Research Article

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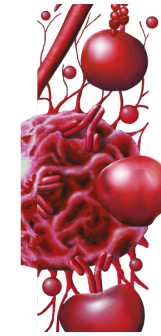
Immunotherapy



PD-1, PD-L1, IDO, CD70 and microsatellite instability as potential targets to prevent immune evasion in sarcomas

Vasiliki Siozopoulou*[,1,2], Evelien Smits[2,3], Karen Zwaenepoel[1,2], Jimmy Liu[1], Abraham Pouliakis[4], Patrick A Pauwels[1,2] & Elly Marcq[2]

[1]Department of Pathology, Antwerp University Hospital, Edegem, 2650, Belgium

[2]Center for Oncological Research, Integrated Personalized & Precision Oncology Network, University of Antwerp, Wilrijk, 2610, Belgium

[3]Center for Cell Therapy & Regenerative Medicine, Antwerp University Hospital, Edegem, 2650, Belgium

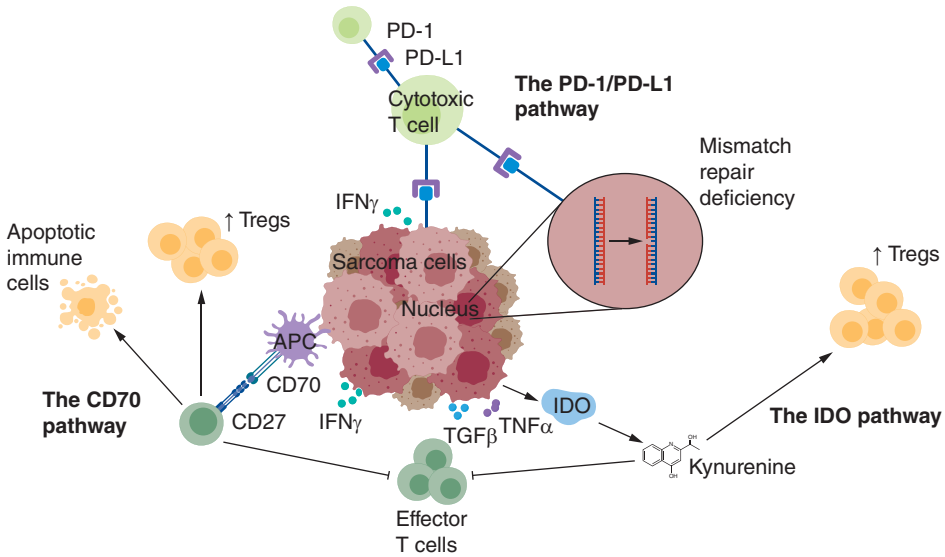
[4]Second Department of Pathology, National & Kapodistrian University of Athens, "Attikon" University Hospital, Athens, 12464, Greece

*Author for correspondence: Tel.: +32 3 821 3059; vasiliki.siozopoulou@uza.be, Vasiliki.siozopoulou@uantwerpen.be

Background: Soft tissue and bone sarcomas are rare entities, hence, standardized therapeutic strategies are difficult to assess. **Materials & methods:** Immunohistochemistry was performed on 68 sarcoma samples to assess the expression of PD-1, PD-L1, IDO and CD70 in different tumor compartments and molecular analysis was performed to assess microsatellite instability status. **Results:** PD-1/PD-L1, IDO and CD70 pathways are at play in the immune evasion of sarcomas in general. Soft tissue sarcomas more often show an inflamed phenotype compared with bone sarcomas. Specific histologic sarcoma types show high expression levels of different markers. Finally, this is the first presentation of a microsatellite instability-high Kaposi sarcoma. **Discussion/conclusion:** Immune evasion occurs in sarcomas. Specific histologic types might benefit from immunotherapy, for which further investigation is needed.

Plain language summary: Sarcomas of the soft tissue and bone are rare cancers. When these cancers spread to other parts of the body, it is hard to find good treatments. Recently, doctors have been using a new type of treatment called immunotherapy to fight several types of cancer. Immunotherapy works by getting one's body's own defense cells to attack the cancer cells. Unfortunately, immunotherapy does not work well for sarcomas and we do not know why. This study was designed to determine if there are certain mechanisms in these tumors that help the cancer cells to hide from defense cells. Determining how to change these mechanisms could make immunotherapy a better treatment for sarcomas in the future.

Graphical abstract:



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Current treatments for sarcoma

Soft tissue and bone sarcomas are rare neoplasms accounting for <1% of all malignancies [1]. Despite their rarity, both benign and malignant soft tissue and bone tumors comprise more than 200 different entities [2]. Diagnostic criteria, prognostic factors and standardized therapeutic strategies are thus difficult to assess for each separate entity. Localized and low-grade tumors are preferably treated with surgery. Neoadjuvant radiation or chemotherapy is applied for large tumors where surgery can be mutilating. Radiation and/or chemotherapy can also be used in an adjuvant setting in cases of unresectable tumors [3,4]. Tumors that are chemosensitive are usually treated with chemotherapy in a neoadjuvant setting, for instance, rhabdomyosarcoma, myxoid liposarcoma, osteosarcoma and Ewing sarcoma [4,5]. A minority of tumors with a known driver oncogenic mechanism can be treated with oncoprotein-specific targeted therapy. For example, *cKIT*/*PDGFβ* mutant driven gastrointestinal stromal tumors are excellent responders to imatinib [6]. Nevertheless, a lack of effective therapy results in a low five-year survival rate of less than 16% for metastatic sarcomas in general [7].

Immunotherapy: immune checkpoints & microsatellite instability

According to the National Cancer Institute (NCI), there are different categories of immunotherapy, including T-cell transfer therapy, monoclonal antibodies, cancer treatment vaccines, immune system modulators and immune checkpoint blockade (ICB) [8]. ICB is nowadays used in tumor treatment protocols, increasing the therapeutic options for many patients with diverse solid tumor types such as melanomas [9], lung carcinomas [10] and clear cell renal carcinomas [11] among others.

PD-1 is a receptor expressed on the surface of immune cells, mainly T cells. Binding to its ligand, PD-L1, results in a signal transduction cascade that functionally shuts down an adequate immune response. Under normal circumstances, normal cells use this interaction with immune cells to escape an excessive immune response [12]. However, tumor cells can hijack this mechanism by overexpressing PD-L1 on their surface. Inhibitors that block the interaction between PD-1 and PD-L1 have shown a substantial and durable response in the treatment of metastatic tumors and were therefore declared the major breakthrough of the last decade.

Next-generation immune checkpoint modulators have also been described [13]. Among them is CD70, a type II transmembrane glycoprotein belonging to the TNF superfamily. It is expressed on antigen-presenting cells and rapidly induced on both T and B cells upon activation of these cells [14]. CD70 has a stimulatory effect, leading to naive and cytotoxic T-cell proliferation. Upon constitutive overexpression, CD70 can facilitate tumor cell evasion from the immune system by three important mechanisms: induction of T-cell apoptosis, T-cell exhaustion and increasing the amount of suppressive Tregs [15–17]. The constitutive overexpression of CD70 on tumor cells and its absence in normal tissue has led to the development of two different anti-CD70 monoclonal antibodies, SGN-CD70A and ARGX-110. Both antibodies are currently being tested in clinical trials [18–20].

Another player in the field is IDO. IDO can suppress immune cell function by reducing levels of tryptophan. There are two major IDO pathways. Tumor cells are shown to upregulate IDO expression, which initiates the breakdown of tryptophan in the tumor microenvironment (TME) into kynurenine. Kynurenine suppresses effector T cells and promotes the activity of Tregs [21–24], which results in an ineffective antitumor immune response. Second, it promotes the activity of Tregs that suppresses the immune response [25]. IDO expression is upregulated in several types of cancer [26]. It has been shown that inhibition of subtype IDO1 can synergize with ICB. The main effect of ICB is removing the negative effect of tumor cells on immune cells, but it also stimulates the production of IDO, which switches off the immune response in a negative feedback loop [27]. Moreover, IDO has been reported to induce resistance to anti-CTLA-4 therapy in mice [28]. Preclinical and clinical trials suggest that IDO-targeting drugs should enhance ICB efficacy [27]. Moreover, IDO expression in tumor cells confers resistance to chemotherapy drugs, independent of its immune regulatory function [29,30]. These three different pathways of immune evasion are schematically represented in Figure 1.

The molecular status of tumors also plays a role in response to immunotherapy [31]. A specific type of hypermutated tumor is those with microsatellite instability (MSI). Given the hypermutated status, those tumors present many neoantigens that can trigger immune responses and have shown very good response rates when treated with ICB [32].

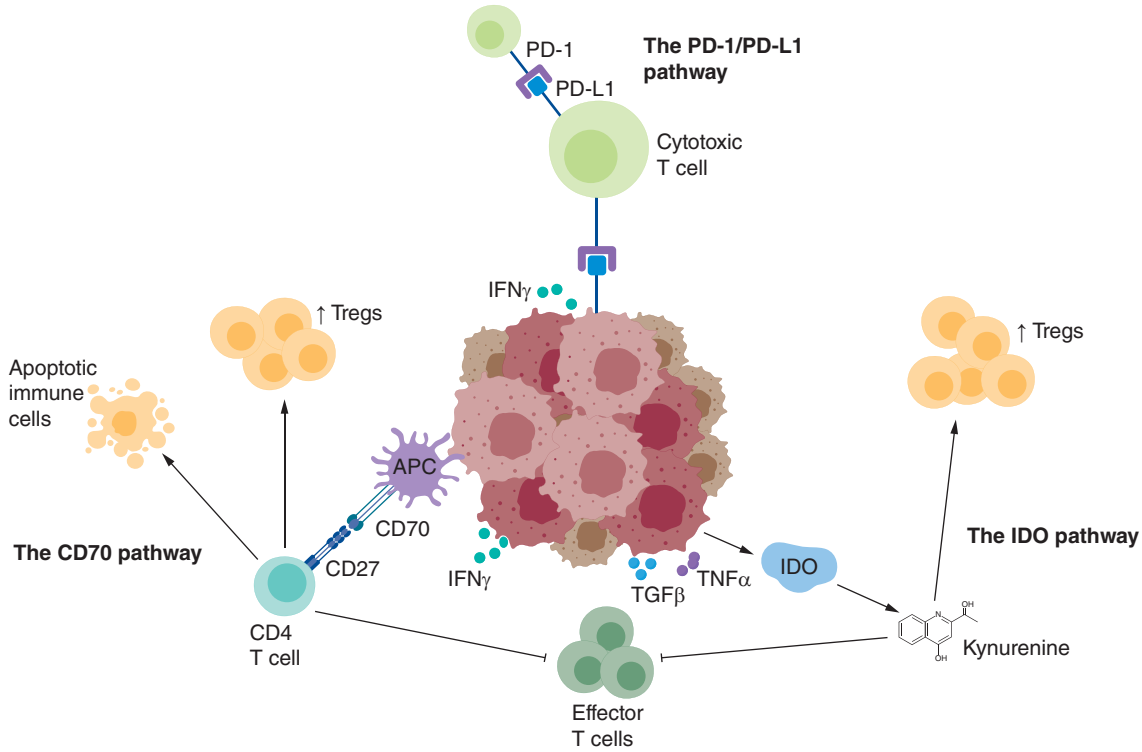

Figure 1. Three pathways of tumor evasion: CD70, PD1/PD-L1 and IDO.

Thus, the US FDA has given approval to pembrolizumab, an anti-PD-1 agent, for pediatric and adult patients with MSI-high or mismatch repair-deficient solid tumors [33]. The role of MSI in immune response and immunotherapy is shown in Supplementary Figure 1.

Immunotherapy is not a standard therapeutic option in sarcomas. Results from clinical trials were not encouraging [31]. The relationship between response and the presence of known immune modulatory biomarkers is not well documented. Moreover, MSI status has not been extensively investigated in sarcomas but, given its pivotal role in selecting patients for immunotherapy, investigation of this biomarker in sarcomas is needed. In summary, the immune checkpoints CD70, IDO, PD-1 and PD-L1 can induce immune evasion, while inhibitors of those proteins are used in clinical trials or in daily routine practice for the treatment of many tumor types. Moreover, patients with MSI-high or mismatch repair-deficient solid tumors can benefit from ICB with pembrolizumab (anti-PD-1) [32]. Hence, this paper, was designed to investigate the expression of the immune checkpoints CD70, IDO, PD-1 and PD-L1 on sarcoma tumor cells and in the TME by means of immunohistochemistry (IHC). Moreover, the microsatellite status of the tumors was also examined by molecular technique.

Materials & methods

Patient selection & samples

Archival formalin-fixed paraffin-embedded (FFPE) tissue samples from 68 patients with soft tissue and bone tumors were retrieved from the Department of Pathology at the Antwerp University Hospital (Antwerp, Belgium). They were collected between 2016 and 2018 from biopsy material as well as excision specimens. The biopsies were fixed in 4% formaldehyde for up to 12 h while the excision samples were fixed for up to 32 h, and were afterward embedded in paraffin on a routine basis. This research was limited to 5 years, as FFPE material older than 5 years may lose its immunoreactivity. Because the quality of the immunohistochemical staining was of great importance, the decision to investigate only samples younger than 5 years was made. Approval was received from the Ethics Committee of the Antwerp University Hospital/University of Antwerp (EC 18/45/517) to use historical samples. As it was a retrospective study on archival material, no informed consent could be obtained.

Immunohistochemistry

Sections of 4-µm thickness were prepared from FFPE tissue blocks and baked for 15 min at 60°C. IDO1 IHC was done with IDO1 AB (SP260 Mab, M5600, SpringBio, NJ, USA 40 min AB1/100) with 4 min pretreatment using protease 1 (Ventana). Staining was performed using the Ultraview detection system on a BenchMark Ultra instrument (Ventana Medical Systems Inc, Roche, AZ, USA). PD-L1 and CD45 IHC were performed on an Omnis instrument (Agilent, CA, USA) with the PD-L1 AB (28-8 clone, Abcam, 20 min AB1/50) and a CD45 AB (clone 2B11+ PD7/26, RTU, Omnis) respectively, in combination with the Envision Flex+ detection system. For PD-L1 an additional 10 min rabbit linker incubation was performed. Pretreatment consisted of a low-pH reagent (Agilent, 30 min 95°C). For CD45, no rabbit incubation was performed. Pretreatment in this case consisted of a high-pH reagent (Agilent, 30 min 95°C). PD-1 IHC was performed using anti-PD-1 (NAT105 clone, Cell Marque, CA, USA 1/200 20 min) on an Omnis Apparatus in combination with the Envision Flex detection kit. Pretreatment consisted of 30 min incubation with low-pH at 95°C. CD70 IHC was performed using the CD27 ligand antibody (clone 301731 R&D systems MAB2738, 1/40 for 20 min) on an Autostainer 48 in combination with the Envision Flex detection kit. Pretreatment was performed on a PT Link instrument using high-pH reagents (Dako, 30 min at 95°C). Upon staining, the sections were counterstained with hematoxylin, washed in reaction buffer, airdried for 15 min at 60°C and further processed using the Coverstainer instrument (Agilent). The tonsil and appendix were used as positive controls for all stainings. All stained slides were assessed and scored independently by two pathologists and one scientist.

First, the immune response of the tumor was evaluated. CD45 staining was done to better understand and evaluate immune infiltrate in the tumor. CD45 is a common leukocyte antigen that is expressed on almost all hematopoietic cells except for mature erythrocytes and megakaryocytes [34]. Immune responses in the different compartments of the TME were assessed (Supplementary Figure 2). The lymphocytic infiltrate in the peritumoral stroma was evaluated, namely the stroma that surrounds the tumor. Peritumoral stroma was defined as the area around the tumor when the tumor reaches the middle of a 20× microscopic field; the lymphocytic infiltrate was evaluated as follows: score 0 = no lymphocytes; score 1 = low lymphocytic density; score 2 = intermediate lymphocytic density; score 3 = high lymphocytic density. Moreover, the presence of lymphoid aggregates within the peritumoral stroma was assessed (no or score 0 and yes or score 1). The presence of tumor-infiltrating lymphocytes (TILs), namely the lymphocytic immune infiltrate in the intratumoral stroma, was also measured as follows: score 0 = no lymphocytes; score 1 = low lymphocytic density; score 2 = intermediate lymphocytic density; score 3 = high lymphocytic density and presence of lymphoid aggregates.

Afterward, the expressions of different immune checkpoints on the lymphocytes of all previously described compartments of the TME were evaluated (i.e., the peritumoral stroma, lymphoid aggregates and intratumoral stroma) but also those expressions on the tumor cells. Expression of each marker in the tissue was divided into five categories (0 = <1%; 1 = 1–<5%; 2 = 5-<10%; 3 = 10-<50%; 4 = ≥50%), as described by Marcq *et al.* [35]. A cutoff value of ≥1% was used to determine the positivity of all samples. Samples were considered to be positive in case of ≥1% positive cells, with specific staining of any intensity (0 = no expression; 1 = weak; 2 = moderate; 3 = strong) and any distribution (membrane and/or cytoplasm). These criteria were used for the IHC scoring of the different markers. All slides were scanned and evaluated using the digital Philips Platform. Pictures were also made using the same platform.

MSI testing

MSI analysis was performed using the Idylla MSI cartridge on an Idylla platform (Biocartis). As input material, one FFPE section was used. This test analyses seven monomorphic biomarkers (*ACVR2A*, *BTBD7*, *DIDO1*, *MRE11*, *RYR3*, *SEC31A* and *SULF2*). When at least two markers were divergent, the sample was considered MSI-high (MSI-H). When none or only one marker was divergent, the sample was considered microsatellite stable (MSS).

Statistics

Statistical analyses were performed via SAS 9.4 for Windows (NC, USA). For descriptive statistics, the complete range of mean value, standard deviation, minimum and maximum values and median are reported when arithmetic variables are presented. For categorical variables, frequencies and relevant percentages are used. For inferential statistics, for the comparison between two groups when arithmetic variables are in question, the *t*-test or the Mann-Whitney U test was applied according to variable normality (examination by the Kolmogorov-Smirnov test). For

more than two categories, analysis of variance or the Kruskal-Wallis test was applied, depending on a positive or negative test for normality, respectively. When categorical variables were the subject of comparison, the chi-square test was applied (and if required, the Fisher exact test, in cases of expected frequency <5 in more than 80 of the cells) and in the case of 2 × 2 contingency tables, odds ratio (OR) and the relevant 95% CI is reported. For the identification of relations between arithmetic variables (e.g., birth weight and gestational age) Pearson correlation was applied and, in cases where normality was not ensured, the Spearman correlation coefficient was calculated. All tests were two-sided and the level of significance was set to $p < 0.05$ for all study tests.

Results

Patient characteristics

The clinicopathological characteristics of the sarcoma patients included in this study are summarized in Table 1. All 68 patients were diagnosed between 2016 and 2018. The age of the 68 patients was computed from birth date to the initial diagnosis date. The age range was from 2 to 88 years old. On average, the age was 50.4 ± 20.5 years (median: 52.6). The majority of the patients were males (n = 44; 64.7%).

A total of 19 patients were diagnosed with a bone tumor, while the remaining 49 presented with a soft tissue tumor. Among the soft tissue tumors, 25 were located in the deep soft tissue, of which 16 were in the extremities, five were on the trunk or back and four were on the head and neck. In 11 patients, the tumor was located on the skin or subcutaneous adipose tissue. Deep locations in the abdomen, mediastinum and retroperitoneum were also involved for six, five and two cases, respectively.

Sarcoma cells elicit immune response & preferentially express CD70

The immunohistochemical expression of the different parameters in different compartments of the tumor, namely in the peritumoral stroma, in the lymphoid aggregates at the periphery of the tumor, in the intratumoral stroma and on tumor cells, are shown in Table 2. All samples were examined for the expression of immune checkpoints IDO, PD-L1, PD-1 and CD70. The immunohistochemical expression of the different antibodies is depicted in Supplementary Figure 3.

The positivity of each marker in the different compartments was evaluated as described in the Methods section. A small 3% of the samples did not have peritumoral tissue available for evaluation. In these specific cases, all parameters referring to this location were excluded from evaluation.

The lymphocytic infiltrate in the peritumoral stroma and in the intratumoral stroma were evaluated first. In the peritumoral stroma, two almost percentage-equal categories were found, the low score category (scores 0 and 1) and the high score category (scores 2 and 3) with a slight superiority of the low score category. Similar results were found for the intratumoral stroma, for which the balance tilted slightly toward the high score category. Based on the CD45 expression, the presence of lymphoid aggregates was also noted. In this series, 46% of tumors had lymphoid aggregates at the periphery of the tumor while the rest did not. In the majority of the cases, IDO expression in the lymphocytes of the peritumoral stroma, lymphoid aggregates, intratumoral stroma or in tumor cells we not observed. In cases where IDO was positive, it was expressed with a low score (score 1). However, almost 5% of lymphocytes in the intratumoral stroma and 3% of tumor cell samples displayed a high IDO score (score 4). Regarding CD70 expression, negative lymphocytes were found in one-third of the cases in the peritumoral stroma, one-third of the cases in the lymphoid aggregates and one-third of the cases in the intratumoral stroma. When positive, the percentage of positivity decreased as the score increased (from 1 to 4). However, CD70 expression on the tumor cells was high (scores 2, 3 and 4), reaching around 55%. PD-L1 was variably expressed in the different compartments. It was usually negative and when positive, it was mostly a score of 1 or 2. Interestingly, in 1.5% of the samples, the tumor cells displayed a high score of 4. PD-1 was more often positive on the lymphocytes than PD-L1 and gave also slightly higher score percentages in these cases. Concerning the tumor cells, PD-1 was negative in the vast majority of the samples.

Correlation among different markers: T-cell exhaustion has a multivariate component

The different markers were correlated to determine any statistical value. One interesting element that arose is the fact that the lymphocytes in the peritumoral stroma frequently expressed PD-L1 ($p = 0.0029$) and, more importantly, PD-1 ($p = 0.0002$). The correlation coefficient between PD-L1 and PD-1 in the peritumoral stroma was $r = 0.65$; $p < 0.0001$. Moreover, a strong correlation of PD-L1 on the tumor cells and PD-1 on the immune cells of the different compartments (peritumoral stroma, lymphoid aggregates and intratumoral stroma) was noted

Table 1. Clinicopathological characteristics of sarcoma patients in this series.

Characteristic	Frequency (n)	Percent (%)
Gender		
Male	44	64.7
Female	24	35.3
Age		
Average	50.4 ± 20.5 years	N/A
Median	52.6	
Tumor location		
Bone	19	27.94
Deep soft tissue extremities	16	23.53
Deep soft tissue trunk and back	5	7.35
Deep soft tissue head and neck	4	5.88
Skin and subcutaneous fat tissue	11	16.18
Abdomen	6	8.82
Mediastinum	5	7.35
Restoperitoneum	2	2.94
Histological type		
Chondrosarcoma	10	14.71
Ewing sarcoma	3	4.41
Osteosarcoma	5	7.35
Angiosarcoma	7	10.29
Kaposi sarcoma	8	11.76
Leiomyosarcoma	5	7.35
Liposarcoma	9	13.24
Myxofibrosarcoma	5	7.35
Rhabdomyosarcoma	3	4.41
Synovial sarcoma	3	4.41
Sarcoma NOS	10	14.7
Grade		
High grade	47	69.12
Low grade	17	25
Not known	4	5.88
Local or metastatic disease		
Local aggressive	15	22.06
Monometastatic disease	11	16.87
Multimetastatic disease	12	17.64
No local recurrence or metastatic disease reported	30	44.12
Oncogenic mechanism		
Oncogenic mechanism known	14	20.59
Not known oncogenic mechanism	46	67.65
Oncogenic virus (HIV)	8	11.76
Survival		
Alive	40	59.70
Death from disease	22	32.84
Death from other cause	6	7.46
Medical history		
No medical history	54	79.41
HIV	5	7.35
Lymphoma and HIV	1	1.47
Lymphoma and other tumors	1	1.47
Epithelial tumor	5	7.35
Melanoma	1	1.47
Syndrome	1	1.47
Therapy		
Excision only	26	38.24
Excision + adjuvant CHMT	7	10.29
Excision + adjuvant RT	10	10.41
Excision + adjuvant CHMT and RT	1	1.47
Excision + adjuvant ICB	1	1.47
Excision + adjuvant targeted therapy	1	1.47
Neoadjuvant CHMT	12	17.64
Follow-up	2	2.94

CHMT: Chemotherapy; ICB: Immune checkpoint blockade; N/A: Not applicable; NOS: Not otherwise specified; RT: Radiotherapy.

Table 2. Immunohistochemical scoring in various tumor environment sites.

Marker	Compartment	Score				
		0	1	2	3	4
CD45	PS	6.4	46	27	20.6	0
	LA	No: 54		Yes: 46		
	IS	16.7	28.8	24.2	30.3	0
	Tumor cells	NA	NA	NA	NA	NA
IDO	PS	33.9	46.4	14.3	5.4	0
	LA	93.1	6.9	0	0	0
	IS	32.3	27.4	19.4	16.1	4.8
	Tumor cells	90.5	4.8	0	1.6	3.2
CD70	PS	33.3	31.6	17.5	12.3	5.3
	LA	34.5	17.2	17.2	24.1	6.9
	IS	32.3	27.4	19.4	16.1	4.8
	Tumor cells	27.7	0	9.2	24.6	23.1
PD-L1	PS	71.9	10.5	14	1.8	1.8
	LA	48.3	13.8	24.1	13.8	0
	IS	67.7	12.9	11.3	8.1	0
	Tumor cells	76.9	6.2	0	9.2	1.5
PD-1	PS	47.4	28.1	15.8	8.8	0
	LA	34.5	6.9	34.5	20.7	3.5
	IS	54.8	14.5	16.1	12.9	1.6
	Tumor cells	98.5	0	1.5	0	0

IS: Intratumoral stroma; LA: Lymphoid aggregates; N/A: Not applicable; PS: Peritumoral stroma.

($p < 0.0001$; $p = 0.0139$ and $p < 0.0001$, respectively). These findings suggest a strong influence of the PD-L1/PD-1 (exhaustion) pathway in the microenvironment of sarcomas. A strong correlation between CD70 and PD-L1 expression in the peritumoral stroma was seen in most of the cases ($p < 0.0001$). Moreover, the presence of lymphocytes in the intratumoral stroma was strongly correlated with the expression of IDO on the tumor cells ($p = 0.0028$). Furthermore, IDO in the peritumoral stroma was correlated with the presence of PD-1 in the same area ($p = 0.0367$) as well as PD-1 and PD-L1 in the lymphoid aggregates located at the periphery ($p = 0.0038$ and $p = 0.0008$, respectively). These findings suggest that multiple pathways involved in T-cell exhaustion are at play in sarcoma samples.

Immune checkpoint expression is correlated with different clinical & pathological parameters

Survival

Patients were divided according to survival status into those patients that were still alive, those that died from the disease and those that died from any other cause (Table 1). None of the IHC parameters as expressed in their numeric form was linked to death ($p > 0.05$ in all cases) with the exception of IDO. Namely, all patients that died from the disease ($n = 22$) had IDO-negative tumor cells. This was statistically significant when correlated with the patients that died from any other cause ($n = 5$), all of which had a positive IDO score in the tumor cells ($p = 0.001237$). The significance of this finding is, however, unclear.

The role of the patient's life status (dead or alive) along with histological type, histological grade, metastatic status, as well as type of therapy were examined. The histological type was not proven to have a role in the life status of patients (OR: 0.6177) and, even when the tumors were grouped into the larger categories of bone sarcomas and soft tissue sarcomas, no difference was detected (OR: 1.05; 95% CI: 0.3–3.2; $p = 0.9319$). On the contrary, the histological grade was important. Namely, of the 27 patients that died, 1 had a grade I tumor (3.7%, a total of 4 patients had grade I), 2 had a grade II tumor (7.4%, a total of 17 patients had grade II) and 22 were grade III (88.9%, a total of 46 patients had grade III); the p-value of the statistical test was 0.0072. The OR for dying from a grade III tumor compared with grade I and II combined was 6.5 (95% CI: 1.7–25.3; $p = 0.0035$) and the risk of death was 52%. In terms of therapy and risk of death, patients under chemotherapy had 6.9-times higher odds of death compared with those that did not receive chemotherapy (95% CI: 2.3–20.5; $p = 0.0004$), but this was probably also due to the high grade of those tumors, while excision and radiotherapy did not achieve a statistical

significant level of evidence for their role in patient survival (p = 0.8840 and 0.3004, respectively). Moreover, high-grade tumors showed more CD70 expression in the lymphoid aggregates (pFisher = 0,0058), indicating that the immune cells in the lymphoid aggregates have an exhausted phenotype [17]. Another interesting finding was that high-grade tumors displayed a stronger intratumoral immune response than low-grade tumors (pFisher = 0,0911), implying a hot inflammation signature. Although this last finding was not statistically significant, it had a tendency toward significance and needs further investigation.

The identification of an oncogenic driver mechanism was not found to have a role in patient survival (p = 0.6461; OR: 1.3; 95% CI: 0.4–3.7) for surviving when a driver alteration is identified. Additional analysis showed that metastasis and multiple metastases were linked to patient death, specifically metastasis (irrelevant of the type: locally, single or multiple) leads to death, showing an OR of 6.6 (95% CI: 2.1–21.0; p = 0.0010) and multiple metastases is linked to a higher OR of 26.8 (95% CI: 3.2–225.2; p < 0.0001). Finally, older age had an important role (p = 0.0237) as patients that eventually died had higher age than survivors (median [Q1–Q3]: 63.7 [52.6–70.8] versus 45.7 [35.8–60.8], respectively).

Histological types

The Box and Whisker plots in Supplementary Figure 4A & B represent the expression of the different parameters in each histological type separately, while Figure 2 displays the different IHC results in relation to histological type. To start, the distribution of lymphocytes was investigated according to tumor type. The presence of lymphocytes in the intratumoral stroma, in other words, the presence of TILs, was significantly correlated with different tumor types (p = 0.0001). The differences in the expression of lymphocytes in the intratumoral stroma when grouping the tumors into two large categories were examined: on the one hand, the bone sarcomas (i.e., Ewing, chondrosarcoma and osteosarcoma) and, on the other hand, the soft tissue sarcomas (i.e., angiosarcoma/Kaposi sarcoma, sarcoma not otherwise specified [NOS], leiomyosarcoma, liposarcoma, synovial sarcoma, myxofibrosarcoma, rhabdomyosarcoma; Figure 3). Accordingly, soft tissue sarcomas display a higher expression of lymphocytes in the intratumoral stroma (p < 0.0001); in more detail, the median value of lymphocytes in the intratumoral stroma was 2 for the soft sarcomas (Q1–Q3: 1–3) while for the bone sarcomas was 0 (Q1–Q3: 0–1). Among the soft tissue sarcomas, the highest score of lymphocytes in the intratumoral stroma was seen in liposarcoma and myxofibrosarcoma with a median of 3, followed by angiosarcoma (including Kaposi sarcoma), leiomyosarcoma, soft tissue sarcoma NOS, rhabdomyosarcoma and synovial sarcoma that displayed a median of 2. Among the bone sarcomas, osteosarcoma and Ewing sarcoma had a median score of lymphocytes in the intratumoral stroma of 1 while chondrosarcomas had a median score of 0.

IDO expression on the tumor cells was seen in six cases. Although not statistically significant, four out of the six cases with IDO-positive tumor cells were bone tumors, namely, three chondrosarcomas and one osteosarcoma. Chondrosarcomas had the highest scores (score 4, n = 2) and osteosarcoma had a score of 3 (n = 1). The three cases with a low score of 1 were a chondrosarcoma, a liposarcoma and a soft tissue sarcoma NOS. The expression of IDO in the lymphocytes in the peritumoral stroma was different for each histologic type (p = 0.0470; Figure 2). Among the different tumor types, leiomyosarcoma tends to express the highest score with a median of 1.5. Myxofibrosarcomas, synovial sarcomas and osteosarcomas displayed the second-highest score with a median of 1. On the other hand, Ewing sarcoma and rhabdomyosarcomas scored usually low with a median of 0. Moreover, leiomyosarcomas and synovial sarcomas had also the highest scores of IDO expression on the lymphocytes in the intratumoral stroma with a median of 2 (p = 0.0197; Supplementary Figure 4A & B respectively).

Although CD70 and PD-L1 expression in the different compartments were not significantly associated with the histological types, myxofibrosarcomas almost always had high scores for PD-L1 in all compartments, especially in the lymphocytes in the intratumoral stroma (median 2.5) and in the tumor cells (median 1; Supplementary Figure 4A). Myxofibrosarcoma was also the only tumor type that expressed CD70 in the lymphoid aggregates with a high score (3 and 4); Supplementary Figure 4A). Still, the highest score for CD70 in tumor cells was in synovial sarcoma (median 4; Supplementary Figure 4A) and, to a lesser extent, in leiomyosarcomas, liposarcomas, angiosarcomas (among which also Kaposi sarcomas) and soft tissue sarcoma NOS (median 3; Supplementary Figure 4B).

On the other hand, PD-1 expression in the different compartments of the tumor was significantly correlated with histological type; namely, the p-value regarding the expression in the lymphocytes in the peritumoral stroma was 0.0004, in the lymphoid aggregates 0.0347 and in the lymphocytes in the intratumoral stroma 0.0074 (Figure 2). Regarding the lymphocytes in the peritumoral stroma, leiomyosarcomas and angiosarcomas gave the highest score with a median of 2. Leiomyosarcomas together with soft tissue tumors NOS showed strong expression for PD-1

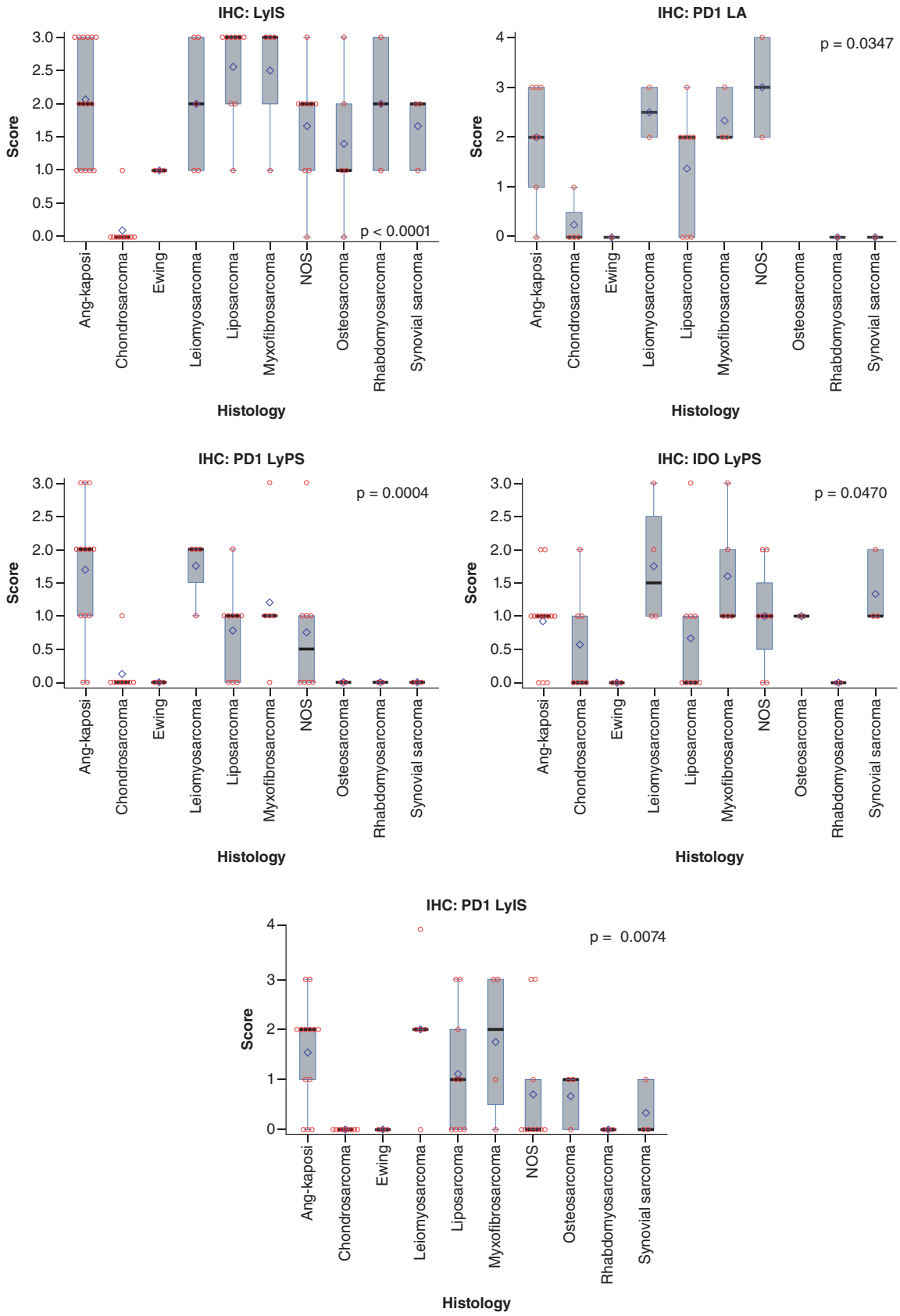

Figure 2. Immunohistochemistry results in relation to histology type. Lower and upper part of gray box indicate quartiles 1 and 3, respectively; the bold solid line within the box indicates median value and the circle mean values, while the lower and upper part of whiskers shows minimum and maximum value after excluding outliers (not shown). Red circles represent the data point.
Ang: Angiosarcoma; IHC: Immunohistochemistry; LA: Lymphoid aggregates; LyIS: Lymphocytes in intratumoral stroma; LyPS: Lymphocytes in peritumoral stroma; NOS: Not otherwise specified.

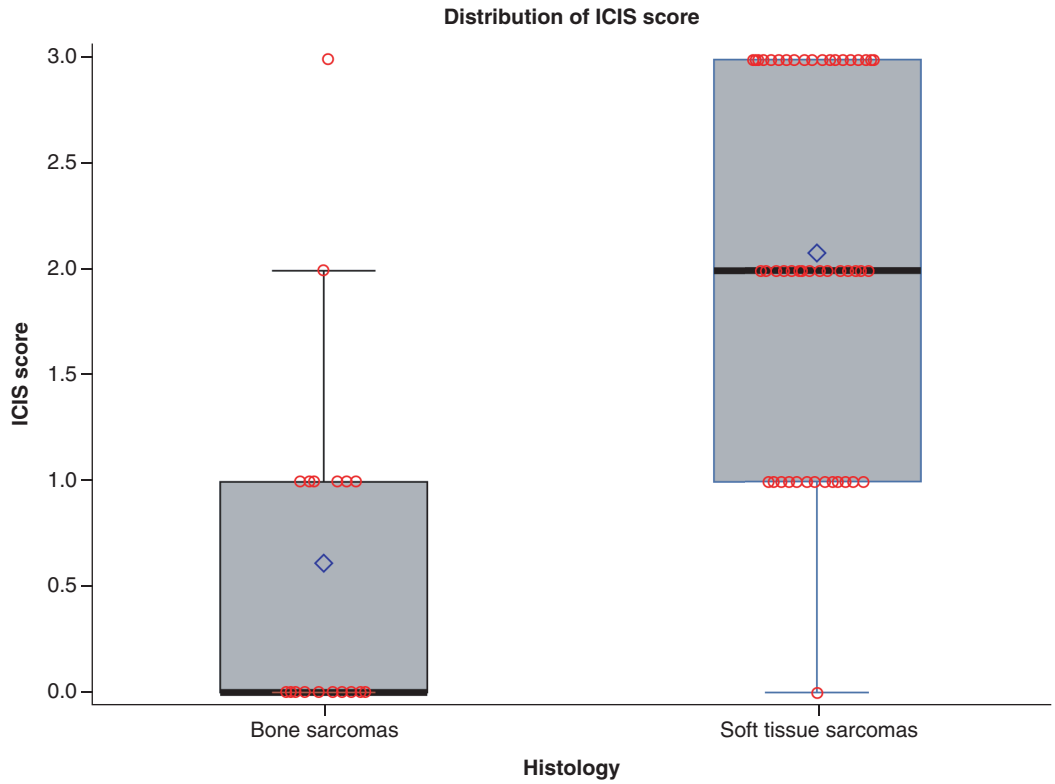

Figure 3. Distribution of lymphocytes in the intratumoral stroma in relation to histological group: bone versus soft tissue sarcoma. Lower and upper part of gray box indicate quartiles 1 and 3 respectively; the bold solid line within box indicates median value and the circle mean values, while lower and upper part of whiskers shows the minimum and maximum values after excluding outliers (not shown). Red circles represent the data point.

in the lymphoid aggregates with scores 2.5 and 3 respectively. High levels of PD-1 in the lymphocytes in the intratumoral stroma were mostly observed in angiosarcoma, leiomyosarcoma and myxofibrosarcoma, all of which had a median of 2. Notably, rhabdomyosarcomas and Ewing sarcomas were the tumors with the lowest expression of the immune checkpoint in general (Supplementary Figure 4A & B, respectively).

Molecular status

The tumors were divided into those that are known from the literature to have an oncogenic driver mechanism (among them sarcomas induced via an oncogenic virus; n = 22) and those with a complex karyotype without a known or a proven oncogenic driver alteration (n = 46). In the case of tumors with a known oncogenic mechanism, this was confirmed by molecular techniques, in the context of initial diagnosis. Those with an oncogenic driver mechanism presented statistically significantly more lymphoid aggregates and had more lymphocytes in the intratumoral stroma than the other category, with a median of 1 versus 0 for the lymphoid aggregates and a median of 2 versus 1 for the lymphocytes in the intratumoral stroma ($p = 0.0104$ and $p = 0.0181$, respectively; Figure 4).

Microsatellite instability

All cases were evaluated for MSI. Only one patient had an MSI-high tumor. The patient was a 45-year-old male with a history of lymphoma and HIV infection, diagnosed with nonmetastatic skin Kaposi sarcoma, low grade, according to the French Federation of Cancer Centers Sarcoma Group classification. This patient was treated with excision and was not reported to show recurrence or to die from the disease. The tissue sample showed a tumor with the presence of a strong immune response in the peritumoral stroma (score 2) and lymphoid aggregates at the periphery, while the lymphocytes that infiltrate in the intratumoral stroma were rather limited (score 1). The tumor displayed a low score (score 1) for CD70 in the peritumoral stroma, in the intratumoral stroma as well as in the tumor cells, while the lymphoid aggregates were negative for this marker. All other markers tested (IDO, PD-1 and PD-L1) were negative in all the tumor environmental sites that were examined.

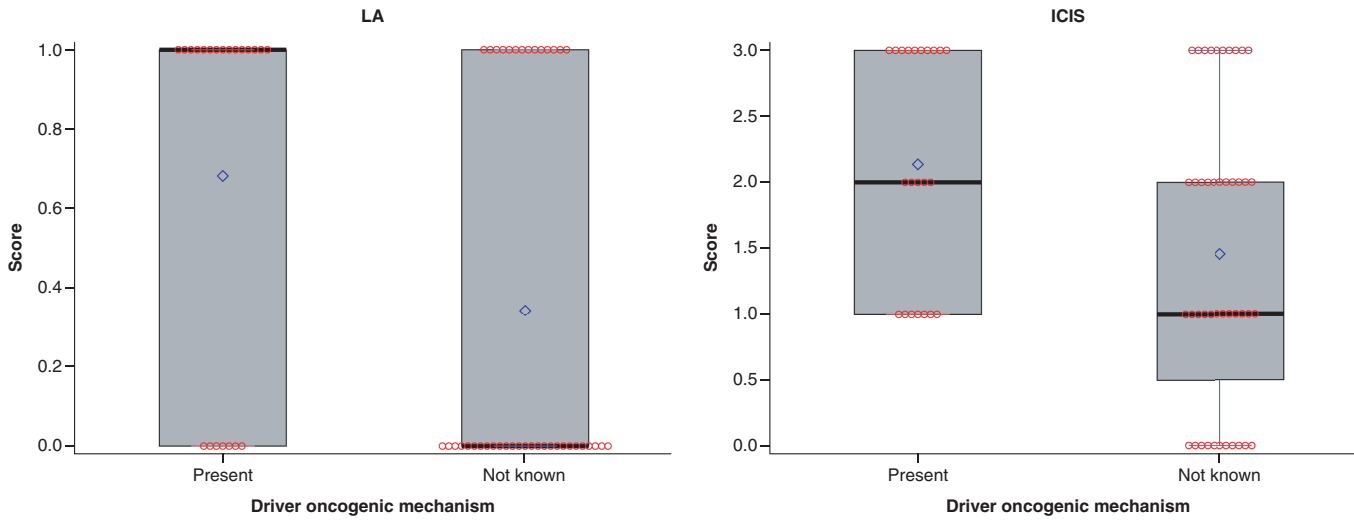

Figure 4. Presence of lymphoid aggregates (left) and lymphocytes in intratumoral stroma (right), as evaluated by means of CD45 immunohistochemistry staining, in relation to presence or not (in this case, mentioned as not known) of driver oncogenic mechanism.
Lower and upper box edges indicate Q1 and Q3, respectively; bold horizontal lines correspond to median values, upper and lower whisker edges indicate minimum and maximum observations and circles within boxes indicate mean values; outliers are not presented. Red circles represent the data point.
LA: Lymphoid aggregates.

Metastatic & nonmetastatic patients

Metastatic disease is difficult to manage with the current treatment protocols, hence, the high mortality rates in this group of patients. Nonmetastatic patients have a better prognosis, still, this is mostly seen in cases where the tumor can be completely excised. However, in some instances, the tumor is locally aggressive, for which primary surgical excision is not possible. In these cases, a neoadjuvant treatment with immunotherapy that will reduce the size of the tumor could be of clinical and prognostic value. This is the reason that investigating the immune profile in nonmetastatic patients is equally important as in metastatic patients.

A comparison was performed between metastatic and nonmetastatic patients to investigate any differences in their immune expression and molecular and clinical profile. No significant differences could be seen in age, gender, histological grade and molecular status between these two categories ($p > 0.05$ for all cases). Among the immunohistochemical markers, CD70 expression in lymphocytes in the peritumoral stroma showed higher expression in the metastatic cases ($p = 0.0107$). IDO expression in the tumor cells was not expressed in this category ($p = 0.0149$). The rest of the immunohistochemical markers displayed no significant statistical difference in metastatic versus nonmetastatic disease. Considering the outcome, 20 (58.8%) of the metastatic patients died, in contrast to the nonmetastatic patients where 5 (17.2%) died (OR: 6.9; 95% CI: 2.1–22.3; $p = 0.0009$). The comparison between metastatic and nonmetastatic patients is shown in Supplementary Table 1.

Discussion

In this article, we describe the expression profile of the immune checkpoints IDO, CD70, PD-1 and PD-L1, as well as the MSI status in tissue samples of diverse sarcoma types, both of the soft tissue and the bone. We investigated the expression of immune checkpoints in the different compartments of the tumor, namely the peritumoral stroma, lymphoid aggregates at the periphery of the tumor, intratumoral stroma as well as the tumor cells. MSI was investigated in the tumor cells.

The tumor and the surrounding microenvironment are closely related and constantly interact. The TME plays a very important role in tumor progression. One of its important elements is the immune cells. PD-1 is expressed by immune cells, mainly cytotoxic T lymphocytes. When it binds to its ligand PD-L1, a switch-off signal results in the downregulation of the immune response [31,36]. In such cases, the presence of PD-1 can indicate an exhausted phenotype. In our samples, we found a strong correlation between the expression of PD-1 and PD-L1 in the peritumoral stroma of the TME. This could mean that the peritumoral stroma in sarcomas might lead to tumor progression, as the tumor cells can escape the inactivated immune system. Moreover, the expression of PD-L1

in the tumor cells was correlated with the expression of PD-1 in the immune cells of the peritumoral stroma, the lymphoid aggregates and the intratumoral stroma, also suggesting an exhausted phenotype. Another feature attributed to tumors is the inflammation signature. Tumors that display a strong immune infiltrate are called 'hot' while those without one are called 'cold' tumors [37]. The strong immune response seen in 'hot' tumors may contribute to the eradication of tumor cells. Undifferentiated sarcomas have been described as 'hot' tumors and this could explain the clinical activity of these tumors to pembrolizumab in clinical trials [38]. In our series, sarcomas NOS (i.e., undifferentiated sarcomas) also showed an inflamed phenotype. Still, among our cases, the tumors with the strongest inflammation signature were myxofibrosarcomas and liposarcomas. Another very interesting conclusion that emerged from our study is that there is a clear difference between soft tissue and bone tumors, as the latest almost always show a lack or paucity of tumor immune cell infiltration, which characterizes the 'cold' phenotype. Many parameters have been implied to be responsible for this phenotype, among others, the absence of T cell priming or activation, lack of tumor antigens and deficit of T cells homing to the tumor bed [39]. In osteosarcomas, immune cell infiltration depends on the plasticity of the tumoral extracellular matrix as well as proteolysis of this matrix through metalloproteinases [40]. T cells may cross the blood vessels, but they cannot progress along the dense and tight fibers of the osteosarcoma extracellular matrix, thus remaining trapped within it [41]. Hence, it is suggested that induced proteolysis of the extracellular matrix could be the key to the success of any immune cell therapy for osteosarcomas [42]. Although ICB therapy seems not to have a direct role in treating 'cold' tumors, combination approaches with traditional treatments such as radiation, chemotherapy and tyrosine kinase inhibitors could contribute to turning these tumors 'hot' [43].

In our series, tumors with an oncogenic driver mechanism (including oncogenic viral mechanisms) correlated with more lymphoid aggregates and more lymphocytes in the intratumoral stroma. We know that desmoid tumors, for instance, which are known to harbor mutations of the *CTNNB1* or the *APC* gene, present lymphoid aggregates at the periphery of the tumor, indicating tertiary lymphoid organs [44]. The role of tertiary lymphoid organs is to recruit activated immune cells in the tumor, participating in antitumor immune response [45]. Still, as discussed, the lymphocytes in the lymphoid aggregates can display an exhausted phenotype. This suggests immune suppression of these cells, indicating that immune cells in tertiary lymphoid organs are in a dynamic process, changing from activated to exhausted. In such cases, a marker of exhaustion may be interesting to be included when analyzing tissue samples. Recognition of the mechanism that drives this transformation could be of benefit to patients with sarcoma with a known oncogenic pathway.

As mentioned, PD-L1 expression was mainly observed in the lymphocytes of the peritumoral stroma and the lymphoid aggregates. Few cases had positive PD-L1 expression in the tumor cells. Among the different tumor types, myxofibrosarcoma had the highest PD-L1 scores, followed by angiosarcoma, osteosarcoma and liposarcoma. These data correlate with the PD-L1 expression profile that is described in sarcomas in the literature. Accordingly, among the tumors with the higher rates of PD-L1 positivity are undifferentiated pleiomorphic sarcomas, angiosarcomas, rhabdomyosarcomas, myxofibrosarcomas, leiomyosarcomas and dedifferentiated liposarcomas [46].

Notably, myxofibrosarcomas also showed high levels of lymphocytes in the intratumoral stroma as well as high levels of PD-1 in these lymphocytes. Although this was not statistically significant, it suggests that those cells represent an exhausted phenotype. Myxofibrosarcomas are aggressive soft tissue tumors and chemotherapy and radiotherapy can be used in neoadjuvant, adjuvant or metastatic settings [47]. Until now, not much has been known about the use of immune checkpoint inhibitory therapy for the treatment of these tumors. In a case report of a patient with metastatic myxofibrosarcoma, the patient showed partial response for 16 months and overall survival for over 29 months with the administration of nivolumab and bevacizumab [48]. In another case study, a patient in a palliative setting receiving chemotherapy for metastatic myxofibrosarcoma showed a partial response with pembrolizumab for 14 cycles [49]. In a multicentric study with nivolumab plus ipilimumab in metastatic sarcomas, one patient with myxofibrosarcoma achieved a complete response [50]. Although in these cases no correlation with PD-L1 expression in the tumor cells was reported, our data in combination with these reports, suggest that PD-L1 expression in myxofibrosarcoma may play a role in response to ICB. Further investigation is needed to evaluate if patients with this tumor type could benefit from this therapeutic approach.

The role of PD-L1 expression as a predictive marker for response to immunotherapy has been a subject of investigation in many clinical trials. The reported results are contradictory, as some trials describe a positive correlation between objective response and progression-free survival and PD-L1 expression in tumor cells, while others could not demonstrate such a correlation [31].

Moreover, we found that myxofibrosarcomas displayed high levels of CD70 in the lymphocytes in the intratumoral stroma. Although statistically not significant, myxofibrosarcomas also showed high CD70 expression on tumor cells. The CD70 signaling pathway increases the cytotoxic T-lymphocyte response and supports memory formation [51]. Transient CD70 expression is present on activated T and B cells and mature dendritic cells. On the other hand, persistent CD70 signaling leads to the exhaustion of T cells [14]. The presence of CD70 in myxofibrosarcomas has not been previously described, but given the consistent positivity in our cases, we suggest that those tumors need further investigation for the possibility of targeting with anti-CD70 antibodies.

Leiomyosarcomas expressed high levels of PD-1 in the lymphocytes of the peritumoral stroma, the lymphoid aggregates as well as in the lymphocytes of the intratumoral stroma. Still, no expression of PD-L1 was noted. Given the lack of PD-L1 in these cases, possible inhibition of the immune system through the PD-L1 and PD-1 interaction seems less likely. Hence, the use of anti-PD-1 or anti-PD-L1 therapy may not have any value for this tumor type. In a phase II clinical trial with pembrolizumab and cyclophosphamide in advanced sarcomas, all of the leiomyosarcomas that were included in the study showed progressive disease [52].

IDO was mostly positive in the lymphocytes in the peritumoral stroma. Few cases had IDO positivity in the lymphoid aggregates and the lymphocytes in the intratumoral stroma. Three cases had high expression of IDO in the tumor cells. Notably, IDO expression in the tumor cells was mostly observed in bone tumors, mainly chondrosarcomas and osteosarcomas. Both chondrosarcomas and osteosarcomas presented IDO positivity also in the lymphocytes in the peritumoral stroma. Still, the highest scores for IDO expression in the lymphocytes in the peritumoral stroma were noted for leiomyosarcomas. Studies of regulatory and effector pathways illuminate IDO as an inflammatory modifier [53]. Moreover, the IDO pathway is possibly an important mechanism for primary resistance to PD-1 inhibition [54]. Notably, PD-1/PD-L1 reverse signaling also induces IDO. Given that clinical trials of IDO and PD-1 inhibitors show promising results, coinhibition of these two targets provides a rationale to evaluate additional combinations of immune checkpoint inhibitors with IDO inhibitors [53]. Although in our series, limited IDO positivity was shown on the tumor cells, the statistical correlation with chondrosarcomas and osteosarcomas was strong.

We investigated the MSI in all our samples, which induces a hypermutated phenotype. MSI-H tumors generate numerous neoantigens and are highly sensitive to ICI therapy regardless of the tumor type and tissue of origin [32]. Mismatch repair deficiency is a very rare event in soft tissue and bone tumors. In a large cohort of almost 6200 patients, MSI-H signature was observed in only 0.3% of the patients, more frequently presenting in uterine endometrial stromal sarcoma, leiomyosarcoma and sarcoma NOS [55]. Other studies refer that sarcomas with myogenic differentiation and undifferentiated sarcomas show the highest percentage of MMR deficiency/MSI among the different soft tissue and bone tumor types [56]. We found one case exhibiting an MSI, that represents nearly 1.5% of our samples. It was a patient with low-grade Kaposi sarcoma through HIV infection. The patient had a history of lymphoma, but he was not known for any other malignancies. To our knowledge, this is the first case of a Kaposi sarcoma showing an MSI. A case of body cavity-based lymphoma associated with Kaposi sarcoma herpesvirus/human herpesvirus type 8 infection, has been documented to display MSI [57]. These data suggest that MSI may be potentially involved in the pathogenesis of HIV-related lesions among which Kaposi sarcomas. Thus, the evaluation of microsatellite status is interesting in these cases, as ICB could be a promising therapy for disseminated disease.

In recent years, immunotherapy has gained ground in the treatment of different types of tumors with good response rates, like melanoma or renal cell carcinoma. This has also been a research field of interest for sarcomas, as many trials have investigated the use of immune checkpoint inhibitors in different sarcoma types. Currently, the success of immune checkpoint inhibitors and immunotherapy, in general, is rather limited in soft tissue and bone malignancies. In our study, we raised a few very important issues that in our opinion have to be taken into account in future research. We pointed out that specific sarcoma types like leiomyosarcomas and myxofibrosarcomas may be good candidates for immunotherapy and this is in line with previous publications, making this finding even stronger. Moreover, we are of the few to investigate CD70 expression in sarcomas and were able to present that myxofibrosarcomas could be considered for further investigation for anti-CD70 treatment. We also make clear that MSI status should be considered in a tumor-agnostic setting. We are the first to present an MSI-H Kaposi sarcoma, offering new therapeutic possibilities. Finally, we showed that bone tumors display a 'cold' phenotype, presumably due to the extracellular matrix. Research on modification of this matrix may transform the immune environment of these tumors.

A limitation of this study is the relatively low number of cases; still, sarcomas are rare entities. Nevertheless, the issues that we raise in this study are important and could be a trigger for further investigation.

Conclusion

In this study of different types of soft tissue and bone sarcomas, immune cells, in general, displayed an exhausted phenotype; still, the driver mechanism is not always known. Differences were found in the expression of immune checkpoints in distinct tumor types. Among the different tumor types, myxofibrosarcomas expressed more PD-L1 tumor cell positivity. This, in correlation with studies that presented good response rates for treatment with PD-1 or PD-L1 inhibitors, makes myxofibrosarcomas realistic candidates for immune checkpoint inhibitor therapy. Moreover, the expression of PD-L1 could be investigated as a possible prognostic biomarker for those tumors. Leiomyosarcomas have a strong infiltrate but no PD-L1 expression. IDO is expressed in the lymphocytes in the peritumoral stroma. While no anti-PD-L1 or anti-PD1 therapy is indicated, IDO inhibition or a combination of IDO inhibitors with ICB could be an option for leiomyosarcomas. Bone sarcomas seem to have a 'cold' inflammation signature, indicating that response to immunotherapy will be poor for this tumor type. Manipulation of tumor matrix and T-cell priming could be explored prior to consideration of ICB therapy. This is also the first description of a case of Kaposi sarcoma that was MSI-high, among the eight cases of Kaposi sarcoma in this series. Kaposi sarcomas require further investigation, as people with disseminated disease may benefit from immune checkpoint inhibitor therapy.

Summary points

- Soft tissue and bone sarcomas display, in general, an exhausted immune cell phenotype; still, the driver mechanism is not always known.
- Myxofibrosarcomas express PD-L1 tumor cell positivity, making these tumors realistic candidates for immune checkpoint inhibitor therapy. Expression of PD-L1 could be investigated as a possible prognostic and predictive biomarker for those tumors.
- Leiomyosarcomas express IDO in the lymphocytes in the peritumoral stroma and have a strong infiltrate but no PD-L1 expression. Thus IDO inhibition or a combination of IDO inhibitors with immune checkpoint blockade could be an option for leiomyosarcomas.
- Bone sarcomas show a 'cold' inflammation signature, thus, response to immunotherapy will be poor for this tumor type.
- Microsatellite instability is a very rare event in soft tissue and bone sarcomas. A case of microsatellite instability-high Kaposi sarcoma was described here.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/imt-2022-0049

Author contributions

Conceptualization: V Siozopoulou, P Pauwels and E Marcq; formal analysis: V Siozopoulou; funding acquisition: P Pauwels and E Smits; investigation: V Siozopoulou, J Liu and E Marcq; methodology: V Siozopoulou and K Zwaenepoel; statistics: A Pouliakis; supervision: E Smits, P Pauwels and E Marcq; visualization: V Siozopoulou; writing the original draft: V Siozopoulou; writing, review and editing: V Siozopoulou, E Smits, K Zwaenepoel, J Liu, A Pouliakis, P Pauwels and E Marcq. All authors read and agreed to the published version of the manuscript.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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