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Soft tissue sarcomas: evaluation of biomarkers and clinical outcomes

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LIST OF ABBREVIATIONS

CI	Confidence Interval
CR	Complete Response
CT	Computed Tomography
DDLS	Dedifferentiated Liposarcoma
DFS	Disease Free Survival
ECOG	Eastern Cooperative Oncology Group
EMT	Epithelial to Mesenchymal Transition
FAK	Focal adhesion kinase
FMS	Fibromyxoid sarcoma
Hb	Hemoglobin
HG	High Grade
LG	Low Grade
LMS	Leiomyosarcoma
LPS	Liposarcoma
MRI	Magnetic Resonance Imaging
MET	Mesenchymal to Epithelial Transition
MLP	Myxoid Liposarcoma
MFS	Myxofibrosarcoma
miRNA	microRNA
mTOR	mammalian Target of Rapamycin
MPNST	Malignant Peripheral Nerve Sheath Tumor
n.r.	Not reached
nRQ	Normalized Relative Quantities
OS	Overall Survival
PI3K	Phosphatidylinositol 3-kinase
PR	Partial Response
SD	Stable Disease
SS	Synovial Sarcoma
STD DEV	Standard Deviation
STS	Soft Tissue Sarcoma
TF	Transcription Factor
ULN	Upper Level of Normal
UPS	Undifferentiated Pleomorphic Sarcoma
ZEB	Zinc finger E-box-binding homeobox

Abstract

Background:

Soft tissue sarcomas (STS) are a rare, heterogeneous and complex group of tumors of mesenchymal origin. A great proportion of patients (pts) with high-risk STS eventually develop metastatic disease, and pts with advanced disease have a median overall survival (OS) of about 12 months [Judson I et al, Lancet Oncol 2014]. STS have a tendency to metastasize to lungs, but may also relapse in other distant organs. Systemic chemotherapy (CT) comprising anthracycline therapy remains to date the standard reference regimen.

Among the over 50 different histological types known, leiomyosarcoma (LMS), undifferentiated pleomorphic sarcoma (UPS) and myxofibrosarcoma (MFS) contain complex genomes characterized by a multitude of rearrangements, amplifications, and deletions. Only few diagnostic and prognostic markers exist, and the accurate diagnosis and prediction of the clinical behaviour of many of these tumors remain a challenge. Some biomarkers, specifically markers related to Epithelial to Mesenchymal transition (EMT) and its reverse process (MET) and microRNAs (miRNAs) may be useful to identify a possible signature with prognostic and diagnostic value, and could also elucidate new possible pathogenic mechanisms. The aims of this study were to describe efficacy and toxicity of CT for advanced STS in a cohort of unselected pts treated at Istituto Oncologico Veneto (IOV), and to evaluate the significance and the prognostic value of the expression of markers linked to EMT/MET processes, related miRNAs and myo-miRNAs in tumors samples.

Patients and methods:

Medical records of pts with advanced STS treated at Istituto Oncologico Veneto from January 2010 to December 2015 were reviewed and clinical data retrieved. Vital status was recorded as of September 30th 2016. OS was estimated with Kaplan-Meier method, and univariate analysis for OS was performed with Cox regression.

Tumor tissues from pts affected by STS referred to the Istituto Oncologico Veneto, Padova, were either retrieved from the Tissue Biobank of the Department of Surgical, Oncological and Gastroenterological Sciences, University of Padova,

or freshly received as tru-cut biopsy or surgical specimen. All the specimens were reviewed by an expert pathologist for confirmation of representativeness of the samples. Samples were collected from November 2014 (date of Ethical Approval) to September 2016. Eligible histological types were LMS, UPS, and MFS.

Total RNA, enriched in low molecular weight molecules, was extracted using NucleoSpin miRNA columns (MN GmbH & Co, Germany) from frozen or fresh tissue samples.

Markers linked to EMT/MET were analyzed by qRT-PCR using primers specific for epithelial markers (E-cadherin and ZO-1), and mesenchymal markers (Snail, Slug, Vimentin, Zeb−1, Zeb−2, and N-cadherin). Expression of alpha-SMA was also evaluated along with Periostin. The expression of specific miRNAs linked to EMT (miR-100-5p-5p), to MET (miR200b-3p, miR30b-5p and miR30c-5p) and myo-miRNAs (miR1, miR133a-3p andmiR133b) was measured. The study was approved by the institutional Ethics Committee and conducted in accordance with the Helsinki Declaration and Good Clinical Practice guidelines. Written informed consent was obtained from all pts.

Results:

Clinical evaluation of pts referred to Medical Oncology Unit 1 – Istituto Oncologico Veneto, IOV - IRCCS.

Overall 405 pts were eligible, of whom 51.4% had advanced or metastatic disease. One hundred-sixty seven patients were taken in charge at Istituto Oncologico Veneto. Median age was 61 years (range 16-89); 87 patients (52.1%) were female, and 54 (32.3%) were aged ≥ 70 years. Out of these, 37 patients (19.8%) did not receive chemotherapy. The prevalent histological types were LPS (24%), LMS (19.8%) and UPS (11.4%). Most pts had ECOG PS 0-1 (71.8%). Globally, median OS was 17.7 months (95%CI 13.59-21.75) and median PFS was 10 months (95%CI 7.9-12.0). Patients receiving only first-line CT were 57 (34.1%); 43 patients (25.7%) received more than two lines of CT. First-line CT regimens were anthracycline-based in 81 patients (62.3%).

Median OS of patients treated with CT was 19.9 months (95%CI 16.4–23.5), while that of untreated patients was 3.3 months (95%CI 1.3-5.2), $p<0.001$. At univariate analysis ECOG PS, anemia and lymphopenia were associated with prognosis. Patients with PS 0 had better median OS compared to those with PS 1

and PS 2-3, respectively 23.5 months (95%CI 18.5–28.5), 15.8 months (95%CI 18.5–28.5), 6.6 months (95%CI 1.0-12.3), p<0,001. Baseline low lymphocyte count was associated with worse survival, with median OS of12.5 months (95%CI 5.4-19.5) compared to patients with neutrophil/lymphocyte ratio <3, who had median OS of 22.8 months (95%CI 15.2-30.4), p=0.005. Baseline anemia was also associated with worse survival, with median OS for anemic patients (Hb<12 g/dL) being 9 months (95%CI 3.2-14.8) and median OS for non-anemic patients being 20.9 months (95%CI 16.6-25.4). No difference in OS was seen according to age, gender, chemotherapy regimen, BMI, albumin levels, or LDH levels.

Grade ≥3 toxicities occurred in 59 patients (45.4%), and 23 patients (17.7%) required hospitalization for toxicity management.

Molecular analysis on samples

Overall, 55 STS samples were fully characterized, specifically 28 LMS, of which 10 low/intermediate grade (LG) and 18 high grade (HG) LMS; 13 myxoid sarcomas (of which 8 MFS/ and 5 low-grade FMS); and 14 UPS.

Correlation of EMT-related markers and miRNA with histological type and grade was assessed. The samples were analysed for the expression of E-cadherin and ZO-1, as epithelial markers, and of Slug, Vimentin, Snail, ZEB-1, ZEB-2, N-cadherin, and Periostin, as markers related to a mesenchymal status. E-cadherin expression was not found in all analysed STS.

Alpha-SMA was significantly expressed in LMS compared to UPS and MFS/FMS (p<0.001), and not significantly different in UPS compared to MFS/FMS. ZEB-1 and ZEB-2 expression was significantly higher in LMS compared to MFS/FMS (p<0.001) and UPS (p=0.001 for ZEB-1, p=0.003 for ZEB-2), whereas no differential expression was measured between UPS and MFS/FMS. Interestingly, ZEB-1 and ZEB-2 were differentially expressed in HG and LG LMS (p=0.038 for ZEB-1, p=0.048 for ZEB-2). Also N-cadherin expression was significantly higher in LMS compared to MFS/FMS (p=0.006) and UPS (p=0.028), whereas no differential expression was measured according to the grading.

As for periostin, this was found to be higher in LMS compared to MFS/SFM (p=0.002), and in UPS compared to MFS/FMS (p=0.005); no difference was observed between LMS and UPS.

miR-1, miR-133a-3p and miR-133b (“myo-miRNAs”) expression was found to be

significantly higher in LMS compared to MFS and UPS (p=0.002), though no difference was observed between HG and LG LMS. All other analysed miRNAs did not show a different expression in the three histological subtypes, nor it was different according to grade., with the exception of miR-100-5p, which was found to be significantly over-expressed in LMS compared to MFS/FMS (p=0.02). An inverse correlation between Slug and myo-miRNAs expression was observed. Also, a direct correlation between ZEB family members, and an inverse correlation between ZEB-1 and miR-200b was observed.

In univariate analysis, high ZEB-1 (≥0.4) was correlated with worse OS (2.3 months, 95%CI 0.9-3.4) vs low ZEB-1 (8.6 months, 1.5 – n.r.), p=0.058. Similarly, high ZEB-2 (≥0.9) was correlated with worse OS (2.2 months, 95%CI 0.9-32.7) vs low ZEB-2 (8.6 months, 95%CI 1.5 – n.r.), p=0.052. High Periostin was also correlated with worse OS (2.2 months, 95%CI 1.2-2.5) vs low Periostin (8.6 months, 95%CI 1.3 – n.r.), p=0.028. In multivariate analysis, grade and periostin and ZEB-1 levels confirmed to be associated with overall survival.

All the other analysed EMT-related markers, as well as all analysed miRNAs, were not correlated with OS nor with PFS.

Conclusions:

Our clinical data confirm that there is a benefit for pts that have been treated for advanced disease compared to those not receiving active treatment. There remains a need for novel effective therapies in metastatic STS, particularly for pts with certain chemo-resistant subtypes.

The analysis on tumor samples highlighted that a "myo-miRNA" signature may serve as potential confirmatory markers in LMS samples with difficult/controversial histological findings Moreover, some biomarkers linked to the mesenchymal phenotype (i.e. ZEB-1, ZEB-2, and Periostin) may have prognostic value.

In light of the findings from this study, we have planned to proceed with validation of such results in a larger sample and to provide a correlation with immunoistochemical staining.

Also, we are planning to verify whether circulating Periostin and N-cadherin may be correlated with outcomes and response to therapy in STS.

INTRODUCTION

1.1 Epidemiology and Classification of Soft Tissue Sarcomas

High-grade soft tissue sarcomas (STS) are a heterogeneous and complex group of tumors of mesenchymal origin that account for approximately 1% of all adult malignancies [Fletcher CDM et al, WHO Classification of tumours of soft tissue and bone 2013; Siegel RL et al, CA Cancer J Clin 2016].

The incidence is estimated to be 4-5 new cases/100.000 persons/year [AIOM-AIRTUM I Numeri del cancro 2016; Jemal A et al, CA Cancer J Clin 2007].

There are about 12,310 new cases of soft tissue sarcoma diagnosed each year in the United States, with 4,990 deaths [Siegel RL et al, CA Cancer J Clin 2016]. Taking into account the overall population, the figures are similar in Italy, where in 2016 it is estimated that 2,100 new cases will be diagnosed, 1,200 in men and 900 in women [AIOM-AIRTUM I Numeri del cancro 2016].

Still, the true incidence of STS remains uncertain to some degree, since issues such as underreporting as well as the changing of classification over time are common.

The overall estimated 5-year survival rate is 65.3% in the US, and the 5-year survival is 18.4% in patients with sarcomas with distant spread [NCI SEER Stat Fact Sheets: Soft Tissue Including Heart Cancer, 2016]. STS account for 1% of deaths due to cancer in both sexes [AIOM LG 2015], with 5-year survival rates in Italy ranging from 61 to 70% (Table 1).

Indeed, a great proportion of patients with high-risk STS eventually develop metastatic disease, and patients with advanced disease have a median overall survival (OS) of about 12 months [Judson I et al, Lancet Oncol 2014]. In particular, high-grade sarcomas show high rates of local recurrence, frequent metastases, and poor prognosis [Zagars CK et al., Cancer 2003].

Table 1. Relative 5-year standardized survival (%) after diagnosis of soft tissue sarcoma, according to geographic area in Italy (years considered 2000-2004, AIRTUM pool) [AIRTUM WG. Epidemiol Prev 2011; 26 (5-6): Suppl. 1]

Male				**Female**			
North-west	North-east	Center	South	North-west	North-east	Center	South
64	63	61	62	66	65	61	70

Approximately 80% of sarcomas originate from soft tissue, and the rest originates from bone.

STS may develop in any part of the body, yet most originate in extremities and in girdles (70%), 10% in the trunk, 10% in the retroperitoneum, 5% in viscera and the remaining 5% in the head and neck region [AIOM, LG 2015].

The histopathologic spectrum of sarcomas is broad, presumably because the embryonic mesenchymal cells from which they arise have the ability to mature into several types of connective tissues, such as striated skeletal and smooth muscle, adipose and fibrous tissue, bone, and cartilage. Although ectodermal in origin, malignant tumors affecting peripheral nerves are included because of similarities in their clinical behaviour, management, and outcome.

As classified by the World Health Organization (WHO), the group of STS includes more than 80 different histologic subtypes [Fletcher CDM et al, WHO Classification 2013]. The WHO classifies most soft tissue sarcomas according to the presumptive tissue of origin, that is the normal tissues which the tumor most closely resembles [Siegel RL et al, CA Cancer J Clin 2016]. Examples include liposarcoma (LPS), synovial sarcoma (SS), leiomyosarcoma (LMS), rhabdomyosarcoma (RMS), fibrosarcoma, and angiosarcoma. In some cases, histogenesis is uncertain and the designation reflects the morphologic appearance of the cells or the architectural pattern (eg, alveolar sarcoma of soft parts, epithelioid sarcoma, clear cell sarcoma, Ewing sarcoma).

The most common subtypes of soft tissue sarcoma in adults are undifferentiated pleomorphic sarcoma – UPS - (previously called malignant fibrous histiocytoma), LPS, and LMS [Wibmer C et al, Ann Oncol. 2010; Toro JR et al, Int J Cancer 2006].

Only few diagnostic and prognostic markers exist, and the cellular origin of several sarcoma subtypes is unknown. Therefore, the accurate diagnosis and the prediction of the clinical behaviour of many of these tumors remain a challenge [van de Rijn M and Fletcher JA, Annu Rev Pathol 2006].

1.2 Diagnosis

When a soft tissue mass is present, and a STS is suspected, ultrasound is often carried out as first imaging approach, yet magnetic resonance imaging (MRI) is the gold standard imaging modality. Computed tomography (CT) performs as

well in retroperitoneal tumours, and it is also useful for staging purposes.

After proper imaging assessment, diagnosis must be ascertained by means of core needle biopsy, possibly by using ≥14 Gauge needles. An excisional biopsy can be performed for superficial lesions less than 3 cm in size. In selected cases, after multidisciplinary discussion, an open biopsy may be needed to complete or confirm diagnosis.

Imaging may also provide information that helps to estimate the malignancy grade (i.e. necrosis) for patients who are candidate for neoadjuvant treatment, in those cases in which assignment of grade on bioptic specimen is difficult. Biopsy should be planned in such a way that the pathway and the scar can be removed by definitive surgery. Histological diagnosis should be made according to the 2013 WHO classification [Fletcher CDM et al, WHO 2013]. A pathological expert validation is required in all cases when the original diagnosis was made outside a reference centre/network [Ray-Coquard I et al, Ann Oncol 2012].

Pathological diagnosis relies on morphology and immunohistochemistry. It should be complemented by molecular pathology, especially when the specific histological diagnosis is doubtful, and/or the clinical pathological presentation is unusual; it may also have prognostic and/or predictive relevance.

The malignancy grade should be provided in all cases in which this is feasible based on available systems, because it has prognostic and predictive meaning. The 'Federation Nationale des Centres de Lutte Contre le Cancer' (FNCLCC) grading system is generally used, which distinguishes three malignancy grades based on differentiation, necrosis and mitotic rate [Trojani M et al, Int J Cancer 1984]. Whenever possible, the mitotic rate should be provided independently. An effort should be made to improve the reliability of mitotic count as actually recorded. Grading cannot be assigned after preoperative medical treatment, by which the tumour tissue undergoes major therapy-related changes.

Tumour site should be properly recorded. Tumour size and tumour depth in relation to the superficial fascia should also be recorded, since they display a prognostic value..

If preoperative treatment is carried out, the pathology report should include an assessment of the histological response of the tumour.

For STS no validated system is available at present to guide pathological response to treatment, since no percentage of residual 'viable cells' is considered to have a

specific prognostic significance, as it happens for osteosarcoma and Ewing sarcoma.

STS can arise in every body anatomic district, and a multidisciplinary approach is mandatory in all cases, involving pathologists, radiologists, surgeons, radiation therapists, medical oncologists, nuclear medicine specialists, and organ-based specialists, as needed. Management should be carried out in reference centres for sarcomas and/or within reference networks sharing multidisciplinary expertise. Such a centralised referral should be pursued as soon as a clinical sarcoma diagnosis is suspected.

This translates in the recommendation of referring all patients having a deep soft tissue mass, or presenting with a superficial lesion of soft tissues having a diameter of >5 cm [ESMO, Ann Oncol 2014].

1.3 Stage classification and risk assessment

In STS the common staging classification adopted is the American Joint Committee on Cancer (AJCC)/International Union against Cancer (UICC) stage classification system [Edge SB et al, AJCC Cancer Staging Manual, 2010].

Such a classification has indeed limited relevance and should be improved. It stresses the importance of the malignancy grade in sarcoma, along with tumour size and tumour depth for limb sarcomas.

Site, tumour resectability and presence of metastases are also important.

For staging purposes, a chest spiral CT scan is mandatory. Regional lymph node metastases are infrequent, with the exception of some histological types, such as epithelioid sarcoma and clear cell sarcoma, for which regional assessment through CT/MRI may be added to the usual staging procedures. Abdomen assessment by means of CT scan is suggested, in particular for some histotypes such as limb myxoid liposarcoma. A brain CT scan may be added for alveolar soft part sarcoma, clear cell sarcoma and angiosarcoma.

Other imaging techniques, such as bone scan, whole-body MRI or PET scan, are optional and may be used in selected cases or as discussed in multidisciplinary team.

1.4 Treatment

Treatment options for STS include surgery, radiotherapy, and systemic anticancer therapy (cytotoxic chemotherapy or targeted cancer agents). Surgery and radiation therapy are the standard initial treatment options for patients with primary resectable STS; however, up to 50% of patients experience recurrence [Wesolowski R et al, Cleve Clin J Med 2010]. Neoadjuvant and/or adjuvant chemotherapy with the combination of an anthracycline (either doxorubicin or epirubicin) and ifosfamide has been shown to improve disease-free survival (DFS), yet with only marginal improvement in overall survival (OS), at the price of high toxicity, therefore a clear indication to (neo)adjuvant treatment is still debated [Pervaiz N et al, Cancer 2008; Gronchi A et al, Ann Oncol 2016].

For patients with advanced unresectable or metastatic STS, chemotherapy is the mainstay of treatment. First-line regimens for metastatic STS in most cases include anthracyclines, alone or in combination with other agents, as recommended by the National Comprehensive Cancer Network [NCCN guidelines "Soft Tissue Sarcoma", version 2.2016] and the European Society of Medical Oncology [ESMO, Ann Oncol 2014], although first-line treatment recommendations may vary by histologic subtype and previous treatment. Other cytotoxic chemotherapy agents that have shown activity in clinical trials are gemcitabine, docetaxel, vinorelbine, pegylated liposomal doxorubicin, temozolomide, trabectedin and eribulin. All these agents can be associated with significant adverse events, including pancytopenia, febrile neutropenia, nausea, alopecia, and fatigue. The two most recently approved cytotoxic drugs are Trabectedin and Eribulin.

Trabectedin is a tetrahydroisoquinoline alkaloid derived from the Caribbean marine tunicate *Ecteinascidia turbinate*, which has been approved for treatment of patients with advanced STS after failure of anthracycline and ifosfamide or in patients with advanced STS for whom these agents are not suitable [Demetri G et al, J Clin Oncol 2016]. Trabectedin has shown a particularly important activity in the treatment of mixoid round cell LPS [Grosso F et al, Ann Oncol 2009] and in general in translocation-related sarcomas [Kawai A et al, Lancet Oncol 2015]. Its antineoplastic activity depends on several mechanisms, including a drug-induced cell cycle arrest and cell death which is increased in cells deficient in homologous

recombination (e.g., cells with mutations of BRCA1/2), an immunomodulatory effect, with selective cytotoxicity against monocytes and tumor-associated macrophages, and a regulation of various transcription factors involved in cell proliferation [D'Incalci M. Future Oncol 2013].

Eribulin, a microtubule-dynamics inhibitor which is a structurally modified analogue of halichondrin B originally isolated from the marine sponge *Halichondria okadai*, has been recently shown to be active in STS. A randomized phase III trial of Eribulin compared to Dacarbazine in advanced STS demonstrated significant improvement in overall survival in patients treated with Eribulin compared to- those assigned to dacarbazine (median 13.5 vs 11.5 months [9.6–13.0]; HR 0.77 [95% CI 0.62–0.95]; p=0.0169) [Schöffski P et al, Lancet 2016]. Benefit with Eribulin seemed greater in patients with LPS, and in light of this finding Eribulin has been approved by FDA for the treatment of metastatic LPS in 2016.

Moreover, a number of targeted cancer agents, including sunitinib and pazopanib, have demonstrated activity in particular STS histologic subtypes. Sunitinib, a multityrosine kinase inhibitor of vascular endothelial growth factor receptor- (VEGFR-) 2, platelet-derived growth factor receptor- (PDGFR-) β, and c-Kit, showed activity in patients with locally advanced or metastatic STS in a non-randomized phase II trial [George S et al, J Clin Oncol 2009]. Pazopanib is a multityrosine kinase inhibitor of VEGFR-1, VEGFR-2, VEGFR-3, PDGFR-α, PDGFR-β, and c-Kit, which has been approved for use as single agent in patients with advanced STS who have received prior chemotherapy [van der Graaf WT et al, Lancet 2012].

Recently Olaratumab, a human anti-platelet-derived growth factor receptor-α (PDGFR-α) monoclonal antibody, has been demonstrated to improve both PFS and OS in association with doxorubicin, compared to doxorubicin alone, in patients with advanced or metastatic soft-tissue sarcoma in a phase II randomized trial [Tap WD et al. Lancet. 2016], and a phase III trial is currently ongoing.

1.5 Clinical and molecular markers in STS

1.5.1 Clinical markers

To date, histological grade, location and size are universally established risk factors for STS metastatic progression. High grade, deeply seated masses and size

greater than 5 cm are considered as adverse prognostic factors.

In addition, some circulating markers such as C-reactive protein, haemoglobin, serum albumin, neutrophils to lymphocyte ratio have been demonstrated to carry a prognostic significance with regard to survival in different types of carcinomas [Al Murri AM et al, Br J Cancer. 2006; Li MX et al, Int J Cancer. 2014; Hu K et al, BMJ Open. 2015]

Only a few studies have investigated the prognostic value of serum biomarkers in bone sarcoma [Aggerholm-Pedersen N et al, Transl Oncol. 2016], and STS [Nakamura T et al, Cancer. 2012].

A prognostic clinical score suggested by the Royal Marsden Hospital (London, UK) ("RMH score") able to estimate prognosis for patients enrolled in phase I trials was recently validated in patients with bone sarcomas enrolled in phase I trials at the MD Anderson Cancer Center (Houston, USA). In this study, independent factors that predicted shorter survival were male sex, more than two metastatic sites, more than three previous therapies, hemoglobin level <10.5 g/dL, platelet count >200 $x10^3$/L, creatinine level ≥1.3 mg/dL, and lactate dehydrogenase level (LDH) > Upper Level of Normal (ULN) [Livingston JA et al, Oncotarget. 2016].

In a retrospective study conducted at the Royal Marsden Hospital on older patients treated with palliative chemotherapy for metastatic STS low lymphocyte count, low albumin, anemia, along with histological subtype ad presence of comorbidity were predictive of poorer survival [Yousaf N et al, Clin Sarcoma Res. 2015].

These studies suggest that some parameters may help clinical decisions, yet such clinical variables have not been validated in patients with STS.

1.5.2 Molecular markers

To date, molecular biomarkers for STS patient stratification that may add prognostic information or help predicting response to treatment are not yet well documented.

The combination of high heterogeneity, both intratumoral and intertumoral, with their rarity has made diagnosis and prognosis of high-grade sarcomas difficult.

A number of markers have been suggested, among which nuclear expression of IGF-1R and chemokine receptor CXCR4, together with age, tumour size and use

of radiotherapy were shown to be associated with worse survival in patients with SS [Palmerini E et al, Orph J Rare Disease. 2014].

Recent research has included the use of next generation sequencing or mass spectrometry imaging to find out possible biomarkers in STS. Microarray-based comparative genomic hybridization and mRNA expression profiling have identified some genomic alterations and candidate genes for discrimination of sarcoma subtypes, for disease progression, and as potential therapeutic targets [Fritz et al, Cancer Res. 2002; Adamowicz et al, Genes Chromosomes Cancer. 2006; Francis et al, Biomed Chromatogr Genomics. 2007], though exploratory in nature so far.

Among possible biomarkers, microRNAs and transcripts related to Epithelial to Mesenchymal Transition (EMT) and Mesenchymal to Epithelial Transition (MET) are promising, and therefore we decided to evaluate such biomarkers in our translational study.

1.5.3 Markers related to Epithelial to Mesenchymal Transition (EMT)

Phenotypic plasticity is the ability of an organism to change its phenotype in response to changes in the environment [Price TD et al, Proc Biol Sci. 2003].

In cancer, phenotypic plasticity involves a process in which cells transiently acquire phenotypic traits of another lineage. The two most commonly studied types of plasticity are epithelial to mesenchymal transition (EMT) and its reverse process, mesenchymal to epithelial transition (MET).

Epithelial cells have an apico-basal axis of polarity and form layers in close contact with each other, whereas mesenchymal cells are loosely organized in a three-dimensional extracellular matrix, in which they are more motile.

EMT is a complex and reversible biological process involving a functional transition of epithelial cells into mobile mesenchymal cells [Kalluri R, Weinberg RA. J Clin. Invest. 2009; Thiery JP et al, Nat Rev Mol Cell Biol. 2006].

Emerging evidence suggests that EMT contributes to metastatization and recurrence in most carcinomas, as well as in sarcoma [Franco-Chuaire ML et al, Invest Clin. 2013; Liang YJ et al, Carcinogenesis. 2013].

In carcinomas, EMT drives invasion and metastatic dissemination, while MET is suggested to play a role in metastatic colonization [Diepenbruck M, Christofori G. Curr Opin Cell Biol. 2016], Figure 1.

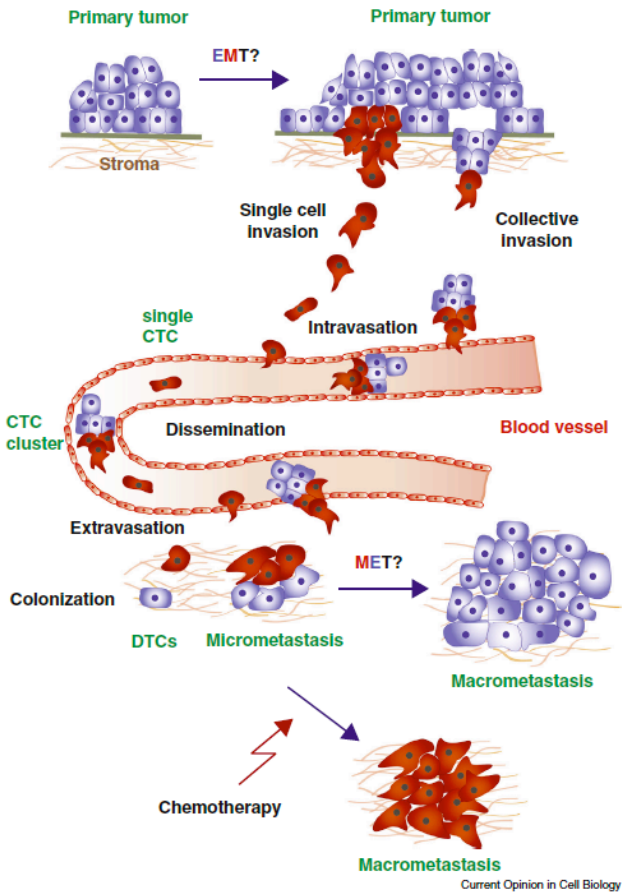

Figure 1: EMT/MET role in tumor invasion and progression.

Induction of EMT in epithelial tumor cells has been shown to enhance migration, invasion and cancer 'stemness'. Induction of MET facilitates metastatic colonization [*taken from Diepenbruck M, Christofori G. Curr Opin Cell Biol. 2016*]

Phenotypic plasticity in sarcomas is not well studied; however, there is evidence that a subset of sarcomas undergo a MET-like phenomenon. The exact mechanisms by which these transitions occur is still largely unknown, yet it is likely that some of the regulators that drive EMT and MET in carcinomas also play a role in sarcomas.

The transcription factors (TF) families Snail (zinc finger proteins Snail and Slug), Zeb (zinc finger and homeodomain proteins ZEB-1 and ZEB-2) and Twist (basic helix–loop–helix proteins E12, E47, Twist1, Twist2 and Id) play a central role in EMT during organogenesis and tumorigenesis and are considered master EMT TF [Thiery JP et al, Cell. 2009; Polyak K et al, Nat Rev Cancer. 2009]. They are potent inducers of the epithelial cell dedifferentiation process by acting as transcriptional repressors of epithelial genes, including E-cadherin, and activators of mesenchymal genes, including N-cadherin [Lamouille S et al, Nat Rev Mol Cell Biol. 2014].

Key molecules related to a more polarized, "epithelial" status are E-cadherin and ZO-1.

E-cadherin is a calcium-dependent cell-cell adhesion molecule with pivotal roles in epithelial cell behavior, tissue formation, and suppression of cancer [van Roy F, Berx G. Cell Mol Life Sci. 2008].

ZO-1, protein of Zonula occludens-1, also known as Tight junction protein-1, is a protein located on the cytoplasmic membrane surface of intercellular tight junctions, with a putative role in signal transduction at cell–cell junctions [Stevenson BR et al, J Cell Biol. 1986].

The most commonly referred mesenchymal markers are vimentin and N-cadherin. Others include alpha-smooth muscle actin (alpha-SMA), desmin, alpha-actin, fibronectin, synaptophysin [Yang J et al, Eur J Cancer. 2014] and periostin.

Vimentin is a type-III intermediate filament normally expressed in mesenchymal cells, and its expression has been reported in epithelial cells involved in organogenesis, wound healing and tumour invasion [Lahat G et al, PLoS One. 2010].

N-cadherin is a transmembrane adhesion glycoprotein whose forced expression leads to downregulation of E-cadherin expression and enhancement of cancer cell motility and migration [Yang J et al, Mol Cell Proteomics. 2010]. N-cadherin has been associated with worse clinical outcomes in some reports [Abufaraj M et al,

World J Urol; Aleskandarany MA et al, Breast Cancer Res Treat. 2015].
Alpha-SMA is the actin isoform typical of vascular smooth muscle cells. Actins are highly conserved proteins that are involved in cell motility, structure and integrity. Alpha-SMA is particularly helpful in diagnosis of smooth muscle differentiation of soft tissue neoplastic masses [Skalli O et al, J Cell Biol. 1986].

Periostin, also known as osteoblast-specific factor 2, is a multifunctional glycoprotein that belongs to the group of matricellular proteins.
It was originally discovered in mesenchymal cells (osteoblasts, osteoblast-derived cells, periosteum).
Periostin serves as a ligand for alpha-V/beta-3 and alpha-V/beta-5 integrins, and through the integrin-binding domains it interacts with several integrin receptors thus affecting the regulation of the intracellular signaling pathways associated with protein kinases PI3K/AKT and focal adhesion kinase (FAK), supporting adhesion and migration of epithelial cells [Gillan L et al, Cancer Research. 2002].
Through its influence on extracellular matrix restructuring and tissue remodeling, it plays major roles in tissue healing, development, and disease [Conway SJ et al, Cell Mol Life Sci. 2014].
Periostin has been found to have a key role in tumorigenesis and EMT in several types of tumors [Chen M et al, J Neurol Sci. 2016]. In many cancers, Periostin binds to integrins on cancer cells, activating signaling pathways that ultimately lead to increased cell survival, invasion, angiogenesis and metastasis.
Periostin has been recently associated with poor outcomes in osteosarcoma, but no data are available so far for STS [Hu F et al, Int J Exp Pathol. 2016].
Because of the connection of EMT with cancer, the attention of the scientific community has been directed towards the search for and identification of effective therapeutic targets. Such targets include signal transduction in tumor cells and the use of microRNAs, which would play a role in EMT-associated phenotypic changes and tumoral progression.

1.5.4 microRNAs (miRNAs)

MicroRNAs (miRNAs), which are small, noncoding RNA molecules of 20–24 nucleotides, have a major role in RNA silencing and post-transcriptional regulation of gene expression, with the capacity to inhibit at the post-

transcriptional and/or transcriptional level through targeting the 3'-untranslated regions of mRNAs.

Early studies have highlighted the role of miRNAs in physiological processes, and how their deregulation can lead to cancer. The causes of abnormal miRNA expression in cancer cells are only partially understood, and suggested mechanisms are abnormalities in miRNA-processing genes and proteins, and epigenetic regulation of miRNA expression [Calin GA, Croce CM. Nat Rev Cancer. 2006]. It has been reported that almost every type of cancer, including sarcoma, displays a specific profile of aberrantly expressed miRNAs that might be used as potential biomarkers or as therapeutic targets [Winter J et al, Nat Cell Biol. 2009; Jiang B et al, J Exp Clin Cancer Res. 2016; Chen W et al, J Exp Clin Cancer Res. 2016; Lauvrak SU et al, Br J Cancer. 2013; Li J et al, Cell Prolif. 2014].

miRNAs are therefore considered attractive candidates that may improve diagnostic, prognostic, and predictive characterization of this group of malignancies [Ryan BM et al, Nat Rev Cancer. 2010].

Recent publications suggest that different histological subtypes of sarcomas may have distinct miRNA expression patterns [Subramanian S et al, Oncogene 2008; Ugras S et al, Cancer Res 2011].

A recent study has shown a miRNA set in high grade sarcomas that could have an important role in the process of sarcomagenesis [Renner M et al, Gene, Chromosome and Cancer. 2012], Figure 2.

In this work, by means of hierarchical clustering of miRNA expression data from 76 sarcoma samples in an unsupervised manner, four main sarcoma subgroups were identified. One subgroup consisted mainly of SS, a second subgroup was formed exclusively by LMS, a third subgroup consisted mainly of mixoid liposarcoma (MLS) and the remaining sarcoma samples, that could not be further subdivided, were composed of dedifferentiated LPS, pleomorphic LPS, UPS, MFS, malignant peripheral nerve sheath tumor (MPNST), SS and MLS, and three samples of LMS. Within this subgroup five out of eight MPNST samples grouped together according to their diagnosis [Renner M et al, Gene, Chromosome and Cancer. 2012].

In this series, some miRNAs, and specifically miR-133a-3p, miR-133b, and miR-1 were found to be over-expressed in LMS, and together with miR-206, have been

called *myo-miRNAs*. They have been shown to play a major role in skeletal muscle proliferation and differentiation [Chen JF et al, Nat Genet. 2006]. Thus, miR-133a/b and miR-1 may be possibly involved in the malignant transformation of smooth muscle cells to LMS.

miR-1 has been suggested to function as a tumor suppressor in a variety of human cancers, since it inhibits proliferation, migration, and invasion, through the repression of several oncogenes (i.e. c-MET, Pim-1) and other molecules such as Vascular Endothelial Growth Factor-A (VEGFA) [Nasser MW et al, J Biol Chem. 2008; Niu J et al, Dis Markers. 2016].

Beyond a putative, direct role of miRNAs in the pathogenesis of STS, there is accumulating evidence on the inter-play between miRNAs and EMT markers in cancer cells [Liang YJ et al, Carcinogenesis. 2013; Fan Z et al, Oncotarget. 2015]. Indeed, several miRNAs promote or suppress EMT through direct or indirect modulation of the expression of EMT-related traits or transcription factors [Ma L et al, Nat Cell Biol. 2010; Williams LV et al, PLoS ONE. 2013; Bracken CP et al, Cancer Res. 2008]. Their role may be sometimes controversial due to the pleiotropic properties some of them display.

Among these, miR-100-5p is believed to be one of the drivers of EMT, yet at the same time it can suppress tumorigenesis, migration and invasion [Chen D et al, PLoS Genet. 2014].

Also, there are data on the role of combined expression of the mir-200 family and ZEB-1 with upregulation of an epithelial gene activator, GRHL2, in driving sarcoma cells to a more epithelial-like state, and such a regulatory network may have a prognostic relevance [Somarelli JA et al, Mol Cell Biol. 2016].

Both miR-1 and miR-200 target Slug, and this way inhibits EMT. Actually, both miR-1 and miR-200 have a role in inhibition of EMT via Slug-dependent and in inhibition of tumorigenesis via Slug-independent mechanisms [Liu YN et al, Oncogene 2013]. Other miRNAs known to be EMT modulators are miR-30 family. In particular, miR-30b has been shown to inhibit EMT by targeting Snail, and by blocking TGF-β1-induced EMT [Zhang J et al, Biochem Biophys Res Commun. 2012; Zhong Z et al, Mol Med Rep. 2014].

In STS, data on the role of miRNAs and correlation with EMT are scarce, and evaluation of such markers could be useful in order to highlight possible diagnostic and/or prognostic miRNAs signatures.

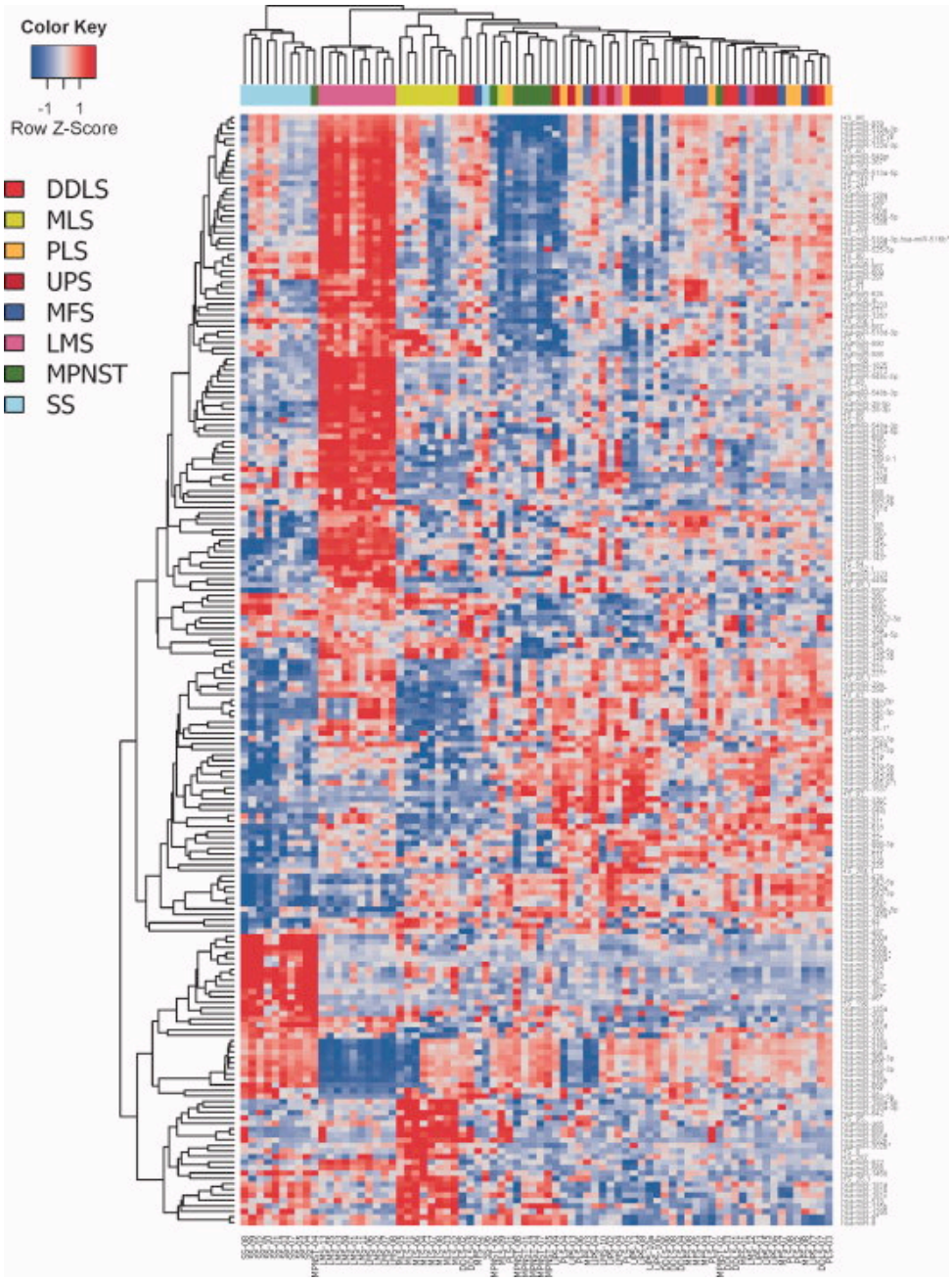

Figure 2: Unsupervised hierarchical clustering of miRNA expression data of 76 untreated, primary high-grade sarcoma samples *[from Renner M et al. Gene, Chromosomes and Cancer 2012]*.

AIM OF THE STUDY

The aims of this study were:

1. to describe efficacy and toxicity of chemotherapy for advanced STS in a cohort of unselected patients treated at Istituto Oncologico Veneto IOV – IRCCS, and to evaluate possible clinical predictors of outcome;
2. to study the expression of biomarkers in different sarcoma histological types in order to evaluate possible histological-specific profiles
3. to evaluate the prognostic relevance of such biomarkers.

PATIENTS AND METHODS

Clinical evaluation of pts referred to Medical Oncology Unit 1 – IOV

This is a mono-institutional exploratory prospective pilot study conducted at the Istituto Oncologico Veneto IOV - IRCCS di Padova.

Patients referred to Medical Oncology 1 Unit, at Istituto Oncologico Veneto, from January 2010 to December 2015 were identified via a prospectively maintained sarcoma database.

Only patients with locally advanced STS, deemed not amenable to surgical resection, or those with metastatic disease were included. Patients with a diagnosis of GIST and primary bone sarcomas were excluded.

Data regarding each patient were retrospectively collected from electronic medical records. Date of diagnosis, age at diagnosis, site of metastasis, chemotherapy agents used, performance status, serum albumin, LDH, lymphocyte and neutrophil count, date of death or last follow-up were collected. Response was evaluated by means of the RECIST criteria, version 1.1 for all the patients in which this was possible.

The clinical outcome of each patient was recorded as alive or dead as of September 30th 2016. Laboratory ranges for blood parameters were as follows; serum albumin (normal range 35 – 50 g/L), LDH (normal range 100–280 U/L), lymphocytes (normal range 1.3 – 3.5 $\times 10^9$/L), neutrophils (normal range 1.80 – 7.80 $\times 10^9$/L) and haemoglobin (normal range, male 13 – 16.5 g/dL, female 11.5 – 15 g/dl).

Translational study

Tumor tissues from patients affected by STS referred to the Istituto Oncologico Veneto, Padova, were either retrieved from the Tissue Biobank of the Department of Surgical, Oncological and Gastroenterological Sciences, University of Padova, or freshly received as tru-cut biopsy or surgical specimen.

Eligible histological types were leiomyosarcoma (LMS), undifferentiated pleomorphic sarcoma (UPS), and fibromyxosarcoma (MFS). Low-grade fibromyxoid sarcomas (FMS) were also analyzed.

All the specimens were reviewed by an expert pathologist for confirmation of representativeness of the samples. Samples were collected from November 2014

to September 2016.

Markers linked to EMT/MET were analysed by qRT-PCR as previously reported [Lignitto et al, Cancer Med. 2014], using primers specific for epithelial markers (E-cadherin and ZO-1), and mesenchymal markers (Snail, Slug, Vimentin, Zeb−1, Zeb−2, and N-cadherin). Expression of alpha-SMA, a smooth muscle-derivation marker widely used for immunohistochemical diagnosis of LMS, was also evaluated, along with Periostin.

The expression of specific miRNAs linked to EMT (miR-100-5p-5p), to MET (miR200b-3p, miR30b-5p and miR30c-5p), and myo-miRNAs (miR-1, miR 133a-3p and 133b) was assessed by MicroRNA locked nucleic acids (LNATM) PCR primer sets (Exiqon A/S, Denmark). These PCR amplification primers offer a high sensitivity, low background and accurate quantification of low microRNA levels. The high specificity allows the discrimination between closely related microRNA sequences.

10 ng RNA of each sample was reverse transcribed to cDNA using miRCURY LNATM Universal reverse trancription (RT) microRNA PCR cDNA synthesis Kit (Exiqon A/S Denmark) according to the manufacturer's protocol. The assays were performed in duplicate on the ABI 7900HT RealTime PCR system (Applied Biosystem, Foster City, CA, USA).

To evaluate differences in efficiencies in RNA isolation, cDNA synthesis and PCR amplification, known spike-ins (RNA spike-ins, Exiqon A/S Denmark) were added into the sample prior to RNA isolation (UniSp2, UniSp4, UniSp5) and prior to cDNA synthesis (UniSp6) following the manufacturer's protocol. The relative quantification (RQ) of miRNAs were standardized to U6 as endogenous control and calculated using 2-ΔΔCt method [Livak KJ and Schmittgen TD, Methods 2001].

These analyses were conducted in the laboratory of Dr. ML Calabrò.

The study was approved by the Institutional Ethics Committee in November 2014, and conducted in accordance with the Helsinki Declaration and Good Clinical Practice guidelines. Written informed consent was obtained from all patients.

Statistical analysis

Clinical evaluation of pts referred to Medical Oncology Unit 1 - IOV

Overall survival was measured from the start of chemotherapy to the time of death and censored at last follow-up. Survival was estimated with the Kaplan–Meier product-limit method, comparisons between groups were performed using the log-rank test. Univariate and multivariate analysis and hazard ratio (HR) were calculated using Cox regression.

Translational study

The distributions of biomarkers among categories of clinical variables, such as grade and histotype, were compared by the Kruskal-Wallis test and relationships among all biological variables were explored by means of the Spearman rank correlation coefficient.

Overall survival was defined as the time from diagnosis until death. Survival probabilities were calculated with the Kaplan-Meier method and the log-rank test was used to test for differences between clinical and biological categories. Each biomarker was dichotomized and classified according to the median value.

Hazard ratios (HR) and their 95% confidence interval (95%CI) based on the Cox proportional hazards model were estimated to test the association between clinical and biological characteristics and the risk of death.

The proportionality assumption was tested by including time-dependent covariates in each model.

Multiple Cox models were used to determine the adjusted association of clinical and biological factors on the probability of failure.

All tests were two-sided, and a $P < 0.05$ was considered statistically significant. Statistical analyses were performed by using SAS version 9.2 (SAS Institute, Cary, NC).

RESULTS

4.1 Clinical evaluation of pts referred to Medical Oncology Unit 1 – Istituto Oncologico Veneto IRCCS.

4.1.1 Patients' characteristics

We identified a total of 405 patients with STS referred to Medical Oncology 1 at Istituto Oncologico Veneto from January 2010 to December 2015.

Two-hundred and eight patients (51.4%) had advanced or metastatic disease. Forty-one patients were seen for consultation only and were treated elsewhere, therefore were excluded from the analysis.

One hundred-sixty seven patients were taken in charge at Istituto Oncologico Veneto. Median age was 61 years (range 16-89), 87 patients (52.1%) were female, and 54 (32.3%) were aged ≥ 70 years.

Out of these, 37 patients (19.8%) did not receive chemotherapy: five patients underwent surgical metastasectomy, 9 patients received palliative radiotherapy only and 21 did not receive any active oncological treatment because of severe comorbidity.

Patients' characteristics are described in Table 2.

4.1.2 Chemotherapy response and toxicity

One hundred-thirty patients were treated for advanced disease at Istituto Oncologico Veneto.

Patients receiving only first-line chemotherapy were 57 (34.1%), patients receiving two lines of chemotherapy were 30 (18%), and 43 patients (25.7%) received more than two lines of chemotherapy.

First-line chemotherapy regimens were anthracycline-based, either doxorubicin single-agent or a combination of epirubicin and ifosfamide) in 81 patients (62.3%). In 13 patients (10%) first-line regimen was high-dose infusional ifosfamide, 12 patients (9.2%) received trabectedin and 24 patients (18.5%) received other regimens (gemcitabine-based regimens, tyrosine-kinase inhibitors, oral cyclophosphamide, and others).

A complete response to first-line therapy was observed in three patients (2.3%), partial response was reported in 33 patients (25.4%), with disease stabilisation (clinical benefit) in further 38 patients (29.2%), whereas progressive disease as best response was reported in 45 patients (34.7%).

Grade ≥3 toxicities occurred in 59 patients (45.4%), and 23 patients (17.7%) required hospitalization for toxicity management. Hematological grade 3 or 4 toxicity developed in 40 patients (30.8%), with febrile neutropenia being the most prevalent (14 patients, 10.8%). Eighteen patients (13.8%) presented grade ≥3 extra-hematological toxicity, mainly mucositis (2 patients), nausea and vomiting (2 patients), cardiac toxicity (1 patient).

4.1.3 Survival

For patients with advanced or metastatic disease, with a median follow-up time of 12.8 months (0.07-83.43), median OS was 17.7 months (95%CI 13.59-21.75), and median PFS was 10 months (95%CI 7.9-12.0); Figures 3 and 4.

Median OS of patients treated with chemotherapy was 19.9 months (95%CI 16.4–23.5), while that of untreated patients was 3.3 months (95%CI 1.3-5.2), p<0.001; Figure 5.

At univariate analysis, ECOG PS, anemia and lymphopenia (neutrophils/lymphocytes ratio ≥3), were associated with prognosis; Table 3.

Patients with PS 0 had better median OS compared to those with PS 1 and PS 2-3, respectively 23.5 months (CI 95% 18.5–28.5), 15.8 months (95%CI 18.5–28.5), 6.6 months (95%CI 1.0-12.3), p< 0.001; Figure 6.

Baseline low lymphocyte count, with neutrophil/lymphocyte ratio ≥3 was associated with worse survival, with median OS 12.5 months (95%CI 5.4-19.5) compared to patients with neutrophil/lymphocyte ratio <3, who had median OS 22.8 months (95%CI 15.2-30.4), p=0.005; Figure 7.

Baseline anemia was also associated with worse survival, with median OS for anemic patients (Hb<12 g/dL) being 9 months (95%CI 3.2-14.8) and median OS for non-anemic patients being 20.9 months (95%CI 16.6-25.4); Figure 8.

No difference in OS was seen according to age, gender, chemotherapy regimen, BMI, albumin levels, or LDH levels.

We found baseline anemia and lymphopenia were associated, OR 5.56 (95%CI 2.11-14-71, p<0.001), and this interaction was considered for multivariate

analysis. In multivariate analysis, ECOG PS and lymphopenia were confirmed to be associated with worse survival; Table 4.

No differences according to all explored variables were observed for PFS.

Table 2: Characteristics of patients with advanced STS treated at IOV (N=167)

		N *(%)*
Age	≥ 70 years	54 *(32.3)*
	<70 years	113
Gender	Female	87 *(47.9%)*
	Male	80 *(52.1%)*
PS	0	66 *(39.5%)*
	1	54 *(32.3%)*
	2	22 *(13.3%)*
	3	11 *(6.5%)*
	NA	14 *(8.4%)*
Histological type	Liposarcoma	40 *(24.0%)*
	LMS	33 *(19.8%)*
	UPS	19 *(11.4%)*
	Synovial Sarcoma	10 *(6.0%)*
	Angiosarcoma	7 *(4.2%)*
	MPNST	6 *(3.6%)*
	Myxofibrosarcoma	5 *(3.0%)*
	Other	47 *(28.1%)*
Site of primary tumor	Limbs	70 *(41.9%)*
	Retroperitoneum	46*(27.5%)*
	Trunk	12 *(7.2%)*
	Other	39*(23.4%)*
Site of metastases	Lung and extra-pulmonary	59*(35.3%)*
	Locally advanced/inoperable	37*(22.2%)*
	Lung (only site)	36*(21.6%)*
	Only extrapulmonary	35*(20.9%)*
Previous treatment for localized disease	None	72 *(43.1%)*
	Previous (neo)adjuvant therapy	32*(19.2%)*
	Metastatic at diagnosis	63 *(37.7%)*
Neutrophil/Lymphocite ratio ≥3 (N=119)	Yes	61 *(51.3%)*
	No	58 *(48.7%)*
Albumin levels (N=88)	Normal	66 *(75%)*
	Low	22 *(25%)*
Anemia (N=135)	Absent	88 *(65.2%)*
	Present	47 *(34.8%)*
LDH (N=85)	Normal	45 *(52.9%)*
	Elevated	40 *(47.1%)*

Table 3: Univariate analysis for OS

Variable	*B*	*SE*	*Wald*	*df*	*p value*	*HR*	*HR 95% CI*
Age (continuous)	0,005	0,007	0,7	1	0,403	1,005	0,993 - 1,018
Histological type							
LMS	0,570	0,752	0,575	1	0,448	1,768	0,405 - 7,715
Liposarcoma	0,580	0,738	0,618	1	0,432	1,786	0,420 - 7,588
MFS	1,527	1,009	2,288	1	0,130	4,603	0,637 - 33,285
MPNSC	0,476	1,002	0,226	1	0,635	1,610	0,226 - 11,478
SS	0,490	0,818	0,359	1	0,549	1,633	0,328 - 8,118
UPS	0,491	0,785	0,392	1	0,531	1,634	0,351 - 7,614
Other	0,565	0,733	0,594	1	0,441	1,760	0,418 - 7,405
PS							
1 vs. 0	0,551	0,232	5,643	1	**0,018***	1,735	1,101 - 2,733
2-3 vs. 0	1,233	0,352	12,282	1	**< 0.001***	3,43	1,722 - 6,833
Site of metastases							
Locally Advanced	-0,645	0,372	3,014	1	0,083	0,525	0,253 - 1,087
Lung and Extra-pulmonary	0,346	0,286	1,460	1	0,227	1,413	0,806 - 2,477
Extra-pulmonary	0,237	0,330	0,518	1	0,472	1,268	0,664 - 2,420
First line CT regimen							
HD-IFO vs. Anthra-Based	0,041	0,379	0,012	1	0,914	1,042	0,495 - 2,190
Trabectedin vs. Anthra-Based	-0,129	0,359	0,130	1	0,719	0,879	0,435 - 1,775
Other vs. Anthra-Based	-0,422	0,317	1,771	1	0,183	0,656	0,352 - 1,221
N/L							
Yes vs. No	0,680	0,247	7,619	1	**0,006***	1,975	1,218 - 3,201
Albumin levels (N=88)							
Low vs. Normal	0,059	0,324	0,033	1	0,855	1,061	0,563 - 2
LDH (N=85)							
High vs. Normal	0,116	0,293	0,158	1	0,691	1,124	0,632 - 1,996
N. lines of treatment							
Two Lines vs. more than two	0,073	0,278	0,068	1	0,794	1,075	0,623 - 1,855
One Lines vs. more than two	0,129	0,243	0,282	1	0,596	1,138	0,707 - 1,831
Anemia							
Yes vs. No	0.446	0.236	3.565	1	**0.059***	1.563	0.983-2.484

*significant factors in univariate analysis

Table 4: Multivariate analysis for OS

Variable	*B*	*SE*	*Wald*	*df*	*p value*	*HR*	*HR 95% CI*	
PS								
1 vs. 0	0,45	0,282	2,538	1	0,111	1,568	0,902	2,728
2-3 vs. 0	0,8	0,411	3,791	1	**0,052***	2,225	0,995	4,979
N/L								
Yes vs. No	0,671	0,307	4,792	1	**0,029***	1,956	1,073	3,568
Anemia								
Yes vs. No	0,944	0,558	2,868	1	0,09	2,571	0,862	7,668
Anemia*N/L	-1,076	0,649	2,753	1	0,097	0,341	0,096	1,215

*significant factors in multivariate analysis

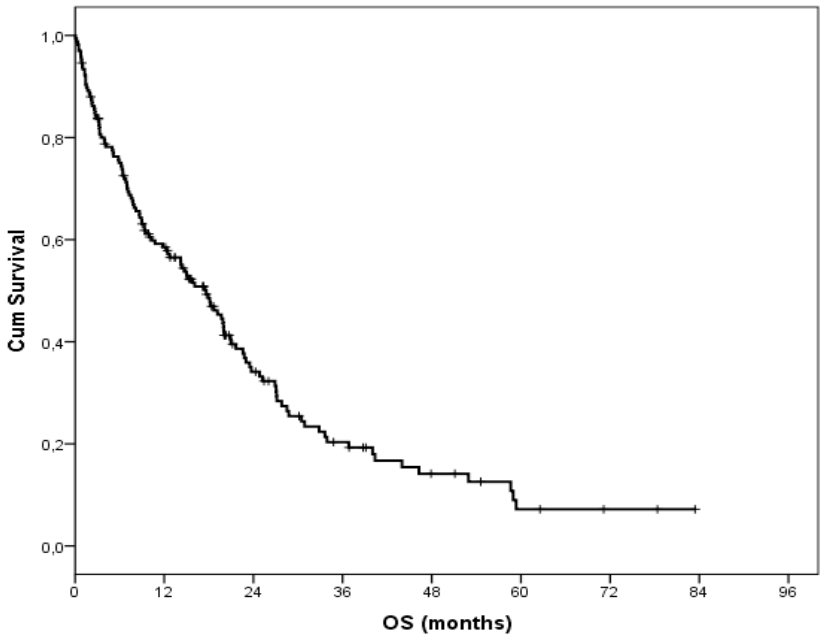

Figure 3: OS for advanced STS patients (N=167; 121 events).

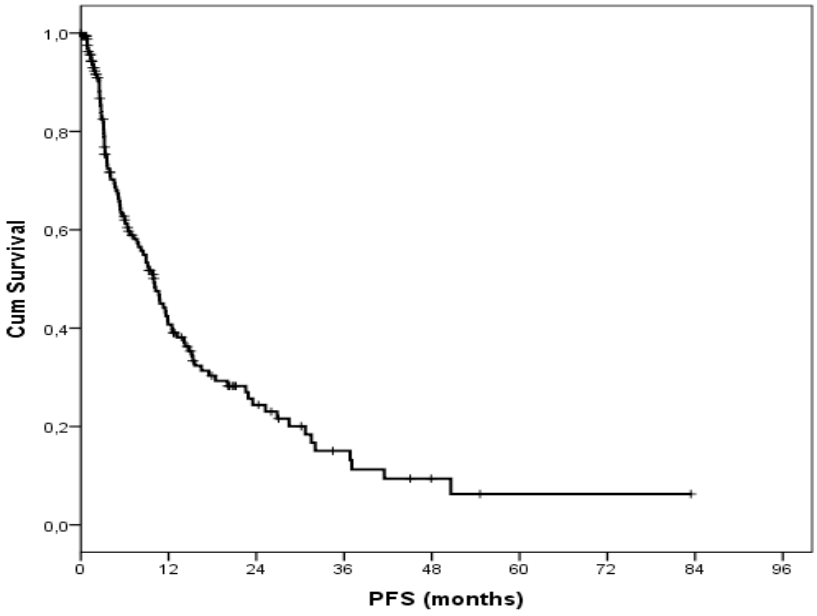

Figure 4: PFS for advanced STS patients (N=167; 106 events).

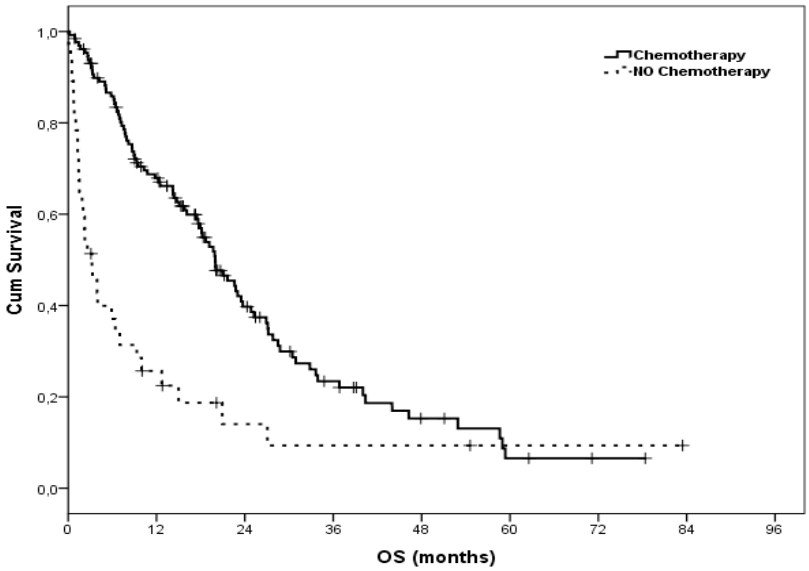

Figure 5: OS according to chemotherapy administration.

OS for patients treated with first-line chemotherapy (thick line, 130 patients, 90 events) and not treated (dotted line, 37 patients, 31 events); p<0.001.

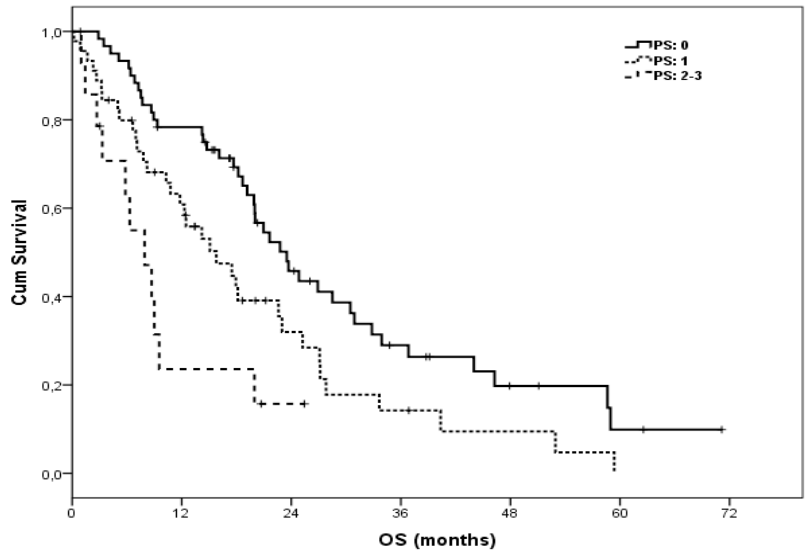

Figure 6: OS according to Performance Status (0 vs 1 vs 2-3).

OS for patients with PS 0 (thick line, 61 patients, 41 events), PS 1 (thin line, 45 patients, 35 events) and PS 2-3 (dotted line, 14 patients, 11 events); p<0.001.

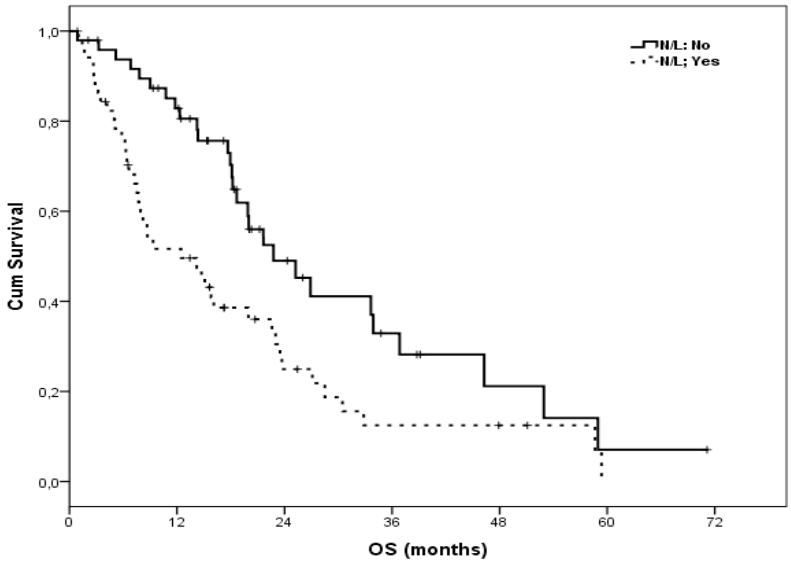

Figure 7: OS according to lymphopenia.

OS for patients with neutrophil/lymphocyte ratio <3 (thick line, 49 patients, 28 events) and for patients with neutrophil/lymphocyte ratio ≥3 (dotted line, 52 patients, 41 events); p=0.005.

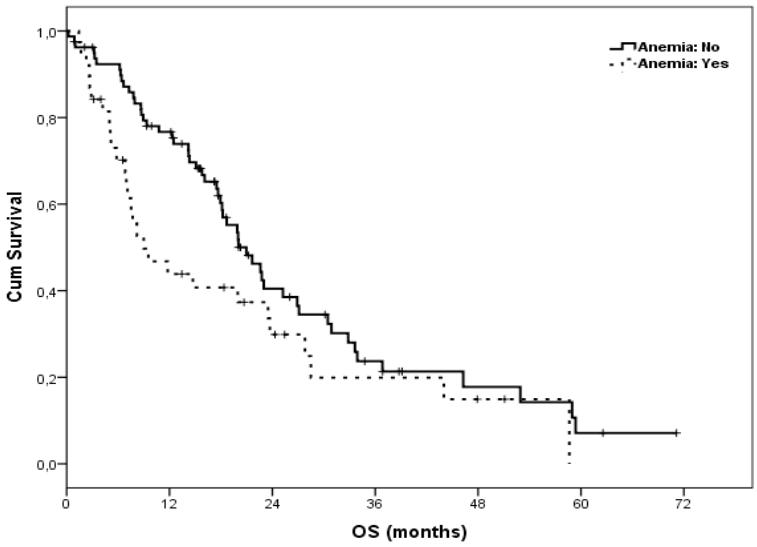

Figure 8: OS according to anemia.

OS for patients with Hb>12 g/dL (thick line, 80 patients, 53 events) and for patients with Hb<12 g/dL (dotted line, 38 patients, 28 events); p=0.057.

4.2 Translational study

4.2.1 Samples

Overall, sixty-seven STS samples were initially included in the translational stydy specifically 30 LMS, of which 10 low/intermediate grade (LG) and 20 high grade (HG) LMS; 22 myxoid sarcomas (of which 17 MFS/ and 5 low-grade FMS); and 15 UPS. The quantitative evaluation of biomarkers was limited by the low amount of RNA extracted from 12 samples. Therefore the complete molecular characterization was carried out in 54 samples, whose characteristics are described in Table 5.

4.2.2 Markers distribution

EMT-related transcripts and microRNAs distribution in our samples are described in Table 6.

Markers' distribution according to tumor grade is shown in Table 7.

Neither EMT-related transcripts nor microRNAs were found to be correlated with tumor grade.

4.2.3 Evaluation of EMT transcripts

The samples were analysed for the expression of E-cadherin and ZO-1, as epithelial markers, and of Slug, Vimentin, Snail, ZEB-1, ZEB-2, N-cadherin, and Periostin, as markers related to a mesenchymal status.

EMT-related transcripts expression for each considered histological type is shown in Figure 10.

E-cadherin expression was not found in all analysed STS.

In LMS, two distinct pattern of expression of EMT-related markers can be observed, with the presence of such patterns in both LG and HG LMS.

In MFS the expression of EMT-related transcripts is constantly low, and grading-independent.

In UPS there appears to be a great variability in EMT-related markers expression.

Table 5: Sample characteristics (N=55)

Variable			
Grade	G1	3	5.45%
	G2	14	25.45%
	G3	38	69.09%
Histotype	LMS	28	50.91%
	MFS	13	23.64%
	UPS	14	25.45%
Metastases at diagnosis	No	46	85.19%
	Yes	8	14.81%
	Missing	1	
Tumor size	≥5 cm	42	80.77%
	<5 cm	10	19.23%
	Missing	3	
Living status	Alive	26	47.27%
	Dead	29	52.73%
Relapse	No	19	38.00%
	Yes	31	62.00%
	Missing	5	

Table 6: Markers distribution in all histological types: (a) EMT-related transcripts and (b) miRNAs

a) EMT-related transcripts

Variable	N	Mean nRQ	Std Dev	Median	Inferior Quartile	Superior Quartile	Min	Max
N_CAD	46	4.5	7.6	1.3	0.2	4	0	32.5
PERIOSTIN	43	4.6	7.4	2.4	0.2	5.9	0	36.8
SLUG	49	1.7	2.1	1.1	0.3	1.9	0	9.7
Alpha-SMA	49	61.5	192.9	1.2	0.3	13.4	0	973.1
SNAIL	46	2.1	3.7	1	0.3	2.5	0	22.5
VIMENTIN	49	2	3.2	1.3	0.6	2.4	0.1	20.9
ZEB-1	45	157.3	404.1	0.4	0.2	95.4	0	1674.2
ZEB-2	45	5.5	19.4	0.9	0.5	3.1	0.1	129.1
ZO_1	49	3.9	8.2	1.1	0.4	2.8	0.1	44.1

b) miRNAs

Variabile	N	Mean nRQ	Std Dev	Median	Inferior quartile	Superior Quartile	Min	Max
miR-1	49	60.7	221.1	1.2	0.2	16.5	0	1500.2
miR-100-5p-5p	49	4.8	9.1	1.8	0.6	5.7	0	59.3
miR-133a-3p	49	49.6	165.6	1.9	0.2	24	0	1096.9
miR-133b	49	54.9	187.7	2.2	0.2	25.8	0	1247.3
miR-200b-3p	44	3	7.3	0.8	0.4	1.9	0	39.6
miR-30b-5p	49	1.7	1.9	1	0.6	1.9	0.1	9.1
miR-30c-5p	49	1.7	2.4	1	0.5	2.1	0.1	15.5

Table 7: Markers distribution according to grade: (a) EMT-related transcripts and (b) miRNAs

a) EMT-related transcripts

Variable	N	Grade	Mean nRQ	Std Dev	Median	Inferior Quartile	Superior Quartile	Min	Max	*p-value (KW)*
N_CAD	13	G1+G2	3.6	6.5	1.2	0.3	2.2	0.1	21	*0.6256*
	33	G3	4.8	8.1	1.4	0.2	5.5	0	32.5	
PERIOSTIN	12	G1+G2	1.9	2.2	1.1	0.3	2.7	0	7.3	*0.2079*
	31	G3	5.7	8.4	2.7	0.2	7.3	0.1	36.8	
SLUG	14	G1+G2	1.6	2.2	1.2	0.3	1.9	0.1	8.8	*0.8505*
	35	G3	1.8	2.1	1.1	0.3	2	0	9.7	
Alpha-SMA	14	G1+G2	108.9	248.9	2.7	0.3	56.8	0	806.5	*0.3518*
	35	G3	42.5	166	0.9	0.2	12.5	0	973.1	
SNAIL	13	G1+G2	2.9	6.1	1	0.3	2.6	0.1	22.5	*0.5746*
	33	G3	1.8	2.2	1.2	0.4	2.2	0	8.9	
VIMENTIN	14	G1+G2	3.3	5.5	1.5	0.6	2.6	0.1	20.9	*0.6027*
	35	G3	1.6	1.3	1.2	0.5	2.2	0.2	5.5	
ZEB-1	13	G1+G2	188.1	461	0.4	0.2	1.8	0	1578.5	*0.5973*
	32	G3	144.8	386	0.5	0.2	129.6	0.1	1674.2	
ZEB-2	13	G1+G2	3.3	6.5	0.6	0.3	1.8	0.1	22.5	*0.1759*
	32	G3	6.4	22.6	1	0.7	3.6	0.1	129.1	
ZO_1	14	G1+G2	6.6	12.3	1.1	0.3	6.8	0.1	44.1	*0.7393*
	35	G3	2.8	5.8	1.1	0.4	2.5	0.2	31.4	

b) miRNAs

Variable	N	Grade	Mean nRQ	Std Dev	Median	Inferior Quartile	Superior Quartile	Min	Max	*p-value (KW)*
miR-1	17	G1+G2	24.4	47.8	3	0.2	7.8	0.1	164.1	*0.5706*
	32	G3	80	271	0.6	0.2	25.4	0	1500.2	
miR-100-5p	17	G1+G2	3.8	5	1.7	0.9	5.9	0	18.2	*0.6290*
	32	G3	5.4	10.7	2.2	0.6	5.4	0	59.3	
miR-133a-3p	17	G1+G2	22.5	43.3	4.9	0.4	20.6	0.1	168.5	*0.4371*
	32	G3	64	202.2	0.8	0.1	35.3	0	1096.9	
miR-133b	17	G1+G2	25.7	50.9	6.6	0.2	25.8	0.1	203.5	*0.4751*
	32	G3	70.4	229.1	1.2	0.2	32	0	1247.3	
miR200b-3p	15	G1+G2	5.9	11.9	0.8	0.5	2.5	0.3	39.6	*0.2298*
	29	G3	1.4	1.7	0.9	0.3	1.9	0	6.8	
miR30b-5p	17	G1+G2	1.8	2.5	1	0.5	1.6	0.1	9.1	*0.7687*
	32	G3	1.6	1.5	1	0.7	1.9	0.1	6.7	
miR30c-5p	17	G1+G2	2.1	3.8	1	0.5	1.4	0.2	15.5	*0.6898*
	32	G3	1.5	1.2	1	0.6	2.3	0.1	4.7	

Figure 10: Global expression profile of EMT-related transcripts according to histological subtype.

EMT-related transcripts expression (nRQ) in LMS, MFS and UPS. Values have been normalized on global mean.

More in detail, as for the expression of the transcripts of ZO-1, Slug, Vimentin and Snail, no significant difference was found in the three histological types. Also, no difference was observed in the expression of such markers between HG and LG LMS; Figure 11.
Alpha-SMA was significantly expressed in LMS compared to UPS and MFS/FMS ($p<0.001$), and not significantly different in UPS compared to MFS/FMS, Figure 12.

ZEB-1 and ZEB-2 expression was significantly higher in LMS compared to MFS/FMS ($p<0.001$) and UPS ($p=0.001$ for ZEB-1, $p=0.003$ for ZEB-2), whereas no differential expression was measured between UPS and MFS/FMS. Interestingly, ZEB-1 and ZEB-2 were differentially expressed in HG and LG LMS ($p=0.038$ for ZEB-1, $p=0.048$ for ZEB-2); Figure 13.
Also N-cadherin expression was significantly higher in LMS compared to MFS/FMS ($p=0.006$) and UPS ($p=0.028$), whereas no differential expression was measured according to the grading; Figure 14.

As for periostin, this was found to be higher in LMS compared to MFS/FMS ($p=0.002$), and in UPS compared to MFS/FMS ($p=0.005$), no difference was observed between LMS and UPS; Figure 15.

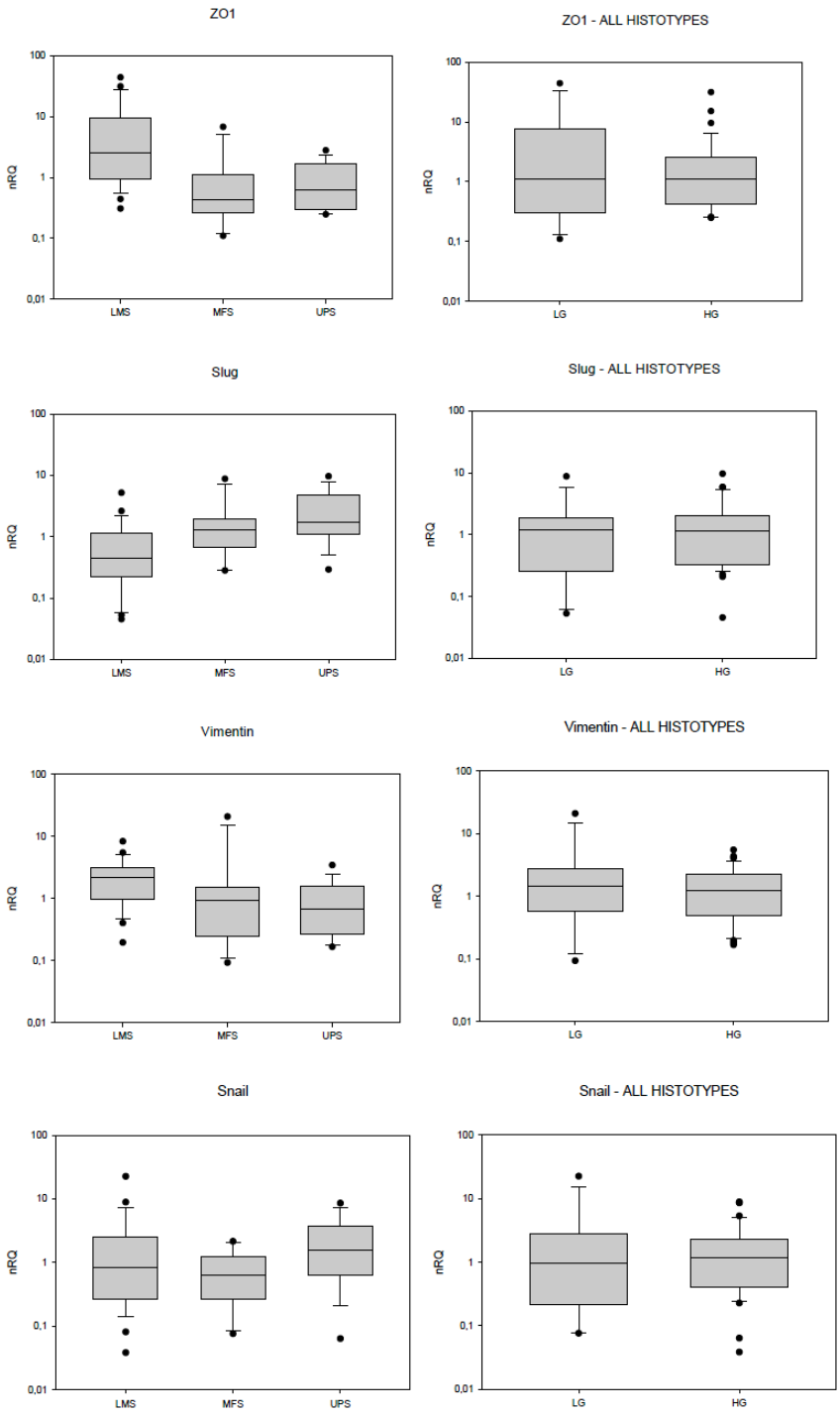

Figure 11: Expression of ZO-1, Slug, Vimentin and Snail.

Box plots describing expression (nRQ) of ZO-1, Slug, Vimentin and Snail according to histological subtypes and grade in all histological types.

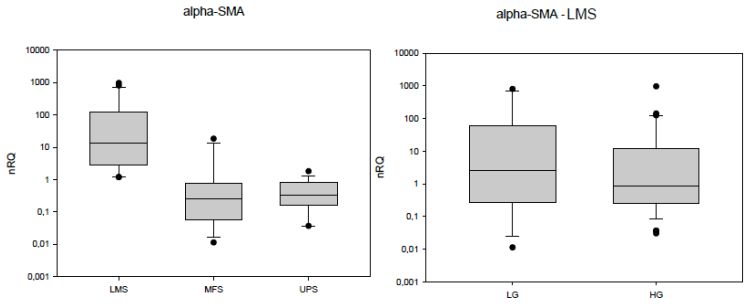

Figure 12: Expression of alpha-SMA.

Box plots describing expression (nRQ) of alpha-SMA according to histological subtypes and grade in LMS.

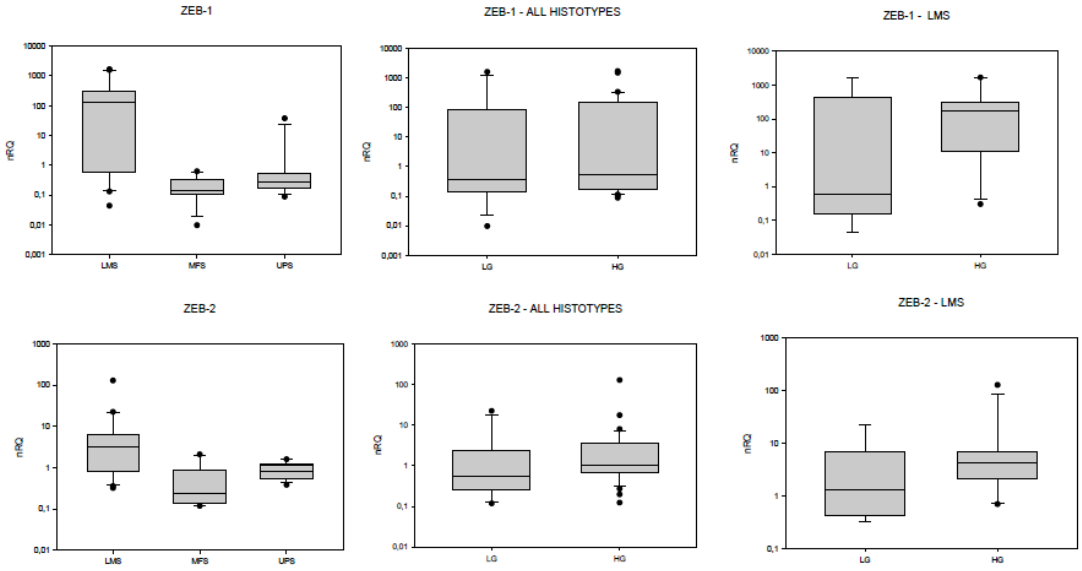

Figure 13: Expression of ZEB-1 and ZEB-2.

Box plots describing expression (nRQ) of ZEB-1 and ZEB-2 according to histological subtypes, and according to grade in all histotypes and in LMS.

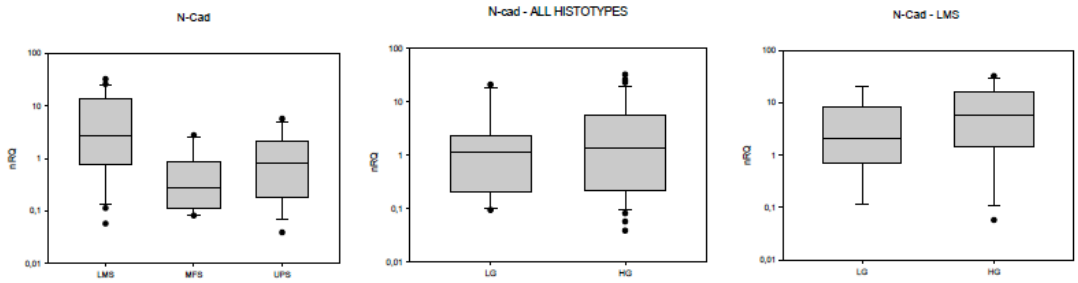

Figure 14: Expression of N-cadherin.

Box plots describing expression (nRQ) of N-cadherin according to histological subtypesand to grade in all histotypes and in LMS.

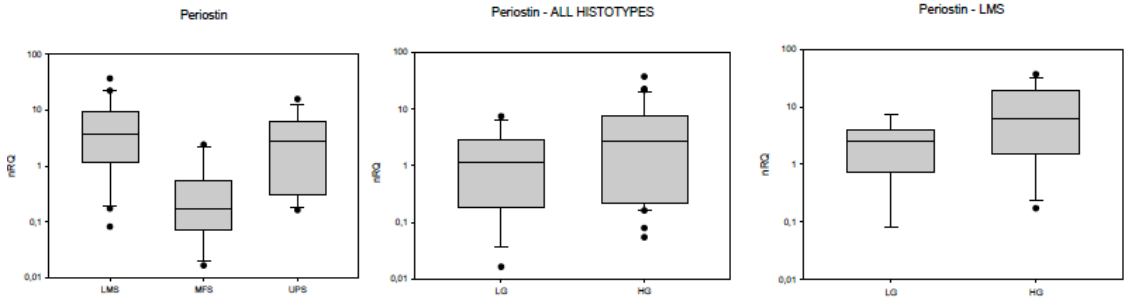

Figure 15: Expression of Periostin.

Box plots describing expression (nRQ) of Periostin according to histological subtypes and to grade in all histotypes and in LMS.

4.2.4 Evaluation of microRNAs

The expression of miRNAs in the three histological types is shown in Figure 16.

In our samples miR-1, miR-133a-3p and miR-133b ("myo-miRNAs") expression was found to be significantly higher in LMS compared to MFS and UPS ($p=0.002$) though no difference was observed between HG and LG LMS; Figure 17.

All other analysed miRNAs did not show a different expression in the three histological subtypes, nor it was different according to grade, with the exception of miR-100-5p, which was found to be significantly over-expressed in LMS compared to MFS/FMS ($p=0.02$); Figures 18-20.

4.2.5 Correlation of EMT-related markers and miRNAs

In our samples, we observed an inverse correlation between Slug and myo-miRNAs expression; Figure 21.

Also, a direct correlation between ZEB family members, and an inverse correlation between ZEB-1 and miR-200b was observed; Figure 22.

Figure 16: Global profile of analysed miRNAs.

Global expression profiles of miRNAs in all analysed miRNAS in LMS, MFS and UPS. nRQ values have been normalized on global mean.

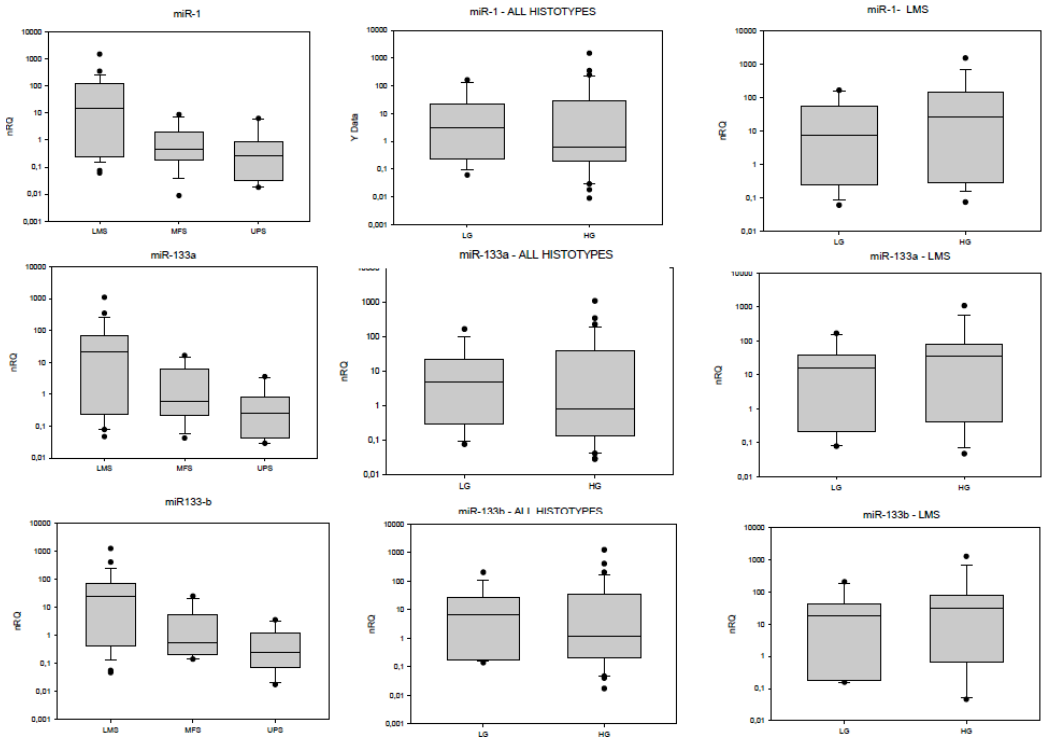

Figure 17: miR-1, miR-133a-3p and miR-133b ("myo-miRNAs") expression according to histological type.

Expression profiles of myo-miRNAs according to histological subtype, and according to grade in all histological subtypes and in LMS.

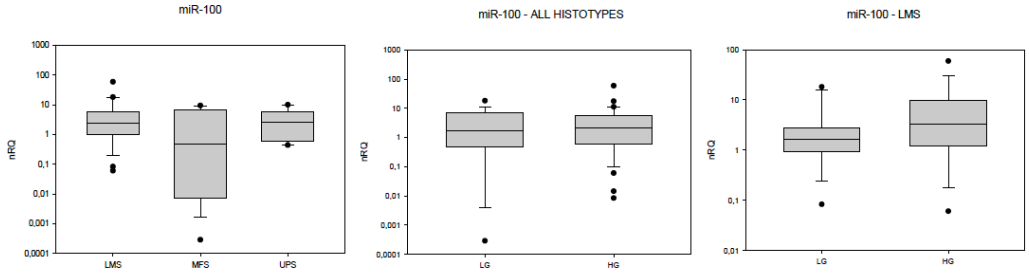

Figure 18: : miR-100-5p expression according to histological type.

Expression profiles of miR-100-5p according to histological subtype, and according to grade in all histological subtypes and in LMS.

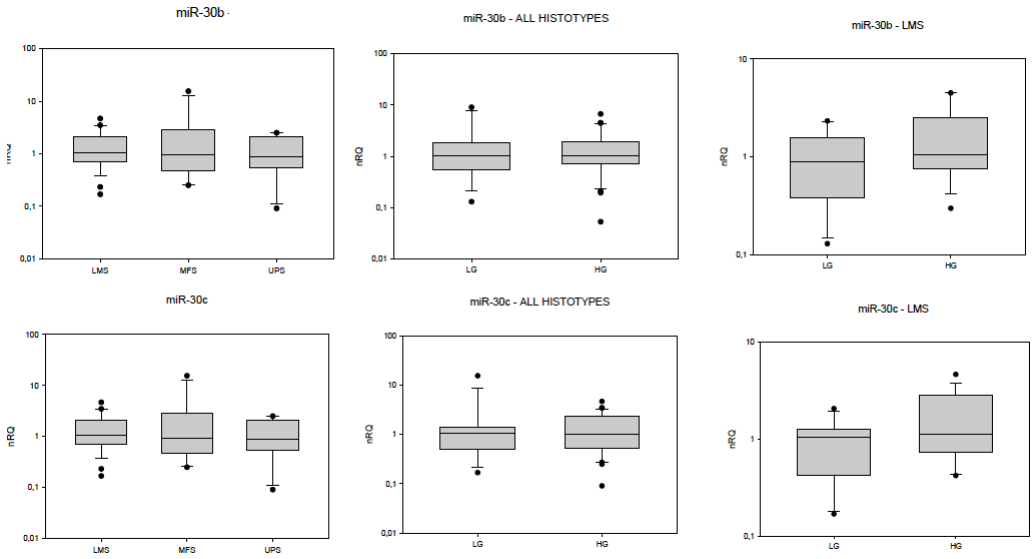

Figure 19: miR-30b and miR-30c expression according to histological type.
Expression profiles of miR-30b and miR-30c according to histological subtype, and according to grade in all histological subtypes and in LMS.

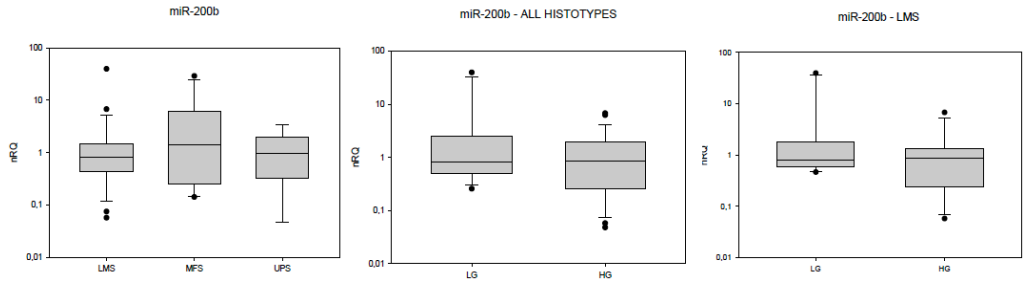

Figure 20: miR-200 expression according to histological type.
Expression profiles of miR-200 according to histological subtype, and according to grade in all histological subtypes and in LMS.

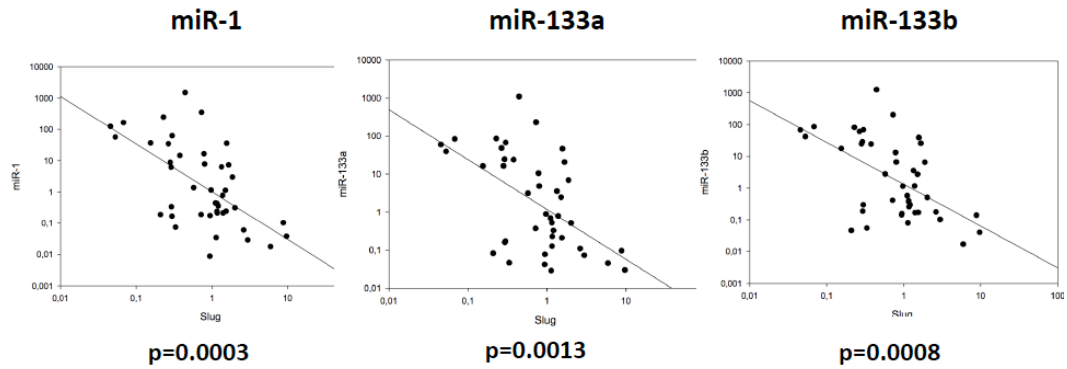

Figure 21: Expression of Slug and myo-miRNAs.

The inverse correlation between myo-miRNAs and Slug is consistent across all myo-miRNAs.

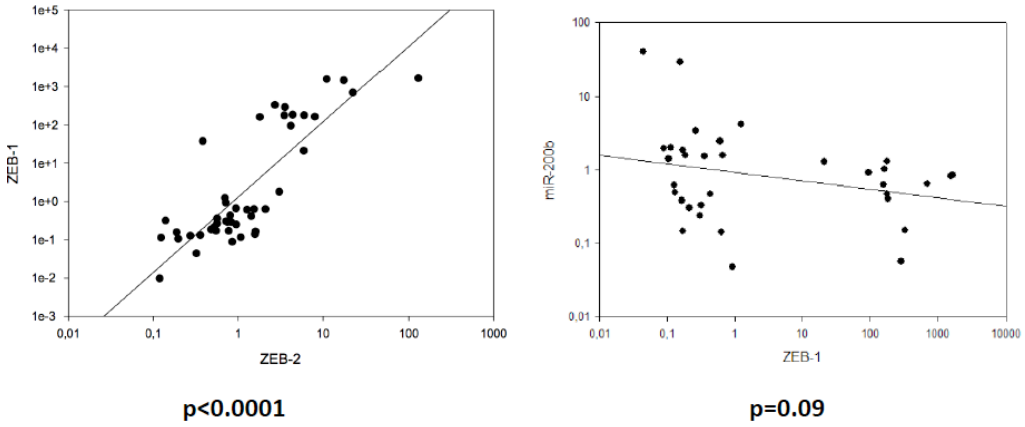

Figure 22: ZEB-family and miR-200b correlation.

The expression shows a direct correlation between ZEB-1 and ZEB-2; there is an inverse correlation, with a trend to statistical significance, between the expression of ZEB-1 and miR-200b.

4.2.6 Correlation of biomarkers with survival

In univariate analysis for OS there was a significant correlation between grade, metastatic stage at diagnosis, histological subtypes and survival; Table 8 and Figure 23.

Taking into consideration biological variables, there was a trend to significant correlation between ZEB-1 and ZEB-2 and OS. Taking as a cut-off threshold the median value, high ZEB-1 (≥0.4) was correlated with worse OS (2.3 months, 95%CI 0.9-3.4) vs low ZEB-1 (8.6 months, 1.5 – n.r.), p=0.058.
Similarly, high ZEB-2 (≥0.9) was correlated with worse OS (2.2 months, 95%CI 0.9-32.7) vs low ZEB-2 (8.6 months, 1.5 – n.r.), p=0.052.
The other marker that was significantly associated with survival in univariate analysis was Periostin.Taking as a cut-off threshold the median value, high Periostin (≥2.4) was correlated with worse OS (2.2 months, 95%CI 1.2-2.5) vs low Periostin (8.6 months, 1.3 – n.r.) , p=0.028; Figure 24.

In multivariate analysis, grade, Periostin and ZEB-1 levels confirmed to be associated with overall survival, Table 9**.**

All the other analysed EMT-related markers, as well as analysed miRNAs, were not correlated with OS, nor with PFS.

Table 8:Univariate analysis for OS – clinical variables.

Variable		Events/n	Median OS (95%CI)	p-value log-rank	HR (95% CI)	(HR) p-value
Grade (n=54)				*0.0050*		
	G1-G2	4/16	- (2.70;-)		0.24 (0.08;0.70)	*0.0091*
	G3	25/38	2.16 (1.32;6.73)		1	
Metastases at diagnosis (n=53)				*0.0261*		
	No	23/45	3.42 (2.03;-)		0.36 (0.14;0.92)	*0.0320*
	Yes	6/8	1.52 (0.11;7.67)		1	
Tumor size (n=51)				*0.1476*		
	≥5 cm	26/42	2.33 (1.47 ;8.01)		2.36 (0.71 ;7.81)	*0.1599*
	<5 cm	3/9	- (0.74 ;-)		1	
Histotype (n=54)				*0.0944*		
	LMS	17/28	2.51 (2.02;7.67)		0.89 (0.39;2.01)	*0.7806*
	MFS	3/12	- (1.32 ;-)		0.27 (0.07 ;1.00)	*0.0498*
	UPS	9/14	2.10 (0.89 ;-)		1	

Table 9:Multivariate analysis for OS.

	HR (95% CI)	(HR) p-value
Grade		
G1-G2	1	
G3	4.64 (1.48;14.52)	0.0083
Histotype		
LMS	1	
MFS	0.27 (0.08;0.96)	0.0437
UPS	0.37 (0.29;1.56)	0.3484
Periostin		
<2.4 (median)	1	
>=2.4	2.22 (0.95;5.20)	0.0660
ZEB-1		
<0.4 (median)	1	
>=0.4	2.33 (1.01;5.38)	0.0473
ZEB-2		
<0.9 (median)	1	
>=0.9	1.95 (0.88;4.31)	0.1009

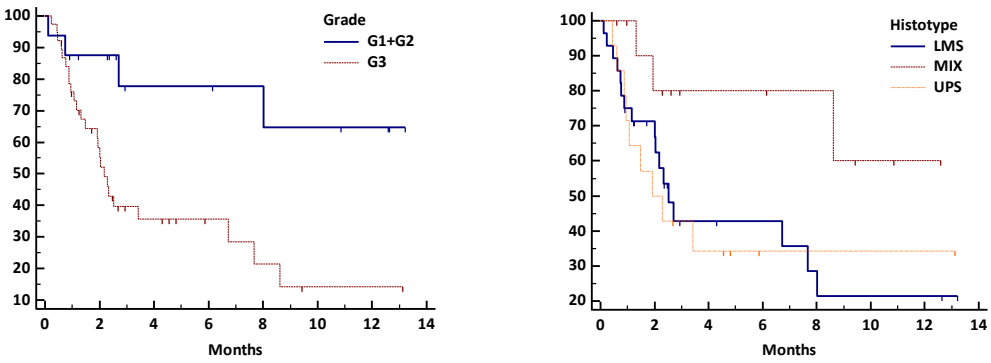

Figure 23: OS according to grade and histological type.

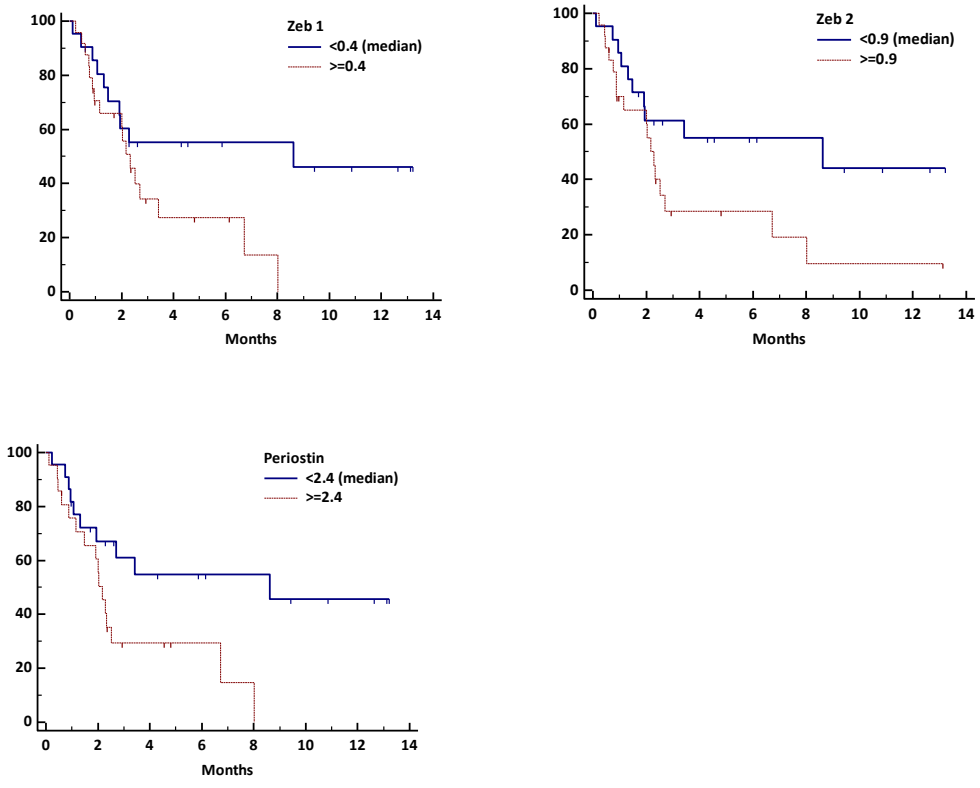

Figure 24: OS according to EMT-markers: a) ZEB-1; b) ZEB-2; c) Periostin.

Discussion

Clinical evaluation of pts referred to Medical Oncology Unit 1 – Istituto Oncologico Veneto IRCCS.

In the retrospective analysis of real world data from 167 patients treated for advanced STS in our Institution in the past 5 years we have found that about one third of patients were aged 70 years and older, with median age of all patients being 61 years. This finding is in line with epidemiologic data, and raises some concerns on best treatment for these patients, given the under-representation of older patients in clinical trials

Of all the patients with STS referred to our Medical Oncology Unit, about 35% were metastatic at diagnosis, and about 65% relapsed after primary tumor treatment, thus confirming the aggressive nature of STS.

Our data confirm the role of anthracyclines in first-line treatment of STS, that allowed to obtain a clinical benefit (complete response + partial response + stable disease) in 57% of patients. Yet, this efficacy results must be weighted against the burden of toxicity. In our study indeed about a half of patients had severe (grade 3 or 4) toxicity, and almost 20% of patients required hospitalization for toxicity-related reasons.

These data suggest a high unmet need for more effective and less toxic treatment options for patients with advanced or metastatic STS.

Globally, overall survival for the whole cohort of patients was in line with literature data and other published series, with a global median OS of 17.7 months

Actually, in our series patients undergoing palliative chemotherapy showed higher OS rates when compared to published data, with median OS for treated patients being 19.9 months.

Randomized trials of first-line chemotherapy have provided median OS for treated patients of about 14 months [Judson I et al, Lancet Oncol. 2014; Chawla SP et al, JAMA Oncol. 2015]. A series from the Royal Marsden Hospital showed a median OS of about 12 month [Karavasilis V e al, Cancer. 2008]. In another recently published Australian series on 253 patients with advanced STS median OS for patients treated with chemotherapy, which were about one third of the whole sample, was 18 months [Bae S et al, Clin Sarcoma Res. 2016].

The retrospective study describing international treatment patterns Sarcoma Treatment and Burden of Illness in North America and Europe (SABINE) study

has detected overall median overall survival rates from diagnosis of metastatic disease of 33.3 months [Leahy M et al, Ann Oncol. 2012], yet this study per inclusion criteria selected only patients not progressing on first line chemotherapy.

Only roughly one fourth of patients received more than two lines of chemotherapy for advanced disease, and this could at least in part explain the difference in survival observed with other recent reports for patients who are able to undergo multiple lines of chemotherapy [Wagner MJ et al, BMC Cancer. 2015].

As for chemotherapy response and toxicity, we were not able to assess the impact of specific chemotherapy agents on toxicity, response or survival due to the heterogeneity of regimes used.

Yet, we could confirm the role of suggested prognostic clinical factor in our series of patients. In particular, better outcomes were associated with good general conditions, as represented by ECOG PS, whereas anemia and lymphopenia were predictors of worse survival. An interaction between anemia and lymphopenia was found, and this could explain results of the multivariate analysis, in which only PS and lymphopenia were predictors of survival.

The lesser role of LDH or albumin levels on outcomes, which have been associated with prognosis in some other studies [Yousaf N et al, Clin Sarcoma Res. 2015], may be related to the high number of missing data for these variables.

Translational study

Soft tissue sarcomas constitute a group of highly aggressive, histologicallyand genetically heterogeneous malignant tumors of mesenchymal origin.

To date, only for some histological types a specific underlying molecular event driving oncogenesis has been recognized thanks to gene expression profiling and other modern techniques.

Despite progress made in the recent years, the vast majority of STS present a pleomorphic morphology, complex karyotypes and expression profiles [Nielsen TO, et al J Clin Oncol. 2010].

Only few diagnostic and prognostic markers exist, and the cellular origin of several sarcoma subtypes is unknown, and this study was aimed at evaluating some potential biomarkers.

In particular, we focused on some biomarkers of EMT and MET, as well as on selected miRNAs, in both HG and LG pleomorphic STS samples.

Most published studies on EMT markers focus on metastatic carcinomas, and studies on sarcomas, namely bone sarcomas and STS, are few.
In our study, EMT markers and explored miRNAs did not correlate with grade of STS. This could probably reflect the fact that STS are mesenchymal neoplasms and as such EMT-markers and EMT-related miRNAs may not well distinguish neoplasms according to grade.

In all LMS samples we found a significant over-expression of alpha-SMA, thus confirming both its role as marker of LMS phenotype, as well as providing indirect evidence for the reliability of analyzed samples.
Also, the expression of myo-miRNAs (miR-1, miR-133a-3p and miR-133b) was significantly higher in LMS compared to MFS and UPS, their expression being consistent and directly correlated in all LMS samples.
In particular, miR-1 and miR-133a have been demonstrated to have a role in repressing isoforms of genes that are normally not expressed in muscle, with targets of miR-1 and miR-133a being up-regulated in rhabdomyosarcomas, thus suggesting a causative role for these miRNAs in the development of rhabdomyosarcomas [Rao PK et al, FASEB J. 2010].
It was shown that miR-133 is involved in muscle development by targeting several genes, and has also been identified as a key factor in cancer development [Yu H et al, Curr Drug Targets. 2014].

Moreover, this study provided some insights on the role of miRNAs in MET. In our samples indeed an inverse correlation between myo-miRNAs and Slug was found, consistently across all myo-miRNAs.
It is known that miR-1 targets Slug, and this way it is suggested to inhibit EMT [Liu YN, et al. Oncogene. 2013]. To the best of our knowledge, miR-133a and miR-133b have not been correlated with Slug-dependent inhibition of EMT yet, therefore this could be a new finding worth of further investigation.

We demonstrated a high expression of such myo-miRNAs in all LMS samples, and demonstrated a positive correlation between the three of them, suggesting a cooperative role in the genesis of LMS.
The consistent expression of myo-miRNAs in all LMS samples may serve both as a diagnostic aid for difficult and/or controversial cases, as well as possible future development of miRNA-based gene therapy.

EMT markers ZEB-1 and ZEB-2 were differentially expressed in LMS and UPS compared to MFS and, globally, both these histological types had worse overall survival compared to MFS.
Interestingly, ZEB-1 and ZEB-2 were highly expressed in HG compared to LG LMS, and this could reflect the role of ZEB-1 and ZEB-2 as key molecules for MET.
N-cadherin expression was found to be significantly higher in LMS compared to MFS and UPS, and Periostin expression was significantly higher in LMS and UPS compared to MFS.
Interestingly, the expression of ZEB-1, ZEB-2 and Periostin was correlated with worse survival in a univariate analysis, suggesting they could be used as prognostic markers.
Indeed, besides known prognostic factors such as grade, or histological subtypes – in our samples UPS had worse overall survival, confirming their undifferentiated, high grade morphology- the only markers significantly associated with prognosis prediction in our study were ZEB-1 and Periostin.

In this study we did not find any correlation between EMT markers or miRNAs and PFS, yet this result must be taken with caution given the low overall number of samples and relatively high number of censored data for PFS.

Also, we found an inverse correlation, with a trend to statistical significance, between the expression of ZEB-1 and miR-200b. This may reflect the mutual role of miR-200 family member and ZEB-1 in driving sarcoma cells to a more epithelial-like state, confirming a recently published finding [Somarelli JA et al. Mol Cell Biol. 2016].

In light of the findings from this study, we have planned to proceed with validation of such results in a larger sample and to provide a correlation with immunoistochemical staining.

It could also be worthwhile to study circulating levels of periostin. In fact, periostin circulating levels have been demonstrated to be associated with distant metastases and poor prognosis in patients with colorectal cancer [Ben QW et al. Int J Oncol. 2009]. It could be therefore interesting to study whether circulating periostin and N-cadherin may be correlated with outcomes and response to therapy also in STS.

Conclusions and perspectives

STS are still a subset of neoplasms in which progress is difficult to achieve due to their rarity, heterogeneity and complexity. Some histological subtypes, even the ones that have higher prevalence such as LMS and UPS, have poor responses to chemotherapy and survival.

Notwithstanding clear selection biases, our clinical, real-world data confirm that there is a benefit for patients that have been treated for advanced disease compared to those not receiving active treatment. Yet, the high rate of toxicity should prompt the search for newer, possibly more active and less toxic regimens.

Our data also confirm the prognostic role of some clinical factors; specifically, the presence of extra-pulmonary metastatic sites, anemia, lymphopenia and elevated LDH were found to be correlated with worse survival.

The analysis on tumor samples highlighted that a "myo-miRNA" signature may serve as potential confirmatory markers in samples with difficult/controversial histological findings.

Moreover, some biomarkers linked to the mesenchymal phenotype, and in particular ZEB-1 and periostin were associated with worse prognosis.

These data suggest the hypothesis that these biomarkers could help clinical decision when indication to treatment is not clear.

Future work will include immunohistochemistry staining of these EMT biomarkers to confirm whether transcripts expression is correlated to protein levels, to possibly be used to help diagnostics and clinical decision.

Also, evaluation of Periostin circulating levels may be studied for their possible role in helping to identify patients at higher risk for progression.

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