VERY LONG CHAIN FATTY ACID SPHINGOMYELIN IN NUCLEAR LIPID MICRODOMAINS OF HEPATOCYTES AND HEPATOMA CELLS: CAN THE EXCHANGE FROM C24:0 TO C16:0 AFFECT SIGNAL PROTEINS AND VITAMIN D RECEPTOR?


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Running Head: nuclear lipid microdomains of hepatoma cells

Abbreviations: CHO, cholesterol; H, hepatocyte; NLR, nuclear lipid rafts; PKCz, Protein Kinase C Zeta; SM, sphingomyelin; STAT-3, signal transducer and activator of transcription-3; VDR, vitamin D receptor.

Abstract

Lipid microdomains localised in inner nuclear membrane are considered platforms for active chromatin anchoring. Stimuli as surgery, vitamin D or glucocorticoid drugs influence there gene expression, DNA duplication and RNA synthesis. Here, we used ultra fast liquid chromatography tandem mass spectrometry to identify sphingomyelin species coupled with immunoblotting analysis
to comprehensively map differences in nuclear lipid microdomains purified from hepatocytes and hepatoma cells. We showed that nuclear lipid microdomains lost saturated very long fatty acid (C24:0) sphingomyelin in cancer cells and enriched in long fatty acid (C16:0) sphingomyelin. We also found that signalling proteins, such as STAT3, Raf1, and PKCζ, were increased and Vitamin D receptor was reduced in cancer cells. Since recent researches showed a shift in sphingolipid composition from C24:0 to C16:0 in relation to cell life, we performed a comparative analysis of properties among C16:0 sphingomyelin, C24:0 sphingomyelin and cholesterol. Our results induced to hypothesise that the enrichment of C16:0 sphingomyelin could determine enhanced dynamic properties of nuclear lipid microdomains in cancer cells with an increased shuttling of proteins signaling molecules.

Key words: Lipid rafts, Nuclear membrane, Hepatoma, Sphingomyelin, Cell signalling proteins, VDR

Introduction

Sphingomyelin (SM) was described as a dominant sphingolipid in mammalian cell membranes indispensable for cell function (Taniguchi and Okazaki, 2014). It contains acyl chains that vary in length from long chain fatty acids (LCFAs) as C16:0 palmitic acid to very long-chain fatty acids (VLCFAs) with 20 or more carbons, including saturated fatty acids (FAs) as 20:0 arachidic acid, 22:0 behenic acid, and 24:0 lignoceric acid, mono- and e poli-VLCFAs (Ohno et al., 2010). VLCFAs are produced from certain LCFAs, provided through diet or generated by FA synthase, and elongated by FA elongase (Soupene and Kuypers, 2008).

SM and cholesterol (CHO) formed functional nanoscale ordered domains, characteristic in particular of the external leaflet of cell membranes whose thickness increased with the length of the acyl chain of SM (Róg and Vattulainen, 2014). Although a high affinity interaction between SM and CHO was suggested (Radhakrishnan et al., 2000; Li et al., 2001), no compelling experimental evidence for the molecular basis of such a specific interaction has been reported in literature,
suggesting that the driving force for the formation of the lipidic domains is provided by the hydrophobic matching condition in membranes (Holopainen et al., 2004). However, it should be mentioned that interactions between SM and CHO were computationally studied in binary mixtures of CHO and SM via molecular dynamic simulation, with a focus on hydrogen bonding by showing that CHO formed more hydrogen bonds with SM than it formed with phosphatidylcholine (Róg and Pasenkiewicz-Gierula, 2006). Atomistic studies of Aittoniemi et al. (2007) indicated that hydrogen bonding alone could not explain the higher affinity of CHO for SM but that one must also consider the contributions of van Der Waals interactions between CHO and the choline groups of SM. In addition, CHO preferred saturated SM, with ordered acyl chains, to establish interactions entropically more favourable, since CHO might increase local entropy and its interactions with disordered acyl chains in unsaturated phospholipids would lead to an ordering effect of cholesterol on acyl chains, thereby decreasing the local entropy (Slotte, 2013). Measurements of sterol bilayer affinity demonstrated that palmitoyl SM was the optimal SM analog for CHO (Slotte, 2013). X-ray scattering data showed that 22:0, 23:0 and 24:0 SM included in a bilayers can lead to transbilayer interdigitation, i.e., the distal part of a long acyl chain from a SM molecule in one leaflet penetrates into the opposing leaflet (Lewin et al., 1985). CHO and SM are present in membranes as lipid microdomains called lipid rafts, extracted as \( l_o \)-phase detergent-resistant membranes whose functional role has long been subject of discussion in the membrane biophysics community. Brown (2006) claimed that the detergent-resistant membrane fractions did not represent lipid rafts present in the cells prior to extraction even if raft-targeting signals identified by detergent-resistant membrane analysis were often required for protein function. Frisz et al. (2013) demonstrated that sphingolipid domains in the plasma membranes of fibroblasts did not contain CHO but that the latest affected the sphingolipid organization via an indirect mechanism that involved the cytoskeleton. Then Honigmann et al. (2014) proposed that alternative interactions were responsible for the strong local trapping of sphingolipid analogue in living nonstimulated cells. However Mollinedo and Gajate (2015) demonstrated that lipid rafts behaved in cell membrane as major
modulators of membrane geometry, lateral movement of molecules, platform for traffic and signal transduction proteins. In addition they they represented the major platforms for signaling regulation in cancer (Mollinedo and Gajate, 2015).

We have previously demonstrated that lipid microdomains, rich in SM and CHO, were present in the inner nuclear membrane and were called nuclear lipid microdomains (NLMs). They played different roles in relation to cell function by acting as platform for vitamin D receptor (VDR) in embryonic hippocampal cells (Bartoccini et al., 2011) and and for glucorticoid drugs in non-Hodgkin’s T cell human lymphoblastic lymphoma (Cataldi et al., 2014). In the liver, NLMs acted as resting place for active chromatin and transcription factors by regulating DNA (Albi et al., 2013) and RNA (Cascianelli et al., 2008; Albi and Villani, 2009) synthesis. No data exist until now about the SM FAs species in NLMs.

Recent research on sphingolipid FAs has been focused on their role in the cell physiopathology. Sassa et al. (2012) described that a shift in sphingolipid composition from C24:0 to C16:0 increased susceptibility to apoptosis in HeLa cells. The role of C16:0 had already been suggested by a study of Zhang et al. (2004) demonstrating that it induced apoptosis in human hepatoma HepG2 cells.

Hepatoma is a leading primary malignancy of the liver, one of the most common cancers worldwide. New therapeutic strategies targeted anti-Signal transducer and activator of transcription 3 (STAT3) protein, a key regulator of inflammation, cell survival, and tumorigensis of liver cells (Hung et al., 2014) and Raf1, that prolonged cell survival and leaded to cancer, even in the absence of oncogenic mutations (Gauthier and Go, 2013). Protein Kinase Cζ (PKCζ) was involved in the hepatocarcinogenesis mechanism by controlling glycogen synthase kinase-3beta (Desbois-Mouthon et al., 2002). Differently Vitamin D3 inhibited hepatocellular carcinoma development (Guo et al., 2013).
To address the role of SM present in NLMs on cell function, we examined the presence of SM species in NLMs purified from hepatocytes and H35 hepatoma cells in relation to signal proteins and Vitamin D3 receptor (VDR).

**Results**

**Nuclear rafts of hepatocytes and hepatoma cells**

Highly purified H and H35 nuclei were used to prepare NLMs. The purification level of the nuclear preparation was similar to that previously reported (Cascianelli *et al.*, 2008). In the nuclei after Barnes treatment, the activity of Glucose-6-Phosphatase was $7\pm2$ nmol/mg protein/min (H) and $6\pm2$ nmol/mg protein/min (H35). The NADH-cytochrome-c reductase activity was undetectable in both preparations. NLM fraction has been obtained from triton solubilisation. In H NLMs the level of protein was $29.40\pm2.21\ \mu g/g$ liver and in H35 NLMs it was $1.31\pm0.02\ \mu g/106$ cells, according to Cascianelli *et al.* (2008) and Bartoccini *et al.* (2011), respectively. No activity of Glucose-6-Phosphatase and NADH-cytochrome-c reductase was detected in both preparations, indicating the absence of cytoplasmic contamination. These data were strongly supported by immunoblot analysis with giantin antibodies, a marker protein for Golgi membrane. The results showed that the band for giantin corresponding to apparent molecular weight of 367 KDa was absent in H NLMs and H35 NLMs (Fig.1). Differently, STAT3, a marker protein for NLMs (Cascianelli *et al.*, 2008) was present in both samples (Fig.1a) even if in H35 NLMs it was expressed 3.43 times in comparison with H NLMs (Fig.1b). To highlighted the level of NLM purification and to exclude the possible chromatin (Chr) and nuclear matrix (NMx) contamination, LaminB was analysed as marker of NLMs whereas Chr and NMx were used as controls. The results showed the absence of LaminB in NMx, the very low level of protein in Chr by indicating the possible presence of small parts of inner nuclear membrane in the sample and a higher content of protein in NLMs as previously reported (Cascianelli *et al.* 2008), prepared from both H and H35 nuclei (Fig.2). The level of CHO was
13.40±0.22 μg/mg protein in H NLMs and 14.65±0.38 μg/mg protein in H35 NLMs, similar to that previously reported (Cascianelli et al., 2008; Bartoccini et al., 2011).

24:0 sphingomyelin shifts to 16:0 sphingomyelin in nuclear lipid rafts of cancer cells

We analysed SM species in NLMs, by using 16:0 SM, 18:1 SM and 24:0 SM external calibrators and the results were compared with those of total nuclear membrane (NM). The results highlighted that in NLMs prepared from cancer cells the value of 16:0 SM increased 6.5 times and that of 24:0 SM decreased 18.65 times in comparison with NLMs from normal cells (Fig.3a). In NM, 16:0 SM increased 1.29 times and 24:0 SM decreased 4.6 times (Fig.3a). As NM contained NLMs, it is possible that the low variations present in NM reflected the changes observed in NLMs. To have a deeper insight of SM species containing saturated or unsaturated fatty acids, we evaluated the areas of all the peaks identified on the basis of their molecular weight and we analysed their values in relation to protein content. A total of twenty-four species were investigated: 16:1 SM; 18:0 SM, 18:2 SM, 20:0 SM, 20:1 SM, 20:2 SM, 20:3 SM, 22:0 SM, 22:1 SM, 22:2 SM, 22:3 SM, 22:4 SM, 24:1 SM, 24:2 SM, 24:3 SM, 24:4 SM, 24:5 SM, 26:0 SM, 26:1 SM, 26:2 SM, 26:3 SM, 26:4 SM, 26:5 SM, 26:6 SM. Seven peaks were detected (Fig. 3b). Significant differences in the levels of various lipid molecular species were found between H NLMs and H35 NLMs. H NLMs resulted richer in 20:0 SM content than H35 NLMs and the lastest were richer in 22:0 SM, 22:1 SM, 22:2 SM, 24:1 SM, 24:2 SM, 24:4 SM content than H NLMs . In the intermediate length acyl chains (20:0 and 22:0) there was the opposite effect to that observed for 16:0 and 24:0. However, 20:0 decreased 3.72 times and 22: 0 increased 2:07 times similar to other fatty acids. Thus we focused the attention on fatty acids with higher variations, such as 16: 0 and 24:0. In NM the changes in SM content between normal and cancer cells were not statistically significant. It is possible that the variations observed in NLMs were not high enough to affect changes in the total NM. Then we compared the changes in the total levels of SM species containing saturated and unsaturated fatty acids. As reported in Fig. 3c, the SM saturated FAs were 1.77 times lower and SM unsaturated FAs
2.42 times higher in NLMs of cancer cells than in NLMs of normal cells. Thus, saturated /unsaturated FAs ratio was 0.71 in H NLMs and 0.17 in H35 NLMs. Among unsaturated FAs, the monounsaturated increased 3.10 times (22:1 SM) and 3.89 (24:1 SM), the two-unsaturated and four-unsaturated increased in a range between 2.16 and 2.42. No significant changes were in NM (Fig.3c). To verify the specificity of SM changes, we analysed phosphatidylcholine (PC) and ceraminde species by using 16:0 18:1 PC, 16:0 24:0 PC, 18:1 18:0 PC, 16:0 ceramide, 20:0 ceramide and 24:0 ceramide as external calibrators. If you compared the total SM species (Fig.3a) with the total PC species (Fig.4a) you could see that PC was higher about 23 times than SM in NM whereas both lipids had similar value in NLMs, as previously published (Cascianelli et al., 2008). The results showed no changes of PC species in both NM and NLMs prepared from normal and cancer cells (Fig.4a). No changes were also present in ceramide species in NM whereas in NLMs 16:0 ceramide decreased 1.5 times and 24:0 ceramide increased 3.13 times (Fig.4b). It is possible that the variations of ceramide species were the results of the changes of substrates for sphingomyelinase, such as SM 16:0 and SM 24:0 (Fig. 3a).

**Comparative Analysis of Properties among C16:0 Sphingomyelin, C24:0 Sphingomyelin and Cholesterol.**

To understand the possible meaning of the FA change in NLM SM of cancer cells, we have studied the properties of C16:0 SM, C24:0 SM and CHO, using molecular modeling calculations. Accordingly, tridimensional models of C16:0 SM, C24:0 SM and CHO were generated *in silico* as detailed in the method section. These models were instrumental to calculate surface descriptors such as the polar surface area ($\text{Å}^2$) and total van der Waals surface area ($\text{Å}^2$), and geometrical descriptors including the topological radius ($\text{Å}$) and topological diameter ($\text{Å}$). Specifically, the topological radius and diameter are respectively defined as the minimum vertex and the maximum vertex of a molecular graph representing the molecule, thereby providing the dimensions of the lipid (Todeschini et al., 2000).
As a result, 16:0 SM (Fig. 5a) is composed of a phosphatidylcholine headgroup, a sphingosine moiety of 18 carbon atoms, and a N-linked fatty acid chain of 16 saturated carbon atoms. Its polar surface area is 118 Å², representing the 10% of the total van der Waals surface area (1173 Å²). The topological radius of 16:0 SM is 17 Å, whereas the topological diameter is 33 Å.

24:0 SM (Fig. 5b) contains a phosphatidylcholine headgroup, a sphingosine moiety of 18 carbon atoms, and a N-linked fatty acid chain of 24 saturated carbon atoms. Its polar surface area is also 118 Å², representing the 8.5% of the total van der Waals surface area (1384 Å²) of the lipid. The topological radius of 24:0 SM is 21 Å, whereas the topological diameter is 41 Å.

CHO (Fig. 5c) is composed of a steroid nucleus with a polar hydroxyl group at C3, and a lipophilic side chain. Its polar surface area is 20 Å², representing the 3% of the total van der Waals surface area (607 Å²) of the lipid. The topological radius of Cholesterol is 8 Å, whereas its topological diameter is 15 Å.

Membrane thickness values are reported in literature for 18:0 SM (46–47 Å) and 24:0 SM (52–56 Å) SM (Maulik et al., 1995; Maulik, et al., 1996). Since membrane thickness is related to lipid chain length, we compared the topological diameter of lipids taken as a value of lipid chain length to infer the thickness of lipid rafts formed by 16:0 SM and 24:0 SM. This approximation resulted in a range of thickness values of 43–44 Å for the membrane composed of 16:0 SM and of 52-56 Å for the membrane composed of 24:0 SM in combination with CHO.

**Signal proteins and vitamin D receptor located in nuclear lipid rafts are different between hepatocytes and hepatoma cells.**

We have tested the possibility that the changes of SM species between H NMs and H35 NMs might be associated with variations of functional protein content. Raf1, PKCζ and VDR expression have been analyzed by immunoblotting with specific antibodies in whole cells and in NLMs. Both samples showed immunoreactivity in correspondence to the bands with apparent molecular weight corresponding to 80 KDa (Raf1), 78 KDa (PKCζ) and 50 KDa (VDR) (Fig. 6a). In whole cells the band density increased 1.15 and 1.89 times for Raf1 and PKCζ respectively and decreased 1.92 times for VDR in cancer cells in comparison with normal cells. An increase of band density of 1.69
and 2.39 times for Raf1 and PKCζ respectively and a decrease of 2.87 times for VDR appeared in H35 NLMs in comparison with H NLMs (Fig.6b).

Discussion

Considerable evidences suggested the implication of sphingolipids in the cancer (Adan-Gokpulut et al., 2013). Nowadays researchers focused the attention on the different species of sphingolipid molecules containing long and very long FAs. In has been demonstrated that cancer cells incorporate and remodel exogenous 16:0 into structural and oncogenic glycerophospholipids, sphingolipids, and ether lipids (Louie et al., 2013). In addition, supplementation of the culture medium with 16:0 modifies the FA composition of Reuber H35 hepatoma cells (Martínez-Cayuela et al., 2000). The shift of sphingolipid composition from very long FAs (24:0) to long FAs (16:0) changes cell function (Sassa et al., 2012).

We have demonstrated that NLMs act as platform for duplication and transcription of active chromatin (Cascianelli et al., 2008; Albi et al., 2013), for VDR (Bartoccini et al., 2011) and desamethasone (Cataldi et al., 2014) resting place to regulate gene expression.

Here we demonstrated for the first time that in NLMs of cancer cells the FAs of SM change from very long FAs (24:0 SM) to long FAs (16:0 SM) in comparison with normal cells. Taking into account the calculated properties of 16:0 SM and 24:0 SM (van der Waals surface area and topological radius), it is possible to envisage a possibility for 24:0 SM stronger than 16:0 SM in making lipid microdomains with CHO. This hypothesis is supported by the larger van der Waals surface area of 24:0 that would allow a better accommodation of CHO underneath the polar headgroup of SM. It has been reported that SM forms bilayers with different thickness, depending on the size of its fatty acid chain such as 46–47 Å for 18:0 SM and 52–56 Å for 24:0 SM (Maulik et al., 1995; Maulik, et al., 1996). Comparing the topological diameters of 16:0 (33 Å), 18:0 (35 Å), 20:0 (37 Å) and 24:0 (41 Å) SM, the thickness of lipid rafts formed by 16:0 and 24:0 SM in combination with CHO can be in a range of 43–44 Å and 52-56 Å, respectively. In addition, our
results indicate that H35 NLMs enrich in unsaturated FAs that are known to increase the area of a lipid and consequently membrane fluidity (Ziegelhöffer et al., 2012). Rapidly growing evidence reinforces the notion that lipid rafts in membranes participate to the recruitment of proteins and lipid signaling molecules (Brown and London, 2000; Róg and Vattulainen, 2014). And so, considering our results, we hypothesized that the narrower thickness of NLMs composed of 16:0 SM and the increase of unsaturated fatty acids might determine enhanced dynamic properties of the NLRs in hepatoma cells, with an increased shuttling of protein molecules. Here we demonstrate in H35 NLMs an increase of proteins involved in hepatocarcinogenesis, such as STAT3 (Hung et al., 2014), Raf1 (Gauthier and Go, 2013) and PKCζ (Desbois-Mouthon et al., 2002) in spite of the reduction of VDR, probably for the reduction of Vitamin D3 that inhibits hepatocellular carcinoma development (Guo et al., 2013). The changes are higher in NLMs than in whole cells, supporting the idea that the modifications of NLMs in cancer cells might influence the content of functional proteins in these microdomains that act as platform for active chromatin (Cascianelli et al., 2008).

In conclusion, we show changes in SM and functional proteins of NLMs in cancer cells. This underlines the importance of focusing the attention on NLMs instead of the global nuclear membrane when the cancer cells are studied.

**Materials and Methods**

**Animals and Cells**

Thirty-day-old Sprague Dawley rats of either sex (Harlan Nossan, Milan, Italy) kept at normal light-dark periods were used. They had free access to pelleted food and water prior to killing between 9 and 10 a.m. All treatments were made according to the international regulation of National Institutes of Health. H35 hepatoma cells were obtained from the European Collection of Animal Cell Cultures (Salisbury, Wilts, United Kingdom).

**Materials**
Dulbecco’s modified Eagle’s medium (DMEM), bovine serum albumin (BSA), dithiothreitol, fetal bovine serum (FBS), phenylmethylsulfonylfluoride (PMSF), methanol, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2-propanol, methyl-tert-butyl ether, formic acid, chloroform, and cholesterol (CHO) were obtained from Sigma Chemical Co. (St. Louis, Missouri, U.S.A.); lipid standards 16:0 SM, 18:1 SM, 24:0 SM, 16:0 18:1 PC, 16:0 24:0 PC, 18:1 18:0 PC, 16:0 ceramide, 20:0 ceramide and 24:0 ceramide were purchased from Avanti (Avanti Polar, Alabaster, AL, USA); anti-Giantin, anti-Signal transducer and activator of transcription 3 (STAT3), anti-Raf1, anti-Protein Kinase ζ (PKCζ) and anti-Vitamin D Receptor (VDR) were obtained from Santa Cruz Biotechnology, Inc. (California, USA); anti-Lamin B was obtained from Oncogene (Boston, MA)

**Rat Liver**

Rat liver was homogenised in 10 mM Tris-HCl buffer, pH 7.4, containing 0.25M sucrose, 1mM EDTA, 0.1% ethanol, 0.1 M PMSF and 0.2 M dithiothreitol by using the Thomas homogenizer; the homogenate was filtered through two layers of surgical gauze and used for hepatocyte nuclei isolation.

**Cell culture**

H35 hepatoma cells were seeded in 25cm² flasks and were grown in monolayer in DMEM enriched with 10% FBS, 2mM of L-glutamine, 100 IU/ml of penicillin, 100 μg/ml of streptomycin and 250 μg/ml of amphotericin B. Cells were maintained at 37 °C in a saturating humidity atmosphere containing 95% air 5% CO₂ and used for hepatoma nuclei isolation.

**Hepatocyte and hepatoma cell nuclei isolation**
H nuclei were isolated from liver homogenate according to Bresnick et al. (1967) in the presence of 1 mM PMSF, as previously described (Albi et al., 1994). Briefly, liver homogenate was centrifuged at 700g for 10 min at 4°C. The procedure was repeated twice and the final pellet was resuspended in 2.4M sucrose containing 1mM MgCl₂, followed by centrifugation at 50000g for 60 min. at 4°C. The pellet, was washed with 0.25M sucrose containing 1mM MgCl₂ and centrifuged at 2000g for 10 min. This method yielded a homogeneous population of hepatocyte nuclei with no contamination from other types of nuclei (Albi et al., 1994).

H35 nuclei were isolated as previously reported (Albi et al., 2005). Briefly the homogenized cells were treated with 1% Triton X-100 in hypotonic buffer (0.5:1 v/v); the cellular suspension was stirred on a vortex mixer for 30 sec. the buffer containing 1.5 M sucrose was added (0.25:1 v/v) and solution was centrifuged at 2000g for 10 min.

The H and H35 cell nuclei were then washed twice with Barnes et al. solution (0.085M KCl, 0.0085 NaCl, 0.0025M MgCl₂, Trichloroacetic acid (TRA)-HCl 0.005M), as previously reported (Rossi et al., 2007). This treatment, during which the nuclei were sedimented at 2000 x g., removed mitochondrial and microsomal contaminations. The nuclei were checked for possible mitochondria and microsome contamination evaluating the activity of a microsomal marker (NADH-cytochrome C-reductase) and Glucose-6-Phosphatase, as previously reported (Albi et al., 2005).

**Purification of Nuclear Membrane**

Nuclear membranes were purified from H and H35 isolated nuclei as previously reported (Albi et al., 1999).

**Purification of Chromatin**

Chromatin was purified from H and H35 isolated nuclei as previously reported (Albi et al., 2003).
**Purification of Nuclear Matrix**

Nuclear Matrix was purified from H and H35 isolated nuclei as previously reported (Albi *et al.*, 2003a).

**Purification of Nuclear Lipid Microdomains**

NLMs were purified from H and H35 cell nuclei according to normal and hepatectomised rats Cascianelli *et al.* (2008), Bartoccini *et al.* (2011). The extraction was carried out with Triton X-100 dissolved in distilled water (10% v/v), on ice. This solution was added to the purified nuclei to a final detergent concentration of 1% (v/v). The extract was placed in a cushion of 80% sucrose with a gradient of 15-40% sucrose on top. After centrifugation overnight floating fractions were carefully collected with a pipette, diluted five times with 25 mM HEPES-HCl, 150 mM NaCl, pH 7.1 and centrifuged at 100000g for 120 min to obtain the pellet containing rafts. To test the purity of NLRs the absence of giantin, as marker protein for Golgi membrane (Satoh *et al.*, 2005), and the presence of STAT3, as specific NLR marker (Cascianelli *et al.*, 2008) were evaluated by immunoblotting analysis.

**Lipid extraction**

Lipid extraction was performed according to Matyash *et al.* (2008) as reported by Lazzarini *et al.*, (2014) with modifications. The pellets of H and H35 NLRs were suspended in Tris 10mM pH 7.4 and diluted with 1mL methanol. 3 mL ultra pure water and 3 mL MTBE were added. Each Sample was vortexed for 1 min and centrifuged at 3000 g for 5 min. The supernatant was recovered. The extraction with MTBE was repeated on the pellet and the supernatant was added to the first. The organic phase was dried under nitrogen flow and resuspended in 500 µL of methanol.

*Ultra Fast Liquid chromatography tandem mass spectrometry (UFLC- MS/MS)*
16:0 SM, 18:1 SM, 24:0 SM, 16:0 18:1 PC, 16:0 24:0 PC, 18:1 18:0 PC, 16:0 ceramide, 20:0 ceramide and 24:0 ceramide standards were prepared according to Matyash et al., (2008). Standards were dissolved in chloroform/methanol (9:1 v/v) at 10μg/mL final concentration. The stock solutions were stored at -20°C. Working calibrators were prepared by diluting stock solutions with methanol to 500:0, 250:0, 100:0, 50:0 ng/ml final concentrations. 20μL of standards or lipids extracted from serum were injected after purification with specific nylon filters (0.2 μm).

Analyses were carried out according to Rabagny et al. (2011) by using Ultra Performance Liquid Chromatography system tandem Mass Spectometer Applied biosistem (Shimadzu Italy s.r.l., Italy). The lipid species were separated, identified and analysed as previously reported (Garcia-Gil et al., 2014). The samples were separated on a Phenomenex Kinetex phenyl-hexyl 100 A column (50 x 4.60 mm diameter, 2.6 μm particle diameter) with precolumn security guard Phenomenex ULTRA phenyl-hexyl 4.6. For SM, column temperature was set at 50°C, flow rate 0.9 mL/min. Solvent A was 1% formic acid; solvent B 100% isopropanol containing 0.1% formic acid. Run was performed for 3 min in 50% solvent B and then gradient to reach 100% B in 5 min. The system has been reconditioned for 5 min with 50% B before the next injection. The SM species were identified by using positive turbo ion spray and modality multipole reaction monitoring. The identification and analysis of cholesterol has been conducted by atmospheric pressure chemical ionization (APCI) in positive ionization conditions and multipole ion scan modality.

**Protein content**

Total protein concentration was determined spectrophotometrically at 750 nm by using bovine BSA as a standard as previously reported (Albi et al., 2008).
**Electrophoresis and Western Blot analysis**

30µg of protein from H and H35 NLRs were submitted to SDS-PAGE electrophoresis in 8% polyacrylamide slab gel for Giantin detection, 10% for STAT3, Raf1 and PKCζ, VDR and Lamin B according to Laemmli (1970). For the electrophoresis image analysis, the gel was stained by Coomassie-blue. The transfer of protein was carried out into nitrocellulose in 90 min according to Towbin *et al.* (1979). The membranes were blocked for 30 min with 5% non-fat dry milk in PBS, pH 7.5, and incubated over night at 4°C with specific antibodies. The blots were treated with horseradish-conjugated secondary antibodies for 90 min. Visualization was performed with the enhanced chemiluminescence kit from Amersham.

**Properties of 16:0 SM, 24:0 SM and Cholesterol.**

3D-Chemical structures of 16:0 SM, 24:0 SM and cholesterol were generated using Maestro v9.5 (Schrödinger, LLC, New York, NY, 2014). Geometries were optimized using semi-empirical calculations with AM1 method and RHF wavefunction. Surface and geometrical properties including polar surface area (Å²), total van der Waals surface area (Å²), topological radius (Å) and topological diameter (Å) were calculated using Canvas v1.7 (Schrödinger, LLC, New York, NY, 2014).

**Statistical analysis**

Data are expressed as mean ± S.D. and *t* test was used for statistical analysis.

**References**


Figure 1. Giantin and STAT3 in Nuclear Lipid Microdomains purified from Hepatocytes and Hepatoma cells.

a) Immunoblot of proteins probed with specific antibodies. The position of Giantin and STAT3 was indicated in relation to the position of molecular size standards; b) The area density evaluated by densitometry scanning and analysis with Scion Image, the data represent the mean ± SD of three separated experiments. H, hepatocyte; H35, hepatoma cell line. * p<0.001 versus H.
Figure 2. LaminB in Nuclear Matrix, Chromatin and Nuclear Lipid Microdomain from Hepatocytes and Hepatoma cells.

a) Immunoblot of protein probed with specific antibody. The position of LaminB was indicated in relation to the position of molecular size standard; b) The area density evaluated by densitometry scanning and analysis with Scion Image, the data represent the mean ± SD of three separated experiments. NMx, nuclear matrix; Chr, chromatin, NLM, nuclear lipid microdomain, H, hepatocyte; H35, hepatoma cell line. * p<0.001 versus NMx and Chr.
Figure 3. Sphingomyelin in Nuclear Membrane and Nuclear Lipid Microdomains purified from Hepatocytes and Hepatoma cells.

a) SM species studied by using 16:0 SM, 18:1 SM and 24:0 SM external calibrators. Data are expressed as nmol/mg protein and represent the mean ± SD of three separated experiments. b) SM species studied by evaluating the areas of all the peaks identified on the basis of their molecular weight. Data are expressed as area/mg protein and represent the mean ± SD of three separated experiments; c) Total saturated and unsaturated fatty acids. Data are expressed as area/mg protein and represent the mean ± SD of three separated experiments. H, hepatocyte; H35, hepatoma cell line.

* p<0.001 versus H.
Figure 4. Phosphatidylcholine and ceramide in Nuclear Membrane and Nuclear Lipid Microdomains purified from Hepatocytes and Hepatoma cells.

a) PC species studied by using 16:0 18:1 PC, 16:0 24:0 PC, 18:1 18:0 PC external calibrators; b) ceramide species studied by using 16:0 ceramide, 20:0 ceramide and 24:0 ceramide standars. Data are expressed as nmol/mg protein and represent the mean ± SD of three separated experiments. H, hepatocyte; H35, hepatoma cell line. * p<0.001 versus H.
Figure 5. Sodels of 16:0 Sphingomyelin, 24:0 Sphingomyelin and Cholesterol.

a) Polar surface area (cyan) and lipophilic surface are (orange) of 16:0 Sphingomyelin; b) Polar surface area (cyan) and lipophilic surface are (orange) of 24:0 Sphingomyelin; c) Polar surface area (cyan) and lipophilic surface are (orange) of Cholesterol.
Figure 6. Raf1, Protein kinase Cζ (PKCζ) and Vitamin D3 Receptor (VDR) in Whole Cells and in Nuclear Lipid Microdomain purified from Hepatocytes and Hepatoma cells.

a) Immunoblot of proteins probed with specific antibodies. The position of proteins was indicated in relation to the position of molecular size standards; b) The area density evaluated by densitometry scanning and analysis with Scion Image, the data represent the mean ± SD of three separated experiments. H, hepatocyte; H35, hepatoma cell line; NLM, nuclear lipid Microdomains. * p<0.001 versus H.