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**Sphingosine kinase-1 is overexpressed and correlates with hypoxia in osteosarcoma:  
relationship with clinicopathological parameters**

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## ABSTRACT

The Sphingosine kinase-1/Sphingosine 1-Phosphate (SphK1/S1P) signaling pathway is overexpressed in various cancers, and instrumental for the adaptation to hypoxia in a number of solid tumor models, but no data are available in osteosarcoma characterized by hypoxia. The objectives of the present study were (i) to assess the contribution of SphK1/S1P signaling in regulating HIF-1 $\alpha$  expression under hypoxia in various osteosarcoma cell models, (ii) quantify SphK1 enzymatic activity in biopsies of osteosarcoma, and (iii) examine the relationship between SphK1, S1P receptor 1 (S1P<sub>1</sub>) and hypoxia (GLUT-1) in 130 cases of osteosarcoma (71 long bones and 59 flat bones) by immunohistochemistry.

In osteosarcoma cells, the accumulation of HIF-1 $\alpha$  under hypoxia was prevented by the inhibition of SphK1. In addition, FTY720 (Fingolimod), which is used for the treatment of relapsing-remitting multiple sclerosis, and targets SphK1 and S1P<sub>1</sub> also prevented HIF-1 $\alpha$  accumulation. Resistance to doxorubicin was observed in hypoxic osteosarcoma cells. Importantly, in contrast to conventional chemotherapy, FTY720 did equally inhibit cell proliferation in both normoxia and hypoxia.

A significant increase in SphK1 activity was observed in cancer as compared to normal bones. In all series of TMA samples, immunohistochemical analysis showed that GLUT-1, SphK1 and S1P<sub>1</sub> were expressed in tumors regardless of the location. SphK1 markedly correlated to the hypoxic marker GLUT-1 suggesting that SphK1 is overexpressed and correlates with intratumoral hypoxia. No correlation was found between GLUT-1 or SphK1 with response to chemotherapy, yet a statistical difference was found with an increased expression of S1P<sub>1</sub> in poor responder patients in long bone osteosarcomas. Importantly, multivariate analyses showed that GLUT-1 was associated with an increased risk of death in flat bone, whereas SphK1 and S1P<sub>1</sub> were associated with an increased risk of death in long bones.

Collectively, our data suggest that SphK1/S1P signaling might represent a potential target to investigate in osteosarcoma patients.

Keywords : FTY720, Fingolimod, Sphingosine kinase, S1P<sub>1</sub>, Osteosarcoma, HIF, GLUT-1

## INTRODUCTION

Although rare, osteosarcoma is the most common malignancy of bone predominantly affecting children and young adults <sup>1,2</sup>. It often occurs in the metaphyses of long bones such as the femur or tibia, and is pathologically characterized by cells with high grade atypia and aberrant osteoid formation <sup>1</sup>. Jaw osteosarcoma accounts for only 6% of all osteosarcomas and develops mainly in the mandible. It is diagnosed approximately two decades later than long bone osteosarcoma and entails a lower risk of lung metastases reported to be 20-25% versus 44-49% for long bone osteosarcomas <sup>3</sup>.

According to the 2020 WHO classification, osteosarcoma can be classified into high grade conventional osteosarcomas, the most common histologic subtype (75% of all cases), periosteal osteosarcoma of intermediate-grade, and low-grade osteosarcomas (paraosteal and intramedullary osteosarcomas). Microscopically, conventional osteosarcoma is highly heterogeneous with cells that produce varying amounts of osteoid/chondroid matrix <sup>4</sup>.

The high complexity of the osteosarcoma genome did not allow for the identification of key molecular therapeutic targets so far <sup>5-7</sup>. As a consequence, osteosarcoma treatment still involves a combination of surgery with either neoadjuvant or adjuvant chemotherapy. 5-year survival has not evolved over the past 4 decades, and does not exceed 70% with combined surgery and chemotherapy. It falls to 25% in patients with metastatic or relapsed osteosarcoma <sup>8-12</sup>.

There are a number of reported mechanisms for treatment resistance in osteosarcoma including genomic aberrations, non coding RNA post-transcriptional regulations or tumor microenvironmental factors such as intratumoral hypoxia <sup>13</sup>. Hypoxia is a feature of many solid human tumors as the aberrant cell proliferation rate is associated with a disequilibrium between oxygen supply and consumption <sup>14</sup>. The many effects of hypoxia on cancer biology include not only promotion of progression and metastasis <sup>15</sup> but also resistance to radiotherapy or chemotherapy <sup>16</sup>. The transcription factor, hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ), has been reported as the master driver of adaptation to hypoxia. A wealth of reports based on immunohistochemical studies of human tumor sections indicate that HIF-1 $\alpha$  is overexpressed in the majority of human cancers and these elevated levels correlate with cancer-related death <sup>17</sup>. A number of studies have analyzed the association of hypoxic markers (HIF-1 $\alpha$ , VEGF or GLUT-1) with prognosis and clinicopathological characteristics of osteosarcoma. Recent meta-

analyses suggest that hypoxia would be associated with lower survival rate, higher microvessel density, metastasis, higher pathologic grade, tumor stage and poor response to chemotherapy<sup>18-20</sup>.

Sphingosine 1-phosphate (S1P) is a pleiotropic phospholipid that regulates proliferation, migration, inflammation or angiogenesis<sup>21</sup>. S1P is produced by phosphorylation of sphingosine by two isoforms of sphingosine kinases (SphK1 and SphK2). Sphingosine kinase 1 (SphK1) is commonly overexpressed in cancer cells and correlates with poor patient prognosis in a number of tumor types<sup>22, 23</sup>. As SphK1/S1P signaling, contributes to malignant progression by controlling proliferation and metastatic potential of cancer cells it represents a potential target for anti-cancer therapy<sup>24</sup>. We previously identified SphK1/S1P signaling as a new modulator of HIF-1 $\alpha$  and HIF-2 $\alpha$  activities under hypoxia in a wide array of cancer cell models (prostate, glioma, breast, lung and renal cell carcinoma) both *in vitro* and *in vivo*<sup>25-27</sup>. FTY720 (Fingolimod) is an analogue of sphingosine that has been approved by the US Food and Drug Administration (FDA) in 2010 for the treatment of relapsing-remitting multiple sclerosis after two phase III clinical studies establishing its efficacy and global safety<sup>28, 29</sup>. It is currently under trial for the treatment of breast carcinoma, glioblastoma and anaplastic astrocytoma (NCT03941743 and NCT02490930). It can be phosphorylated *in vivo* to form FTY720-phosphate (FTY720-P), a mimetic of S1P, interacting with several S1P receptors but preferentially inducing internalization and degradation of S1P<sub>1</sub><sup>30</sup>. Some of its actions are also attributed to its unphosphorylated form notably inhibition of SphK1 activity as suggested by several independent studies<sup>31-33</sup>. Antitumoral properties of FTY720 have already been evidenced in several cancer animal models<sup>32, 34-37</sup>. We previously reported that FTY720 decreases both HIF-1 $\alpha$  and HIF-2 $\alpha$  expression in a number of cellular and animal models of renal cell carcinoma<sup>38</sup>. Collectively, these data suggest that targeting the SphK1/S1P signaling represents a strategy that could potentially be exploited in therapeutic approaches to decrease hypoxia in cancer<sup>39, 40</sup>.

The objectives of this work was to assess i) the role of the SphK1/S1P signaling in various cellular models (U-2OS, SaOS-2, MG-63 and 143B) osteosarcoma cell lines under hypoxia, ii) the SphK1 enzymatic activity in human osteosarcoma samples versus non tumoral bones, and iii) the expression of GLUT-1, SphK1 and S1P<sub>1</sub> in 130 cases of osteosarcomas (long and flat bone

samples) as the link between SphK1/S1P signaling and hypoxia has never been investigated in human tissues.

## **MATERIALS AND METHODS**

### **Patient and tumor characteristics**

Tissue microarrays (TMAs) were prepared from the diagnostic biopsies of 130 patients coming from Toulouse (AGB), Lille (SA), Nantes (LG), Marseille (CB), Paris (FL), Tours (GDP). TMAs (triplicate sampling of 1 mm) were established at the Institut Universitaire du Cancer biobank of Toulouse (AGB). For all cases, the TMA cores have been selected in the most cellular areas based on the diagnostic biopsy slide stained with Hematoxylin Eosin (H&E). All OS samples were reviewed and reclassified by the accredited pathologists (SA, CB, FL, GDP, AGB) of the GFPO (French Group of Bone Pathologists), according to the WHO 2020 classification.

The TMAs were then stored at the certified NF 96–900 cancer biobank of Toulouse (BB-0033-00014) where the immunohistochemistry study was conducted (GLUT1, SphK1, S1P<sub>1</sub>). According to the French law, the biobank cancer collection was declared to the Ministry of High Education and Research (DC-2020-4074) and a transfer agreement was obtained (AC-2020-4031) after titres by ethical committees. All patient records and information were anonymized and de-identified before analysis. Informed consent was obtained from all patients and the use of the biological specimens was approved by the local institutional review board.

Patient and tumor characteristics, and response to treatment are described in Table 2 for the 130 patients included in the tissue microarray study. We studied 59 flat bone osteosarcomas (mandibular and other locations: pelvis, ribs) and 71 long bone osteosarcomas.

To complete our exploration we used 12 other surgical biopsies coming from the OS Toulouse collection and 10 normal bone samples also stored at the certified NF 96–900 cancer biobank of Toulouse (BB-0033-00014). These samples were used to quantify the SphK1 kinase enzymatic activity.

### **Immunohistochemistry**

SphK1 immunohistochemistry was performed manually on deparaffinized FFPE tissue sections heat pre-treated (95°) using Envision TRS Low pH buffer (pH 6, GV80511-2, Agilent Technologies, CA, USA) during 30 minutes. Sections were subsequently incubated with 3% hydrogen peroxide for 20 minutes to block endogenous peroxidase. The primary mouse monoclonal SphK1 antibody (1/300 in Envision Flex diluent (K800621-2), Agilent Technologies), was applied during 1 hour at room temperature and visualized using the Envision Flex DAB detection kit (Agilent Technologies). The tissue slides were counterstained using hematoxylin (Agilent Technologies) for 20 minutes at room temperature.

S1P<sub>1</sub> immunohistochemical stains were automated using the Discovery ULTRA (Roche, Ventana Medical Systems, Innovation Park Drive, Tucson, AZ USA). After dewaxing, tissue slides were heat pre-treated using a CC1 (pH8) buffer (05424569001, Roche) during 32 minutes at 100°C. The slides were blocked for endogenous peroxidase activity using the CM inhibitor (32 minutes at 37 °C) (Roche). The primary S1P<sub>1</sub> antibody <sup>41</sup>, , used at 1/100 in Ventana Diluent (Roche) was incubated for 20 minutes at 36 °C. The target was then linked using the OmniMap anti-rabbit (05269679001, Roche, Tucson, AZ, USA) HRP conjugated secondary antibody and visualized using the ChromoMap DAB detection kit (05266645001, Roche). The tissue slides were counterstained using hematoxylin (05277965001, Roche) for 8 minutes followed by post-coloration using the Bluing reagent for 4 minutes at room temperature (05266769001, Roche).

Finally, GLUT1 immunohistochemistry was performed using the Benchmark ULTRA (Roche, Ventana Medical Systems, Tucson, AZ, USA). After dewaxing, tissue slides were heat pre-treated using a CC1 (pH8) buffer (05424569001, Roche) for 64 minutes at 98°C. The slides were blocked for endogenous peroxidase activity and incubated with ready-to-use primary anti-GLUT1 polyclonal antibody (06419178001, Roche) for 32 minutes. The target was then visualized using the OptiView DAB detection kit (06396500001, Roche). The tissue slides were counterstained using hematoxylin (05277965001, Roche) for 8 minutes followed by post-coloration using Bluing reagent for 4 minutes at room temperature (05266769001, Roche).

All slides were then dehydrated (ethanol and xylene) and mounted using xylene-based mounting. Isotype negative control immunoglobulin stains were included for all markers as quality control. The staining for each target was evaluated using two methods: the percentage of stained cells, without considering the intensity of the staining, altered by the preanalytical treatment of histological sections and the IRS Score (semi quantitative immunoreactive score). This latter scoring system multiplies staining intensity (4 grades) by the percentage of cells positive for the marker (5 grades) and results in a scoring of 0 to 12 <sup>42, 43</sup>. Immunoreactivity was considered positive if detected in >1% of cells per core of 1mm, irrespective of staining intensity. A double-blind examination by two pathologists, one expert in bone sarcoma, was performed for the interpretation of the immunohistochemistry results.

### **Chemicals and reagents**

Culture medium was from Life Technologies (Saint Aubin, France). Serum was from Perbio (Brebieres, France). [ $\gamma$ -32P]ATP was from Perkin-Elmer (Courtaboeuf, France). Silica gel 60 TLC plates were from VWR (Fontenay sous Bois, France). FTY720 (fingolimod) was from Enzo



Life Science (Villeurbanne, France). SKI II (CAS Number 312636-16-1) SphK1 inhibitor, doxorubicin and all other reagents were from Sigma-Aldrich (Saint-Quentin Fallavier, France).

### **Cell lines**

Human U-2OS, MG-63 and SaOS-2 osteosarcoma cell lines were obtained from ATCC (Molsheim, France). Human 143B cell line was kindly supplied by Dr F. Lecanda (CIMA, Pamplona, Spain). Cells were cultured in RPMI containing 10% fetal bovine serum at 37°C in 5% CO<sub>2</sub> humidified incubators. Cell lines were routinely verified by the following tests: morphology examination, growth analysis and mycoplasma detection (MycoAlert™, Lonza, Basel, Switzerland). All experiments were started with low-passaged cells (<25 times). Hypoxia (0,1% O<sub>2</sub>, 5% CO<sub>2</sub>, 94,5% N<sub>2</sub>) was achieved using an InVivo<sub>2</sub> hypoxic workstation (Ruskin, Bridgend, UK).

### **Sphingosine kinase-1 enzymatic activity**

SphK1 activity was performed as previously described <sup>44</sup>, and determined in the presence of 50 µM sphingosine, 0.25% Triton X-100 and [ $\gamma$ -<sup>32</sup>P] ATP (10 µCi, 1 mM) containing 10 mM MgCl<sub>2</sub>. The labeled S1P was separated by thin layer chromatography on silica gel 60 with 1-butanol/ethanol/acetic acid/water (80:20:10:10, v/v) and visualized by autoradiography. Activity was expressed as picomoles of S1P formed/min/mg of protein.

### **Western-blot analysis and antibodies**

Mouse anti-HIF-1 $\alpha$  (BD Biosciences, Le Pont de Claix, France) was used as primary antibody. Proteins were visualized by an enhanced chemiluminescence detection system (GE Healthcare, Vélizy-Villacoublay, France) using anti-mouse horseradish peroxidase-conjugated IgG (Bio-Rad, Hercules). Equal loading of protein was confirmed by probing the blots with anti- $\alpha$ -tubulin or anti- $\beta$ -actin antibodies (Sigma-Aldrich). Densitometry quantitation was determined with Image J software 1.53i using the area under the peak method (NIH, Bethesda, MD).

### **Quantitative Real Time PCR**

Total RNA was isolated using the RNeasy minikit (Qiagen, Courtaboeuf, France) and 1 µg were reversed transcribed to cDNA using the SuperScript First Stand Synthesis System (Invitrogen). Quantitative real time PCR was performed using the MESA Blue PCR Master mix (Eurogentec). Reactions were performed using hSphK1 (forward 5'-CTGGCAGCTTCCTTGAACCAT-3'; reverse, 5'-TGTGCAGAGACAGCAGGTTCA-3').  $\beta$ -actin-specific primers (forward primer, 5'-GCAAAGACCTGTACGCCAAC-3'; reverse primer, 5'-AGT

ACTTGCCTCAGGAGGA-3'), TBP-specific primers (forward primer, 5'-TGACCTAAAGACCATTGCACTTCG -3'; reverse primer, 5'-CGTGGTTCGTGGCTCTCTTATC -3'). For analysis, all genes were normalized to expression of zeta polypeptide (YWHAZ) gene as endogenous control.

### **Cell viability Assay**

The MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used to determine cell death as previously described <sup>45</sup>. Briefly, cells were seeded at 5,000 cells/well in 24-well plates and allowed to attach overnight. After 72 h of treatment, cells were incubated at 37 °C and 5% CO<sub>2</sub> with 25 µL MTT solution (5 mg/mL; Sigma-Aldrich) for 4 h. After solubilization with 500 µL of lysis buffer (DMSO), formazan was quantified by spectrophotometry with a microplate reader at 570 nm absorbance. The GI<sub>50</sub> values corresponding to the concentration that caused 50% inhibition of cell proliferation were calculated from dose-response curves obtained by nonlinear regression analysis. All the results were calculated from data obtained in three independent experiments.

### **Statistical analysis**

The statistical significance of differences between the means of two groups was evaluated by unpaired Student's t test. All statistical tests were two-sided, and the level of significance was set at P<0.05. Calculations were done using Prism 9 (GraphPad Software, San Diego, CA).

For the overall survival analysis, the outcome was the occurrence of death and the time was defined as the time between the date of diagnosis and the date of death (event) or the date of last follow-up (censorship). For the analysis of metastasis progression-free survival (MPFS), the outcome measure was the occurrence of metastasis and / or the occurrence of death. The time was defined as the time between the date of diagnosis and the date of metastatic progression, if presence of metastases, or the date of death (event) or the date of last follow-up (censorship). Patients who locally relapsed as their first event were considered to be censored data, in order to avoid the bias related to the quality of the surgical resection margins. All survival rates were estimated by the Kaplan-Meier method with 95% confidence intervals (CI). The association between the presence of markers expressed in percentage of cells (for a 10% increase in labeled cells) or using the IRS score (for an increase in the score of 1) and the occurrence of death or metastases / death was estimated using a Cox proportional hazards model with an adjustment for age. The association between the presence of markers

and response to chemotherapy was estimated using logistic regression with adjustment for age. These multivariate analyzes were performed using SAS 9.4 software.

## RESULTS

### The SphK1/S1P signaling regulates HIF-1 $\alpha$ accumulation in osteosarcoma cell lines

In agreement with work conducted by others <sup>46-51</sup>, hypoxia was associated with increased HIF-1 $\alpha$  accumulation in U-2 OS, MG-63 and SaOS-2 cell lines peaking at 4-6h of treatment (Figure 1A). Because we previously reported that the SphK1/S1P pathway is a regulator of both HIF-1 $\alpha$  and HIF-2 $\alpha$  during hypoxia in multiple cancer cell lineages <sup>25-27, 38</sup>, we evaluated its relevance with regard to HIF-1 $\alpha$  expression in osteosarcoma cells. In line with our previous findings in renal cell carcinoma models <sup>27</sup>, we found an increase in SphK1 and S1P receptor 1 (S1P<sub>1</sub>) mRNA expression after 60 minutes of hypoxia in the U-2 OS cell model (Figure 1B). We next directly assessed SphK1 enzymatic activity. Hypoxia caused a rapid 2 fold increase in S1P production with a peak at 2-4h, returning to basal levels by 16h of treatment (Figure 1C). To validate that SphK1/S1P dependent-signaling was causal in the accumulation of HIF-1 $\alpha$ , we used SKI-II, a specific inhibitor of SphK1 activity <sup>52</sup>, and FTY720 that inhibits both SphK1 activity and S1P<sub>1</sub> expression <sup>30-33</sup>. Both SKI-II and FTY720 dose-dependently inhibited HIF-1 $\alpha$  accumulation in U-2 OS cells (Figure 1D and E). These data suggesting that SphK1/S1P signaling is required for the regulation of HIF-1 $\alpha$  in osteosarcoma are in line with our previous findings in prostate and renal cell carcinoma cell models <sup>25-27, 38</sup>.

Highly significant resistance to chemotherapies used in clinic including etoposide, cisplatin and doxorubicin have been reported in cellular models of osteosarcoma under hypoxic conditions <sup>48, 51</sup>. In line with the aforementioned studies, hypoxia significantly reduced the sensitivity of U-2 OS, SaOS-2, MG-63 and 143B cells to doxorubicin with GI<sub>50</sub> values exhibiting from 3 to over 13-fold increase under hypoxia versus normoxic conditions (Table 1). In contrast, FTY720 remarkably reduced cell proliferation to the same extent in normoxic and hypoxic conditions in all osteosarcoma cell lines suggesting that efficacy of FTY720 was related to its anti-HIF-1 $\alpha$  effect.

### Sphingosine kinase-1 activity is overexpressed in osteosarcoma biopsy samples

Sphingosine kinase-1 (SphK1) enzymatic activity was assessed in human biopsies (10 non-tumor and 12 tumor individual samples). As a whole, mean SphK1 activity was 432 pmol/min/mg (95% CI: 77.4-835) in tumor samples versus 6.3 (95% CI: 0.45-12.1) in non-cancerous tissue accounting for a statistically significant ( $p = 0.023$ ) increase in osteosarcoma

tissue as compared to normal bone, suggesting that S1P is abundantly generated in osteosarcoma tissues (Figure 2).

### **Description of the patient population**

The demographic, clinical and histological data of the 130 patients are summarized in Table 2. The median age at diagnostic was 22 years (range, 6 to 84). Seventy nine (79) of the patients (60.8%) were >18 years of age and 80 (61.5%) were males. Fifty nine (59) osteosarcomas were located in the flat bones (mandibular and other locations) (45.4%) and 71 in the limbs (54.6%). All osteosarcomas were high-grade, conventional subtypes with a higher proportion of conventional osteoblastic (46.2%) compared to chondroblastic (24.6%). The tumors were localized in 89 (68.5%) of cases and there were 41 (31.5%) of metastatic patients.

We compared the clinical parameters between the two distinct groups: flat versus long bone osteosarcomas. The latter were significantly more common in young people under the age of 18 ( $p<0.001$ ) and in men ( $p=0.0068$ ). Osteosarcomas localized to the mandible/flat bone were mainly seen in older patients ( $p<0.001$ ). Patients with osteosarcoma localized in the long bones had more metastatic progression than those with tumors localized in the flat bones ( $p=0.012$ ). The response to chemotherapy was significantly better in patients with long bone osteosarcoma ( $p=0.0064$ ). Metastases were significantly more common in long bone osteosarcomas ( $p=0.012$ ), and the response to chemotherapy was significantly better in patients with long bone osteosarcomas compared to other locations ( $p = 0.0064$ ).

### **Immunohistochemistry analysis of GLUT-1, SphK1 and S1P<sub>1</sub> expression**

GLUT-1 expression was found in 49.3% of all patients ( $n=98$ ) with an IRS score of 6.31 (Table 3). The staining pattern of GLUT-1 was mostly membranous, with some staining or light dusting in the cytoplasm, as is characteristic of GLUT-1 immunohistochemistry (Figure 3C, D). A significantly higher percentage of cells stained for GLUT1 and a higher IRS score were associated to long bone ( $p=0.0116$  and  $p=0.0353$ , respectively) versus flat bone osteosarcomas (Table 3). SphK1 and S1P<sub>1</sub> expressions were found in 67.3% of all patients ( $n=96$ ) with an IRS score of 4.547, and in 64.2% of all patients ( $n=111$ ) with an IRS score of 4.3, respectively (Table 3). Of note, there were no statistical differences of SphK1 and S1P<sub>1</sub> (percentage of stained cells or IRS score) between the osteosarcoma sites (Table 3). The staining pattern of SphK1 (Figure 3E, F) was cytoplasmic as we previously reported in prostate and lung cancer <sup>22, 23, 53</sup>. The

staining pattern of S1P<sub>1</sub> (Figure 3G, H) was membranous and cytoplasmic as we previously reported in kidney cancer <sup>41</sup>. As shown in Table 4, although the strength of linear correlation between SphK1 and GLUT-1 was weak, it was statistically extremely significant for all osteosarcomas ( $p=0.005$  for percentage of stained cells and  $p=0.001$  for IRS score). Similar statistical correlation, albeit to a lesser extent, was found for the long bone (percentage of stained cells and IRS score) and the flat bone osteosarcoma groups (IRS score only). On the contrary, no statistical correlations were found between SphK1 and S1P<sub>1</sub> or between GLUT-1 and S1P<sub>1</sub>.

### **Clinical parameters and biomarkers association with treatment response**

To study the association with response to chemotherapy, we evaluated the correlation between the extent of response to treatment assessed by the histological grading scale of Salzer-Kuntschik et al. <sup>54</sup> and clinical parameter or each biomarker (GLUT-1, SphK1 or S1P<sub>1</sub>). A good response to chemotherapy was defined as <10% viable tumor cells in the surgical specimen, while a poor response was defined as  $\geq 10\%$  viable tumor cells in the surgical specimen. For all osteosarcomas, patients with a good response were younger ( $p<0.0001$ ) (Supplementary Table 1) and there was no association between the response to treatment and the expression of GLUT-1, SphK1 or S1P<sub>1</sub> (Table 5). With regards to long bone osteosarcomas, statistical differences were found with an increased expression of S1P<sub>1</sub> in poor responder patients (Table 5).

### **Clinical parameters and biomarkers associated with survival in the two subgroups (long bones versus flat bones)**

Survival analyses showed no statistically significant difference in overall survival between long bone and flat bone osteosarcoma patients ( $p=0.3307$ ; Supplementary Figure 1). The analysis of metastasis-free survival also showed no statistically significant difference between long bone and flat bone patients ( $p=0.468$ ; Supplementary Figure 1). Age-adjusted multivariate analyses are presented in Table 6.

Regarding overall survival for the flat bone osteosarcomas, the expression of GLUT1 expression (% of stained cells and IRS score) were associated with an increased risk of death HR= 1.20 (95%CI: 1.00-1.44) and HR= 1.15 (95%CI: 1.00-1.32), respectively. With regard to long bones, the expression of GLUT1 expression (% of stained cells and IRS score) were respectively

associated with an increased risk of death HR= 1.16 (95%CI: 0.98-1.38) and HR= 1.14 (95%CI: 1.00-1.29) with borderline significance (0.08 and 0.06, respectively).

SphK1 expression was associated with a significant increase in the risk of death HR=1.25 (95%CI: 1.03-1.51) only in the long bone patients group (based on IRS score). Similarly, in long bone osteosarcomas, S1P<sub>1</sub> expression was associated with an increased risk of death HR= 1.29 (95%CI: 1.00-1.66) at the limit of significance (based on % of stained cells) with regard to overall survival. Concerning metastasis-free survival, S1P<sub>1</sub> expression was associated with a significant increase in HR=1.23 (95%CI: 1.02-1.48) for patients in the long bone osteosarcomas group. No statistical difference was found for S1P<sub>1</sub> expression expressed as IRS score.

Finally, no significant differences were found in the good responders to chemotherapy according to the expression of markers expressed as % of stained cells or IRS score for all markers: GLUT1, SphK1 and S1P<sub>1</sub>.

## DISCUSSION

Hypoxia is a characteristic of solid tumors and the adaptation of cancer cells to hypoxia is instrumental in the development of aggressive phenotype and associated with a poor prognostic in patients <sup>55</sup>. At the cellular level, the adaptation to hypoxia is predominantly mediated by the hypoxia-inducible factors (HIFs), consisting of an oxygen-sensitive  $\alpha$ -subunit and a constitutively expressed  $\beta$ -subunit, that regulate the expression of target genes promoting angiogenesis such as VEGF, glycolysis such as GLUT-1, acidosis such as CA-IX, metastasis, increased tumor growth and resistance to treatments <sup>17</sup>. Immunohistochemical-based analyses (HIF-1 $\alpha$ , VEGF, GLUT-1, CA-IX...) have shown a connection between hypoxia and outcome of tumor therapy. In osteosarcoma, initial studies examined VEGF expression, which was found to be associated with poor outcome (DFS, OS) and metastasis <sup>56-58</sup>. As a target gene of HIF transcription factors, VEGF was also correlated with HIF-1 $\alpha$  expression <sup>57, 58</sup>. Most studies have examined the expression of HIF-1 $\alpha$  suggesting that it would be associated with higher pathologic grade and tumor stage, higher MVD (microvascular density), higher rate of metastasis, poorer overall survival, poorer disease-free survival and poor response to chemotherapy <sup>47, 49, 50, 58-63</sup>. Three meta-analyses recently concluded that HIF-1 $\alpha$  expression might be an effective predictive factor of poor prognosis in osteosarcoma <sup>18-20</sup>. As pointed out by the authors, the results of these meta-analyses should be interpreted with caution as some major biases could not be excluded such as the limited sample size of nearly all studies, the fact that most of the selected studies have been carried out in China proposing that similar studies have to be conducted in populations of other origins, the heterogeneity across the assessment of HIF-1 $\alpha$  expression (different antibodies or dilutions that could affect the sensitivity) as well as non uniform criteria including different cut-off values defining high HIF-1 $\alpha$  level of expression. Fewer studies have examined GLUT-1 expression in canine and human osteosarcomas leading to conflicting results <sup>64-66</sup> ranging from no statistical correlation between GLUT-1 expression and DFS, survival time or percentage of necrosis in dogs <sup>64</sup> to strong GLUT-1 staining in both primary sites and metastases <sup>65</sup> and association with shorter DFS <sup>66</sup>. In addition, these studies of GLUT-1 expression are also limited by the size of the samples (n = 10 to 44). More recently, a study examining the expression of plasma GLUT-1 established that GLUT-1 was overexpressed in patients with osteosarcoma (n=42) as compared to healthy



volunteers (n=38). In addition, levels of GLUT-1 mRNA were significantly higher in tumor tissues than in adjacent healthy tissues of osteosarcoma patients <sup>67</sup>.

In this work, we found that GLUT-1 was expressed in both long and flat bones with a higher expression of GLUT-1 in long bones. Although no correlation was found with response to chemotherapy, GLUT-1 expression was associated with a higher risk of death (OS) in flat bones and a tendency for death or metastatic progression (MPFS). These data thus suggest that hypoxia might indeed be an effective predictive factor of poor prognosis in osteosarcoma, in line to the previously reported studies with HIF-1 $\alpha$  <sup>47, 49, 50, 58-63</sup>, the transcription factor that regulates GLUT-1 under hypoxia.

We also examined the relationship between hypoxia (GLUT-1) and the SphK1/S1P signaling, which has been previously documented by our team to be central for the adaptation to hypoxia in a wide array of cellular (prostate, glioblastoma, lung, breast, renal cell carcinoma) and animal (prostate, renal cell carcinoma) models <sup>25-27, 38</sup>. SphK1/S1P signaling is commonly upregulated in cancer cells <sup>24</sup> and correlates with poor patient prognosis in a number of tumor types (reviewed in <sup>68</sup>). The expression of SphK1 in osteosarcoma had never been reported until this work. A recent study found that S1P levels in serum samples from osteosarcoma patients (n=17) are decreased after chemotherapy <sup>69</sup>, suggesting that S1P could represent a potential biomarker of chemotherapeutic efficacy. As the authors did not assess serum S1P levels in healthy controls, they could not demonstrate that S1P content is in fact increased in osteosarcoma patients.

In our tumor collection, both SphK1 and S1P<sub>1</sub> were expressed in long and flat bones together with GLUT-1. A strong correlation was essentially seen between GLUT-1 and SphK1, regardless of the location (flat versus long bones), which indicates that SphK1 is activated by hypoxia *in vivo*. No association between the response to treatment and the expression of GLUT-1, SphK1 or S1P<sub>1</sub> with the exception of long bone osteosarcomas where statistical differences were found with an increased expression of S1P<sub>1</sub> in poor responder patients. These data regarding response to chemotherapy should be interpreted with caution as the different groups of patients (long versus flat bone osteosarcomas) are not treated with the same chemotherapy regimen. Importantly, age-adjusted multivariate analyses showed that GLUT-1, SphK1 and S1P<sub>1</sub> were associated with an increased risk of death (OS) depending on the osteosarcoma location (long versus flat bones). Interestingly, S1P<sub>1</sub> was the only biomarker associated with increase risk of death (OS), increased risk of death or metastasis (MPFS) and

predictive of poorer response to chemotherapy in long bones. However, one should be cautious in interpreting these findings on bone tissues as the staining intensity of S1P<sub>1</sub> was weaker when compared to the robust GLUT-1 and SphK1 antibodies.

The strong expression of SphK1 in tissues was confirmed by the quantification of SphK1 enzymatic activity, that we originally reported in prostate cancer patient samples <sup>22</sup>, on 12 osteosarcoma compared to 10 non-tumoral bone samples. We found that SphK1 activity was dramatically augmented in osteosarcoma with over a 50-fold increase in S1P production (431 pmoles/min/mg versus 6.6,  $p=0.023$ ). Measuring enzymatic activity in human biopsies is technically demanding and hardly amenable to routine use as a prognostic factor, yet it provides the most accurate information by directly quantifying activity of an enzyme when compared to mRNA or protein assessment.

The relationship between SphK1/S1P signaling and hypoxia was further validated *in vitro* in various osteosarcoma cell lines exposed to hypoxic conditions. Our data suggesting that SphK1/S1P signaling is required for the regulation of HIF-1 $\alpha$  in osteosarcoma are in line with our previous findings in prostate and renal cell carcinoma cell models <sup>25-27, 38</sup>. Interestingly, in contrast to classical chemotherapy, the dual SphK1 and S1P<sub>1</sub> inhibitor FTY720 remarkably reduced cell proliferation to the same extent in normoxic and hypoxic conditions in all osteosarcoma cell lines suggesting that efficacy of FTY720 was related to its anti-HIF-1 $\alpha$  effect.

Collectively, our data (*in vitro*, enzymatic assessment of SphK1 activity in tumors and SphK1 and/or S1P<sub>1</sub> expression in tissues) suggest that SphK1/S1P signaling might represent a potential target in osteosarcoma patients considering that the therapeutic drug FTY720 (also known as Fingolimod, and marketed by Novartis as Gilenya<sup>TM</sup>) is already used in clinic for multiple sclerosis and now considered for repurposing in cancer.

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## LEGENDS TO THE FIGURES

### **Figure 1. Pharmacological inhibition of SphK1 prevents HIF-1 $\alpha$ accumulation in osteosarcoma.**

**A**, human osteosarcoma U-2 OS, MG-63 and SaOS-2 cancer cells were incubated under normoxia or hypoxia up to 8h. Cells were lysed and HIF-1 $\alpha$  expression was analyzed by immunoblotting with an anti-HIF-1 $\alpha$  antibody. Similar results were obtained in three independent experiments. **B**, relative mRNA expression of SphK1 was measured in normoxic (Nx) and hypoxic U-2 OS cells. *Columns*, mean of at least three independent experiments. **C**, U-2 OS cells were incubated under hypoxia for the indicated times. Cell lysates were tested for SphK1 activity. *Points and columns*, mean of five independent experiments; bars, SEM. \*\*,  $P < 0.01$ . Basal SphK1 activity was  $290 \pm 18$  pmol/mg/min. **D**, HIF-1 $\alpha$  expression was analyzed on U-2 OS cells were treated with the indicated concentrations of SKI-II and incubated under for 6h. Similar results were obtained in three independent experiments. **E**, U-2 OS cells were treated with the indicated concentrations of FTY720 and incubated under hypoxia for 6h. Cell lysates were assayed for HIF-1 $\alpha$  expression, including untreated normoxic cells (Nx). Similar results were obtained in three independent experiments.

### **Figure 2. SphK1 activity in human osteosarcoma biopsy samples**

**A**, S1P content of 10 non-tumoral and 12 tumoral biopsies as shown by Thin Layer Chromatography. **B**, SphK1 enzymatic activity of biopsy samples expressed as pmol/min/mg of protein. *Columns*, individual SphK1 activity values and means with 95%CI. Mean SphK1 activities were 6.3 (95% CI: 0.45-12.1) and 432 (95% CI: 77.4-835) pmol/mg/min for non-tumoral and tumoral biopsy samples, respectively.

### **Figure 3. Representative patterns of GLUT-1, SphK1 and S1P<sub>1</sub> expression in osteosarcoma**

**A**, Histological representation of one spot of an osteoblastic osteosarcoma included in TMAs (H&E magnification X 5), **B**, Representation at a magnification X 25 of the zone framed in **A**; **C and D**, GLUT-1 staining in the same spot (magnification x5 and 25 respectively); **E and F**, SphK1 staining in the same spot (magnification x5 and 25 respectively); **G and H**, S1P<sub>1</sub> staining in the same spot (magnification x5 and 25 respectively).

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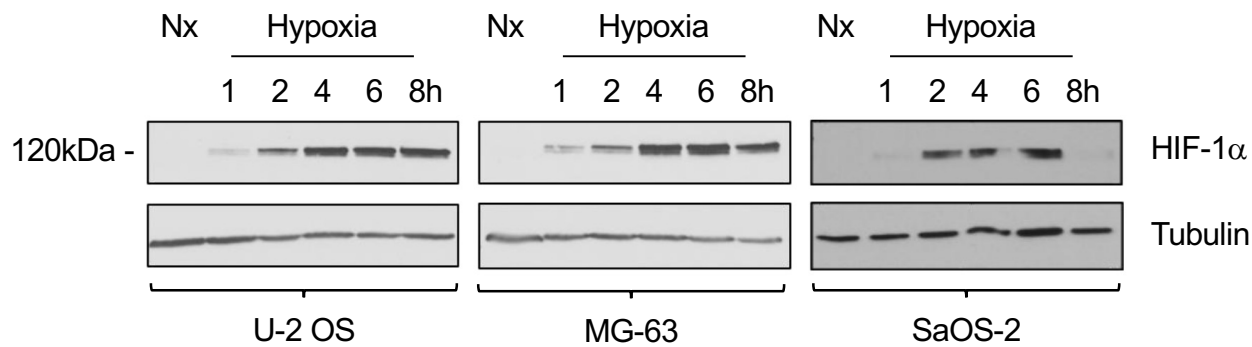
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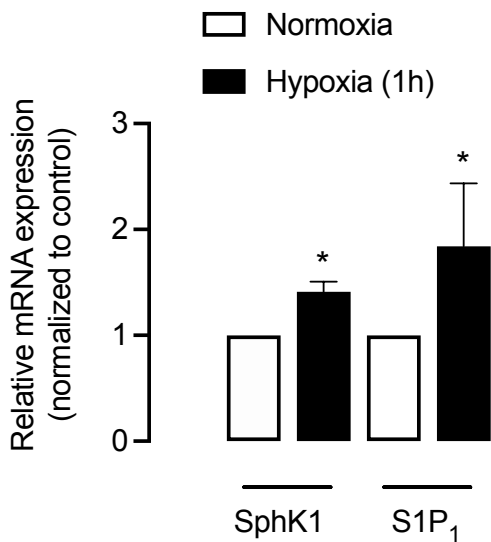
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**Figure 1**

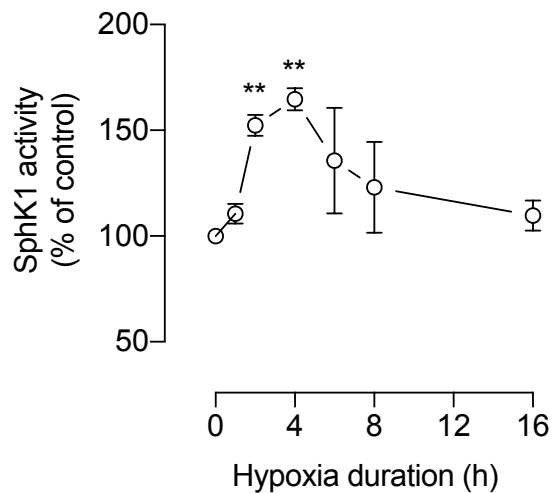
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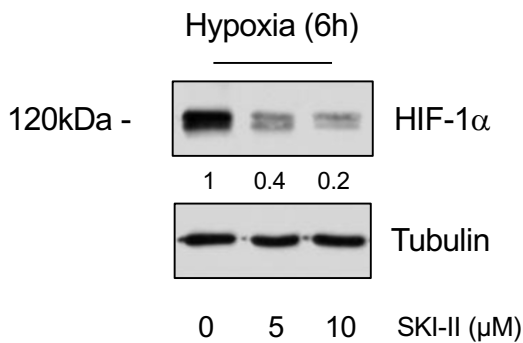
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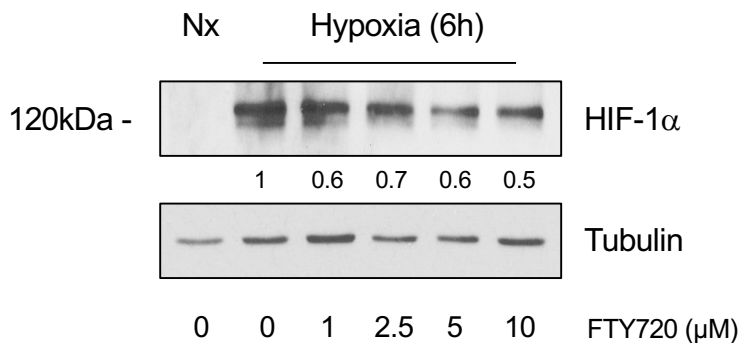
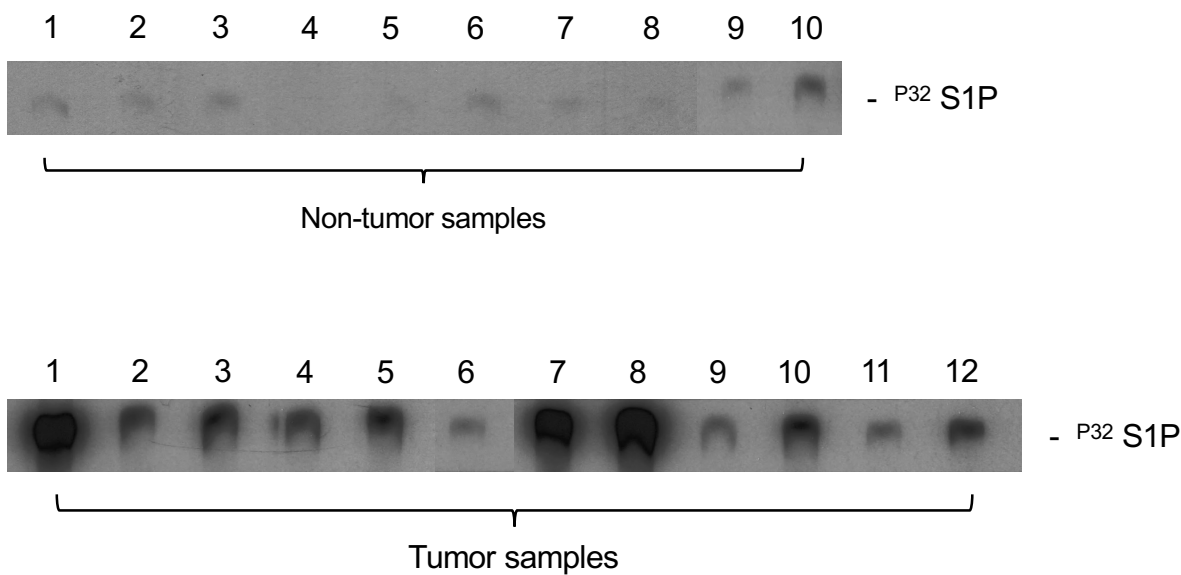
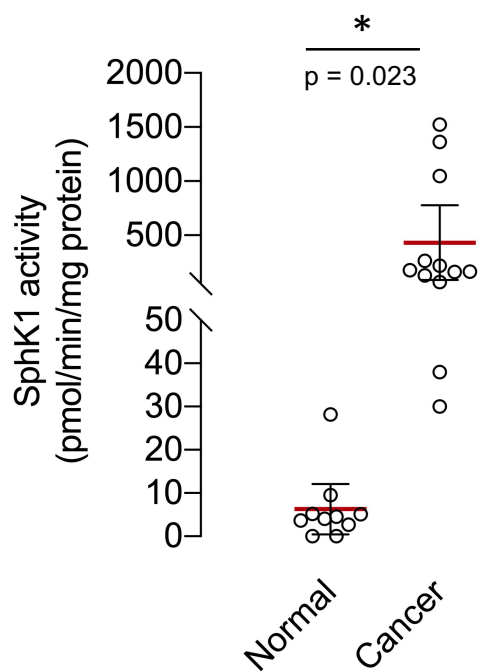


Figure 2

A.



B.



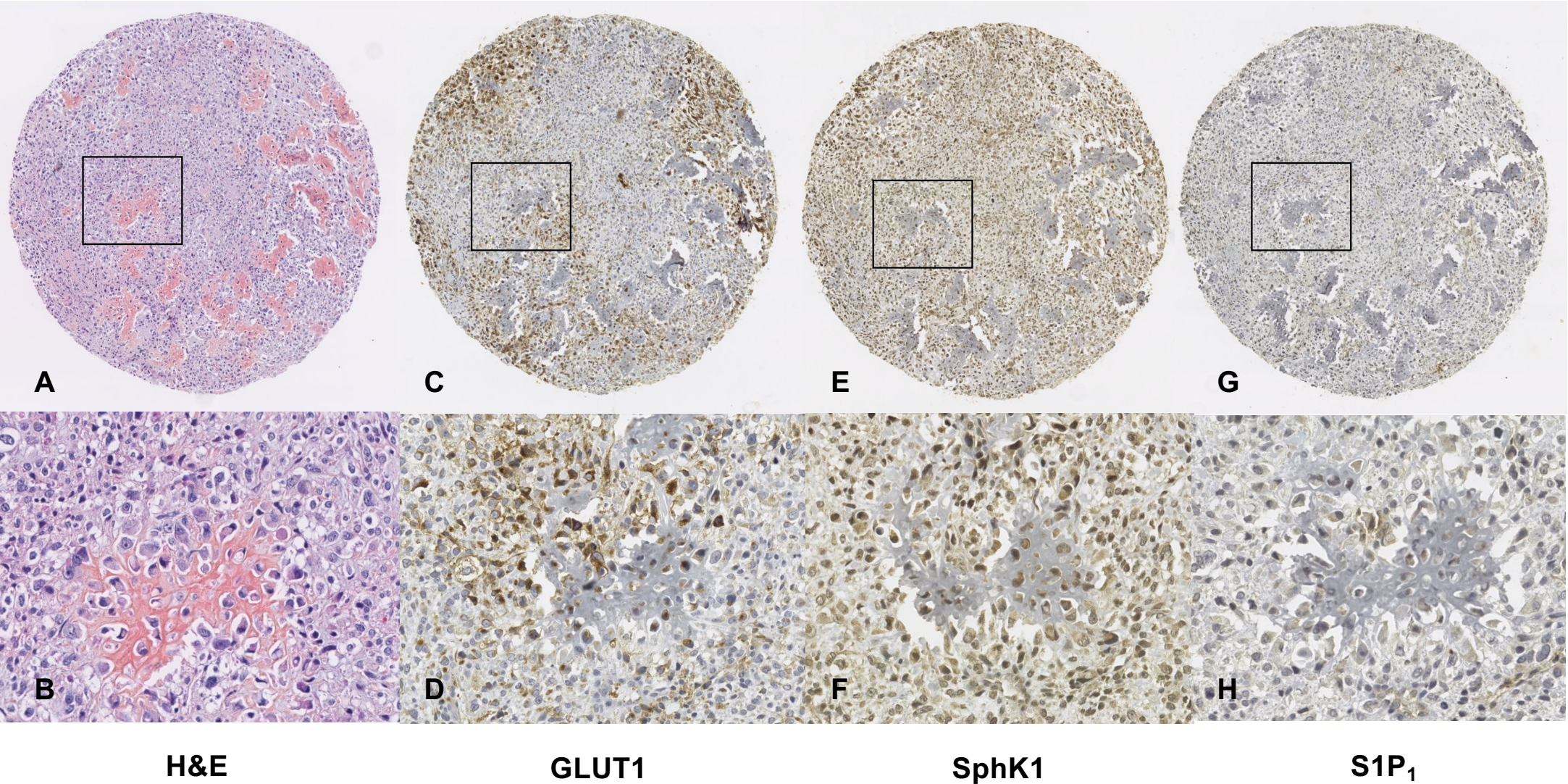
**Table 1.** Cytotoxicity of doxorubicin and Fingolimod (FTY720) on a representative panel of osteosarcoma cell lineages (GI<sub>50</sub> [μM], 72 h, MTT assay). [a]

	Doxorubicin			FTY720		
	Normoxia	Hypoxia	<i>FOLD</i>	Normoxia	Hypoxia	<i>FOLD</i>
U-2 OS	0.066	0.195	<i>3</i>	4.21	4.21	<i>1</i>
SaOS-2	0.073	1.00	<i>14</i>	3.20	3.23	<i>1</i>
MG-63	0.025	0.507	<i>20</i>	1.52	1.50	<i>1</i>
143B	0.017	0.134	<i>8</i>	1.11	1.22	<i>1</i>

[a] The GI<sub>50</sub> values represent the concentration of compound causing 50% inhibition of cell growth. Mean of at least three independent experiments.



**Figure 3**





**Table 2** : Patient and tumor characteristics

Features	All		Long Bones		Flat Bones		p value
	n 130	% 100	n 71	% 54.6	n 59	% 45.4	
<b>Age (Years)</b>							
Median (Range)	22.4 (5.7-83.9)		15.8 (6.6-82.8)		39 (5.7-83.9)		<0.0001
<18y	51	39.2	41	31.5	10	7.7	<0.0001
≥18y	79	60.8	30	23.1	49	37.7	
<b>Gender</b>							0.0068
Male	80	61.5	51	39.2	29	22.3	
Female	49	37.7	20	15.4	29	22.3	
Unknown	1	0.8	0	0.0	1	0.8	
<b>Site</b>							
Flat bones	59	45.4					
Long bones	71	54.6					
<b>Histological type</b>							0.8099
Chondroblastic	32	24.6	11	8.5	21	16.2	
Osteoblastic	60	46.2	47	36.2	13	10.0	
Fibroblastic	25	19.2	7	5.4	18	13.8	
Other	10	7.7	4	3.1	6	4.6	
Unknown	3	2.3	2	1.5	1	0.8	
<b>Metastatics vs Non Metastatic</b>							0.012
Localized	89	68.5	42	32.3	47	36.2	
Metastatic	41	31.5	29	22.3	12	9.2	
<b>Response to chemotherapy</b>							0.0064
Good	53	40.8	39	30.0	14	10.8	
Poor	43	33.1	20	15.4	23	17.7	
Unknown	34	26.2	12	9.2	22	16.9	

**Table 3** : Immunohistochemical results for GLUT-1, SphK1 and S1P<sub>1</sub>

	All					Long Bones					Flat Bones					p value
	AVG	SD	Median	Min	Max	AVG	SD	Median	Min	Max	AVG	SD	Median	Min	Max	
GLUT1																
n=98						n=54					n=44					
% of stained cells	49.29	31.73	60	0	100	55.37	32.95	65	0,00	100	41.82	28.8	50	0	90	0.0116
IRS score	6.31	4.03	6	0	12	7.06	4.03	9	0,00	12	5.39	3.88	6	0	12	0.0353
SphK1																
n=96						n=52					n=44					
% of stained cells	67.28	28.29	80	0	100	70.77	26.7	80	0,00	100	63.16	29.83	80	0	90	0.1835
IRS score	4,57	2.7	4	0	12	4.85	2.55	6	0,00	12	4.25	2.86	3	0	12	0.1828
S1P <sub>1</sub>																
n=111						n=63					n=48					
% of stained cells	64.23	23.14	70	0	100	64.29	23.6	70	0,00	100	64.17	22.77	70	0	90	0.9279
IRS score	4.3	2.52	3	0	12	4.25	2.67	3	0,00	12	4.35	2.33	4	0	12	0.6881

**Table 4** : Correlation between GLUT-1, SphK1 and S1P<sub>1</sub> markers

<b>All OS</b>							
<b>correlation coefficient (r)</b>	GLUT1 % cells	SphK1 % cells	S1P <sub>1</sub> % cells	<b>correlation coefficient (r)</b>	GLUT1 IRS score	SphK1 IRS score	S1P <sub>1</sub> IRS score
GLUT1 % cells	NA	0.3019	0.009351	GLUT1 IRS score	NA	0.3498	-0.01708
SphK1 % cells		NA	0.1387	SphK1 IRS score		NA	-0.01275
S1P <sub>1</sub> % cells			NA	S1P <sub>1</sub> IRS score			NA
<b>p value</b>	GLUT1 % cells	SphK1 % cells	S1P <sub>1</sub> % cells	<b>p value</b>	GLUT1 IRS score	SphK1 IRS score	S1P <sub>1</sub> IRS score
GLUT1 % cells	NA	0.005	0.9287	GLUT1 IRS score	NA	0.001	0.8702
SphK1 % cells		NA	0.1922	SphK1 IRS score		NA	0.9051
S1P <sub>1</sub> % cells			NA	S1P <sub>1</sub> IRS score			NA
<b>Long Bones</b>							
<b>correlation coefficient (r)</b>	GLUT1 % cells	SphK1 % cells	S1P <sub>1</sub> % cells	<b>correlation coefficient (r)</b>	GLUT1 IRS score	SphK1 IRS score	S1P <sub>1</sub> IRS score
GLUT1 % cells	NA	0.3608	-0.1556	GLUT1 IRS score	NA	0.2902	-0.1024
SphK1 % cells		NA	0.1765	SphK1 IRS score		NA	-0.1345
S1P <sub>1</sub> % cells			NA	S1P <sub>1</sub> IRS score			NA
<b>p value</b>	GLUT1 % cells	SphK1 % cells	S1P <sub>1</sub> % cells	<b>p value</b>	GLUT1 IRS score	SphK1 IRS score	S1P <sub>1</sub> IRS score
GLUT1 % cells	NA	0.0127	0.2706	GLUT1 IRS score	NA	0.0478	0.4699
SphK1 % cells		NA	0.2153	SphK1 IRS score		NA	0.3466
S1P <sub>1</sub> % cells			NA	S1P <sub>1</sub> IRS score			NA
<b>Flat Bones</b>							
<b>correlation coefficient (r)</b>	GLUT1 % cells	SphK1 % cells	S1P <sub>1</sub> % cells	<b>correlation coefficient (r)</b>	GLUT1 IRS score	SphK1 IRS score	S1P <sub>1</sub> IRS score
GLUT1 % cells	NA	0.1979	0.174	GLUT1 IRS score	NA	0.3948	0.09562
SphK1 % cells		NA	0.1661	SphK1 IRS score		NA	0.2053
S1P <sub>1</sub> % cells			NA	S1P <sub>1</sub> IRS score			NA
<b>p value</b>	GLUT1 % cells	SphK1 % cells	S1P <sub>1</sub> % cells	<b>p value</b>	GLUT1 IRS score	SphK1 IRS score	S1P <sub>1</sub> IRS score
GLUT1 % cells	NA	0.2336	0.2703	GLUT1 IRS score	NA	0.0142	0.5469
SphK1 % cells		NA	0.3058	SphK1 IRS score		NA	0.2039
S1P <sub>1</sub> % cells			NA	S1P <sub>1</sub> IRS score			NA



**Table 5:** GLUT-1, SphK1 and S1P<sub>1</sub> expression of good and poor responders to chemotherapy

		Good					Poor					p value
All OS		n=40					n=30					
		AVG	SD	Median	Min	Max	AVG	SD	Median	Min	Max	
<b>GLUT1</b>												
% of stained cells		57.75	31.73	70	0	100	45.00	29.8	50	0	90	0.1945
IRS score		7.4	3.85	9	0	12	5.67	3.94	6	0	12	0.0587
		n=38					n=31					
<b>SphK1</b>												
% of stained cells		69.74	25.09	80	0	100	68.39	25.96	80	0	90	0.9506
IRS score		4.76	2.8	5	0	12	4.45	2.39	4	0	8	0.7955
		n=45					n=36					
<b>S1P<sub>1</sub></b>												
% of stained cells		57.78	23.54	60	0	90	66.11	24.06	70	0	90	0.2325
IRS score		3.69	2.2	3	0	12	4.58	2.71	3	0	12	0.0787
Long Bones		n=39					n=20					p value
		AVG	SD	Median	Min	Max	AVG	SD	Median	Min	Max	
<b>GLUT1</b>												
% of stained cells		58.97	31.89	70	0	100	56.67	30.86	60	0	90	0.8059
IRS score		7.59	3.78	9	0	12	7.00	4.05	9	0	12	0.6482
		n=28					n=13					
<b>SphK1</b>												
% of stained cells		75.00	17.53	80	0	100	69.23	29.85	80	0	90	0.744
IRS score		5.00	2.33	6	0	12	4.54	2.37	4	0	8	0.6947
		n=34					n=18					
<b>S1P<sub>1</sub></b>												
% of stained cells		56.18	23.10	60	0	90	71.67	23.33	80	0	90	0.0038
IRS score		3.47	2.22	3	0	12	5.22	2.94	6	0	12	0.0098
Flat Bones		n=11					n=15					p value
		AVG	SD	Median	Min	Max	AVG	SD	Median	Min	Max	
<b>GLUT1</b>												
% of stained cells		54.55	32.67	60	0	90	33.33	24.4	40	0	70	0.0569
IRS score		6.91	4.18	9	0	12	4.33	3.44	6	0	9	0.0993
		n=10					n=18					
<b>SphK1</b>												
% of stained cells		55	36.59	70	0	90	67.78	23.65	75	0	90	0.5847
IRS score		4.1	3.93	3	0	12	4.39	2.48	3	0	8	0.4539
		n=11					n=18					
<b>S1P<sub>1</sub></b>												
% of stained cells		62.73	25.33	80	20	90	60.56	24.13	65	0	90	0.7043
IRS score		4.36	2.11	4	2	8	3.94	2.36	3	0	9	0.6521

**Table 6:** Age-adjusted multivariate analyses

	GLUT1 (% of cells)*			SphK1 (% of cells)*			n
	n	HRa [IC 95%]**	p	n	HRa [IC 95 %]**	p	
<b>Death (Overall survival)</b>							
All OS	98	1.03 [0.93 - 1.15]	0.57	96	1.04 [0.88 - 1.15]	0.96	111
Long Bones	54	0.92 [0.80 - 1.06]	0.24	52	1.10 [0.90 - 1.36]	0.36	63
Flat Bones	44	1.20 [1.00 - 1.44]	0.05	44	0.90 [0.74 - 1.11]	0.33	48
<b>Death or metastatic progression (MPFS)</b>							
All OS	98	1.05 [0.96 - 1.15]	0.31	96	1.03 [0.91 - 1.16]	0.68	111
Long Bones	54	0.97 [0.87 - 1.08]	0.58	52	1.06 [0.90 - 1.25]	0.46	63
Flat Bones	44	1.16 [0.98 - 1.38]	0.08	44	0.95 [0.79 - 1.16]	0.63	48
	n	ORa [IC 95%]	p	n	ORa [IC 95%]	p	n
<b>Good response to chemotherapy</b>							
All OS	70	1.09 [0.92 - 1.29]	0.34	69	0.98 [0.79 - 1.21]	0.82	81
Long Bones	44	0.92 [0.72 - 1.19]	0.53	41	1.04 [0.73 - 1.47]	0.83	52
Flat Bones	26	1.32 [0.95 - 1.84]	0.1	28	0.82 [0.62 - 1.10]	0.19	29

\* for a 10% increase of stained cells

\*\* Age-adjusted

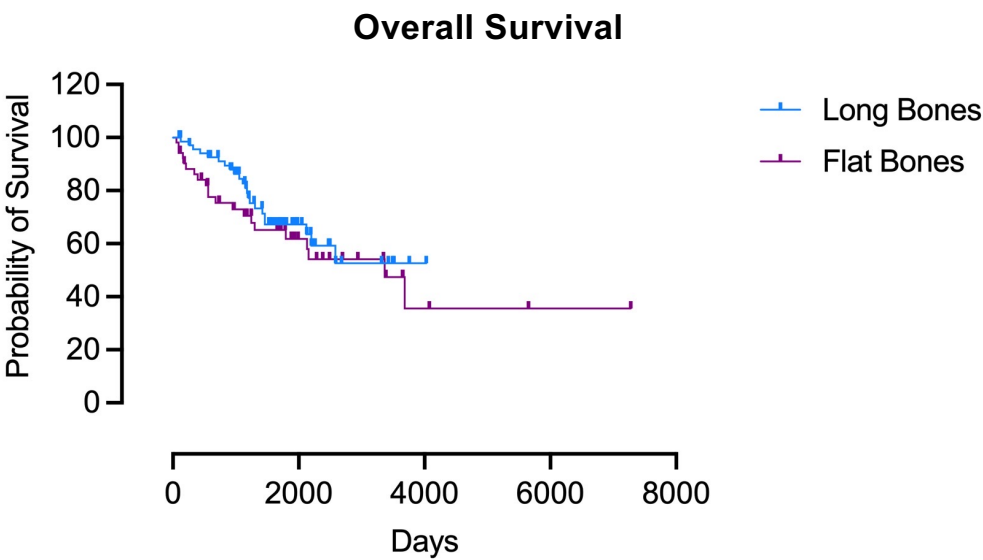
S1P <sub>1</sub> (% of cells)*			GLUT1 (IRS Score)*			SphK1 (IRS Score)*			S1P <sub>1</sub> (IRS Score)*		
HRa [IC 95 %]**	<i>p</i>	n	HRa [IC 95%]	<i>p</i>	n	HRa [IC 95%]	<i>p</i>	n	HRa [IC 95%]	<i>p</i>	
1.09 [0.94 - 1.27]	0.27	98	1.05 [0.96 - 1.14]	0.27	96	1.07 [0.93 - 1.23]	0.35	111	1.05 [0.94 - 1.18]	0.4	
1.29 [1.00 - 1.66]	0.05	54	0.97 [0.86 - 1.09]	0.58	52	1.25 [1.03 - 1.51]	0.02	63	1.07 [0.92 - 1.23]	0.39	
0.92 [0.75 - 1.13]	0.42	44	1.15 [1.00 - 1.32]	0.04	44	0.90 [0.71 - 1.14]	0.39	48	1.02 [0.84 - 1.24]	0.86	
1.01 [0.96 - 1.26]	0.17	98	1.05 [0.98 - 1.13]	0.17	96	1.03 [0.92 - 1.16]	0.6	111	1.07 [0.97 - 1.18]	0.18	
1.23 [1.02 - 1.48]	0.03	54	0.99 [0.90 - 1.08]	0.87	52	1.13 [0.96 - 1.35]	0.15	63	1.09 [0.97 - 1.21]	0.14	
0.91 [0.74 - 1.11]	0.35	44	1.14 [1.00 - 1.29]	0.06	44	0.94 [0.77 - 1.16]	0.58	48	1.01 [0.83 - 1.24]	0.89	
OR [IC 95%]	<i>p</i>	n	ORa [IC 95%]	<i>p</i>	n	ORa [IC 95%]	<i>p</i>	n	OR [IC 95%]	<i>p</i>	
0.88 [0.71 - 1.09]	0.24	70	1.09 [0.95 - 1.25]	0.23	69	0.95 [0.77 - 1.17]	0.63	81	0.89 [0.73 - 1.08]	0.22	
0.72 [0.52 - 1.01]	0.06	44	0.98 [0.80 - 1.19]	0.82	41	0.94 [0.66 - 1.34]	0.73	52	0.80 [0.62 - 1.03]	0.08	
1.07 [0.6 - 1.51]	0.7	26	1.20 [0.94 - 1.54]	0.13	28	0.93 [0.71 - 1.23]	0.62	29	1.10 [0.78 - 1.55]	0.6	

**Supplementary Table 1:** Clinical parameters of good and poor responders to chemotherapy

		Good		Poor		
All OS		n=53	%	n=43	%	p value
<b>Age (Years)</b>						
Median (Range)	16 (6-65)			31 (8-73)		<0.0001
<18y	35	36.5		13	13.5	0.0004
≥18y	18	18.8		30	31.3	
<b>Gender</b>						0.827
Male	34	35.4		27	28.1	
Female	19	19.8		15	15.6	
Unknown	0	0		1	1.0	
<b>Metastatics vs Non Metastatic</b>						0.5774
Localized	35	36.5		26	27.1	
Metastatic	18	18.8		17	17.7	
<b>Progression</b>						0.1911
0	33	34.4		21	21.9	
1	20	20.8		22	22.9	
<b>Recurrence</b>						0.2037
0	45	46.9		32	33.3	
1	8	8.3		11	11.5	
<b>Death</b>						0.0131
0	45	46.9		28	29.2	
1	8	8.3		15	15.6	
Long Bones						
		n=39	%	n=20	%	p value
<b>Age (Years)</b>						
Median (Range)	15 (6-47)			22 (8-60)		0.0137
<18y	30	50.9		9	15.3	0.0049
≥18y	9	15.3		11	18.6	
<b>Gender</b>						0.6501
Male	27	45.8		15	25.4	
Female	12	20.3		5	8.5	
<b>Metastatics vs Non Metastatic</b>						0.233
Localized	24	40.7		9	15.3	
Metastatic	15	25.4		11	18.6	
<b>Progression</b>						0.0838
0	23	39		7	11.9	
1	16	27.1		13	22	
<b>Recurrence</b>						0.2037
0	34	57.6		14	33.3	
1	5	8.5		6	11.5	
<b>Death</b>						0.027
0	33	55.9		13	22	
1	6	10.2		7	11.9	
Flat Bones						
		n=14	%	n=23	%	p value
<b>Age (Years)</b>						
Median (Range)	31 (6-65)			36 (15-73)		0.1924
<18y	5	13.5		4	10.8	0.2187
≥18y	9	24.3		19	51.4	
<b>Gender</b>						>0.9999
Male	7	18.9		12	32.4	
Female	7	18.9		10	27	
Unknown	0	0		1	2.7	
<b>Metastatics vs Non Metastatic</b>						0.7569
Localized	11	29.7		17	46	
Metastatic	3	8.1		6	16.2	
<b>Progression</b>						0.5274
0	10	27		14	37.8	
1	4	10.8		9	24.3	
<b>Recurrence</b>						0.9829
0	11	29.7		18	48.7	
1	3	8.1		5	13.5	
<b>Death</b>						0.1829
0	12	32.4		15	40.5	
1	2	5.4		3	8.1	

**Supplementary Figure 1**  
Kaplan-Meier survival curves for overall survival (**A**) and metastasis progression-free survival (**B**) according to osteosarcoma location.

**A.**



**B.**

