of PYR depending on the mutation [29,39,47] and confirm the advantages deriving from the investment of patients' fibroblasts for the decision on the use of PYR as therapeutic drug.

References

1. Bateman K.S., Cherney M.M., Mahuran D.J., Tropak M., and James N.G. (2011) Crystal structure of β-Hexosaminidase B in Complex with Pyrimethamine, a Potential Pharmacological Chaperone *J Med Chem* 54:1421-1429
2. Beck M. (2010) Therapy for lysosomal storage disorders *IUMB Life* 62:33-40
3. Leinekugel P., Michel S., Conzelmann E. and Sandhoff K. (1992) Quantitative correlation between the residual activity of β-Hexosaminidase A and arysulfatase A and the severity of the resulting lysosomal storage disease *Hum Genet* 88:513-523
4. Futerman A.H. and van Meer Gerrit (2004) The cell biology of lysosomal storafe disorders *Nat Rev Mol Cell Bio* 5:554-565
5. Jeyakumar M., Dwek R.A., Butters T.D. and Platt F.M. (2005) Storage solutions: treating lysosomal disorders of the brain *Nat Rev Neuro* 6:1-12
6. Sillence D.J. and Platt F.M. (2003) Storage disease: new insights into sphingolipid functions *Trends in Cell Biology* 13:195-203
7. Meikle P.J., Hopwood J.J., Calague A.E. and Carey W.F. (1999) Prevalence of Lysosomal Storage Disorders *JAMA* 281:249-254
8. Desnick RJ, Schuchman EH (2002) Enzyme replacement and enhancement therapies: lessons from lysosomal disorders. *Nat Rev Genet* 3: 954-966.
9. Grabowski GA, Hopkin RJ (2003) Enzyme therapy for lysosomal storage disease: principles, practice, and prospects. Annu Rev Genomics Hum Genet 4: 403-436.
10. Bengtsson BA, Johansson JO, Hollak C et al (2003) Enzyme replacement in Anderson-Fabry disease. *Lancet* 361: 352.
11. Dhami R, Schuchman EH (2004) Mannose 6-phosphate receptor-mediated uptake is defective in acid sphingomyelinase-deficient macrophages: implications for Niemann-Pick disease enzyme replacement therapy. *J Biol Chem* 279: 1526-1532.
12. D'Azzo A (2003) Gene transfer strategies for correction of lysosomal storage disorders. *Acta Haematol* 110: 71-85.
13. Cheng SH, Smith AE (2003) Gene therapy progress and prospects: gene therapy of lysosomal storage disorders. *Gene Ther* 10: 1275-1281.
14. Cachon-Gonzalez MB, Wang SZ, Lynch A et al (2006) Effective gene therapy in an authentic model of Tay-Sachs-related diseases. *Proc Natl Acad Sci U S A* 103: 10373-10378.
15. Futerman AH, Sussman JL, Horowitz M et al (2004) New directions in the treatment of Gaucher disease. *Trends Pharmacol Sci* 25: 147-151.
16. Cox T, Lachmann R, Hollak C et al (2000) Novel oral treatment of Gaucher's disease with N-butyldeoxynojirimycin (OGT 918) to decrease substrate biosynthesis. *Lancet* 355: 1481-1485.
17. Lachmann RH (2003) Miglustat. Oxford GlycoSciences/Actelion. *Curr Opin Investig Drugs* 4: 472-479.
18. Weinreb NJ, Barranger JA, Charrow J et al (2005) Guidance on the use of miglustat for treating patients with type 1 Gaucher disease. *Am J Hematol* 80: 223-229.

19. Patterson MC, Vecchio D, Prady H et al (2007) Miglustat for treatment of Niemann-Pick C disease: a randomised controlled study. *Lancet Neurol* 6: 765-772.
20. Platt FM, Neises GR, Reinkensmeier G et al (1997) Prevention of lysosomal storage in Tay-Sachs mice treated with N-butyldeoxynojirimycin. *Science* 276: 428-431.
21. Jeyakumar M, Butters TD, Cortina-Borja M et al (1999) Delayed symptom onset and increased life expectancy in Sandhoff disease mice treated with N-butyldeoxynojirimycin. *Proc Natl Acad Sci* U S A 96: 6388-6393.
22. Yoshiyuki Suzuki,Y., Ogawa S., Sakakibara Y. (2009) Chaperone Therapy for Neuronopathic Lysosomal Diseases: Competitive Inhibitors as Chemical Chaperones for Enhancement of Mutant Enzyme Activities *Perspect Med Chem* 3 : 7-19
23. Leandro P., Gomes C.M. (2008) Protein misfolding in conformational disorders : rescue of folding defects and chemical chaperoning *Mini Rev Med Chem* 8 :901-911
24. Arakawa T., Ejima D., Kita Y., Tsumoto K (2006) Small molecule pharmacological chaperones: from thermodynamic stabilization to pharmacological drugs. Biochim Biophys Acta 1764:1677-1687
25. Ulloa-Aguirre A., Janovick J.A., Brothers S.P., Conn P.M. (2004) Pharmacological rescue of conformationally-defective proteins: implications for the treatment of human disease Traffic 5:821-837
26. Desnick R.J. and Schuchmann E.H. (2002) *Nat Rev Genet* 3:954-966
27. Desnick R.J: (2004) *J. Inherit Metab Dis* 27:385-410
28. Maegawa GH, Tropak M, Buttner J, Stockley T, Kok F, et al. (2007) Pyrimethamine as a Potential Pharmacological Chaperone for Late-onset Forms of GM2 Gangliosidosis. *J Biol Chem*. 282:9150–9161
29. Tropak MB and Mahuran DJ. (2007) Lending a helping hand, screening chemical libraries for compounds that enhance β-hexosaminidase A activity in GM2 gangliosidosis cells FEBS *J*. 274(19):4951-61.
30. Bodensteiner D., Scott C.R., Sims K.B., Shepherd G.M., Cintron R.D., and Germain D.P. (2008) Successful reinstitution of agalsidase beta therapy in Fabry disease patients with previous IgE antibody or skin test reactivity to the recombinant enzyme *Gener Med* 10:353-358;
31. Benjamin E.R., Khanna R., Schilling A., Flanagan J.J., Pellegrino L.J. et al (2012) Co-administration with the pharmacological chaperone AT1001 increases recombinant human α-galactosidase A tissue uptake and improves substrate reduction in Fabry mice *Mol Ther* 20:717-26
32. Ashe KM, Bangari D, Li L, Cabrera-Salazar MA, Bercury SD, Nietupski JB, Cooper CG, Aerts JM, Lee ER, Copeland DP, Cheng SH, Scheule RK, Marshall J. (2011) Iminosugar-based inhibitors of glucosylceramide synthase increase brain glycosphingolipids and survival in a mouse model of Sandhoff disease. Plos ONE doi: 10.1371/journal.pone.0021758
33. Mark B.L., Mahuran D.J., Cherney M.M., Zhao D., Knapp S., James M.N. (2003) Crystal structure of human beta-hexosaminidase B: understanding the molecular basis of Sandhoff and Tay-Sachs disease *J Mol Biol* 327:1093-1109

34. Kolter T. and Sandhoff K. (2006) Sphingolipid metabolism disease *BBA-Biomembranes* 1758:2057-2079
35. Tropak MB, Reid SP, Guiral M, Withers SG, Mahuran DJ (2004) Pharmacological enhancement of beta-hexosaminidase activity in fibroblast from adult Tay-Sachs and Sandhoff patients. *J Biol Chem* 279:13478-13487
36. Kongsaeree P, Khongsuk P., Leartsakulpanich U., Chitnumsub P., Tarnchimpoo B., Walkinshaw MD., Yuthavong Y. (2005) Crystal structure of dihydrofolate reductase from Plasmodium vivax: pyrimethamine displacement linked with mutation-induced resistance *Proc Natl Acad Sci USA* 282:13046-13051
37. Clarke JTR, Mahuran DJ, Sathe S, Kolodny EH, Rigat BA, Raiman JA, Tropak MB (2011) An open-label Phase I/II clinical trial of pyrimethamine for the treatment of patients affected with chronic GM2 gangliosidosis (Tay-Sachs or Sandhoff variants) *Mol Genet Metab* 102:6-12
38. Maegawa GHB, Tropak M, Buttner J, Stockley T, Kok F, Clarke JTR, and Mahuran DJ (2007) Pyrimethamine as a potential pharmacological chaperone for late-onset forms of GM2 gangliosidosis *JBC* DOI:10.1074/jbc.M609304200
39. Osher E, Fattal-Valevki A, Sagie L, Urshanski N, Amir-Levi Y, et al. (2010) Pyrimethamine increases β-hexosaminidase A activity in patients with Late-onset Tay Sachs *Mol Genet Metab* 102:356-363
40. Tropak MB, Bukovac SW, Rigat BA, Yonekawa S, Wakarchuk W and Mahuran DJ (2010) A sensitive fluorescente-based assay for monitoring GM2 ganglioside hydrolysis in live patient cells and their lysates *Glycobiology* 20:356-365
41. Arfi A, Bourgoin C, Basso L, Emiliani C, Tancini B et al. (2005) Bicistronic lentiviral vector corrects β-hexosaminidase deficiency in transuced and cross-corrected human Sandhoff fibroblast. *Neurobiology of Disease* 20:583-593
42. Mencarelli S., Cavalieri C., Magini A., Tancini B., Basso L., et al (2005) Identification of plasma membrane associated mature β-hexosaminidase A, active towards GM2 ganglioside, in human fibroblast *FEBS Letters* 579:5501-5506
43. Leroy, J. G., Ho, M. W., MacBrinn, M. C., Zielke, K., Jacob, J., and O'Brien, J. S. (1972) I-cell disease: biochemical studies, *Pediatr Res* 6, 752-757.
44. Aureli, M., Loberto, N., Lanteri, P., Chigorno, V., Prinetti, A., and Sonnino, S. (2010) Cell surface sphingolipid glycohydrolases in neuronal differentation and aging in culture, *J Neurochem*.
45. Aureli M, Loberto N, Lanteri P, Chigorno V, Prinetti A, Sonnino S. (2011) Cell surface sphingolipid glycohydrolases in neuronal differentiation and aging in culture .J Neurochem. 2011 Mar;116:891-9.
46. Aureli M, Loberto N, Chigorno V, Prinetti A, Sonnino S. (2011) Remodeling of sphingolipids by plasma membrane associated enzymes.Neurochem Res 36:1636-44.
47. Sandhoff K. (2001) The GM2-gangliosidoses and the elucidation of the beta-hexosaminidase system. *Adv. Genet*. 44, 67–91.
48. Neote K, McInnes B, Mahuran DJ and Gravel RA. (1990) Structure and distribution of an Alu-type deletion mutation in Sandhoff disease. *J Clin Invest* 86:1524-31.

49. Zampieri S, Cattarossi S, Oller Ramirez AM, Rosano C, Lourenco CM, Passon, N, et al. (2012) Sequence and copy number analyses of HEXB gene in patients affected by Sandhoff disease : functional characterization of 9 novel variants. *PLoS One* 7:e41516.

5. Theme IV

Cell surface sphingolipid glycohydrolases modulation during tumor irradiation.

Introduction

GLYCOHYDROLASES

Glycohydrolases are a widespread group of enzymes involved in glycosphingolipids (GSLs) metabolism, that hydrolyse the glycosidic bonds. The glycohydrolases are ubiquitous enzymes, being found in lysosomes, plasma membranes (PM), cytosol and blood plasma [1-5]. Glycohydrolases are found in essentially all domains of life. In bacteria and prokaryotes, they are found both as intracellular and extracellular enzymes largely involved in nutrient acquisition. One of the important occurrences of glycohydrolases in bacteria is the enzyme β-galactosidase (LacZ), which is involved in regulation of expression of the Lac-operon in Escherichia Coli. In higher organisms glycohydrolases are found within the endoplasmic reticulum (ER) and Golgi apparatus where they are involved in processing of *N*-linked glycoproteins, and in the lysosomes as enzymes involved in the degradation of carbohydrate structures. Deficiency in specific lysosomal glycohydrolases can lead to a range of lysosomal storage disorders that result in developmental problems or death.

THE REGULATION OF GSLs COMPOSITION AT THE LEVEL of PM

Changes in the GSLs composition of the PM in a certain cell type would lead to very important biological consequences, thus all mechanisms possibly contributing to these changes have a high functional significance. The classical view on sphingolipid (SL) metabolism implies the vescicular transport of neo-biosynthesized SLs from the ER and the Golgi apparatus to the PM (reviewed in [6]). Changes in the activities of enzymes of the biosynthetic pathway have been associated with the changes in GSLs expression that are correlated with biological events such as neoplastic transformation or neuronal differentiation. However, other mechanisms could be responsible for a local regulation of the GSLs composition of PM on restricted PM areas (Figure 1):

i. Both catabolic and biosynthetic enzymes for SLs have been found associated with the PMs. "Signaling" sphingomyelinases (SMase) are resident in, or translocated to, the PM, being able to convert PM sphingomyelin (SM) into ceramide (Cer) [7,8]. Conversely, a PM-associated SM synthase enzyme activity (SMS2), genetically distinct from the Golgi enzyme, has been identified [9]. Thus, the SM/Cer ratio can be locally modulated possibly in response to physiological events, leading to profound consequence on the organization of SLs-enriched membrane areas. In the case of GSLs, a specific PM associated sialidase (Neu3) has been identified and cloned [10,11,12], and its role in modifying the ganglioside composition at the cell surface, acting as well on GSLs molecules present on the surface of adjacent cells (i.e., in a "trans" fashion), has been proven [13,14,15].

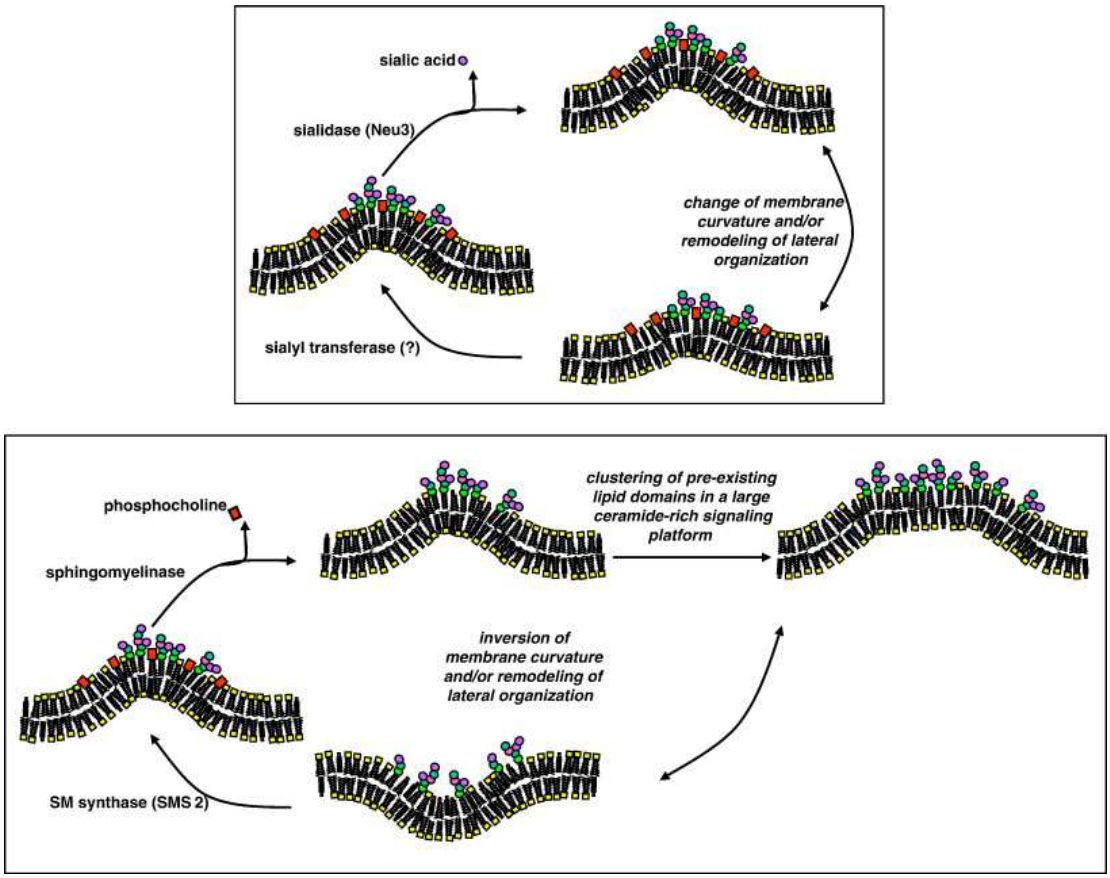


Fig. 1 Regulation of SLs-enriched membrane domains composition by phosphorylation/dephosphorylation ("SM cycle") and glycosylation/deglycosylation.

Moreover, the presence of sialyltransferase activities at the cell surface has been also reported [16-20]. For example, it was recently reported the presence CMP-NeuAc:GM3 sialyltransferase (Sial-T2), that is able to sialylate GM3 at the PM (cis-catalytic activity) by using both the exogenous and endogenous donor (CMP-NeuAc) and acceptor (GM3) substrates [21]. Thus, glycosylation/deglycosylation cycles might be very important mechanisms responsible for rapid and possibly transient changes of the PM-GSLs composition, in analogy to that proposed for the "SM cycle" (Figure 1). The presence of other active glycohydrolases such as β-glucosidase (β-Glc), β-galactosidase (β-Gal) and β-hexosaminidase (β-Hex) [13,22] in the PM has been demonstrated, implying that local hydrolysis of GSLs at the cell surface might represent a general mechanism for the control of GSLs composition.

ii. GSLs can be released from the cell surface in different forms, including shedding vesicles [23-25], whose controlled release from specific glycolipid-enriched membrane areas could represent a further way to modify the lipid membrane domain composition and organization.

MEMBRANE GLYCOHYDROLASES

PM glycoconjugates show changes during development and neoplastic transformation [26,27] suggesting a specific role in cell–cell recognition. Glycosyl epitope changes include many different residues, such as GlcNAc, GalNAc in N-linked structures or sialyl residues in either protein N-linked, O-linked or lipid linked structures, so that cell surface glycohydrolases could be one of the natural candidate for PM glycosylation modification.

For this reason, although in the past little attention was paid to glycohydrolases acting on cellular compartment different from lysosomes, there is now increasing interest on PM associated glycohydrolases: in this compartment they can be involved, together with glycosyltransferases, in GSLs oligosaccharide modification processes regulating cell to cell and/or cell–environment interactions in both physiological and pathological mechanisms [15,28,29] .

Glycohydrolases are the enzymes involved in GSLs metabolism. The presence of glycohydrolases on the PM, where they can be involved in the modifications of the cell surface GSLs or of glycoproteins, has been demonstrated [30]. This class of enzymes are taken into account as they can participate in the modulation of signal transduction processes.

Some of the PM associated glycohydrolases are identified and their activity has been studied for characterization. They are:

- ✓ β-Hexosaminidase
- ✓ Sialidase
- ✓ β-galactosidase
- ✓ β-glucosidase
- ✓ Sphingomyelinase

The presence of β-Hex A (Figure 2) in the external leaflet of PM has been demonstrated in cultured fibroblasts [22]. Immunological and biochemical characterization of the membrane-associated β-Hex indicated that this enzyme has the same structure of that in lysosomes.

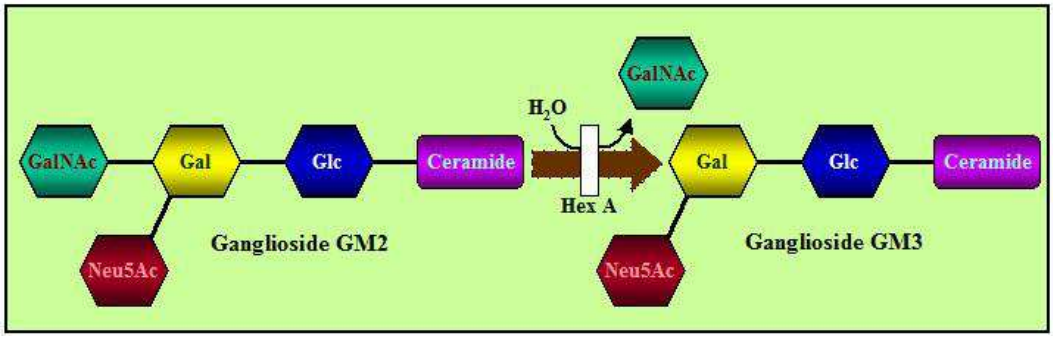


Fig.2 Reaction catalyzed by β-Hex A.

The linkage of sialic acid in sialic acid containing molecules is hydrolyzed by several different sialidases (Figure 3). Taking in account all the available data, Neu3 can be considered an ubiquitous enzyme being expressed at different levels in all the PM of tested normal and pathological human tissues such as human brain [31] normal and colon rectal carcinoma tissue, hepatic tumor and kidney carcinoma [32-36]. In addition its expression and activity were also found in normal and pathological cell lines such as erytroid and erytroleukemic cells [37-39], human fibroblasts [40], rat and mouse neurons, neuroblastoma cells [41], human breast ductal cancer T47D cells, colon carcinoma CaCo2 cells, human colorectal adenocarcinoma HT29 cells, different type of ovarian cancer cells, human cervix adenocarcinoma HeLa cells [36]. It has been demonstrated the presence of Neu3 in the lipid rafts and its co-localization with gangliosides: the non-random distribution of Neu3 at the cellular surface introduces the possibility that the biological effects of this enzyme might be due to the local reorganization of GSLs-based signaling units. Remarkably, Neu3 is capable to modulate the cell surface glycolipid composition because of *trans* interactions, thus modifying the surface of neighboring cells [15].

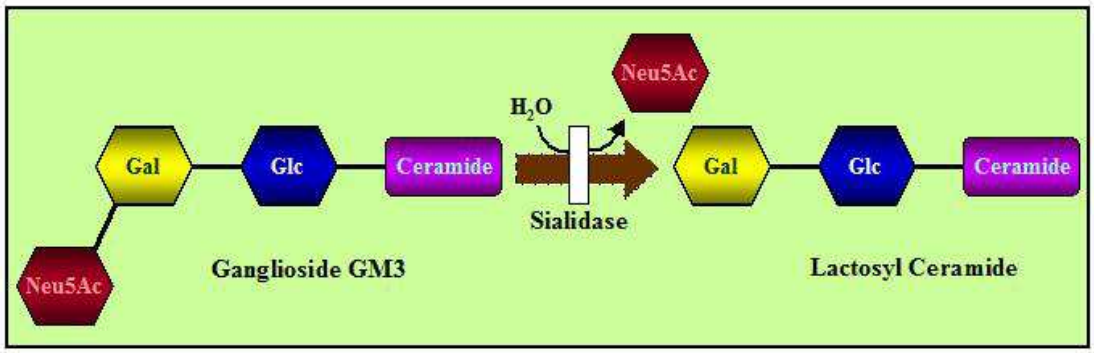


Fig.3 Reaction catalyzed by sialidase.

PM associated β-Gal (Figure 4) has been found in all cell lines studied for the activity of Neu3. In human fibroblasts its expression is up regulated by Neu3 [13]. The β-Gal activity was measured during neuronal cell differentiation and aging, in the total cell lysate and in the PM from rat cerebellar granule cells.

Both these activities resulted up regulated during cell differentiation. As expected the β-Gal activity associated to the cell PMs was much less than that found in total cell homogenate. The total cell activity remained constant during differentiation, then increased 4 fold along aging in culture. Instead, cell surface activity increased 10 fold during differentiation, to then duplicate during the neuronal senescence [42]. β-Gal activity has been proposed as senescent marker of different cell lines [43-46]. The information on the behavior of PM associated enzyme in rat cerebellar granule cells suggests that its activity could be used as a good marker for both

neuronal differentiation and neuronal aging. The identity of the protein, or proteins, responsible for the β-Gal activity present at the cell surface is still unknown, however in living human fibroblasts has been verified the presence of a β-Gal activity which displays a *trans* activity, with no necessity of detergents or activator proteins, on lactosylceramide (LacCer) immobilized on cell culture plates, suggesting that on the cell surface is present at least an enzyme having a β-galactocerebrosidase-like activity [30].

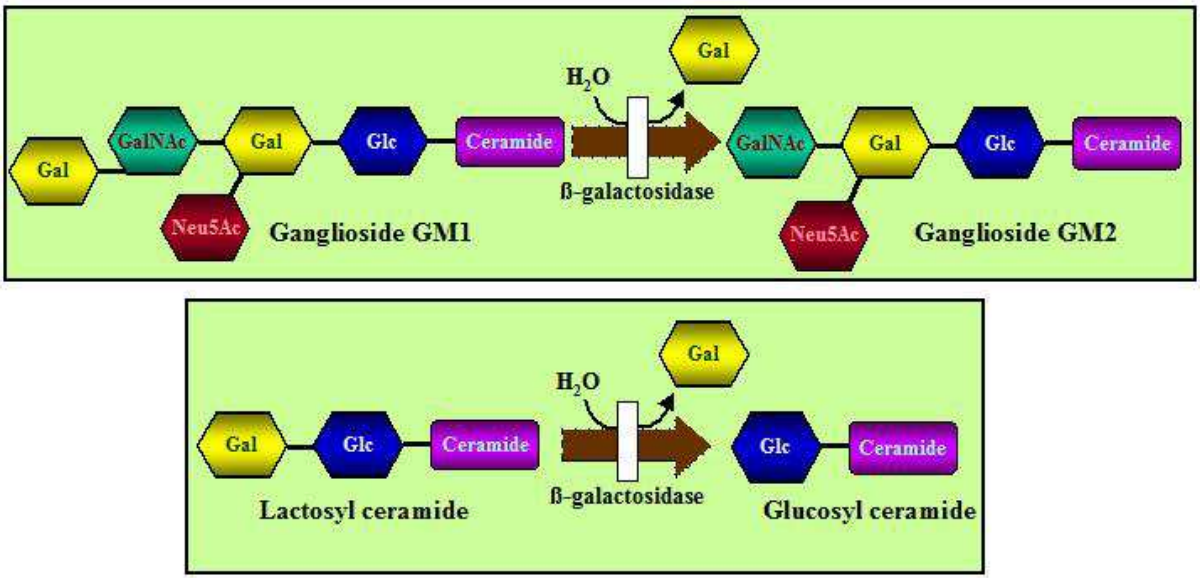


Fig.4 Reaction catalyzed by β-Gal.

Concerning β-Glc (Figure 5), at least three different enzymes have been described: a β-glucocerebrosidase sensitive to the inhibition of Conduritol B Epoxide (CBE) normally described associated to the lysosomes [47], a cytosolic β-Glc [48] and a non-lysosomal β-glucosylceramidase (GBA2). GBA2 has been found associated to endosome vescicles or to the PM, it is insensitive to CBE whereas is specifically inhibited by N-(5-adamantane-1-yl-methoxy)pentyl)-deoxynojirimycin (AMP-DNM) [49,50]. Recently it has been described that a CBE-sensitive β-Glc activity is present in the PMs [30]. Both CBE-sensitive and CBE-insensitive β-Glc activities were found associated to the cell surface of rat cerebellar granule cells in culture, where they progressively increase along the differentiation and the aging process [42].

Total β-Glc activity associated to the PMs of human living fibroblasts works on natural substrates *in trans* fashion [30] and the activity is connected to Neu3 activity. In fact, in human fibroblasts over-expressing Neu3, the β-Glc activity associated to the PM was much higher of that found in normal fibroblasts. Surprisingly this is mainly due to an up-regulation of the CBE-sensitive β-glucosidase [30].

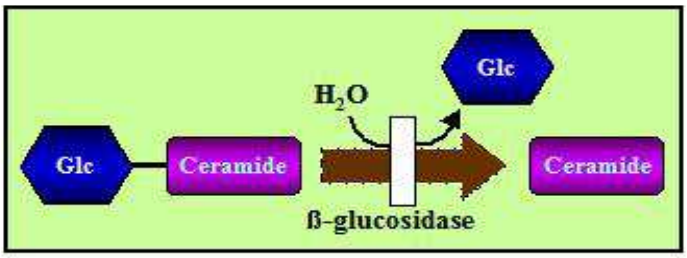


Fig.5 Reaction catalyzed by β-Glc.

Three different SMases are available in eukaryotic cells [51]: secreted SMase, acid SMase and neutral SMase (nSMase). Secreted SMase exerts its activity in the extracellular environment. Acid SMase are principally located in the lysosomes and few evidences reports about its association to PM. Neutral SMase identify a family of different enzymes that catalyze the hydrolysis of SM into Cer at pH 7.4 and are Mg^{2+}-dependent enzymes. Among the three recently cloned mammalian neutral SMases, only nSMase2 and nSMase3 display *in vivo* activity. nSMase3 is reported to localize to the endoplasmic reticulum and the Golgi compartment [52,53] while the subcellular localization of nSMase2 remains not fully determined. nSMase2 was first described as a Golgi-associated protein potentially involved in the Golgi secretory vesicle trafficking [54], however other studies have indicated a PM localization of overexpressed GFP-, FLAG- or V5-tagged-nSMase2 in various cancer cell lines [55-57]. This enzyme is characterized by the presence of two palmitoylation sites that were reported to be important for PM localization [58]. Furthermore, stimuli such as TNFα, H_2O_2 and cell confluence seemed to induce nSMase2 translocation from the Golgi to PM lipid rafts [51].

Therefore the glycol-hydrolytic enzymes, responsible for the catabolism of the SLs, are not only recovered in the classical cellular compartment deputed to the catabolism (the lysosomes) but are also associated to other cellular district. In some case the same lysosomal enzyme has been found associated to other compartments, as is the case of the β-Hex A associated to the PM; in some others, enzymes codified by different genes have been found in different regions of the cell. This last is the case of the sialidases Neu2, Neu3 and Neu4 with respect to the lysosomal enzyme Neu1, or the case of the non-lysosomal β-glucosylceramidase GBA2.

Considering the presence of lysosomal glycohydrolases at the cell surface, two aspects need to be clarified: how lysosomal deriving enzymes can reach the PM and how they can be active at the cell surface where the pH conditions are far from those of the lysosomes? It is known that a regulated fusion of lysosomes with the PM might represent a general mechanism of repairing for the PM [59], and could be responsible of the presence of lysosomal enzymes facing at the cell surface. However this enzymatic "translocation" could not be accidental and the fusion of lysosomal membranes with the PM could be the way for all the lysosomal glycolipid-metabolizing enzymes

to reach the cell surface where, together with specific and different membrane associated enzymes, play an active role in remodeling the glycolipid content and pattern of the external leaflet of the PM.

At the cell surface the pH conditions are usually considered near to the neutrality, whereas the lysosomal pH is much more acidic. For some glycohydrolases described associated to the PM has been evaluated the optimum of pH to which they play their catalytic activity. Both the β-Hex A and Neu3 (respectively codified by the same and by a different gene with respect to the corresponding lysosomal enzyme) has been found working in acidic condition (pH 4.45 and pH 4.2 respectively). This information suggests that modulation systems, capable to modify the pH conditions, even locally, could be present at the cell surface. One hypothesis for this theory may be the action of proton pumps present at the PM level for which has already been demonstrated a role in the acidification of the tumor extracellular environment [60-62].

GLYCOSYLATION and CANCER MALIGNANCY

Aberrant glycosylation, at the cell surface level, has been observed in essentially all type of human cancer. This event contributes to deep alterations in the interactions between tumor cells and the extracellular environment, which underline many of the features that defined several tumor phenotype, including self-sufficiency in growth signals, insensitivity to growth-inhibitor stimuli, evasion from programmed cell death (apoptosis), sustained angiogenesis, and tissue invasion and metastasis (Figure 6) [27]

Alteration in glycan expression in cancer is usually a consequence of modification in the metabolic pathway of glycoconjugates, as a result of change in the biosynthesis, catabolism and intracellular trafficking of GSLs and glycoproteins.

Like reported above, changes of the glycoconjugate oligosaccharide structures and in particular changes of GSLs, can be obtained at the cell surface by the activities of PM-glycohydrolases.

Recent study indicate that the aberrant glycosylation could be a result of initial oncogenic phenomena, as well a key event for the induction of invasion and metastasis.

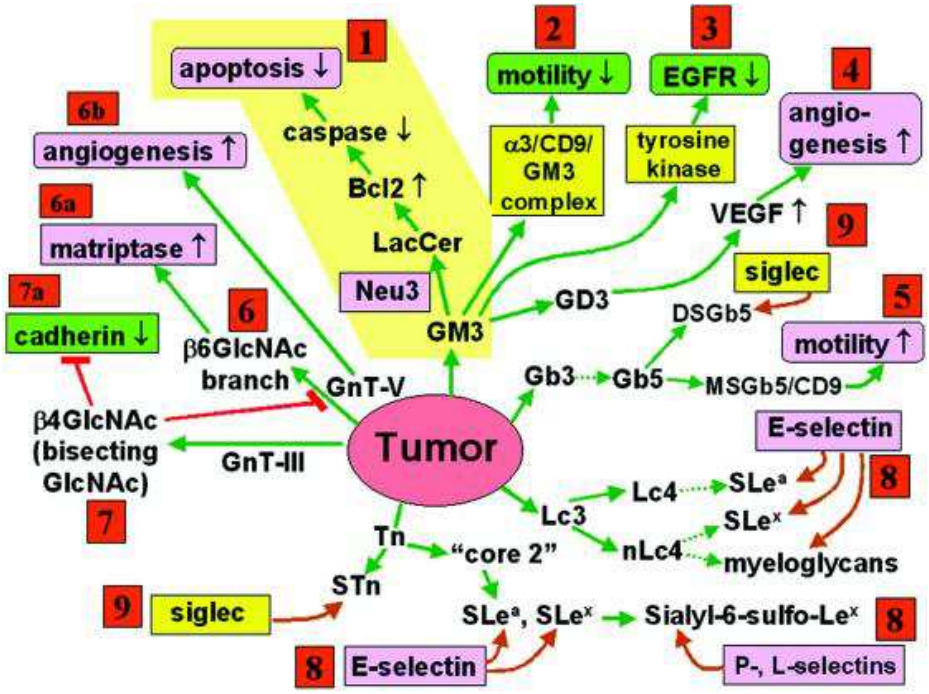


Fig. 6. Glycosylation defining malignancy (invasive and metastatic phenotype of tumors) (from [27]). Tumor cell malignancy is defined by several key phenotypes: apoptosis (route 1), motility (routes 2 and 5), EGF receptor tyrosine kinase (route 3), angiogenesis (routes4and6b), matriptase (matrix-destroyingenzyme)activity (route 6a), self-adhesion (through cadherin) (route 7a), adhesion to ECM (through integrin), adhesion to ECs and platelets (through E- or P-selectin) (route 8), adhesion to blood cells and other parenchymatous cells (through siglecs) (route 9). Each phenotype is up- or down-regulated by different status of glycosylation. Phenotypes with 1 or 2 and green color inhibit tumor invasiveness. Those with 1 or 2 and pink color promote invasiveness. Glycosyl epitopes capable of binding to specific ligands (pink color without arrow) promote invasiveness. Ligands with yellow color have variable or unclear effect on invasiveness.

Another important point of discussion regard the promotion or inhibition of the tumor progression depending on the glycosylation. It has been demonstrated that high expression of some glycosyl epitopes leads to a promotion of invasion of metastasis (i.e. β6GlcNAc branching in *N*-linked structure; sialyl-Tn in *O*-linked structure; sialyl-Le^x, sialyl-Le^a, and Le^y in either *N*-linked, *O*-linked, or lipid-linked structure; GM2, GD3, and sialyl-Gb5 in lipid-linked structure) while expression of other epitopes leads to an inhibition of tumor malignancy (β4GlcNAc, competitive with β6GlcNAc; histo-blood group A and B competitive with sialylated structures including sialyl- Le^x and sialyl-Le^a; Gb5 competitive with sialyl-Gb5) (Figure 6).

Very little is known about the mechanism of these glycosyl epitopes related to the expression of the respective glycosyltransferase genes, and how this epitopes are able to induces invasive and metastatic phenotypes in tumor cells.

However for some glycohydroles we have an important information and confirmations on their mechanisms of action in defining the cancer malignancy.

In particular, a specific role for sialidase Neu3 has been proposed in intestinal and prostatic tumor cells resistant to therapy. These cells express higher quantity of Neu3 with respect to those belonging to the tissue around the tumor. Neu3 hydrolizes the PM ganglioside GM3, maintaining high the PM LacCer level. LacCer has been shown to act as anti-apoptotic compound, thought the increased Bcl-2 and decrease of caspase expression, and this is believed to be a system devised by the tumor to escape apoptosis and to proliferate [36,63].

On the other hand, the SM is one of the main SL component of the PM, and the production of pro-apoptotic Cer by SM hydrolysis by activation of SMases, has been reported to participated to the tumor reduction upon treatment of with ionizing radiations.

EFFECT of IONIZING RADIATION

Radiotherapy represents the therapeutic strategy for the treatment of an increasing number of tumors. Recently various schemes of radical radiation therapy, based on the excellent results of phase I-II study using the hypofractionation in different cancer diseases, have been developed. Thanks to the application of conformal/intensity-modulated techniques in combination with precise Image-guided localization of the target, radiotherapy dose escalation has been shown to improve disease control. Therefore, it should be possible to use a larger dose per fraction without increasing the risk of serious late injury to the normal tissues. In the last years, studies of radiobiology focused to provide rationale for evaluating proteins known to play an important role in the control of the apoptotic pathway as potential biomarkers. Despite some limitations, such as time-consuming procedures, low resolution due to heterogeneity of proteins and post-translational modifications, proteomic approach could be used to study molecular biomarkers to predict and follow the radiotherapy response.

Irradiation of cancer cells leads to cell death by different mechanisms as summarized in Figure 7 (extracted from [26]). DNA damage (1), mitochondrial damage (2) and superoxide production (3) bring mainly to necrosis, whereas it has been demonstrated that also Cer is involved in cell death after irradiation via the induction of apoptosis (4)

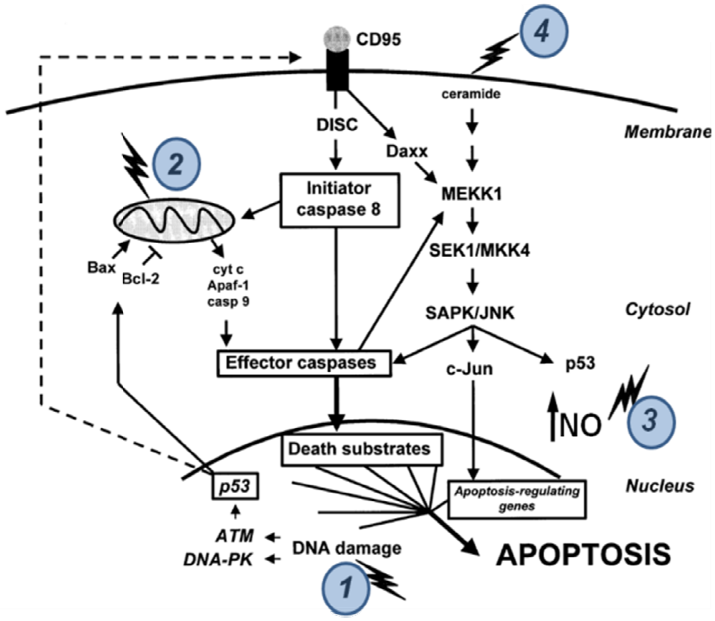


Fig.7 Irradiation induced apoptotic-pathways. Irradiation of cancer cells leads to cell death by different mechanisms: 1. DNA DAMAGE: irradiation may induce irreparable DNA damage causing brakes in DNA strands or leading to replicational and transcriptional errors. In both cases DNA damage initiate apoptosis via p53-dependent mechanisms. 2.MITHOCONDRIAL DAMAGE 3.OXIDATIVESTRESS Irradiation leads to reactive oxygen species formation, bringing mainly to necrosis 4.PM MODIFICATION: it has been demonstrated that also Cer is involved in cell death after irradiation via the induction of apoptosis . Scientific literature reports that Cer-mediated apoptosis, caused by the cell irradiation, is due to the activation of cell surface SMase and the following activation of the Cer-dependent phosphorylation cascade.

So far, an effect of ionizing radiations on the PM activities of glycosidases has never been reported, however some data in the literature suggested that these enzymes could contribute to the formation of pro-apoptotic Cer under some circumstances. It has been demonstrated that in human fibroblasts the over-expression of Neu3 causes the onset of apoptosis due to an increase in the PM Cer production by a parallel increase of expression/activity of β-Gal and β-Gluc as cellular response [13,30]. Activation of glycosidases by ionizing radiations could change the ratio between the apoptotic Cer and the antiapoptotic/proliferative LacCer (Figure 8). This could explain why some tumors are more respondents to irradiation than others. PM SMases has been reported in the above described apoptotic process [64].

The Cer produced by SM or glycolipid hydrolysis forms large Cer-enriched membrane platforms, instrumental for the operation and modulation of cell signaling processes.

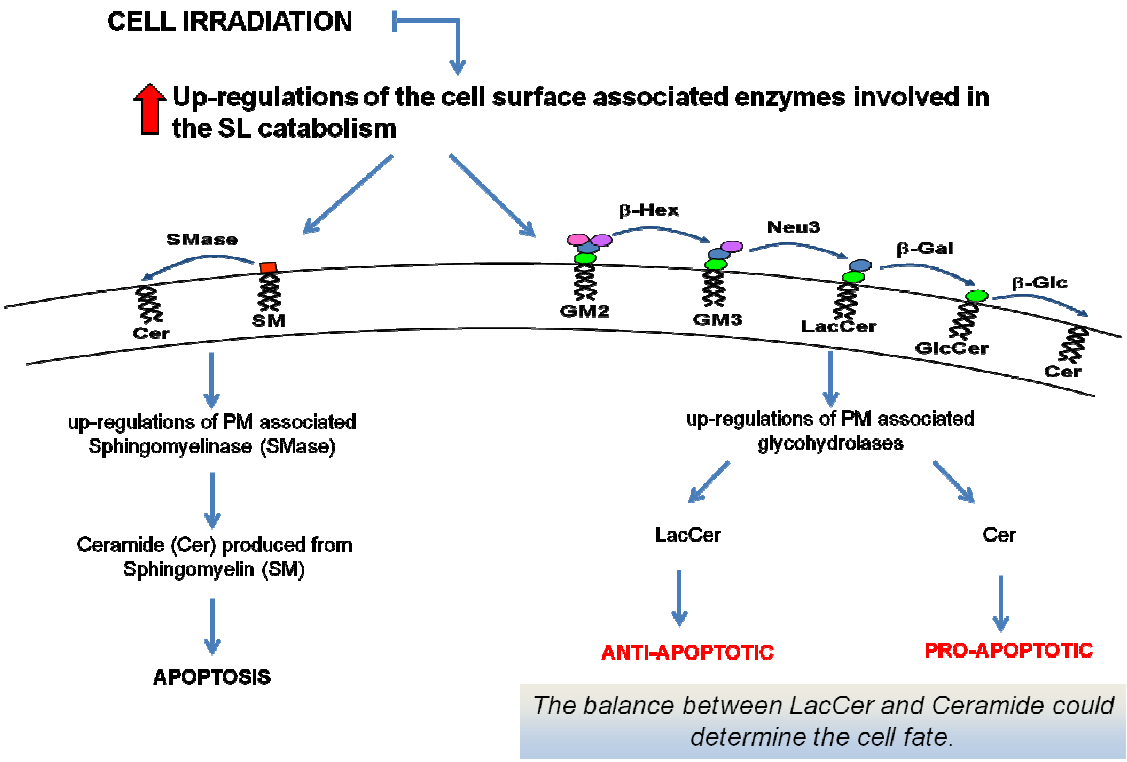


Fig. 8 Up-regulation of the cell surface associated enzymes involved in SLs-catabolism after cell irradiation.

Aim

The aim of this study is to understand the role played by the SL glycohydrolases in the production of Cer in order to develop, by *in vitro* studies, therapeutic strategies that combine radiotherapy with different approaches. These findings could allow the modulation of the cell surface associated glycohydrolases and/or the cell surface glycoconjugate composition**.**

Matherials and Methods

MATERIALS

Commercial chemicals were of the highest purity available, common solvents were distilled before use and water was doubly distilled in a glass apparatus. Trypsin, KCl, Trypan blue, reagents for cell culture were from Sigma Chemical Co. (St Louis, MO, USA). MEM, DMEM and RPMI-1640 as well as fetal calf serum (FCS) were purchased from EuroClone (Leeds, UK); AM-calcein, DMEM-F12 were from Invitrogen (Carlsbad, CA, USA). 6-hexadecanoylamino-4-methylumbelliferone (H-MUB) 4-methylumbelliferone (MUB), 4-methylumbelliferyl-β-D-galactopyranoside (MUB-Gal) and 4-methylumbelliferyl-β-D-glucopyranoside (MUB-Glc) were from Glycosynth (Warrington, UK).
6-hexadecanoylamino-4-methylumbelliferyl-phosphorylcholine (H-MUB-PC) was from Moscerdam Substrates (Oegstgeest, Netherland). Hoechst 33258 (2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole trihydrochloride trihydrate) was from Thermo Scientific (Rockford, IL, USA). High-performance silica gel thin-layer plates (HPTLC Kieselgel 60) were from Merck GmbH (Milan, Italy).
The preparation of isotopically labeled [3-^{3}H(sphingosine)]GM3 (specific radioactivity, 2.3 Ci/mmole) and of [1-^{3}H]sphingosine (specific radioactivity, 2.2 Ci/mmole) has been described in detail [65].
Human normal and pathological fibroblasts were prepared as described [66]. Pathological cells were prepared from a 7 year old Niemann-Pick type A patient (NPD). These cells had an acidic SMase activity on the artificial substrate H-MUB-PC lower than 9% of the activity present in normal human fibroblasts and a six fold increase of sphingomyelin content [67]. Tumor cell line T47D from breast tumor, HT29 and CaCO2 from colon carcinoma, SHSY-5Y from neuroblastoma tumor; A2780, A2780/HPR and A2780/Cis from ovary tumor [68-70] were available in the laboratory.

METHODS

Cell Cultures

CaCO2 were cultured and propagated in MEM; A2780 and T47D in RPMI-1640. All of the cultures medium were supplemented with 10% FCS (heat-inactivated for all cell lines except fibroblasts), 1% glutamine, 1% penicillin/streptomycin. The cells were cultivated as monolayer in a humidified atmosphere at 37°C and 5% CO_2.

Irradiation of cells

The most widely used technique for mathematically modeling the effect of radiation on cancer cell survival, is linear quadratic (LQ) formalism. The core formula involved is an expression which

correlate the probability of survival, S, of a population of mammalian cells following exposure to an individual radiation dose, d, as follows:

$$S= \exp[-\alpha D-\beta D2]$$

where α and β are tumor- or normal tissue-specific parameters. Rearrangement of the equation to account for the total impact of n individual fractions of radiation exposure yields another common expression for the biologically effective dose (BED):

$$BED=nd(1+/\alpha/\beta)$$

The units of BED are Gray (Gy), the standard unit of radiation dose. The BED is a convenient metric for comparing the relative impact of a given schedule of radiation dose on a given tumor or tissue, as long as the α/β ratio is known or closely estimated. The usual value for tumors is approximately 10 Gy, but intraoperative methods for delivering hypofractionated partial breast irradiation suggested that single doses of up to 21 Gy were tolerated for small tumors (≤2.5 cm) with excellent results regarding tumor control. In this study, preliminary experiments allowed to increase this dose without a very acute toxicity effect on the mammalian cells irradiated. We irradiated the cell lines with 6 Mev photons (direct field) with a linear accelerator. We utilized a dose escalation scheme delivering four dose of 10 Gy, 20 Gy, 40 Gy and 60 Gy, with enzymatic activity evaluated after every fraction (from 10 min to 48 hr after irradiation). The same enzymatic analysis was performed simultaneously on non- irradiated cells.

Determination of cell viability and apoptosis

At the end of the enzyme assays and at different time after irradiation cell viability was assessed by calcein staining and by Trypan blue exclusion [42], whereas cell apoptosis was determined by Hoechst 33258 fluorescent staining. For the calcein staining, cells were washed with PBS, the plate was then put on ice and 200 µl of a calcein-AM solution (6.25 µg/ml in PBS) were added to each well. Cells were incubated for 15 minutes at 37°C, 5% CO_2. Calcein-AM was then removed and 100 µl of PBS and 50 µl of 1% Triton X-100 were added to each well. Plate was stirred at RT for 15 minutes and then the fluorescence was detected by a microplate reader (Victor, Perkin-Elmer).

The number of living and dead cells has been determined by counting cells after Trypan blue staining, as previously described [71].

For the detections of apoptosis, control and irradiated cells were stained with a PBS solution containing 10 µg/ml of Hoechst 33258 at room temperature for 10 minutes in the dark and then cells were washed three times with PBS and analyzed by fluorescence microscope. Due to the chemical properties of the Hoechst 33258 in the condition mentioned above, only the apoptotic

cells show the nuclear staining [72]. This was confirmed by the evaluations of the changes of nuclear chromatin morphology. A minimum of 700 cells were scored for the incidence of chromatin apoptotic changes (condensation, compaction along the periphery of the nucleus and segmentations) as previously described [73].

Plasma Membrane (PM) associated hydrolase assays

We recently described a simple method [42], that allows to measure the activity of several glycohydrolases associated with the PMs of intact living cells. This method is based on the observation that the fluorogenic substrates commonly used for the *in vitro* assay of glycohydrolase activity (i.e., MUB-Glc, MUB-Gal) are not taken up by living cells [50]. In fact, under the appropriate experimental conditions, we did not observe any fluorescence associated with the cells. Moreover, the artificial substrates were not subjected to spontaneous, non-enzymatic hydrolysis nor hydrolyzed in observable entity by secreted enzymes. Thus, their hydrolysis under these experimental conditions is due exclusively to the PM associated enzymatic activities [30,42].

PM associated β-galactosidase (β-Gal-ase), β-glucosidase (β-Glc-ase) and SMase activities were determined in control or irradiated living cells plated in 96-well microplate at the density of $33X10^3$ cells/cm^2 by a **h**igh throughput assay (HTA). The β-Gal-ase and β-Glc-ase activities were assayed using the artificial substrates MUB-Gal and MUB-Glc solubilized in DMEM-F12 without phenol red at pH 6 at the final concentrations of 250 μM and 6 mM respectively. The SM-ase activity was assayed using the artificial substrates H-MUB-PC solubilized in the same DMEM-F12 reported for the other enzymes at the final concentration of 100 μM. At different times (from 30 min to 2 hours) aliquots of the medium were analyzed by fluorimetry in a microplate reader (MUB: λ_{ex}: 355 nm /λ_{em}: 460nm ; H-MUB: λ_{ex}:405 nm /λ_{em}:460nm) after adding 15 volumes of 0.25 M glycine (containing 0,3% Triton X-100 for SM-ase assay), pH 10.7. MUB and H-MUB were used to establish the calibration curve in order to quantify the substrates hydrolysis.

Control experiments were performed also in irradiated cells in order to exclude any activity released from lysosomes and/or from other intracellular sites. To this purpose, each enzymatic assay was performed on aliquots of medium previously maintained in contact with cells for different times. Moreover, to exclude any intracellular enzymatic degradation of the fluorigenic substrates, the absence of fluorescence associated with the cells was evaluated at the end of each enzymatic assay by fluorescent microscopy and by fluorimetric analysis of the lysed cells in 0.25 M glycine (pH 10.7) [30,42].

Activity of PM sialidase Neu3 was determined on the total homogenate using 6 μM [3-^{3}H(*sphingosine*)]GM3 as a substrate in the presence of detergent Triton X-100 [13]. This

experimental condition strongly reduced the activity of the other sialidases [74]. The enzymatic reaction was stopped by adding chloroform/ methanol (2:1 by vol) and the reaction mixture was analyzed by HPTLC using the solvent system chloroform/methanol/0.2% aqueous $CaCl_2$, 55:45:10 by vol. After chromatography, the separated radioactive lipids were detected and quantified by radioactivity imaging performed with a Beta-Imager 2000 instrument (Biospace, Paris, France), and the radioactivity associated with individual lipids was determined with the specific β-Vision software provided by Biospace.

Cell treatments with tritiated lipids

Treatment of cell cultures with [1-^{3}H]sphingosine. [1-^{3}H]sphingosine was administered to 20, 40 and 60 Gy irradiated and control T47D cells in order to metabolically label all SLs as previously described [75]. [1-^{3}H]sphingosine dissolved in methanol was transferred into a sterile glass tube, and dried under a nitrogen stream; the residue was then solubilized in an appropriate volume of pre-warmed (37°C) cell medium to obtain the desired final concentration ($3x10^{-9}$ M). After a 2 hour incubation (pulse), the medium was removed, cells were washed and incubated up to 72 hr (chase) with cell medium not containing radioactive precursor. After chase the cells were collected and radioactive lipids were analyzed as described below.

Treatment of cell cultures with [3-^{3}H(sphingosine)] GM3. Isotopically labeled [3-^{3}H(sphingosine)]. GM3 was administered to both T47D control and irradiated cells (40 Gy dose) after 24, 48 and 72 hours from irradiation. [3-^{3}H(*sphingosine*)]GM3 dissolved in propan-1-ol/water, 7:3 (v/v), was transferred into a sterile tube and dried under a nitrogen stream. The residue solubilised in the cell culture medium without serum at the final concentration of $4.5x10^{-6}$ M. In order to follow both the lysosomal and PM metabolism of the gangliosides a part of the cells were pre-incubated with 50 μM chloroquine for 30 minutes in complete cell cultures medium in agreement with previously reported protocol [13]. After removal of the medium and rapid washing of cells, 1 ml of the medium containing the radioactive lipid were added to each dish and the cells were incubated at 37°C in the presence or not of 50 μM chloroquine for 4 hr. At the end of incubation, cells were washed fourth times with complete cell culture medium and then collected with PBS.

Radioactive lipid analyses

Cells were lyophilized and subjected to lipid extraction and SL analyses. Total lipids from lyophilised cells were extracted with chloroform/methanol/water 20:10:1 by vol, followed by

chloroform/methanol 2:1 by vol. The total lipid extracts were subjected to a two-phase partitioning by adding 20% water to the lipid extract; the total lipid extract, the aqueous and organic phases were analysed by HPTLC (1000 dpm/lane). [^{3}H]SLs of total extracts and organic phases were separated using the solvent system chloroform-methanol-water 110:40:6 by vol, and those of aqueous phases with chloroform-methanol-0.2% aqueous $CaCl_2$, 50:42:11 by vol. For the specific determination of the Cer content the radioactive lipids of the organic phases were separated using the solvent system hexane-chloroform-acetone-acetic acid 20:70:20:2 by vol. [^{3}H]SLs were identified by referring to the position of standards in the chromatogram and quantified by radioimaging after 48-96 hours of acquisition (β-Imager 2000, Biospace, Paris, France).

Statistics

All the experiments have been performed in fivefold and repeated three times. Data are presented as the mean values ± standard deviations and were tested for significance employing ONE or Two way ANOVA with Bonferroni post-test analysis, as specified in figure legend. The level of significance was set at $p<0.05$.

Other analytical methods

The radioactivity associated with lipid extracts was determined by liquid scintillation counting.

The protein assays were carried out using the DC Protein Assay kit (Biorad).

Results

Here it is reported the first detailed information on the activation of several cell surface glycosidases in normal and pathological cells subjected to irradiation, an event accompanied by the production of PM Cer and the onset of apoptotic cell death [76].

i. PM glicohydrolases activity

Three cell lines (T47D cells from breast cancer, CaCO2 from colon carcinoma, and A2780 from ovary tumor) were analyzed for the presence of β-Glc, β-Gal, sialidase Neu3 and SMase activites associated to the PM. The PM enzyme activities were determined in living cells under condition that excluded contamination from intracellular activity.

As reported in Table 1, all of the tested cells showed a characteristic basal activity of the four enzymes to the PM.

Table 1 Specific cell surface enzymes activity in non irradiated tumor cell lines.

Cells	β-glucosidase pmoles/10^6 cells/hour	β-galactosidase pmoles/10^6 cells/hour	Neu3 pmoles/mg cell protein/hour	Sphingomyelinase
Normal fibroblasts	8,598±342	776±87	289±9	1,100±200
T47D	731±120	447±53	57±4	423±51
Caco-2	1,621±206	3,133±405	3,203±324	690±79
A2780	462±90	240±50	123±16	294±21

Each value is the mean of three independent experiments performed in five-fold replicate ± SD. B-gucosidase, β-galactosidase and sphingomyelinase activities were determined in living cells on artificial substrates. Sialidase Neu3 activity was determined in the cell homogenate on ganglioside GM3. Sphingomyelinase activity was determined at pH 6 and is associated to both neutral and acidic activities.

ii. Effect of ionizing radiations on cell PM associated activity in tumor cells

Afterwards we analyzed the same tumor cell lines to assess the effect of irradiation on β-Glc, β-Gal, sialidase Neu3 and SMase activites associated to the PM.

As show in Figure 8, after 72h from cell irradiation (dose of 20/40/60 Gy) we observed a general increase, even if at different level for each cell line tested, with a significant difference for each enzyme considerate.

T47D CTRL PM activity (pmoli/10^6cells x h)	
β-Gal	447±53
β-Glc	731±120
SMase	4,23±0,5
NEU3	57±4,2

CaCO2 CTRL PM activity (pmoli/10^6cells x h)	
β-Gal	447±53
β-Glc	731±120
SMase	4,23±0,5
NEU3	57±4,2

A2780 CTRL PM activity (pmoli/10^6cells x h)	
β-Gal	447±53
β-Glc	731±120
SMase	4,23±0,5
NEU3	57±4,2

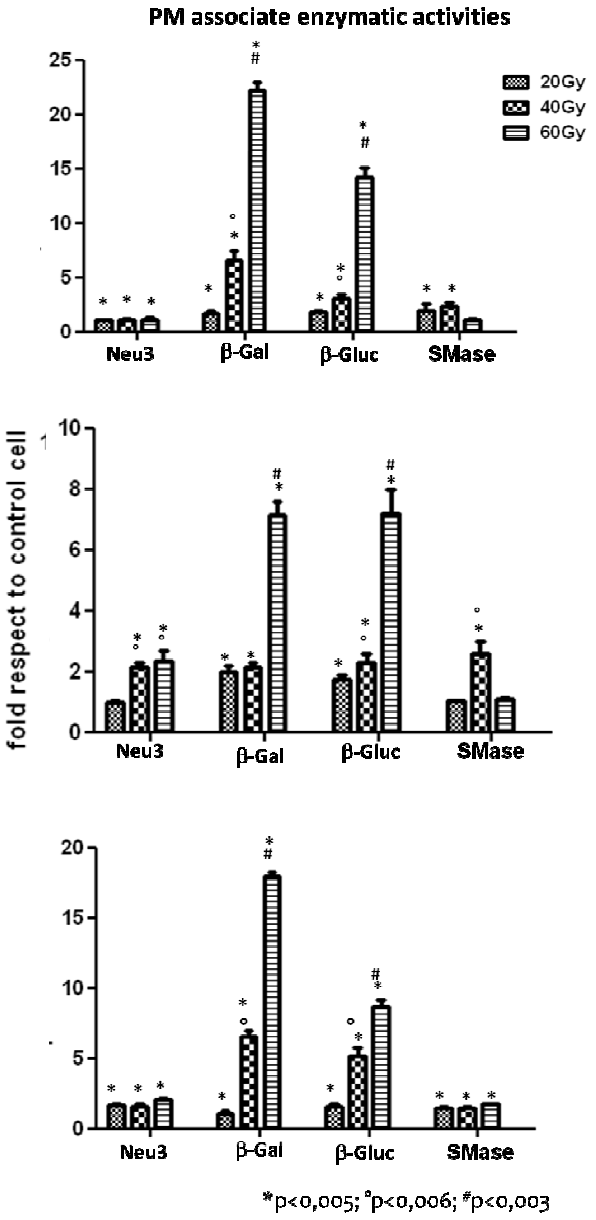


Fig. 8 Effect of ionizing radiations on cell PM associated activity in tumor cells. Effect of a dose of 20/40/60 Gy on the enzymes β-glucosidase, β-galactosidase, sialidase Neu3 and sphingomyelinase associated to the cell surface of breast tumor line T47D, of intestinal carcinoma cell lines CaCO2, of ovary tumor cell lines A2780. Enzyme activity associated to the plasma membranes was determined 72 hours from cell irradiation and is expressed as increase of activity with respect of non irradiated cells (control cells). Basal enzyme activities as reported in Table 1. Data represent the mean ± SD, n = 3 independent experiments, 3 replicates for each experiment. Two Way ANOVA followed by Bonferroni post-tests *p<0.0001 versus control cells, + p<0.001 vs the same enzyme in the previous cell line.

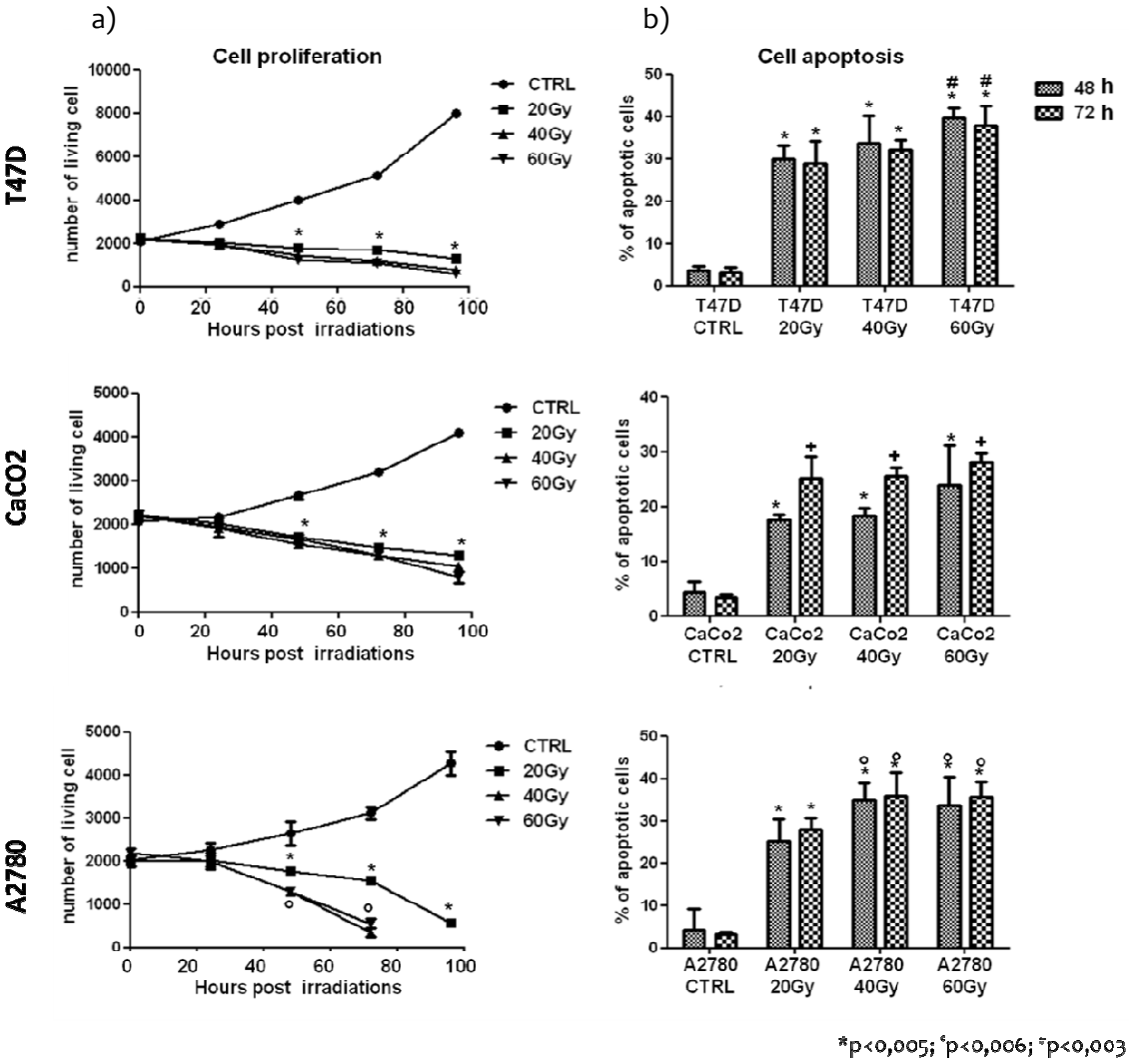
For A2780, T47D and Caco2 cells we observed a strong increase in the β-Gal and β-Glc activities (2-5 fold at 20 Gy; 2-7 fold at 40 Gy and 8-25 fold at 60 Gy). The activity of β-Gal and β-Glc increased

progressively with the dose. Instead the behavior of Neu3 and SMase was somehow different. In irradiated cells, both enzymes showed activity about two times higher of that the control cells (1.5-2.5 fold for all the treatments) . Nevertheless, the change of activity could not be modified by increasing dose of irradiation.

iii. Effect of ionizing radiations on cell death and apoptosis

Cells were irradiated up to 60 Gy and microscopically analyzed by Tripan Blue assay or by calcein staining. Figure 9 shows that the number of living cells progressively decrease along 96h in culture after receiving 20, 40, and 60 Gy, respectively.

Correspondently, the percent of apoptosis moved from 5% in control cells to 30-35% after 48/72h after irradiation. These results on cell growth and apoptosis suggest that the maximum effect due to irradiation is already obtained at the dose of 20 Gy.



*p<0,005; °p<0,006; #p<0,003

Fig. 9 Effect of ionizing radiations on cell death. Cells were irradiated with doses of 20, 40 and 60 Gy and assayed for cell growth, percent of apoptosis and plasma membrane sphingolipid hydrolases. Panel a, number of living cells determined by trypan-blue exclusion assay up to 96 h from cell irradiation. Panel b, percent of apoptosis determined at 24, 48 and 72 h from cell irradiation. Data represent the mean ± SD, n = 2 independent experiments, 3 replicates for each experiment. Two Way and one Way (in separated graph not shown) ANOVA followed by Bonferroni post-tests.

iv. Effect of ionizing radiations on Cer production.

Cell SLs were metabolically labeled with [1-H^3]sphingosine, submitted to irradiation, and the radioactive lipids analyzed for changes in their content with respect to the control. Figure 10 shows the tritium-imaging of TLC separation of lipids extracted from irradiated and control T47D, CaCO2 and A2780 after metabolic labeled with [1-H^3]sphingosine. Clearly, the double band corresponding to the Cer (due to the heterogeneity in acyl chains) displays a higher intensity in irradiated cells with respect to the control cells. Quantification of the spots showed that Cer significant increased. We calculated that the Cer increased up 3 times respect to the control after 72h, independently from the radiation dose. No significant differences were detected for other SLs between irradiated and control cells (data not shown), suggesting that the majority of this Cer is produced in lysosome.

v. Effect of ionizing radiations on Cer production at the PM level.

To better understand the role played by the SLs glycohydrolases in the production of Cer at the PM level after irradiation-dependent activation, T47D cells were subjected to irradiation (40 Gy) in normal condition or in conditions that block lysosomal activities (50 µM chloroquine) and then metabolic labelled with [^{3}H]GM3.

After a pulse with radioactive GM3, total lipids were extracted and analyzed for radioactivity content after TLC separation. The GM3 administered to the cells is taken up to the cells becoming component of PM. In the absence of chloroquine, GM3 enters the cell metabolic pathway of SLs and reach the lysosomes by membrane endocytosis. Under these conditions we found, in addition to a relatively large amount of unmetabolized GM3, LacCer, GlcCer, and Cer as catabolic products. We found also radioactive sphingomyelin, due to the recycling of sphingosine. This latter result is the proof that GM3 reached the lysosomes. Cells subjected to 40 Gy irradiation showed a similar catabolic pattern and a quantity of Cer 10-35% higher than that of control (Figure 11).

In the presence of chloroquine, lysosomes are not active and GM3 cannot be intracellularly catabolysed. Under these conditions, GM3 was again transformed into LacCer, GlcCer and Cer, but

no radioactive sphingomyelin could be observed in the radioactive lipid mixture. This result confirms that any change of the GM3 structures occurs at the cell surface, likely by sequential activity of sialidase Neu3, β-Gal and β-Glc. These cells showed a 150% increase of Cer after 40 Gy irradiation, reaching 200% after 72h irradiation. The increase of the cell Cer content in irradiated cells was followed by a significant reduction of GlcCer. This confirms the previous results obtained in fibroblasts [13] indicating that Cer is produced also at the PM and that its quantity depends by the activity of PM hydrolases.

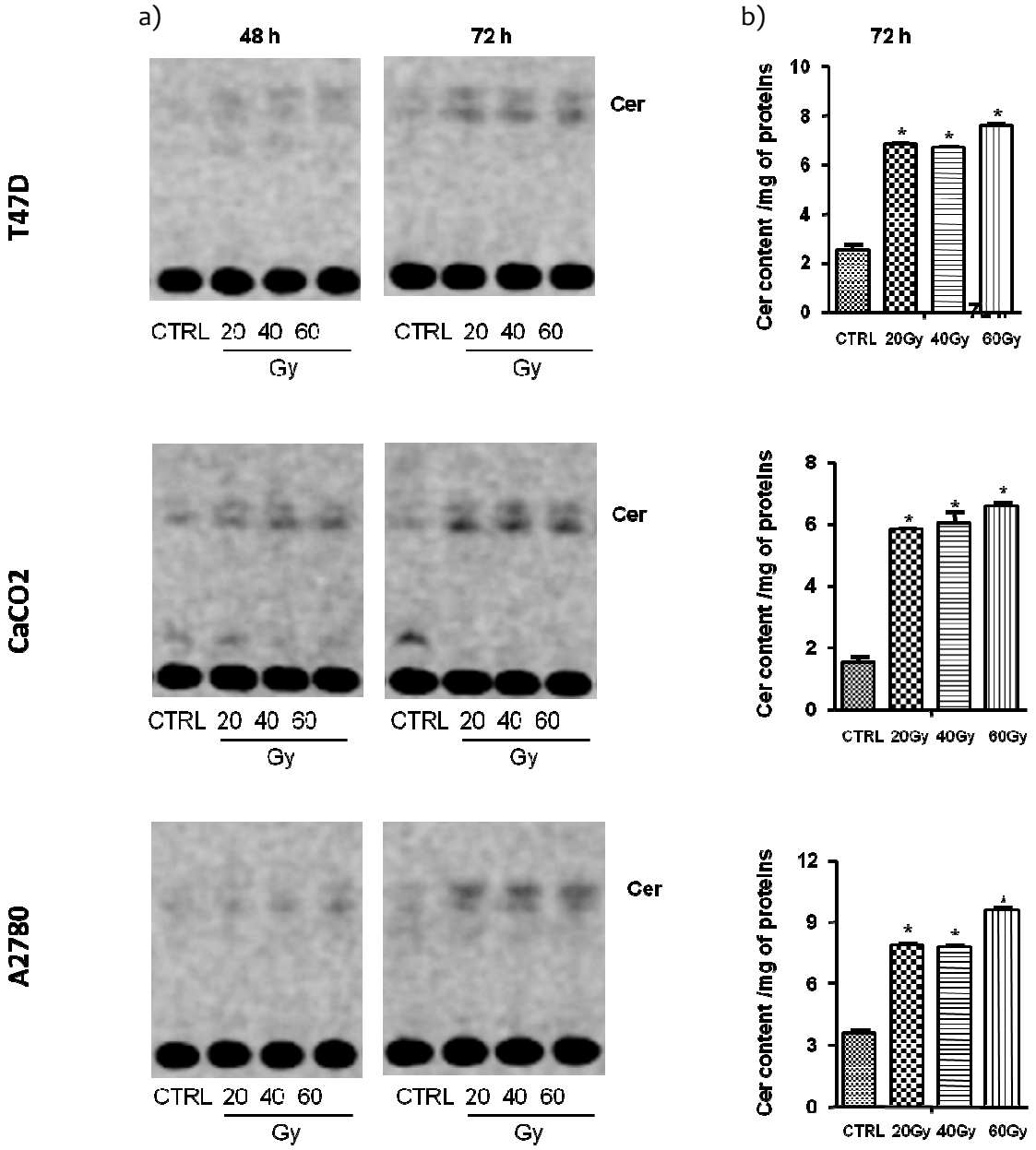


Fig. 10 Metabolic tritium labeling. Cells were metabolically labeled with [1-^{3}H]sphingosine and submitted to irradiation up to 20/40/60 Gy. Panel A, TLC chromatographic separation of the total radioactive lipids extracted from cells tritium labeled and subjected to irradiation. Extraction was performed 48/72 hr from irradiation. Total extract was separated on HPTLC silica gel 100 plates using the solvent system hexane-chloroform-acetone-acetic acid 20:70:20:2 by volume optimal for the ceramide separation. 1000 dpm were applied in each line and after separation submitted to 3 day counting for digital radio-imaging. Panel B, radioactivity quantification of ceramide spots reported in panel A (72h); data are expressed as increase of radioactivity with respect of non irradiated cells. Data represent the mean ± SD, n = 2 independent experiments, 3 replicates for each experiment. Two Way ANOVA followed by Bonferroni post-tests *p<0.004 versus control cells.

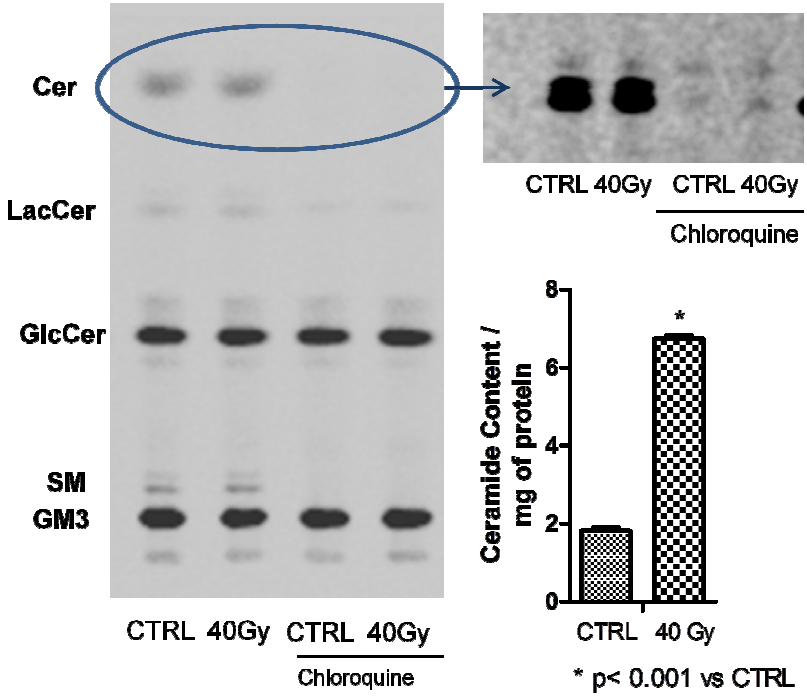


Fig. 11 Administration of isotopically tritium labeled GM3 to breast tumor cells T47D. Cells were maintained in culture in the presence of chloroquine to prevent lysosomal activity, and incubated with [3-^{3}H (sphingosine)]GM3 in the absence of serum. After a short pulse and irradiation with a dose of 40 Gy, the radioactive lipids were extracted and separated by TLC. Separation was carried out using the solvent system chloroform: methanol:water, 110:40:6 by vol. After TLC separation and radio-imaging, lipids were quantified for radioactivity content.Data represent the mean ± SD, no2 independent experiments, 3 replicates for each experiment. Two Way ANOVA followed by Bonferroni post-tests *p< 0.0003 versus control cells.

Discussion

SLs hydrolases are known from long time as lysosomal catabolic enzymes necessary to remove the hydrophilic head from SLs yielding Cer. Cer is then hydrolyzed by ceramidase to sphingosine that can be recycled or completely catabolysed, outside of lysosomes, to water and carbon dioxide [77].

In recent years a more detailed information became available on the association of some SL hydrolases to the PMs [78]. The availability of a simple methodology capable to measure the enzyme activities associated with PM in living cells, with no interference of the lysosomal activities, allowed to better understand the behavior of these enzymes [42]. Some of these enzymes become PM components as a consequence of fusion processes between lysosomes and PM, but some have a structure different from that of the lysosomal enzymes as for glucocerebrosidase GBA2, sialidase Neu3 and probably the neutral SMase [78]. The action of these enzymes to modifying the SL structure has been considered a system to modify the PM organization and properties, mainly related to the cell signaling processes [79]. An increase of activity of PM SMase is claimed to be responsible for the formation of large Cer-rich platforms instrumental for the cell signaling [80]. Activation of SMase by TNF-α leads to the Cer dependent phosphorylation cascade ending with cell apoptosis [81]. Activation of the PM SMase by the ionizing radiations used for radiotherapy of tumors, is well described to participate to the cell death and tumor reduction, through cell apoptosis caused by an increase of PM Cer [64,73]. Previously it has been showed that in human fibroblasts an increased expression of sialidase Neu3 was associated with an increase of activity of β-glucosidase and β-galactosidase, resulting in an increase of PM Cer and cell death by apoptosis [13].

Here we reported data on the behavior of PM glycosidases in cells treated with ionizing radiations, and present the first information on their possible participation to Cer production and cell death in radiotherapy treatments.

We treated several cell lines from breast, ovary, colon and neuroblastoma tumors. All of the cells showed basal PM activities of sialidase Neu3, β-Glc, β-Gal and SMase (table 1) that increased after cell irradiation. Nevertheless, the quantitative changes of these activities were different in the cell lines as reported in Figure 8.

A tremendous increase of activity of β-Glc and β-Gal was observed after treatment. The activity increased with the radiation dose and with time after the treatment. Neu3 and SMase reached the maximum value already at 20 Gy to remain then constant up to 60 Gy. Cells, at different dose or time from the treatment showed a similar mortality and similar percentage of apoptotic cells. This suggest that over a certain increase of dose and time from the treatment, the role of PM hydrolase activities, become less relevant. As described for the SM pathway, these signaling

mechanisms may be distinct from the p53-dependent apoptosis. Remarkably, the T47D cell line is characterized by high expression levels of mutant p53 [82].

To proof that, in parallel with an increase of the glycohydrolase activities, more Cer is actually produced in the PM, we administered isotopically tritiated lipids to tumor cells and we followed the fate of radioactivity under different experimental conditions. We first administered tritiated sphingosine in order to metabolically label all SLs, so that we could follow changes of the radioactive SL pattern after cell irradiation. These changes were originated by changes of all the cell SL metabolic enzymes, including lysosomal hydrolases whose activities are much higher of those associated with the PM. The radioactive pattern displayed changes mainly related to the content of neutral glycolipids, with the exception of Cer that triplicated in quantity. This suggests that SL hydrolases are probably activated by the ionizing radiations, but does not exclude an up-regulations of the CERs enzymes, as previously described [83,84]. To better understand the site and mechanism of Cer production in irradiated cells, tritiated GM3 was administered to T47D breast cancer cells. Our idea was to follow the production of PM Cer, due to the sequential activities of Neu3, β-Gal and β-Glc, under conditions that block lysosomal activity. To avoid lysosomal catabolism, we administered GM3 in the presence of chloroquine, a well known inhibitor of lysosomal activity, and followed its metabolic fate. In the absence of chloroquine, we observed a minor but statistically significant increase of Cer. The experimental conditions used, that privileged the permanence of administered ganglioside at the PM with respect to the intracellular localization and the high dilution of radioactive Cer deriving from the catabolism of exogenously administered ganglioside into non radioactive endogenous Cer, does not allow to overlap the result obtained by steady-state metabolic labeling of all SLs with tritiated sphingosine. In the presence of chloroquine, we had an high increase of Cer. We calculated that the absolute quantitative increase of Cer determined in the presence of chloroquine corresponds to the minor increase of total cell Cer in the absence of chloroquine. This suggests that under our experimental conditions, the Cer formed in lysosomes is rapidly catabolysed to sphingosine and that the observed increase of Cer is largely due to the production of a more time existing PM Cer.

The increase of PM Cer in cells subjected to ionizing radiations was shown to participate to the onset of cell death [85]. The activation of SMase has been regarded as the main mechanism for the production of pro-apoptotic Cer. The information presented in this paper that ionizing radiations also activate PM glycohydrolases suggests that PM Cer can derive by all PM SLs. This requires additional care for developing radiotherapy protocols. As reported in Figure 2, tumor cells show different patterns of enzyme activities. Thus, it would be necessary to know which is the ratio between LacCer and Cer at the end of irradiation. LacCer is an antiapototic compound and it could cancel or reduce the apoptotic effect of Cer.

The involvement of glycosidases in PM Cer production increases several times the number of enzymes that can participate to induce cell apoptosis and adds a new knowledge on the apoptotic mediated cell death induced by ionizing irradiations. We summarized this in Figure 6. The fact that PM glycosidases can participate to the induction of apoptosis in tumor cells open the possibility to design drugs capable to modulate their activity. This would allow to develop new chemotherapy treatments in combination with radiotherapy. Moreover the actions of the cell surface associated glycosidases are not confined to the GSLs metabolism but also to other cell surface associated glycoconjugates. Their modifications during the cell irradiation need to be further study in the future.

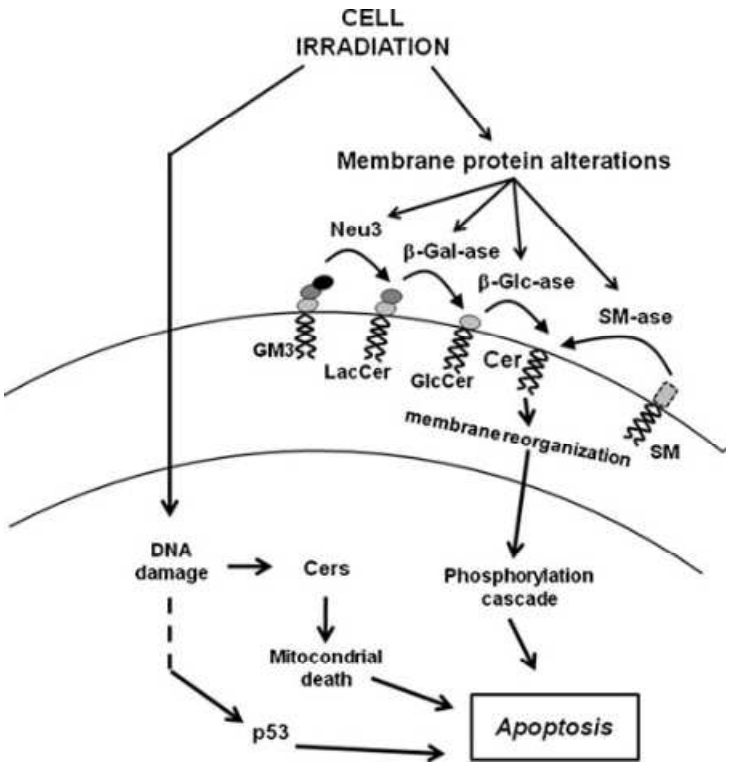


Fig. 12 Simplified scheme of the effects of ionizing radiations on cells in culture (from [76])

References

1. Lombardo, A., Caimi, L., Marchesini, S., Goi, G. C., and Tettamanti, G. (1980) Enzymes of lysosomal origin in human plasma and serum: assay conditions and parameters influencing the assay, *Clin Chim Acta 108*, 337-346.
2. Weng, S., and Spiro, R. G. (1996) Endoplasmic reticulum kifunensine-resistant alpha-mannosidase is enzymatically and immunologically related to the cytosolic alpha-mannosidase, *Arch Biochem Biophys 325*, 113-123.
3. Gopalan, V., Glew, R. H., Libell, D. P., and DePetro, J. J. (1989) The dual effects of alcohols on the kinetic properties of guinea pig liver cytosolic beta-glucosidase, *J Biol Chem 264*, 15418-15422.
4. Willemsen, R., Brunken, R., Sorber, C. W., Hoogeveen, A. T., Wisselaar, H. A., Van Dongen, J. M., and Reuser, A. J. (1991) A quantitative immunoelectronmicroscopic study on soluble, membrane-associated and membrane-bound lysosomal enzymes in human intestinal epithelial cells, *Histochem J 23*, 467-473.
5. Venerando, B., Goi, G. C., Preti, A., Fiorilli, A., Lombardo, A., and Tettamanti, G. (1982) Cytosolic sialidase in developing rat forebrain, *Neurochem Int 4*, 313-320.
6. van Meer, G., Voelker, D. R., and Feigenson, G. W. (2008) Membrane lipids: where they are and how they behave, *Nat Rev Mol Cell Biol 9*, 112-124.
7. Levade, T., and Jaffrezou, J. P. (1999) Signalling sphingomyelinases: which, where, how and why?, *Biochim Biophys Acta 1438*, 1-17.
8. Goni, F. M., and Alonso, A. (2002) Sphingomyelinases: enzymology and membrane activity, *FEBS Lett 531*, 38-46.
9. Huitema, K., van den Dikkenberg, J., Brouwers, J. F., and Holthuis, J. C. (2004) Identification of a family of animal sphingomyelin synthases, *Embo J 23*, 33-44.
10. Hasegawa, T., Yamaguchi, K., Wada, T., Takeda, A., Itoyama, Y., and Miyagi, T. (2000) Molecular cloning of mouse ganglioside sialidase and its increased expression in neuro2a cell differentiation, *J Biol Chem 275*, 14778.
11. Wada, T., Yoshikawa, Y., Tokuyama, S., Kuwabara, M., Akita, H., and Miyagi, T. (1999) Cloning, expression, and chromosomal mapping of a human ganglioside sialidase, *Biochem Biophys Res Commun 261*, 21-27.
12. Miyagi, T., Wada, T., Iwamatsu, A., Hata, K., Yoshikawa, Y., Tokuyama, S., and Sawada, M. (1999) Molecular cloning and characterization of a plasma membrane-associated sialidase specific for gangliosides, *J Biol Chem 274*, 5004-5011.
13. Valaperta, R., Chigorno, V., Basso, L., Prinetti, A., Bresciani, R., Preti, A., Miyagi, T., and Sonnino, S. (2006) Plasma membrane production of ceramide from ganglioside GM3 in human fibroblasts, *FASEB J 20*, 1227-1229.
14. Kalka, D., von Reitzenstein, C., Kopitz, J., and Cantz, M. (2001) The plasma membrane ganglioside sialidase cofractionates with markers of lipid rafts, *Biochem Biophys Res Commun 283*, 989-993.
15. Papini, N., Anastasia, L., Tringali, C., Croci, G., Bresciani, R., Yamaguchi, K., Miyagi, T., Preti, A., Prinetti, A., Prioni, S., Sonnino, S., Tettamanti, G., Venerando, B., and Monti, E. (2004) The plasma

membrane-associated sialidase MmNEU3 modifies the ganglioside pattern of adjacent cells supporting its involvement in cell-to-cell interactions, *J Biol Chem* 279, 16989-16995.

16. Preti, A., Fiorilli, A., Lombardo, A., Caimi, L., and Tettamanti, G. (1980) Occurrence of sialyltransferase activity in the synaptosomal membranes prepared from calf brain cortex, *J Neurochem* 35, 281-296.
17. Matsui, Y., Lombard, D., Massarelli, R., Mandel, P., and Dreyfus, H. (1986) Surface glycosyltransferase activities during development of neuronal cell cultures, *J Neurochem* 46, 144-150.
18. Durrie, R., Saito, M., and Rosenberg, A. (1988) Endogenous glycosphingolipid acceptor specificity of sialosyltransferase systems in intact Golgi membranes, synaptosomes, and synaptic plasma membranes from rat brain, *Biochemistry* 27, 3759-3764.
19. Durrie, R., and Rosenberg, A. (1989) Anabolic sialosylation of gangliosides in situ in rat brain cortical slices, *J Lipid Res* 30, 1259-1266.
20. Iwamori, M., and Iwamori, Y. (2005) Changes in the glycolipid composition and characteristic activation of GM3 synthase in the thymus of mouse after administration of dexamethasone, *Glycoconj J* 22, 119-126.
21. Crespo P. M., Demichelis V. T., Daniotti J. L. (2010) Neobiosynthesis of Glycosphingolipids by Plasma Membrane-associated Glycosyltransferases *J. Biol. Chem*. 285, 29179–29190
22. Mencarelli, S., Cavalieri, C., Magini, A., Tancini, B., Basso, L., Lemansky, P., Hasilik, A., Li, Y. T., Chigorno, V., Orlacchio, A., Emiliani, C., and Sonnino, S. (2005) Identification of plasma membrane associated mature beta-hexosaminidase A, active towards GM2 ganglioside, in human fibroblasts, *FEBS Lett* 579, 5501-5506.
23. Kong, Y., Li, R., and Ladisch, S. (1998) Natural forms of shed tumor gangliosides, *Biochim Biophys Acta* 1394, 43-56.
24. Deng, W., Li, R., and Ladisch, S. (2000) Influence of cellular ganglioside depletion on tumor formation, *J Natl Cancer Inst* 92, 912-917.
25. Chigorno, V., Giannotta, C., Ottico, E., Sciannamblo, M., Mikulak, J., Prinetti, A., and Sonnino, S. (2005) Sphingolipid uptake by cultured cells: complex aggregates of cell sphingolipids with serum proteins and lipoproteins are rapidly catabolized, *J Biol Chem* 280, 2668-2675.
26. Hakomori, S. (1996) Tumor malignancy defined by aberrant glycosylation and sphingo(glyco)lipid metabolism, *Cancer Res* 56, 5309-5318.
27. Hakomori, S. (2002) Glycosylation defining cancer malignancy: new wine in an old bottle, *Proc Natl Acad Sci U S A* 99, 10231-10233.
28. Lukong, K. E., Seyrantepe, V., Landry, K., Trudel, S., Ahmad, A., Gahl, W. A., Lefrancois, S., Morales, C. R., and Pshezhetsky, A. V. (2001) Intracellular distribution of lysosomal sialidase is controlled by the internalization signal in its cytoplasmic tail, *J Biol Chem* 276, 46172-46181.
29. Cordero, O. J., Merino, A., Paez de la Cadena, M., Bugia, B., Nogueira, M., Vinuela, J. E., Martinez-Zorzano, V. S., de Carlos, A., and Rodriguez-Berrocal, F. J. (2001) Cell surface human alpha-L-fucosidase, *Eur J Biochem* 268, 3321-3331.

30. Aureli, M., Masilamani, A. P., Illuzzi, G., Loberto, N., Scandroglio, F., Prinetti, A., Chigorno, V., and Sonnino, S. (2009) Activity of plasma membrane beta-galactosidase and beta-glucosidase, *FEBS Lett 583*, 2469-2473.
31. Kopitz, J., Sinz, K., Brossmer, R., and Cantz, M. (1997) Partial characterization and enrichment of a membrane-bound sialidase specific for gangliosides from human brain tissue, *Eur J Biochem 248*, 527-534.
32. Monti, E., Preti, A., Venerando, B., and Borsani, G. (2002) Recent development in mammalian sialidase molecular biology, *Neurochem Res 27*, 649-663.
33. Miyagi, T., Wada, T., Yamaguchi, K., Shiozaki, K., Sato, I., Kakugawa, Y., Yamanami, H., and Fujiya, T. (2008) Human sialidase as a cancer marker, *Proteomics 8*, 3303-3311.
34. Miyagi, T., Wada, T., and Yamaguchi, K. (2008) Roles of plasma membrane-associated sialidase NEU3 in human cancers, *Biochim Biophys Acta 1780*, 532-537.
35. Ueno, S., Saito, S., Wada, T., Yamaguchi, K., Satoh, M., Arai, Y., and Miyagi, T. (2006) Plasma membrane-associated sialidase is up-regulated in renal cell carcinoma and promotes interleukin-6-induced apoptosis suppression and cell motility, *J Biol Chem 281*, 7756-7764.
36. Kakugawa, Y., Wada, T., Yamaguchi, K., Yamanami, H., Ouchi, K., Sato, I., and Miyagi, T. (2002) Up-regulation of plasma membrane-associated ganglioside sialidase (Neu3) in human colon cancer and its involvement in apoptosis suppression, *Proc Natl Acad Sci U S A 99*, 10718-10723.
37. Venerando, B., Fiorilli, A., Croci, G., Tringali, C., Goi, G., Mazzanti, L., Curatola, G., Segalini, G., Massaccesi, L., Lombardo, A., and Tettamanti, G. (2002) Acidic and neutral sialidase in the erythrocyte membrane of type 2 diabetic patients, *Blood 99*, 1064-1070.
38. Tringali, C., Lupo, B., Anastasia, L., Papini, N., Monti, E., Bresciani, R., Tettamanti, G., and Venerando, B. (2007) Expression of sialidase Neu2 in leukemic K562 cells induces apoptosis by impairing Bcr-Abl/Src kinases signaling, *J Biol Chem 282*, 14364-14372.
39. Tringali, C., Anastasia, L., Papini, N., Bianchi, A., Ronzoni, L., Cappellini, M. D., Monti, E., Tettamanti, G., and Venerando, B. (2007) Modification of sialidase levels and sialoglycoconjugate pattern during erythroid and erytroleukemic cell differentiation, *Glycoconj J 24*, 67-79.
40. 40.Chigorno, V., Cardace, G., Pitto, M., Sonnino, S., Ghidoni, R., and Tettamanti, G. (1986) A radiometric assay for ganglioside sialidase applied to the determination of the enzyme subcellular location in cultured human fibroblasts, *Anal Biochem 153*, 283-294.
41. Schengrund, C. L., and Repman, M. A. (1982) Density-dependent changes in gangliosides and sialidase activity of murine neuroblastoma cells, *J Neurochem 39*, 940-947.
42. Aureli, M., Loberto, N., Lanteri, P., Chigorno, V., Prinetti, A., and Sonnino, S. (2010) Cell surface sphingolipid glycohydrolases in neuronal differentation and aging in culture, *J Neurochem*.
43. Coates, P. J. (2002) Markers of senescence?, *J Pathol 196*, 371-373.
44. Dimri, G. P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E. E., Linskens, M., Rubelj, I., Pereira-Smith, O., and et al. (1995) A biomarker that identifies senescent human cells in culture and in aging skin in vivo, *Proc Natl Acad Sci U S A 92*, 9363-9367.

45. Severino, J., Allen, R. G., Balin, S., Balin, A., and Cristofalo, V. J. (2000) Is beta-galactosidase staining a marker of senescence in vitro and in vivo?, *Exp Cell Res 257*, 162-171.
46. Geng, Y. Q., Guan, J. T., Xu, X. H., and Fu, Y. C. Senescence-associated beta-galactosidase activity expression in aging hippocampal neurons, *Biochem Biophys Res Commun 396*, 866-869.
47. Neufeld, E. F. (1991) Lysosomal storage diseases, *Annu Rev Biochem 60*, 257-280.
48. Daniels, L. B., Coyle, P. J., Chiao, Y. B., Glew, R. H., and Labow, R. S. (1981) Purification and characterization of a cytosolic broad specificity beta-glucosidase from human liver, *J Biol Chem 256*, 13004-13013.
49. Boot, R. G., Verhoek, M., Donker-Koopman, W., Strijland, A., van Marle, J., Overkleeft, H. S., Wennekes, T., and Aerts, J. M. (2007) Identification of the non-lysosomal glucosylceramidase as beta-glucosidase 2, *J Biol Chem 282*, 1305-1312.
50. Overkleeft, H. S., Renkema, G. H., Neele, J., Vianello, P., Hung, I. O., Strijland, A., van der Burg, A. M., Koomen, G. J., Pandit, U. K., and Aerts, J. M. (1998) Generation of specific deoxynojirimycin-type inhibitors of the non-lysosomal glucosylceramidase, *J Biol Chem 273*, 26522-26527.
51. Clarke, C. J., Truong, T. G., and Hannun, Y. A. (2007) Role for neutral sphingomyelinase-2 in tumor necrosis factor alpha-stimulated expression of vascular cell adhesion molecule-1 (VCAM) and intercellular adhesion molecule-1 (ICAM) in lung epithelial cells: p38 MAPK is an upstream regulator of nSMase2, *J Biol Chem 282*, 1384-1396.
52. Corcoran, C. A., He, Q., Ponnusamy, S., Ogretmen, B., Huang, Y., and Sheikh, M. S. (2008) Neutral sphingomyelinase-3 is a DNA damage and nongenotoxic stress-regulated gene that is deregulated in human malignancies, *Mol Cancer Res 6*, 795-807.
53. Krut, O., Wiegmann, K., Kashkar, H., Yazdanpanah, B., and Kronke, M. (2006) Novel tumor necrosis factor-responsive mammalian neutral sphingomyelinase-3 is a C-tail-anchored protein, *J Biol Chem 281*, 13784-13793.
54. Stoffel, W., Jenke, B., Block, B., Zumbansen, M., and Koebke, J. (2005) Neutral sphingomyelinase 2 (smpd3) in the control of postnatal growth and development, *Proc Natl Acad Sci U S A 102*, 4554-4559.
55. Kim, W. J., Okimoto, R. A., Purton, L. E., Goodwin, M., Haserlat, S. M., Dayyani, F., Sweetser, D. A., McClatchey, A. I., Bernard, O. A., Look, A. T., Bell, D. W., Scadden, D. T., and Haber, D. A. (2008) Mutations in the neutral sphingomyelinase gene SMPD3 implicate the ceramide pathway in human leukemias, *Blood 111*, 4716-4722.
56. Karakashian, A. A., Giltiay, N. V., Smith, G. M., and Nikolova-Karakashian, M. N. (2004) Expression of neutral sphingomyelinase-2 (NSMase-2) in primary rat hepatocytes modulates IL-beta-induced JNK activation, *FASEB J 18*, 968-970.
57. Marchesini, N., and Hannun, Y. A. (2004) Acid and neutral sphingomyelinases: roles and mechanisms of regulation, *Biochem Cell Biol 82*, 27-44.
58. Tani, M., and Hannun, Y. A. (2007) Neutral sphingomyelinase 2 is palmitoylated on multiple cysteine residues. Role of palmitoylation in subcellular localization, *J Biol Chem 282*, 10047-10056.

59. Reddy, A., Caler, E. V., and Andrews, N. W. (2001) Plasma membrane repair is mediated by Ca(2+)-regulated exocytosis of lysosomes, *Cell 106*, 157-169.
60. Cardone, R. A., Casavola, V., and Reshkin, S. J. (2005) The role of disturbed pH dynamics and the Na+/H+ exchanger in metastasis, *Nat Rev Cancer* 5, 786-795.
61. Chiche, J., Ilc, K., Laferriere, J., Trottier, E., Dayan, F., Mazure, N. M., Brahimi-Horn, M. C., and Pouyssegur, J. (2009) Hypoxia-inducible carbonic anhydrase IX and XII promote tumor cell growth by counteracting acidosis through the regulation of the intracellular pH, *Cancer Res* 69, 358-368.
62. Casey, J. R., Grinstein, S., and Orlowski, J. (2010) Sensors and regulators of intracellular pH, *Nat Rev Mol Cell Biol 11*, 50-61.
63. Kawamura, S., Sato, I., Wada, T., Yamaguchi, K., Li, Y., Li, D., Zhao, X., Ueno, S., Aoki, H., Tochigi, T., Kuwahara, M., Kitamura, T., Takahashi, K., Moriya, S., Miyagi, T.Plasma membrane-associated sialidase (NEU3) regulates progression of prostate cancer to androgen-independent growth through modulation of androgen receptor signaling. Cell Death Differ. (2011).
64. Haimovitz-Friedman, A., Kan, C. C., Ehleiter, D., Persaud, R. S., McLoughlin, M., Fuks, Z., Kolesnick, R. N.Ionizing radiation acts on cellular membranes to generate ceramide and initiate apoptosis. J Exp Med. 180, 525-535 (1994).
65. Sonnino, S., Chigorno, V., Tettamanti, G.Preparation of radioactive gangliosides, 3H or 14C isotopically labeled at oligosaccharide or ceramide moieties. Methods Enzymol. 311, 639-656 (2000).
66. Leroy, J. G., Ho, M. W., MacBrinn, M. C., Zielke, K., Jacob, J., O'Brien, J. S.I-cell disease: biochemical studies. Pediatr Res. 6, 752-757 (1972).
67. Aureli, M., Prioni, S., Mauri, L., Loberto, N., Casellato, R., Ciampa, M. G., Chigorno, V., Prinetti, A., Sonnino, S.Photoactivable sphingosine as a tool to study membrane microenvironments in cultured cells. J Lipid Res. 51, 798-808 (2010).
68. Behrens, B. C., Hamilton, T. C., Masuda, H., Grotzinger, K. R., Whang-Peng, J., Louie, K. G., Knutsen, T., McKoy, W. M., Young, R. C., Ozols, R. F.Characterization of a cis-diamminedichloroplatinum(II)-resistant human ovarian cancer cell line and its use in evaluation of platinum analogues. Cancer Res. 47, 414-418 (1987).
69. Osmak, M., Eljuga, D.The characterization of two human cervical carcinoma HeLa sublines resistant to cisplatin. Res Exp Med (Berl). 193, 389-396 (1993).
70. Appierto, V., Cavadini, E., Pergolizzi, R., Cleris, L., Lotan, R., Canevari, S., Formelli, F.Decrease in drug accumulation and in tumour aggressiveness marker expression in a fenretinide-induced resistant ovarian tumour cell line. Br J Cancer. 84, 1528-1534 (2001).
71. Mehlen, P., Rabizadeh, S., Snipas, S. J., Assa-Munt, N., Salvesen, G. S., Bredesen, D. E.The DCC gene product induces apoptosis by a mechanism requiring receptor proteolysis. Nature. 395, 801-804 (1998).
72. Kubbies, M.:Flow cytometric recognition of clastogen induced chromatin damage in G0/G1 lymphocytes by non-stoichiometric Hoechst fluorochrome binding. Cytometry. 11, 386-394 (1990).

73. Santana, P., Pena, L. A., Haimovitz-Friedman, A., Martin, S., Green, D., McLoughlin, M., Cordon-Cardo, C., Schuchman, E. H., Fuks, Z., Kolesnick, R.Acid sphingomyelinase-deficient human lymphoblasts and mice are defective in radiation-induced apoptosis. Cell. 86, 189-199 (1996).
74. Wang, P., Zhang, J., Bian, H., Wu, P., Kuvelkar, R., Kung, T. T., Crawley, Y., Egan, R. W., Billah, M. M.Induction of lysosomal and plasma membrane-bound sialidases in human T-cells via T-cell receptor. Biochem J. 380, 425-433 (2004).
75. Prinetti, A., Basso, L., Appierto, V., Villani, M. G., Valsecchi, M., Loberto, N., Prioni, S., Chigorno, V., Cavadini, E., Formelli, F., Sonnino, S.Altered Sphingolipid Metabolism in N-(4-Hydroxyphenyl)-retinamide-resistant A2780 Human Ovarian Carcinoma Cells. J. Biol. Chem. 278, 5574-5583 (2003).
76. Aureli M., Bassi R., Prinetti A., Chiricozzi E., Pappalardi B., Chigorno V., Di Muzio N., Loberto N. and Sonnino S.. Ionizing radiation increase the activity of cell surface glycohydrolases and plasma membrane ceramide content. *Glycoconj J.* 2012 (doi: 10.1007/s10719-012-9385-2)
77. Kolter, T., Sandhoff, K.:Sphingolipids-their metabolic pathways and the pathobiochemistry of nerodegenrative diseases. Angew. Chem. Int. Ed. 38, 1532-1568 (1999).
78. Aureli, M., Loberto, N., Chigorno, V., Prinetti, A., Sonnino, S. Remodeling of Sphingolipids by Plasma Membrane Associated Enzymes. Neurochem Res. (2010).
79. Sonnino, S., Aureli, M., Loberto, N., Chigorno, V., Prinetti, A. Fine tuning of cell functions through remodeling of glycosphingolipids by plasma membrane-associated glycohydrolases. FEBS Lett. 584, 1914-1922 (2010).
80. Goni, F. M., Alonso, A.Biophysics of sphingolipids I. Membrane properties of sphingosine, ceramides and other simple sphingolipids. Biochim Biophys Acta. 1758, 1902-1921 (2006).
81. Huschtscha, L. I., Bartier, W. A., Ross, C. E., Tattersall, M. H.Characteristics of cancer cell death after exposure to cytotoxic drugs in vitro. Br J Cancer. 73, 54-60 (1996).
82. Vojtesek, B., Lane, D. P.Regulation of p53 protein expression in human breast cancer cell lines. J Cell Sci. 105 (Pt 3), 607-612 (1993).
83. Deng, X., Yin, X., Allan, R., Lu, D. D., Maurer, C. W., Haimovitz-Friedman, A., Fuks, Z., Shaham, S., Kolesnick, R.Ceramide biogenesis is required for radiation-induced apoptosis in the germ line of C. elegans. Science. 322, 110-115 (2008).
84. Mesicek, J., Lee, H., Feldman, T., Jiang, X., Skobeleva, A., Berdyshev, E. V., Haimovitz-Friedman, A., Fuks, Z., Kolesnick, R.Ceramide synthases 2, 5, and 6 confer distinct roles in radiation-induced apoptosis in HeLa cells. Cell Signal. 22, 1300-1307
85. Verheij, M., Bartelink, H.Radiation-induced apoptosis. Cell Tissue Res. 301, 133-142 (2000).