Aberrant sphingomyelin ³¹P-NMR signatures in giant cell tumour of bone

Journal:	<i>Biochemistry and Cell Biology</i>
Manuscript ID	bcb-2020-0599.R1
Manuscript Type:	Article
Date Submitted by the Author:	20-May-2021
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Keyword:	Giant cell tumour of bone, sphingomyelin synthase, sphingomyelinase, ³¹ P-NMR, glycerophospholipids
Is the invited manuscript for consideration in a Special Issue? :	Not applicable (regular submission)

Aberrant sphingomyelin ³¹P-NMR signatures in giant cell tumour of bone

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Abstract

An understanding of the biochemistry of the giant cell tumour of bone (GCTB) provides an opportunity for the development of prognostic markers and identification of therapeutic targets. Based on metabolomic analysis, we proposed glycerophospholipid metabolism as the altered pathway in GCTB and the objective of this study was to identify these altered metabolites. Using phosphorus-31 nuclear magnetic resonance spectroscopy (^{31}P -NMR), sphingomyelin was determined as the most dysregulated phospholipid in tissue samples from six patients with GCTB; subsequently, enzymes related to its biosynthesis and hydrolysis were examined using immunodetection techniques. High expression of sphingomyelin synthases 1 and 2, but low expression of neutral sphingomyelinase 2 (nSMase2), was found in GCTB tissues compared to non-neoplastic bone tissues. Sphingomyelin/ ceramide biosynthesis is dysregulated in GCTB due to alterations in the expression of SMS1, SMS2, and nSMase2.

Key Words: Giant cell tumour of bone, sphingomyelin, ceramide, sphingomyelin synthase, sphingomyelinase, neutral sphingomyelinase 2, bone cancer, giant cell-rich bone tumours, ^{31}P -NMR, glycerophospholipids.

39 Introduction

40 Giant cell tumour of bone (GCTB) is one of the most prevalent epiphyseal and meta-
41 epiphyseal neoplasms, which is locally aggressive, recurrent, and has high metastatic
42 potential to the lung (Montgomery *et al.*, 2019; Muheremu and Niu, 2014; Santini-Araujo
43 E., 2015). This neoplasm accounts for 6% of the primary bone cancer and 20% of the
44 benign tumour cases reported in patients between 20 and 50 years of age in the long
45 bones with knee involvement, and it exhibits a slight predominance in women (Lin *et al.*,
46 2019; Montgomery *et al.*, 2019). The histological composition of this tumour includes the
47 following three cell populations: a) multi-nucleated osteoclast-like giant cells (GCs), b)
48 mononuclear monocyte-macrophage cells (MCs), and c) stromal cells (SCs), which are
49 the neoplastic elements (Noh and Park, 2018; Schaefer and Hornick, 2018). The main
50 pathway involved in the pathogenesis of GCTB is linked to high levels of receptor activator
51 of nuclear factor-kappa B ligand (RANKL) in SCs, which stimulates formation of GCs from
52 monocytes and the differentiation of osteoclastic precursors, thereby activating osteolysis
53 (Wu *et al.*, 2015).

54 The specific mutation of the H3F3A gene on chromosome 1 is useful for differentiating
55 this tumour from chondroblastoma, and this may be a potential biomarker for GCTB
56 (Yamamoto *et al.*, 2020). The mutant histone H3G34W, encoded by the H3F3A gene, is
57 characterized by the exchange of the amino acid glycine (G) by tryptophan (W) at position
58 34 (p.Gly34Trp) (Yamamoto *et al.*, 2020). An antibody directed against the H3G34W
59 mutation, with high sensitivity and specificity for GCTB, exhibits diffuse nuclear staining
60 and is positive in 91% of the GCTB samples, but not in chondroblastoma and other types
61 of giant cell-rich bone tumours (Amary *et al.*, 2017; Behjati *et al.*, 2013).

Currently, exploration of the cancer metabolome is of significance in the discovery of diagnostic and predictive markers, since the phenotypic changes can be directly related to their underlying biochemical changes (Armitage and Barbas, 2014). Recently, our research group described the metabolomic profile of GCTB, which indicated glycerophospholipid metabolism as one of the main deregulated pathways (Martínez-López *et al.*, 2017). Glycerophospholipids and sphingolipids are structurally complex saponifiable lipids that form integral parts of cellular membranes and plasma lipoproteins. They play an important role as intracellular signalling molecules during responses to growth factors, differentiation, migration, and cell death (D'Angelo *et al.*, 2018; Hannun and Obeid, 2008; Iqbal *et al.*, 2017; Lordan *et al.*, 2017). Specifically, sphingomyelin (SM) is a bioactive lipid and is the most abundant component of the plasma membrane. SM is capable of forming lipid rafts that regulate the signalling by membrane receptors (Bienias *et al.*, 2016), owing to its ubiquity and abundance. Therefore, defects in SM biosynthesis can directly or indirectly influence the normal physiology of the cell and lead to a neoplastic transformation.

SM levels are crucial for cellular function; therefore, their biosynthesis and cellular concentrations are strictly regulated. SM hydrolysis by sphingomyelinases (SMases) increases the concentration of ceramide (Cer), another bioactive lipid, involved in cellular proliferation, growth, and apoptosis (Bienias *et al.*, 2016; Kolesnick, 2002). The levels of SM and Cer are regulated by the concerted action of two enzyme families, SMases, which convert SM into Cer, and sphingomyelin synthases, which convert Cer into SM. SM biosynthesis is altered in certain types of epithelial tumours (Kozar *et al.*, 2018; Li *et al.*, 2017; Nagahashi *et al.*, 2016; Separovic *et al.*, 2017; Sun and Leung, 1974), with an

increase in SM and a decrease in Cer levels in hepatocarcinoma and ovarian cancer (Kozar *et al.*, 2018; Li *et al.*, 2017), although the specific changes depend on the type of tumour (D'Angelo *et al.*, 2018). In the case of mesenchymal tumours, their lipid profile and bioactive lipids are not well understood, because the focus has been on the sensitivity to antineoplastic therapies, as observed in reports of haematopoietic malignancies (Biswal *et al.*, 2000; Ekiz and Baran, 2010; Ricci *et al.*, 2006). The role of sphingolipids in cancer is explored; however, until now, their contribution in most mesenchymal tumours remains unclear. Therefore, the aim of this study was to analyse SM/Cer biosynthesis in GCTB, a tumour of mesenchymal origin.

Materials and methods

Study population Six non-recurrent GCTB samples were obtained from surgical intralesional excision from patients treated in the UMAE “Dr. Victorio de la Fuente Narváez”, Instituto Mexicano del Seguro Social (IMSS) in Mexico City. The diagnosis was based on clinical, radiological, and histopathological criteria. According to the Enneking staging, all GCTB patients were categorised as IA, which corresponds to low-grade, intra-compartmental lesions, without metastasis or pulmonary dissemination (Enneking *et al.*, 2003). For the control, six non-neoplastic cancerous bone tissue samples obtained from the iliac crest were used. The tissues were dissected by a pathologist with expertise in musculoskeletal pathology and then stored at -80°C. The medical histories of the patients were examined and the samples of patients with metabolic, infectious, or concomitant systemic pathologies and that of those, who received local or systemic adjuvant therapies were discarded. All patients signed a consent authorisation according to the standardised protocols of treatment and surgery, and the clinical information is summarised in Table 1.

The Bioethics Committee of the Escuela Nacional de Medicina y Homeopatía del Instituto Politécnico Nacional approved the procedures of this study. This study was conducted in accordance with the Declaration of Helsinki.

Phospholipid extraction and phosphorus-31 nuclear magnetic resonance (³¹P-NMR) spectroscopy Each frozen tissue sample was weighed (300 mg per sample) and placed in a microtube. Then, 1 mL of a mixture of CDCl₃:MeOD (2:1 v/v) was added (Folch *et al.*, 1957). The tissues were homogenised using zirconium beads in the Bead Bug Homogenizer (Benchmark Scientific; Sayreville, NJ, USA) for 3 min at 180 Hz. The samples were centrifuged at 13,500 × *g* for 10 min at 4°C, and the organic fraction (600 µL per sample) was collected in an NMR tube. Triphenylphosphine was added to achieve a final concentration of 0.1 mM. ³¹P-NMR hydrogen-decoupled spectra were obtained using the Bruker Avance 400 NMR spectrometer (Bruker Biospin, Karlsruhe, Germany). The analyses were performed at 25°C with the probe PABBO BB-1H/D Z-GRD for ³¹P at 161.98 MHz using the pulse sequence zgpg30 and 30° pulse, sweep width of 12931.0 Hz, repetition time of 0.63 s, 20000 scans, and acquisition time of 0.6335 s. Spectra were processed using the Bruker's TopSpin software v.3.1 (Bruker Biospin, Karlsruhe, Germany), by applying 1.0 Hz of line broadening. The identification of phospholipids was conducted according to the methods described in earlier reports (Pierce and Komoroski, 1993).

Data analysis NMR profile The ³¹P-NMR spectra were aligned and the dimensionality reduction was performed by lowering the original data points (32 k points) in bins of 0.04 ppm width (Anderson *et al.*, 2011). To compensate for variations arising due to difference in source cells and extraction, all the bins were normalised using the weight of the

respective sample extract. The alignment, 'binning', and normalisation were performed using the MNova software v.10.1 (Mestrelab Research, Santiago de Compostela, Spain). Since the assigned signals were normalised with sample weights and internal standard, the spectral intensities were compared to obtain a ratio between phospholipids.

Protein extraction and immunoblotting

Proteins were extracted from tissues using urea/thiourea lysis buffer containing 7 M urea, 2 M thiourea, and 4% (w/v) CHAPS detergent in 30 mM Tris-HCl pH 8.5, and Halt Protease Inhibitor Cocktail (Thermo Scientific, Waltham, MA USA). The tissues were homogenised using zirconium beads and processed in the Bead Bug Microtube Homogenizer (Benchmark Scientific; Sayreville, NJ, USA) for 3 min at 180 Hz. Extracts were centrifuged at $13,000 \times g$ for 10 min at 4°C and precipitated with cold acetone (3:1 ratio) at -20°C overnight. Total protein was quantified using the BCA method. Then, 10 µg of protein per sample was subjected to SDS-PAGE on 10% acrylamide/bisacrylamide (30:1) gels at 120 V. The resolved proteins were transferred onto a nitrocellulose membrane, 0.45 µm (Bio-Rad; Hercules, CA, USA) for 40 min at 24 V using the Towbin transfer buffer, 25 mM Tris, 0.192 mM glycine pH 8.6, and 10% methanol, using a trans-blot SD semi-dry electrophoretic transfer cell (Bio-Rad; Hercules, CA, USA). The membranes were blocked using phosphate-buffered saline-tween 0.25% (PBS-T) with 1% casein for 1 h. Membranes were then incubated with the mouse monoclonal anti- β -tubulin (1:6000; Santa Cruz Biotechnology, Dallas, TX, USA), rabbit polyclonal anti-sphingomyelin synthase 1 (anti-SMS1) (1:6000; Thermo Scientific, Waltham, MA USA), rabbit polyclonal anti-sphingomyelin synthase 2 (anti-SMS2) (1 : 6000; Invitrogen, Carlsbad, CA, USA), and anti-neutral sphingomyelinase 2 (anti-nSMase2) (1 : 6000; GeneTex, Irvine, CA, USA) for 1 h at room

temperature (25°C). After rinsing with PBS-T for 30 min, membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit IgG (1: 6000; Sigma-Aldrich, St. Louis, MO, USA) antibodies for 1 h at room temperature. The blots were developed using the Clarity Western ECL substrate (Bio-Rad, Hercules, CA, USA) for autoradiography at 1-min exposure. The autoradiographs were scanned with the EpiChemi Darkroom photo-documenting device (UVP; Upland, California, USA). Densitometric analysis was performed using the Image Studio Lite software v.5.2, LI-COR (Biosciences, Lincoln, Nebraska, USA).

Immunohistochemistry Tissue samples were fixed with 10% formalin-PBS, embedded in paraffin, and sliced in 5 µm-thick sections for further analysis. The tissue sections were de-paraffinised by heating the slides at 55°C for 30 min, followed by three washes using xylol and gradual hydration with 100%, 95%, 85%, and 75% aqueous-ethanol. The tissues were incubated with mouse monoclonal anti-β-tubulin (1:200; Santa Cruz Biotechnology, Dallas, TX, USA), anti-rabbit polyclonal anti-sphingomyelin synthase 1 (anti-SMS1) (1:200; Thermo Scientific, Waltham, MA USA), rabbit polyclonal anti-sphingomyelin synthase 2 (anti-SMS2) (1:200; Invitrogen, Carlsbad, CA, USA), and anti-sphingomyelin phosphodiesterase 2 (anti-nSMase2) (1:200; GeneTex, Irvine, CA, USA). Immuno-labelling was detected using the Histostain-Plus IHC Kit (Thermo Fisher, Waltham, MA, USA), according to the manufacturer's instructions. Staining intensity was determined using the IHC Profiler integrated in the ImageJ software v.1.46r (National Institutes of Health, Bethesda, USA) (Varghese *et al.*, 2014).

Statistical analysis The data matrix of NMR was analysed using multivariate statistics of the SIMCA software v13.0.3 (Umetrics, Umea, Sweden). The data were normalised

using logarithmic transformation and Pareto scaling. Principal Component Analysis (PCA) was used for the general description of the system and the supervised method of Partial Least Squares Discriminant Analysis (PLS-DA) was used for modelling using the same parameters of centring and scaling. For immunoblot densitometric analysis, the results were expressed as mean $\pm$ standard error (SEM) values from at least three independent experiments (n=3). The differences among groups were analysed using two-way ANOVA and the Tukey's *post hoc* test. The GraphPad Prism software v.6.0 (GraphPad, La Jolla, CA, USA) was used for statistical analysis. The significance was set at $P < 0.05$.

Results

Evaluation of phospholipids (PLs) using ^{31}P -NMR spectroscopy The ^{31}P -NMR spectra exhibited adequate resolution that allowed us to distinguish five metabolites (PLs) in GCTB, which were expressed in significantly higher concentrations compared to those in the non-neoplastic bone (Figure 1). The most noteworthy PL signals were identified at approximately -1.0 and 0.5 ppm, which corresponded to phosphatidylcholine (PC) at -0.84 ppm, phosphatidylserine (PS) at -0.02 ppm, sphingomyelin (SM) at 0.05 ppm, phosphatidylethanolamine (PE) at 0.24 ppm, and plasmalogen (PEp) at 0.32 ppm. A chemometric analysis was used to accurately assess the difference in the levels of PLs between the GCTB and non-neoplastic bone tissue samples. The ^{31}P -NMR spectra were binned, and each bin was considered as a variable for construction of a data matrix with the respective sample (Figure S1). A remarkable difference was observed between the non-neoplastic tissue and the GCTB samples in the PCA (Figure 2A). The first principal component (PC1) separated the samples and accounted for 74.3% of the variance within the data (represented as circles in blue colour, Figure 2A), while the second principal

component (PC2) accounted for 5.6% of the variance (green circles). Modelling with PLS-DA (Figure 2B) confirmed the differences between the GCTB and control groups. In the scoring graphs, the signals of some PLs determined the separation of two sample groups. According to the loading plots, the signal intensity of PC, PE, PEP, and SM was remarkably high in the GCTB samples compared to that in the non-neoplastic bone samples. A pseudospectrum (Figure 2C) was constructed with the loading coefficients. The high and red signals represent the variable importance in the projection (VIP) of the chemical shift in the PLS-DA model. Finally, the GCTB model parameters were validated using a permutation test (200 permutations) with a correlation of $R^2 = 0.80$ and the predictive capability of $Q^2 = 0.75$ (Figure 2D). This result supports the hypothesis of an altered SM metabolism. The control group exhibited a lower variance in the VIP for these metabolites. In contrast, the GCTB group exhibited a higher variance for SM, which is related to signalling and apoptosis, thereby indicating variations in this tumour, SM is one of the main substrates of the Cer synthesis. An additional non multivariate analysis of the VIP spectral intensities regarding the ratios of SM, PS, PE and PEP compared to main phospholipid PC was performed (Figure 3), The PS/PC and SM/PC ratios were significantly higher in control samples while PE/PS ratio were lower in controls and Pp/PC was not different in both groups (Table S1).

SMS1, SMS2, and nSMase2 protein expression The metabolic conversion of Cer to SM is catalysed mainly by SMS1 in the Golgi and by SMS2 in the Golgi and the cell membrane. We analysed the expression of both enzymes (Figure 4A) and observed that the relative expression of SMS1 (20.526 fold; $p = 0.0030$) and SMS2 (29.338 fold; $p=0.0066$) were significantly higher in GCTB compared to those in non-neoplastic bone

tissue (control group) (Figure 4B). The conversion of SM to Cer is catalysed by three different types of SMases that are activated according to the pH of the microenvironment. However, in this study, neutral sphingomyelinase 2 (nSMase2) was selected, since it is the best-characterized SMase of the three enzymes (Tani and Hannun, 2007), and it participates in bone mineralisation and cartilage development (Aubin *et al.*, 2005; Stoffel *et al.*, 2019; Stoffel *et al.*, 2007). Interestingly, a significant decrease in the relative expression of nSMase2 was observed in GCTB compared to that in the non-neoplastic bone tissue samples (0.0403 fold; $p = 0.0014$) (Figure 4B). These results suggest a possible selective up-regulation of SMS1 and SMS2, and down-regulation of nSMase2 in GCTB.

SMS1, SMS2, and nSMase2 immunohistochemical analysis The relative expression of SMS1, SMS2, and nSMase2 was analysed using immunohistochemistry (Figure 5). Previously, the tumour tissue was stained with hematoxylin and eosin (H&E) to identify the cell types (Figure 5A and 5B). Numerous multinucleated GCs of the osteoclast type (asterisks) and mononuclear SCs (arrows) are present, in addition to the abundant vascular component, which are all characteristic elements of GCTB. Immunostaining for SMS1 revealed mild cytoplasmic immunoreactivity in GCs, while the staining in SC was scarce (Figure 5C), compared to that in non-neoplastic bone, where it was localised in osteocytes (Figure 5D). We observed that the highest immunoreactivity for SMS2 was in the GC cytoplasm (Figure 5E), and the intensity was moderate compared to that in the control tissue (Figure 5F), where the intensity demonstrated a decreased presence. nSMase2 was expressed in the cytoplasm of both GCs and SCs, at about 50% for both cellular elements (Figure 5G). The expression was evident in the control tissue as well

(Figure 5H). Image analysis and the semi-quantitative comparisons of the optical density scores were performed using the ImageJ software and the IHC Profiler complement (Varghese *et al.*, 2014). The results of this analysis are shown as histograms of pixel density. The upper histogram in each panel corresponds to the GCTB, while the lower histogram corresponds to the non-neoplastic tissue (Figure 5). The semi-quantitative analysis of the images showed a low IHC optical density score for SMS1 and SMS2 of the GCTB compared to the non-neoplastic bone tissue; and a low score for nSMase2 in both samples.

Discussion

Has been shown that the phospholipid content increase with cell transformation and carcinoma progression (Cheng *et al.*, 2016). For example, an increased of phosphocholine, glycerophosphocholine, and free choline, has been detected by ¹H MRS (proton magnetic resonance spectroscopy (¹H-MRS)) in breast, prostate, ovarian, endometrial, cervical, and brain cancers compared to non-cancerous healthy tissues (Glunde *et al.*, 2011). In relation to enzymes involved in phospholipid metabolism can be highlighted the overexpression and elevated activity of choline kinase as factors that contribute to increased phosphocholine and choline in various tumor tissues including breast, ovarian, colorectal, prostate, lung, endometrial, and pancreatic cancer (Cheng *et al.*, 2016).

Concerning mesenchymal tumours such as bone sarcomas, the phospholipid metabolism is scarcer. Until now it is known that in osteosarcoma, the expression of phospholipase A2 which hydrolyses the ester bond of the sn-2 position membrane phospholipids preferably PC releasing free fatty acids and lysophospholipid, is higher at mRNA and

269 protein levels in patients with metastasis. (Liang *et al.*, 2015). Consistently, in MG-63
270 osteosarcoma cell line have been shown the cytidine 5'-diphosphocholine (CDP-choline)
271 pathway is the only route for PC biosynthesis, while silencing Choline kinase- α (CK α) in
272 these cells reduce the cell proliferation and mineralization as well as slight decrease the
273 phosphocholine concentration (Li *et al.*, 2014).

274 Unfortunately, there is limited knowledge on the metabolic changes in tumours of
275 mesenchymal origin, such as GCTB. In this study, we focussed on the evaluation of SM
276 biosynthesis, since it was the most important variable, based on the multivariate analysis
277 and the extraction of the VIP from the data obtained using ^{31}P -NMR spectroscopy.

278 These results are concordant with the earlier reports. It is presently accepted that
279 enzymatic dysregulation in the SM metabolism in signalling plays a vital role in the
280 tumours of epithelial and haematological origin (Ogretmen, 2018). This explains the
281 reason for increased research interest in SM biosynthesis, because there are no reports
282 on its metabolic state in GCTB.

283 The enzymes which are directly responsible for the synthesis of SM include the ceramide
284 choline phosphotransferases or sphingomyelin synthases (SMSs). The reaction begins
285 with the transfer of a phosphocholine residue from phosphatidylcholine (PC) to the
286 primary hydroxyl group of the ceramide, resulting in SM and diacylglycerol (DAG)
287 (Holthuis and Luberto, 2010). Sphingomyelin phosphodiesterases or sphingomyelinases
288 (SMases) hydrolyse the phosphodiester bond of SM, thereby generating Cer and
289 phosphocholine (Bienias *et al.*, 2016). Owing to the concerted action of these two enzyme
290 families, SM concentrations remain balanced in the intracellular compartments. The Golgi
291 apparatus and the plasma membrane are the major sites of SM synthesis (D'Angelo *et*

al., 2018). Therefore, it is inevitable for these enzymes (SMS1 and SMS2) to be distributed in the cytoplasm (see Figure 4). Similarly, the hydrolytic enzyme is also commonly observed to be distributed in the cytoplasm, as SM and Cer, being two bioactive lipids, are strictly regulated with respect to both the time of synthesis and distribution. SM is an essential modulator of the structural properties of the plasma membrane, which influences the accumulation of proteins involved in cell proliferation. Therefore, it is not surprising that the dysregulation of its biosynthetic and hydrolytic enzymes promotes tumourigenesis.

Our results indicated that the relative overexpression of SMS1 and SMS2 in GCTB explained the increase in the intensity of the ^{31}P -NMR signal of SM, however the lower SM/PC ratio in GCTB indicates a possible increase in SM utilization in other processes not dependent on ceramide synthesis. The low SM/PC ratio has not yet been described in GCTB, high levels of SM are usually described as markers of high replicative rate and rapid invasion of other neoplastic tissues, GCTB is considered benign although locally aggressive. This finding is consistent with the increased expression of SMS2 in patients with breast cancer, who exhibit metastases to the lymph node (Zheng et al., 2019) as well as in patients with chemo-resistant leukaemia (Lafont et al., 2011). Leukemic cells and GCs share a haematopoietic origin; thus, the highest contribution of SM in GCTB is attributed to GCs, as suggested by our immunohistochemical results (Figure 4).

Regarding the sphingomyelinases which hydrolyse SM to ceramide and diacylglycerol these enzymes have been implicated in various cancer pathologies (Shanbhogue and Hannun, 2020). aSMase (acid sphingomyelinase) has been involved in metastasis activation of melanome cells and in the mechanism of various anti-cancer drugs as

gemcitabine or sunitib (Adada [et al.](#), 2016). Alk-SMase (alkaline sphingomyelinase) shown decreased activity in biopsies of colorectal carcinoma, while alk-SMase knock-out mice enhanced colonic tumorigenesis (Adada [et al.](#), 2016). Among nSMases family consisting of nSMase1, nSMase2 and nSMase3, the second one is the most studied in cancer pathophysiology (Clarke, 2018). Since nSMase-2 which participates in bone mineralisation and cartilage development (Aubin *et al.*, 2005; Stoffel *et al.*, 2019; Stoffel *et al.*, 2007) we observed its down-regulation in GCTB. This result is in line with the results of previous reports on decreased nSMase2 expression in breast and prostate cancer owing to the loss of heterozygosity in chromosome 16q22.1, where nSMase2 gene is localised (Filippova [et al.](#), 1998). A homozygous loss of nSMase2 was identified in a mouse osteosarcoma cell line and when homozygosity was restored, the cell growth was suppressed (Kim [et al.](#), 2008). Curiously, our results also revealed low basal levels of SMS1 and SMS2 in the healthy tissue samples, which were almost undetectable under the same conditions as the GCTB tissue samples. This is in line with the results from reports on various cell types, which showed similar results, though with slight variations among the different cell types (Holthuis and Luberto, 2010; Shakor *et al.*, 2011).

In summary, our results indicated an overexpression of SMS1 and SMS2 and downregulation of nSMase2 in GCTB, which favoured a disturbance of synthesis of SM and a consequent imbalance in their cellular levels. This dysregulation could be related to mechanisms already described for bone cells. For example, the knockdown of SMS2 inhibits the expression of RANKL mRNA in primary osteoblasts, which results in the suppression of its differentiation to osteoclasts (Yoshikawa [et al.](#), 2019). Taken together, this may suggest that in GCTB, the elevation of SMS2 increases the expression of RANKL

and thus results in a greater proliferation of GCs during osteoclastogenesis. Another remarkable cellular process linked to SMase activity is apoptosis (Hannun and Obeid, 2008; Kolesnick, 2002; van Blitterswijk *et al.*, 2003). The synthesis of Cer by SMase activity facilitates death-receptor clustering at the membrane surface (Goni and Alonso, 2009; Gulbins *et al.*, 2004), especially nSMase2 activity, which is responsible for synthesis of Cer in the internal leaflet of the plasma membrane (Takeda *et al.*, 1999; van Blitterswijk *et al.*, 2003). This results in rapid increase in the Cer levels, resulting in the formation of blebbing in the plasma membrane and subsequently, the apoptotic bodies (van Blitterswijk *et al.*, 2003). These lead to the Cer-dependent induction of apoptosis and suppression of cell proliferation (Ekiz and Baran, 2010; Maguer-Satta, 1998). Based on our results, it can be inferred that in GCTB, the expression of nSMase2 is down-regulated; hence, it is plausible to observe a low concentration of Cer in these tissue samples.

Recently, the relevance of the nSMase2 in the exosome pathway was elucidated. An exosome is a vesicle secreted by the cells that transfers cargo like proteins, glycoproteins, lipids, and nucleic acids into target cells, thereby modulating cell communication (Pegtel and Gould, 2019). In cancer, exosomes are related to tumour progression because of their influence on mechanisms such as migration, invasion, evasion of immune cells, and formation of a metastatic niche (Osaki and Okada, 2019). Exosomal transfer depends on nSMase2 activity, and when this enzyme is suppressed, the metastatic capacity of cancer cells decreases due to inhibition of angiogenesis (Kosaka *et al.*, 2013). Although, the exosome liberation from GCTB is not well understood, its importance in osteoclastogenesis and osteogenesis as well the association between osteosarcoma cells and the microenvironment cells are established (Kosaka *et al.*, 2013; Perut *et al.*,

2019). It is assumed that the imbalance in nSMase2 may also be related to exosome release in GCTB; therefore, future studies are necessary to validate these hypotheses. An optimal SM/Cer ratio is essential for determination of cell fate, whether survival or death, and changes in the metabolism can interrupt this balance and promote tumourigenesis, as probably is the case in GCTB. Recently, has been revised that specific levels of SM depend on the type of cancers (Codini *et al.*, 2021). Increased levels have been found in hepatocellular carcinoma while lower were found in prostate and oesophageal cancer, interestingly, sphingomyelin levels appear to be inversely correlated with the aggressiveness of breast and colorectal cancer cell lines. Finally, the lower ratio of SM/PC indicates a dysregulation of the levels of SM and overexpression of SMS-1, 2 and downregulation of nSMase founded in this study will be useful in understanding the behaviour and progression of mesenchymal tumours, specifically GCTB.

Acknowledgements

We acknowledge BEIFI scholarship for the student Tayde Quiroz-Acosta and CONACYT support register 2019-000006-01NACV-00402 for Yazmin Montserrat Flores-Martinez.

Competing interest statement

The authors declare that there is no interest conflict.

Authors contribution

Conceived and designed the experiments: Nury Pérez-Hernández, Elizabeth Pérez-Hernández, Angel Ernesto Bañuelos-Hernández;

Performed the experiments: Tayde Quiroz-Acosta, Elvia Becerra-Martínez:

382 Statistical analysis: Angel Ernesto Bañuelos-Hernández;

383 Wrote the paper: Yazmin M. Flores-Martinez, Nury Pérez-Hernández;

384 All authors read and approved the final manuscript.

385 **Funding statement**

386 We acknowledge financial support for this work from the SIP-IPN 20210180.

387 **Data availability statement**

388 The data used to support the findings of this study are available from the corresponding
389 author upon request.

Draft

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585

Table 1 *Clinical characteristics of patients with GCTB and controls*

	<i>GCTB</i>	<i>Controls</i>
<i>Number</i>	6	6
<i>Mean age</i>	34	45
<i>Male/female</i>	(4/2)	(3/3)
<i>Tumour localisation</i>	Femur and tibia	NA
<i>Enneking staging</i>	IA	NA

590

591 [Figure Captions]

592 **Figure 1** Stacking of ^{31}P -NMR spectra of GCTB and non-neoplastic bone. Assignments:
593 -0.84 ppm phosphatidylcholine (PC), 0.02 ppm phosphatidylserine (PS), 0.05 ppm
594 sphingomyelin (SM), 0.24 ppm phosphatidylethanolamine (PE), and 0.32 ppm
595 plasmalogen (PE_p), *pure SM.

596 **Figure 2** (A) Score plot of PCA. (B) Score plots of PLS-DA. (C) Load map of the PLS-DA,
597 intensity of colour represents the variable importance in the projection (VIP) phospholipids
598 or differentials; (X-axis) chemical shifts, (Y-axis) probability and correlation. (D) Statistical
599 validation of the PLS-DA model using permutation analysis, $R^2=0.80$ is the explained
600 variance and $Q^2=0.75$ is the predictive ability of the 200-permutations model.

601 **Figure 3** Ratios of the spectral intensities of the VIP phospholipids. Proportions were
602 analysed by two-way ANOVA followed by Sidak's *post hoc* test at 0.05% significance
603 ($n=6$, * $p<0.05$, ** $p<0.01$, **** $p<0.001$).

604 **Figure 4** *Expression of SMS1, SMS2, and nSMase2 analysed using western blot.* Total
605 protein extracts of GCTB and non-neoplastic bone tissue, β -tubulin expression was used
606 as the control. Representative images of triplicate experiments, mean $\pm$ SEM, ANOVA,
607 Tukey's *post hoc* test, $p=0.005$.

608 **Figure 5** *Expression of SMS1, SMS2, and nSMase2 by immunohistochemistry.* (A, B)
609 Representative micrographs showing the H&E staining of GCTB, (H&E x20, x40).
610 Cytoplasmic immunoreactivity in both GCs and SCs of GCTB for (C) SMS1, (E) SMS2,
611 and (G) nSMase2, mild to moderate intensity (x20, x40 in box). Cytoplasmic

immunoreactivity of osteocytes in non-neoplastic bone for (D) SMS1, (F) SMS2, and (H) nSMase2 (x20, x40 in box). The histograms indicate density of the pixels (X-axis) versus the pixel count (Y-axis).

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Figure 1 Stacking of ^{31}P -NMR spectra of GCTB and non-neoplastic bone. Assignments: -0.84 ppm phosphatidylcholine (PC), 0.02 ppm phosphatidylserine (PS), 0.05 ppm sphingomyelin (SM), 0.24 ppm phosphatidylethanolamine (PE), and 0.32 ppm plasmalogen (PE_p), *pure SM.

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Figure 2 (A) Score plot of PCA. (B) Score plots of PLS-DA. (C) Load map of the PSL-DA, intensity of colour represents the variable importance in the projection (VIP) phospholipids or differentials; (X-axis) chemical shifts, (Y-axis) probability and correlation. (D) Statistical validation of the PLS-DA model using permutation analysis, R2=0.80 is the explained variance and Q2=0.75 is the predictive ability of the 200-permutations model.

Figure 3 Ratios of the spectral intensities of the VIP phospholipids. Proportions were analysed by two-way ANOVA followed by Sidak's *post hoc* test at 0.05% significance (n=6, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.001$).

Figure 4 Expression of SMS1, SMS2, and nSMase2 analysed using western blot. Total protein extracts of GCTB and non-neoplastic bone tissue, β -tubulin expression was used as the control. Representative images of triplicate experiments, mean $\pm$ SEM, ANOVA, Tukey's *post hoc* test, $p=0.005$.

Figure 5 Expression of SMS1, SMS2, and nSMase2 by immunohistochemistry. (A, B) Representative micrographs showing the H&E staining of GCTB, (H&E x20, x40). Cytoplasmic immunoreactivity in both GCs and SCs of GCTB for (C) SMS1, (E) SMS2, and (G) nSMase2, mild to moderate intensity (x20, x40 in box). Cytoplasmic immunoreactivity of osteocytes in non-neoplastic bone for (D) SMS1, (F) SMS2, and (H) nSMase2 (x20, x40 in box). The histograms indicate density of the pixels (X-axis) versus the pixel count (Y-axis).