ONCOIMMUNOLOGY
2021, VOL. 10, NO. 1, e1896658 (12 pages)
https://doi.org/10.1080/2162402X.2021.1896658

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ORIGINAL RESEARCH

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Influence of tumor-infiltrating immune cells on local control rate, distant metastasis, and survival in patients with soft tissue sarcoma

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ABSTRACT

Soft tissue sarcomas (STS) are considered non-immunogenic, although distinct entities respond to anti-tumor agents targeting the tumor microenvironment. This study's aims were to investigate relationships between tumor-infiltrating immune cells and patient/tumor-related factors, and assess their prognostic value for local recurrence (LR), distant metastasis (DM), and overall survival (OS).

One-hundred-eighty-eight STS-patients (87 females [46.3%]; median age: 62.5 years) were retrospectively analyzed. Tissue microarrays (in total 1266 cores) were stained with multiplex immunohistochemistry and analyzed with multispectral imaging. Seven cell types were differentiated depending on marker profiles (CD3+, CD3+ CD4+ helper, CD3+ CD8+ cytotoxic, CD3+ CD4+ CD45RO+ helper memory, CD3 + CD8+ CD45RO+ cytotoxic memory T-cells; CD20 + B-cells; CD68+ macrophages). Correlations between phenotype abundance and variables were analyzed. Uni- and multivariate Fine&Gray and Cox-regression models were constructed to investigate prognostic variables. Model calibration was assessed with C-index. IHC-findings were validated with TCGA-SARC gene expression data of genes specific for macrophages, T- and B-cells.

B-cell percentage was lower in patients older than 62.5 years ($p = .013$), whilst macrophage percentage was higher ($p = .002$). High B-cell ($p = .035$) and macrophage levels ($p = .003$) were associated with increased LR-risk in the univariate analysis. In the multivariate setting, high macrophage levels ($p = .014$) were associated with increased LR-risk, irrespective of margins, age, gender or B-cells. Other immune cells were not associated with outcome events.

High macrophage levels were a poor prognostic factor for LR, irrespective of margins, B-cells, gender and age. Thus, anti-tumor, macrophage-targeting agents may be applied more frequently in tumors with enhanced macrophage infiltration.

ARTICLE HISTORY
Received 11 October 2020
Revised 29 January 2021
Accepted 24 February 2021

KEYWORDS
Soft tissue sarcoma; immune cell profile; macrophages

Introduction

Soft tissue sarcomas (STS) account for less than 1% of primary malignancies, yet constitute a heterogenous group of over 50 different histological subtypes.[1] Systemic treatment options – apart from conventional chemotherapy and some tyrosine kinase inhibitors – are still limited, with 20–25% 3-year survival rates in the metastatic setting.[2] Although STS was the first tumor entity successfully treated with an early form of "immunotherapy" in 1891, [3] novel immunotherapeutic agents such as immune checkpoint inhibitors largely failed to achieve encouraging results as observed in different malignant tumors, including melanoma and non-small cell lung cancer.[4,5] Despite the fact that STS are generally considered non-immunogenic, distinct entities, including undifferentiated pleomorphic sarcoma (UPS) and dedifferentiated liposarcoma seem to respond to a certain degree to immunotherapeutic agents.[4] Yet, the underlying mechanisms leading to differences in response rates based on the tumor microenvironment (TME) are largely unknown.[6] Tumor-infiltrating immune cells (TIICs), including tumor-infiltrating lymphocytes (TILs) and monocytes (TIMs), are regarded as a response of the host to tumor-related antigens.[7,8] Expression levels of specific cell lineages have been associated with altered outcomes in various tumor entities.[7,9–12] High levels of CD20 + B-cells, for example, are associated with improved disease-specific survival (DSS) in STS with wide resection margins.[7] Moreover, high CD3 + T-cell and CD8+ cytotoxic-T-cell levels constitute positive prognostic factors for overall survival (OS), disease-free survival, and DSS in STS.[13] Apart from TILs, also TIMs – especially macrophages – have gained attention during the last years due to their dual role in cancer.[12,14] The potential role of macrophages in STS tumorigenesis and progression has already been

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investigated, although with partially contradictory results. In leiomyosarcoma, for example, high levels of macrophages[15,16] have been associated with both poor disease-specific survival[16] and overall survival.[15]

In STS, several prognostic factors for local recurrence (LR), distant metastasis (DM), and OS have been identified in the past, allowing risk-stratification of patients and enabling decision-making toward (neo)-adjuvant radiotherapy and/or chemotherapy.[17–21] However, the role of markers derived from the tumor's immunological profile is still poorly understood.

Therefore, the aims of the present study were to analyze differences in TIICs depending on tumor- and patient-related factors, and to assess whether the amount of TIICs has a prognostic influence on LR- and DM-risk, as well as OS.

Materials and Methods

Any adult patients (≥18 years at the time of surgery), having undergone surgery with curative intent for STS of extremities and trunk between 1998 and 2016 at a single institution were potentially eligible. Subsequently, patients with incomplete demographic, clinical, treatment- or outcome-related information were excluded. Further exclusion criteria were missing informed consent, wrong primary tumor diagnosis that had been adjusted during histopathological reevaluation, and unavailability of representative tumor tissue. Of initially 442 patients treated with STS within the defined time period, 220 had to be excluded, as at least one exclusion criterion applied. Of the remaining 202 patients, 4 treated with neoadjuvant radiotherapy and 10 with neoadjuvant chemotherapy were excluded, as the immune phenotype abundances could have been altered by these preoperative treatments. Thus, 188 patients (87 females [46.3%]; median age: 62.5 years; interquartile range [IQR]: 49.5–75 years) with extremity and trunk STS were finally eligible (Figure 1). Median follow-up was 46.5 months (IQR: 19–99 months).

Clinical Data

Variable patient age was divided into three groups, i.e. 18–35 years (teenagers and young adults), 36–70 years (adults), and >70 years (elderly). Margins were defined according to the *Union International Contre le Cancer* (UICC) guidelines, [22] i.e. R0 being negative margins with at least 1 mm of healthy tissue between tumor and surface, R1 defining marginal margins with less than 1 mm of healthy tissue between tumor and surface, and R2 being microscopically positive margins. For statistical analysis, R1 and R2 margins were grouped together against R0 margins. The maximum diameter of the tumor upon definite surgery was taken as tumor size. Histological subtypes were defined according to the *World Health Organization Classification of Tumors of Soft Tissue and Bone* 2013[23] and divided into six different categories, i.e. synovial sarcoma, myxofibrosarcoma, leiomyosarcoma, malignant peripheral nerve sheath tumor (MPNST), UPS and "others" (i.e. seldom diagnoses). Grading was defined according to the *French Federation of Cancer Centers Sarcoma Group* (*FNCLCC*) into three categories (i.e. G1, G2, and G3). Upon statistical analysis, low-grade tumors (i.e. G1 and G2) were grouped against high-grade (i.e. G3) STS.

Patients underwent regular postoperative follow-up check-ups, with clinical examinations, local magnetic resonance imaging (MRI) scans and computed tomography (CT) scans of the thorax (alternating with chest x-rays) performed three-monthly for the first three postoperative years, then biannually up to the end of the 5th postoperative year, and annually thereafter. Local recurrences were defined as recurrent tumors within the original tumor bed, either diagnosed on local MRI scans or upon re-resection. Distant metastases were defined as

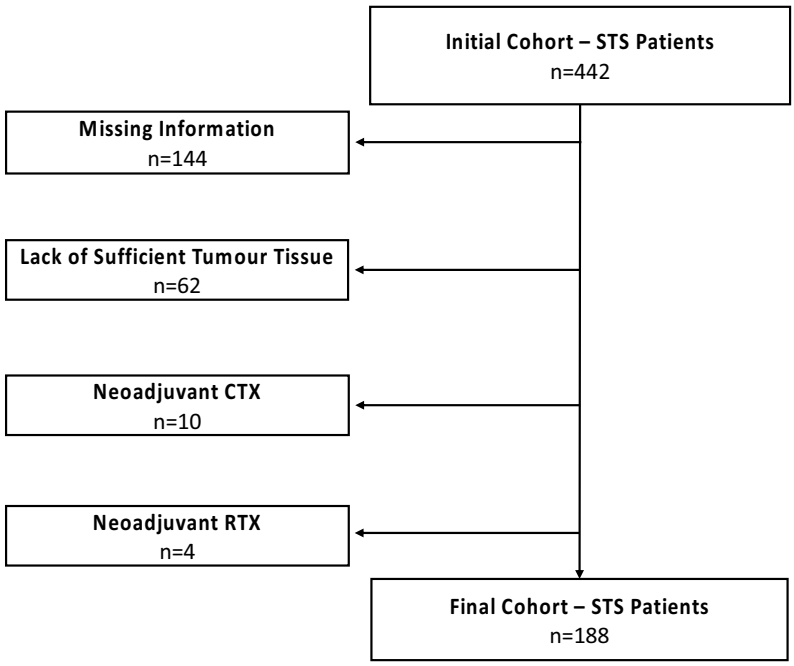

Figure 1. Flow-chart showing patient and sample selection.

disseminated lesions of the original tumor seen on MRI, chest X-rays or CT-scans during follow-up. Time to last follow-up was defined as the time interval from surgery to last follow-up or death. Time to LR and DM were defined as the time interval from surgery to diagnosis of either LR or DM or last follow-up/death. The current study was approved by the local institutional review board (IRB-approval number: 29–205 ex 16/17). All patients included had given their consent to have their tumor tissue analyzed at time being treated for STS.

Construction of Tissue Microarrays

One-hundred eighty-eight paraffin-embedded tumor samples were reevaluated by two specialized soft tissue tumor pathologists (IB and BLA). Representative areas with high tumor-cell abundance were marked on the FFPE specimens. Subsequently, paraffin blocks were punched within the marked areas of interest into seven cores of 4 µm thickness per tumor on average. Per tumor, six cores (± 3 cores) on average were created, resulting in a total of 1266 cores analyzed. The cores were transferred to a recipient paraffin block to construct the TMAs. 3–5 µm sections were cut from each TMA-paraffin block to perform multiplex immunohistochemistry. A simplification of the entire data analysis workflow is visible in Figure 2.

Multiplex Immunohistochemistry

Multiplex immunohistochemistry (MP-IHC) with six antibodies and DAPI was performed, using the following reagents: CD4 (EPR6855, *Abcam plc, Cambridge, UK*), CD8 (C8/144B, *Abcam plc, Cambridge, UK*), CD20 (L26, *Abcam plc, Cambridge, UK*), CD3 (LN10, *Leica Biosystems Inc., Vienna, Austria*), CD45RO (UCHL1, *Cell Signaling Technology Europe, B.V., Leiden, The Netherlands*), CD68 (PG-M1, *Dako Agilent Pathology Solutions, Agilent, Vienna, Austria*). Tyramide-signal-amplification (TSA) kit (*Akoya Biosciences, Marlborough, USA*) technique was used for MP-IHC.

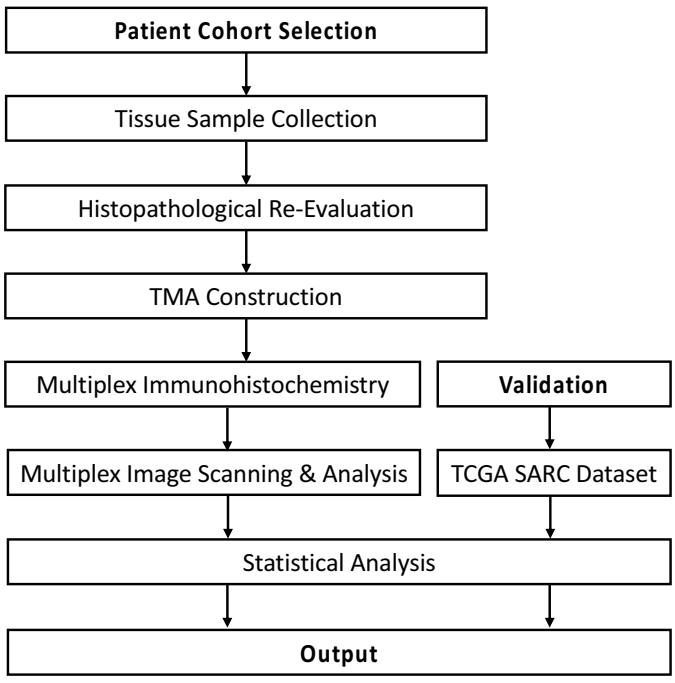

Figure 2. Flow-chart depicting the data analysis workflow.

Staining, Scanning & Image Analysis

Staining of all 5 TMA slides was performed with the autostainer system Bond RX (Leica Biosystems Inc., Vienna, Austria). Slides were then scanned with the Vectra® 3 (Akoya Biosystems, Marlborough, USA; software version 3.0.7) microscope. Whole-slide scans were taken at 4x magnification to locate and select individual TMA cores. Multispectral images of identified TMA cores were then recorded at 20x magnification, resulting into one image color channel for each stained antibody. Images were processed with inForm software (Akoya Biosystems, Marlborough, USA; software version 2.4.8), including spectral unmixing and removal of autofluorescence.

Resulting multispectral images were evaluated using HALO® Image Analysis Platform (Indica Labs, Albuquerque, NM, US; Version 3.1.1076.342). Single recorded images at 20x magnification are stitched together into a continuous field of view of the whole TMA slide. Individual cells were then identified using the DAPI nucleus staining by setting a threshold for nucleus size, roundness and signal intensity. For the six fluorescence-labeled markers, thresholds were set according to staining intensity of the entire cell. Based on the predefined marker combinations, seven different cell phenotypes were differentiated in all TMA cores: T-cells (CD3+), helper T-cells (CD3+, CD4+), cytotoxic T-cells (CD3+, CD8+), helper memory T-cells (CD3+, CD4+, CD45RO+), cytotoxic memory T-cells (CD3+, CD8+, CD45RO+), B-cells (CD20+), and macrophages (CD68+; Figure 3A & 3B). Finally, phenotypes were automatically counted in each TMA core individually and later combined for cores of the same patient. These phenotype abundances, represented as percentages of the total number of cells in each TMA core, constitute the final MP-IHC features used for subsequent analysis.

TCGA-SARC Database

In order to validate the IHC findings, expression data and clinical data for *The Cancer Genome Atlas Sarcoma* (TCGA-SARC) dataset (the *Genomic Data Commons* (GDC) version) was downloaded from the *UCSC Xena browser* (*University of California, Santa Cruz, California, USA*).[24] Gene expression data for primary tumor samples of 259 patients with STS were available. Three cases with incomplete follow-up data were subsequently excluded, resulting in 256 profiles finally analyzed. Gene expression units used were log2(fpkm+1) and treated as continuous variables, as previously suggested.[25,26] *CD19, MS4A1 (Membrane Spanning 4-Domains A1; CD20), CD22* and *CD79A* were used as genes characteristic for B-cells, while *CD68,ITGAM (Integrin Subunit Alpha M; CD11B)* and *CD163* were chosen as genes characteristic for macrophages. *CD3D, CD3E, CD52* and *CD6* were selected as genes characteristic for T-cells. As the TCGA-SARC dataset does not incorporate many clinical parameters such as grading, the statistical analysis focused on differences in gene expression depending on patient age and histological subtype, as well as on their prognostic impact on OS.

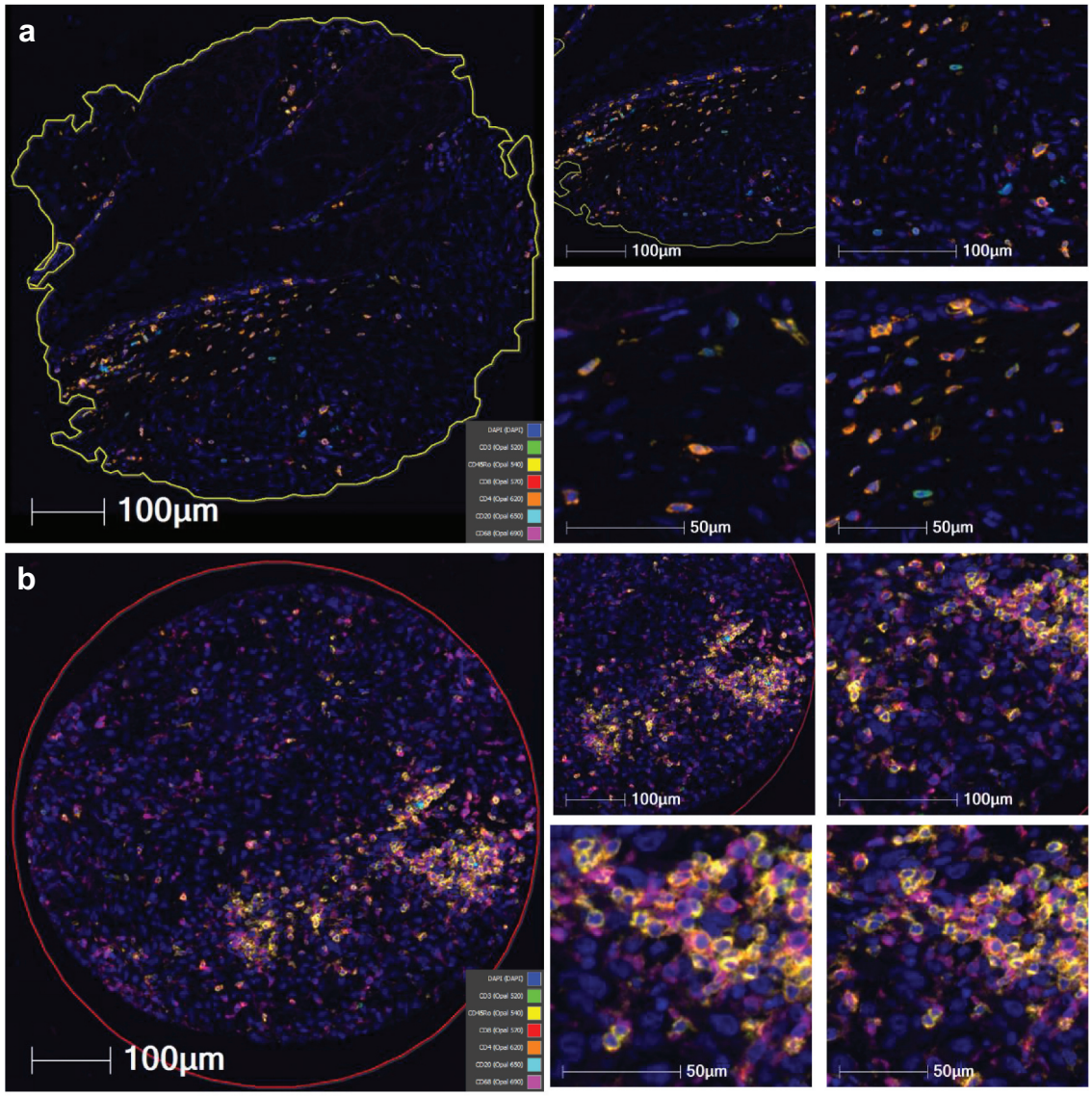

Figure 3A & B. Multispectral image of two undifferentiated pleomorphic sarcomas at different magnifications (clockwise: x12; x20; x40; x60; x80). Note the high positivity for CD68+ in sample depicted in **panel B** in comparison to **panel A.**

Statistical Analysis

Means (with standard deviations [SD]) and medians (with interquartile ranges [IQR]) of clinical data as well as phenotype abundances were provided for normally and non-normally distributed variables, respectively. Normality of continuous variables was assessed with Shapiro–Wilk test. Spearman's rank correlation coefficients were used to assess correlations between different immune cell markers. Wilcoxon rank-sum test and Kruskal Wallis test with post hoc Dunn test (using *Benjamini-Hochberg* adjustment for multiple comparisons[27]) were used to analyze differences in non-normally distributed variables between binominal or categorical clinical variables, respectively. Uni- and multivariate *Fine & Gray* competing risk-regression models were used to assess time-dependent influences of variables on the development of LR and DM, with death as the competing event. The impact of variables on OS was estimated with uni- and multivariate Cox regression analyses. Apart from demographic variables patient age and gender, factors with a p-value of ≤0.1 in the univariate analyses were included in the multivariate models. Immune cell phenotypes were both analyzed as continuous and categorical variables in the univariate time-to-event analyses. For multivariate time-to-event models, the continuous variable pattern was used. The median of each immune cell phenotype was used as the cutoff value defining "low" and "high" phenotype abundance. Harrell's C-index was calculated for each multivariate model to assess its goodness of fit. A C-index close to 1 would indicate perfect calibration, whilst a C-index of 0.5 indicates no predictive discrimination between observed and predicted outcomes.[28] For all analyses, a p-value of <0.05 was considered statistically significant.

Results

The most common histological subtypes were myxofibrosarcomas (n = 76; 40.4%), followed by UPS (n = 32; 17.0%). The majority of STS were G3 (n = 129; 72.9%), whilst G1 (n = 17; 9.6%) and G2 STS (n = 31; 17.5%) amounted to less than a quarter of cases. Further tumor- and treatment-related

variables are detailed in Table 1. CD68+ macrophages and CD3 + T-cells constituted the most frequent cell population, with a median percentage of 2.93 (IQR: 0.85–9.30) and 2.65 (IQR: 1.02–6.70), respectively, over all histological subtypes (Table 1; Figure 4).

Correlation between immune cell markers

According to the Spearman's correlation analysis, all immune cell phenotype abundances were positively associated with each other, except for CD68+ macrophages and CD20 + B-cells, as well as CD68+ macrophages and CD3 + T-cells (Table 2).

Table 1. General patient features.

		Count
Age (in years, median + IQR)		62.5 (49.5–75.0)
Gender	*Male*	101 (53.7%)
	Female	87 (46.3%)
Tumor Size (in cm, mean + SD)		8.5 ± 5.3
Location	*Upper Limb*	47 (25.1%)
	Lower Limb	123 (65.8%)
	Trunk	17 (9.1%)
Depth	*Superficial*	56 (29.8%)
	Deep	105 (55.8%)
	Superficial + Deep	27 (14.4%)
Grade	*G1*	17 (9.6%)
	G2	31 (17.5%)
	G3	129 (72.9%)
Margins	*R0*	143 (76.1%)
	R1	41 (21.8%)
	R2	4 (2.1%)
Histology	Myxofibrosarcoma	76 (40.4%)
	Synovial Sarcoma	12 (6.4%)
	UPS	32 (17.0%)
	Liposarcoma	22 (11.7%)
	Other	25 (13.3%)
	Leiomyosarcoma	21 (11.2%)
Follow-Up (in months; median + IQR)		46.5 (19–99)
Status	*Alive*	120 (63.8%)
	Died	68 (36.2%)
TIIC (in percent; median + IQR)*	*CD3 + T-cells*	2.65 (1.02–6.70)
	CD3+ CD4+ Helper T-cells	0.25 (0.06–0.89)
	CD3+ CD8+ Cytotoxic T-cells	0.34 (0.08–1.71)
	CD3+, CD4+, CD45RO+ Helper memory T-cells	0.06 (0.00–0.28)
	CD3+ CD8+ CD45RO+ Cytotoxic memory T-cells	0.05 (0.00–0.30)
	CD20 + B-cells	0.01 (0.00–0.07)
	CD68+ Macrophages	2.93 (0.85–9.30)

Legend: *IQR – interquartile range; SD – standard deviation; TIIC – tumor-infiltrating immune cells*
**Overall absolute percentages per cell line*

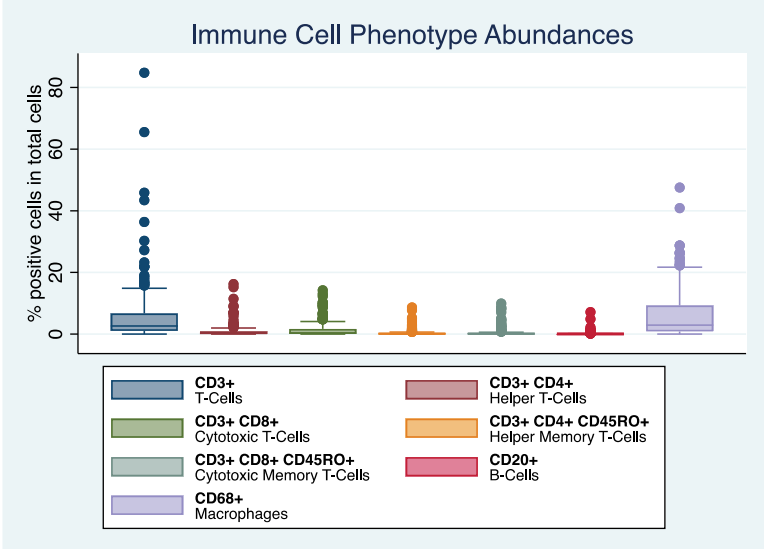

Figure 4. Overall phenotype abundance.

Table 2. Correlation of immune cell phenotype abundances. Spearman's rho indicates strong positive correlation between different TIICs.

	T-Cells	Helper T-Cells	Cytotoxic T-cells	Helper memory T-cells	Cytotoxic memory T-cells	B-cells
Helper T-Cells	0.645*					
Cytotoxic T-cells	0.668*	0.746*				
Helper memory T-cells	0.371*	0.768*	0.526*			
Cytotoxic memory T-cells	0.505*	0.736*	0.704*	0.842*		
B-cells	0.316*	0.438*	0.308*	0.561*	0.483*	
Macrophages	0.198**	0.545*	0.449*	0.598*	0.569*	0.202***

Legend: Spearman's rho given; *p-value < 0.0001; ** *p*-value = 0.140; ***p-value = 0.118

Difference in immuno-profile between patient subpopulations

Clinico-pathological associations with immune cell phenotype abundances and well-known prognostic factors resulted in the following findings: Whilst the percentage of CD20 + B-cells was significantly lower in patients older than 62.5 years of age (0.00% [IQR: 0.00–0.03%] vs. 0.02% [0.00–0.08%]; p = .013), the percentage of CD68+ macrophages was significantly higher (5.16% [IQR: 1.77–11.8%] vs. 1.9% [IQR: 0.64–4.83%]; p = .002). Other cell types did not significantly differ depending on median patient age. Furthermore, the percentage of CD3 + T-cells was significantly lower in tumors larger than the mean size of 8.5 cm in comparison to those smaller than 8.5 cm (1.74% [IQR: 1.04–5.61%] vs. 3.62% [1.05–8.65%]; p = .029), whilst the other immune cells did not differ depending on tumor size. In comparison to low-grade tumors, high-grade STS had significantly higher levels of CD3+ CD4 + T-helper cells (0.33% [IQR: 0.11–1.00%] vs. 0.14% [IQR: 0.03–0.48%]; p = .021), CD3+ CD4+ CD45RO+ helper memory T-cells (0.11% [IQR: 0.01–0.40%] vs. 0.04% [IQR: 0.00–0.18%]; p = .036), CD3+ CD8+ cytotoxic T-cells (0.46% [IQR: 0.12–1.72%] vs. 0.18% [0.03–1.87%]; p = .050), and CD3 + CD8+ CD45RO+ cytotoxic memory T-cells (0.08% [IQR: 0.01–0.39%] vs. 0.01% [IQR: 0.00–0.15%]; p = .002). Marginally significant differences were found for CD68 + macrophages (low-grade vs. high-grade tumors: 1.94% [IQR: 0.65–5.16%] vs. 3.56% [IQR: 0.98–10.54%]; p = .054) and CD3 + T-cells (low-grade vs. high-grade tumors: 1.87% [0.62–5.15%]; p = .063). No significant difference was found for CD20 + B-cells (p = .666). Furthermore, there were significant overall differences between histological subtypes and CD3 + T-cells (p = .019), CD3+ CD4 + T-helper cells (p < .001), CD3+ CD8+ cytotoxic T-cells (p < .001), CD3 + CD4+ CD45RO+ helper memory T-cells (p < .001), CD3 + CD8+ CD45RO+ cytotoxic memory T-cells (p < .001), CD20 + B-cells (p = .031), and CD68+ macrophages (p < .001; Figure 5). Gender, tumor depth and location revealed no statistically significant difference with regards to immune cell count (all p > .05).

Prognostic impact of phenotype abundances and clinical variables

Besides R1/2 margins (p = .037), higher amounts of CD68 + macrophages (p = .003) and CD20 + B-cells (p = .035) were significantly associated with increased risk of LR (Figure 6). Notably, no such association was found when using cutoff values for "high" (above median) and "low" (below median) abundance of CD68+ macrophages (p = .273) and CD20 + B-cells (p = .883; Supplementary Table 1). Other immune cells, neither as continuous or categorical variables, showed no significant association (Supplementary Table 1).

In the multivariate model, the positive association of CD68 + macrophages (p = .014), independent from R1/2 margins, CD20 + B-cells, gender, and patient age (all p > .05) remained significant (Table 3). C-index of 0.727 indicated a good model calibration.

Large tumor size (p = .006) and UPS (p = .028) were significantly associated with a higher DM-risk in the univariate setting, whilst other demographic and tumor related-variables, as well as phenotype abundances (both as continuous and categorical variables), showed no statistically significant association (all p > .05; Supplementary Table 1). In the multivariate model, large tumor size (p = .002) and UPS (p = .009) remained significantly associated with higher risk of DM, independent of grading, patient age, or gender (all p > .05; Table 4). Model calibration was good, with a C-index of 0.696.

CD3+ CD8+ CD45R0+ cytotoxic memory T-cell phenotype abundance above the median was associated with worse OS in the univariate analysis (p = .035; Supplementary Table 1). None of the other immune cell phenotype abundances or demographic variables showed a significant association with OS in the univariate analysis (Supplementary Table 1). In the multivariate model, age over 70 years at time of surgery (p = .046), large tumor size (p = .006) as well as synovial sarcoma (p = .012), and histological subtypes labeled as "other" (p = .034) were independently associated with worse OS, irrespective of gender or grading (all p > .05; Table 5). C-index of 0.728 indicated a good model calibration.

CD68+ macrophages and CD20 + B-cells associated with higher LR-risk in myxofibrosarcoma

Subgroup analysis of the multivariate model for LR was performed for myxofibrosarcoma, being the most frequent histological subtype in the present study and one with high immune cell abundance. In this model, the negative association between high CD68+ macrophages and LR-risk prevailed (p = .012), irrespective of gender, age, margins, or CD20 + B-cells (Table 3). Of note, in myxofibrosarcoma, high CD20 + B-cell levels were also associated with increased LR-risk (p = .045; Table 3). C-index for this model was 0.642.

Validation of IHC with TCGA-SARC database

In the TCGA-SARC dataset, patients older than 62.5 years had significantly higher levels of macrophage-characteristic genes *CD68* (0.93 [IQR: 0.49–1.56] vs. 0.60 [IQR: 0.24–1.33], p = .003),*ITGAM* (2.26 [IQR: 1.47–2.98] vs. 1.66 [IQR: 0.97–2.89], p = .02), and *CD163* (4.35 [IQR: 3.40–5.64] vs. 3.76 [IQR:

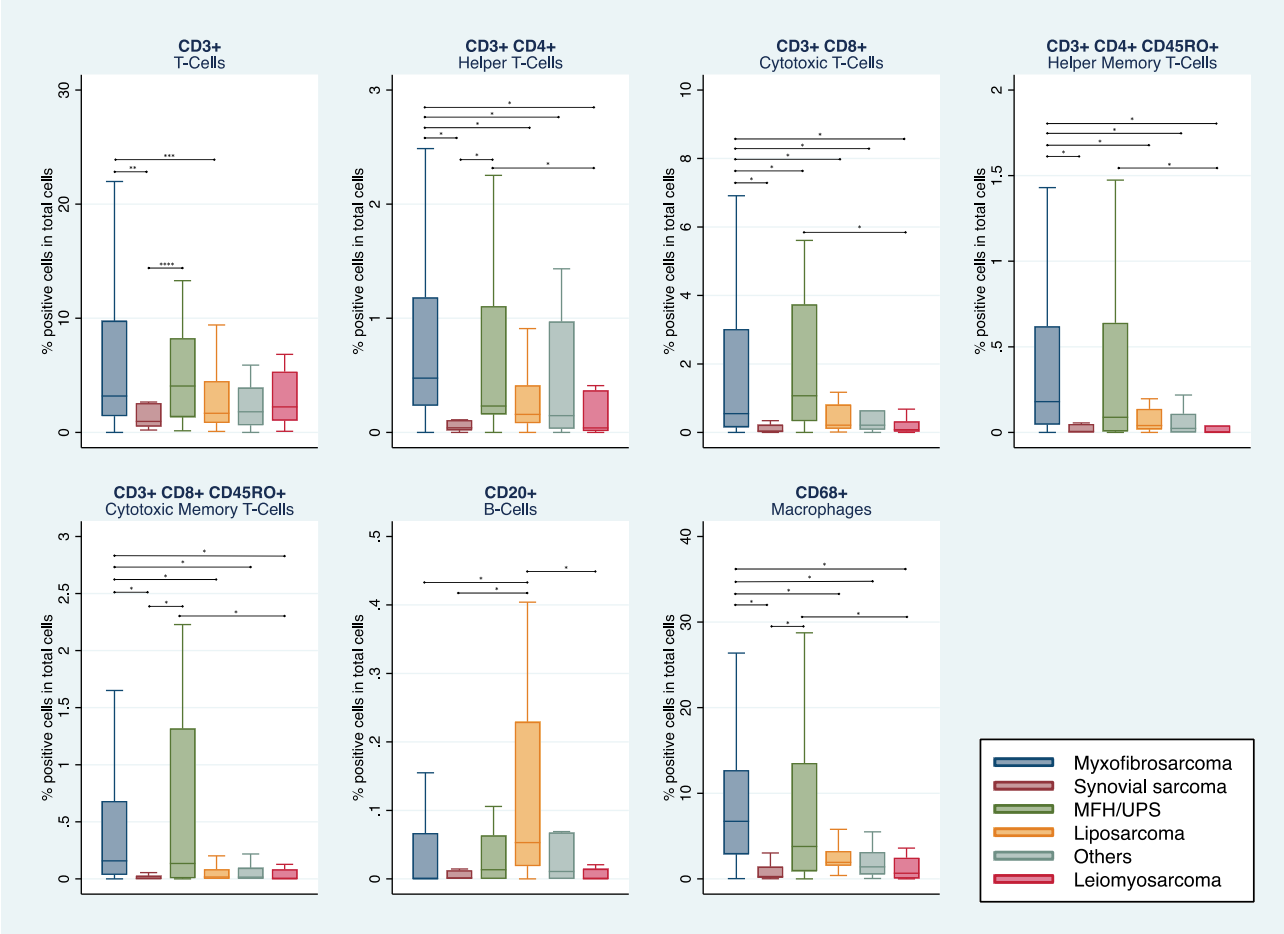

Figure 5. Difference in immune cells depending on histological subtypes. Significant differences outlined with lines and asterisks (*p < .05; **p = .054; ***p = .068; ****p = .062). Note the different scales in each plot.

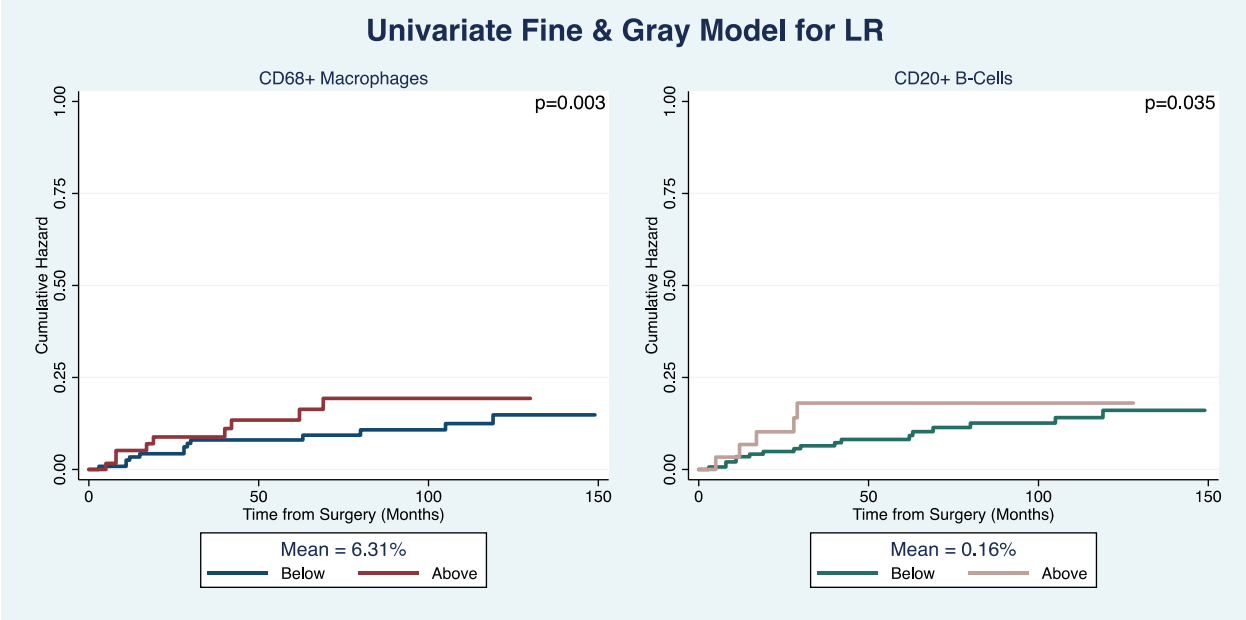

Figure 6. Univariate fine & gray model for local recurrence with death as competing event. High amounts of CD68+ macrophages (left panel) and CD20 + B-cells (right panel; separated at mean value for graphical display) are associated with increased risk of LR.

Table 3. Multivariate competing risk regression model for local recurrence, for all histological subtypes (top) and for myxofibrosarcomas only (bottom), with death as competing event.

Local Recurrence		Multivariate Model – All Histological Subtypes			
			95%CI		
		HR	Lower	Upper	p-value
Gender	*Male*	1			0.154
	Female	0.501	0.193	1.297	
Age at Surgery	*18 – 35 years*	1			
	36 – 70 years	1.035	0.139	7.690	0.973
	> 70 years	1.537	0.195	12.103	0.683
Margins	*R0*	1			0.051
	R1/2	2.436	0.995	5.963	
CD20+ B-cells		1.344	0.762	2.371	0.307
CD68+ Macrophages		1.054	1.010	1.099	**0.014**
		Subgroup Analysis – Myxofibrosarcoma			
Gender	*Male*	1			**0.045**
	Female	0.181	0.034	0.959	
Age at Surgery	*18 – 35 years*	N/A	N/A	N/A	N/A
	36 – 70 years	1			0.548
	> 70 years	1.663	0.316	8.749	
Margins	*R0*	1			0.831
	R1/2	0.740	0.047	11.766	
CD20+ B-cells		1.951	1.014	3.755	0.045
CD68+ Macrophages		1.073	1.016	1.134	0.012

Table 4. Multivariate competing risk regression model for distant metastasis, with death as competing event.

Distant Metastasis		Multivariate Model			
			95%CI		
		HR	Lower	Upper	p-value
Gender	*Male*	1			0.375
	Female	1.291	0.734	2.272	
Age at Surgery	*18 – 35 years*	1			
	36 – 70 years	1.074	0.264	4.368	0.921
	> 70 years	0.860	0.206	3.594	0.836
Grading	*G1/2*	1			
	G3	1.188	0.654	2.159	0.571
Tumour size (in cm)		1.070	1.026	1.116	**0.002**
Histology	*Myxofibrosarcoma*	1			
	Synovial sarcoma	1.662	0.510	5.421	0.399
	UPS	2.866	1.303	6.300	**0.009**
	Liposarcoma	0.388	0.125	1.205	0.101
	Others	1.204	0.501	2.896	0.678
	Leiomyosarcoma	1.961	0.947	4.060	0.070

Table 5. Multivariate cox regression analysis for overall survival.

Overall Survival		Multivariate Model			
			95%CI		
		HR	Lower	Upper	p-value
Gender	*Male*	1			0.794
	Female	0.935	0.563	1.551	
Age at Surgery	*18–35 years*	1			
	36–70 years	1.706	0.469	6.203	0.417
	> 70 years	3.888	1.026	14.740	**0.046**
Grading	*G1/2*	1			0.595
	G3	1.190	0.626	2.261	
Tumor size (in cm)		1.063	1.018	1.111	**0.006**
Histology	*Myxofibrosarcoma*	1			
	Synovial sarcoma	3.512	1.314	9.383	**0.012**
	UPS	1.856	0.933	3.691	0.078
	Liposarcoma	0.467	0.146	1.492	0.199
	Others	2.356	1.069	5.195	**0.034**
	Leiomyosarcoma	1.427	0.638	3.192	0.386

2.13–5.05], p = .002) in comparison to patients younger than 62.5 years, being in line with the IHC findings. Furthermore, levels of B-cell-characteristic gene *CD22* were significantly lower in patients older than the median of 62.5 years (0.18 [IQR: 0.09–0.37] vs. younger than 62.5 years: 0.25 [IQR: 0.10–0.66], p = .030). Abundance of all genes analyzed significantly differed depending on histological subtype (p < .05), with B-cell characteristic genes *CD19, MS4A1* and *CD79A* being present at higher levels in liposarcoma, and T-cell and macrophage-characteristic genes at high levels in myxofibrosarcoma and MFH/UPS compared to other histological subtypes (Figure 7).

For B-cell characteristic genes, univariate Cox-regression analysis revealed no significant impact of *CD19* (p = .122), *MS4A1* (p = .144), *CD22* (p = .907), or *CD79A* (p = .088) expression on OS. Likewise, expression of macrophage-specific genes *CD68* (p = .940),*ITGAM* (p = .315), and *CD163* (p = .248) was not significantly associated with altered OS. Contradictory to the IHC findings, however, high expression levels of T-cell characteristic genes *CD3D* (HR: 0.848; 95%CI: 0.736–0.976; p = .022), *CD3E* (HR: 0.845; 95%CI: 0.724–0.987; p = .034), and *CD52* (HR: 0.835; 95%CI: 0.734–0.951; p = .007) were significantly associated with a better OS, whilst no difference was found for *CD6* (p = .105).

Discussion

In this study, we described a comprehensive picture of immune cell infiltration to sarcoma and its clinical impact on important clinical endpoints. As a result, we observed significant differences in the abundance of tumor-infiltrating immune cells between soft tissue sarcoma subtypes, with overall higher amounts seen in myxofibrosarcoma and UPS. Furthermore, the tumors' immune profile changed with patients' age, with lower amounts of CD20 + B-cells and higher amounts of CD68 + macrophages present in older individuals. These findings were confirmed by TCGA-SARC gene expression analysis. Moreover, higher amounts of tumor-infiltrating immune cells were observed in high-grade tumors. Both high amounts of CD68+ macrophages and CD20 + B-cells correlated with increased risk of LR. The only immune cell type independently associated with patients' prognosis was CD68+ macrophages, with higher percentages correlating with increased risk of LR, independent of margin status, patient age, gender or CD20 + B-cells. Furthermore, in the subgroup analysis for myxofibrosarcoma, high CD68+ macrophage- and CD20 + B-cell-levels were independently associated with higher LR-risk, irrespective of margins, gender, or age.

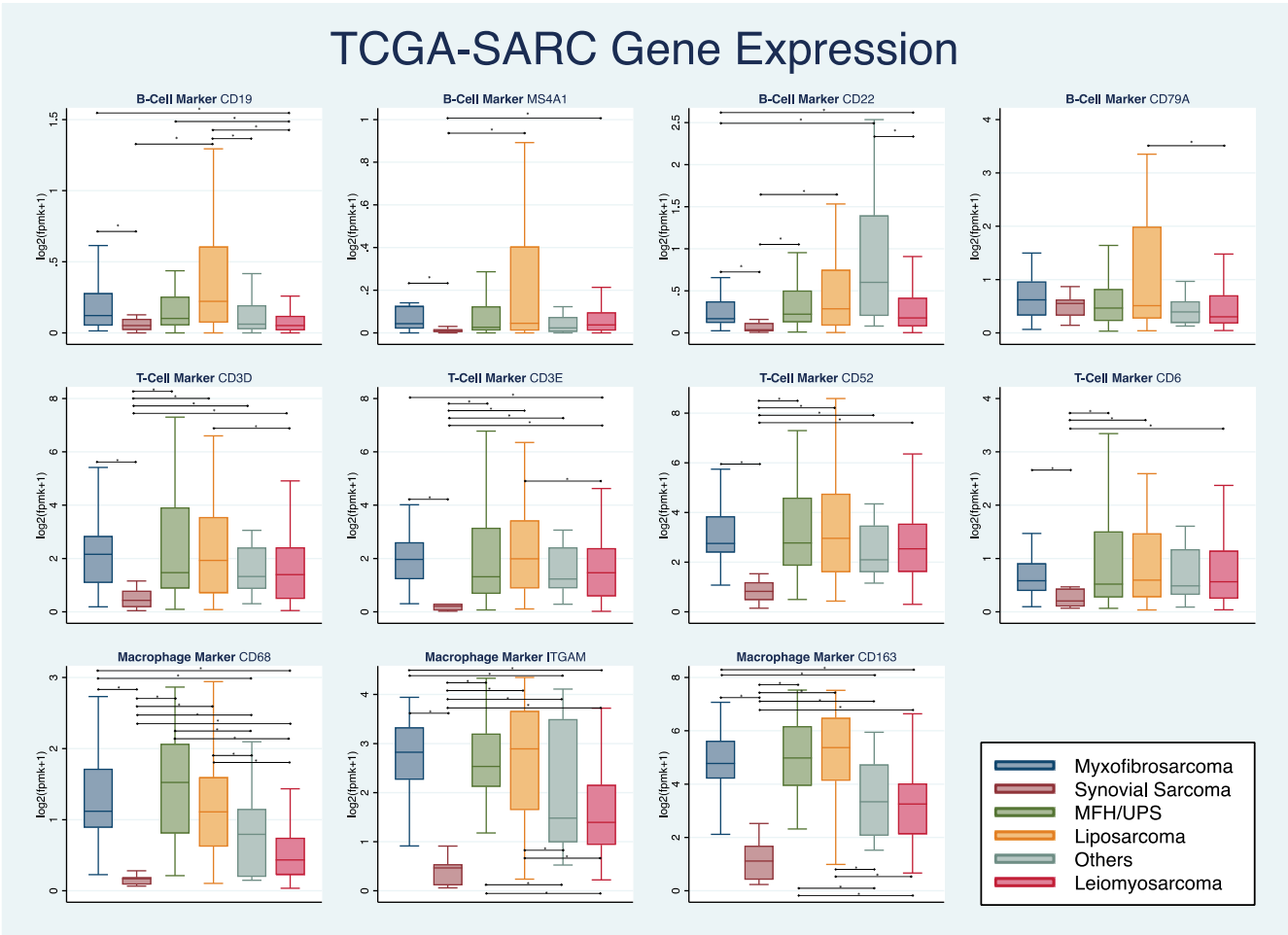

Figure 7. Difference in gene expression levels depending on histological subtypes based on the TCGA-SARC dataset. Significant differences outlined with lines and asterisks (*p < .05). Gene expression is presented as normalized data. Note the different scales in each plot.

As reported by others, [6,29] we observed a significant positive correlation between all immune cell phenotype abundances, except for CD20 + B-cells and CD68+ macrophages, and CD3 + T-cells and CD68+ macrophages. However, in contrast to previous reports, [7] we neither observed a statistically significant positive prognostic impact of increased CD20 + B-cell concentration nor a negative prognostic influence of elevated CD3 + T-cell concentration on patient overall survival. The lack of prognostic impact could be related to the different analysis of the immune cell density in our cohort, taking the percentages of phenotype abundance rather than defining a cutoff value for low and high values of the respective tumor-infiltrating cells.[7] However, when using cutoff values to define high and low immune cell phenotype abundance, we neither were able to demonstrate additional significant associations. Furthermore, the overall percentage of tumor-infiltrating immune cells apart from CD3 + T-cells and CD68+ macrophages was rather low in our study, again highlighting the per tendency low immunogenicity of STS.[4] However, contrary to the study by *Dancsok et al.*, [30] we discovered a significant positive association between advanced tumor grade and high levels of tumor-infiltrating immune cells, with 6 out of 7 phenotypes showing marginal or statistically significant differences between G1/G2 and G3 STS. Notably, the amount of CD68+ macrophages was overall higher than the one of TILs, an observation already made by others.[6,31] Considering that TAMs usually harbor surface molecules and properties inducing angiogenesis, tumor growth and metastasis formation whilst suppressing immune-related responses, they constitute potential anti-tumor targets. However, monocytes and macrophages are rather insensitive to conventional DNA-damaging chemotherapeutics, as they are considered quiescent.[32,33] Notably, specific chemotherapeutic agents, as trabectedin, do not only affect the tumor itself by interfering with late S and G2 phases of the cell cycle, [34,35] but also alter the TME, especially monocytes and macrophages.[32] By triggering the activation of caspase, a key effector molecule of the extrinsic apoptotic pathway in monocytes, apoptosis of macrophages is induced by trabectedin.[14,36] Correspondingly, *Germano et al.* discovered that not only the density of CD68+ macrophages is reduced by 50% in tumors of mice treated with trabectedin but also the production of chemokine (C-C motif) ligand 2 (CCL2) as parameter of monocyte-recruitment, as well as density of micro-vessels.[37] In this respect, it seems reasonable that immune checkpoint inhibitors have rather low efficacy in STS, whilst agents targeting macrophages, like trabectedin, can achieve promising anti-tumor responses.

Furthermore, we found a correlation between elevated CD68+ macrophage abundance and advanced patient age over all histological subtypes. Corresponding to previous observations that STS with complex karyotypes are more likely to promote phagocytic inflammation than translocation-associated STS, [31,38] highest amounts of CD68+ macrophages were seen in UPS and myxofibrosarcoma, whilst very low levels were present in synovial sarcoma. These findings were also confirmed at the genomic level using the TCGA-SARC dataset.

In our study, high levels of CD68+ macrophages were independently associated with an increased risk of LR, irrespective of margin status, patient age, gender, or CD20 + B-cell count. Moreover, in the subgroup analysis for myxofibrosarcoma, not only high CD68+ macrophage levels but also high CD20 + B-cell levels were associated with higher LR-risk. Contrary to our findings, a protective effect of high CD20 + B-cell levels in STS regarding disease-specific survival, [39] metastasis-free survival, [6] and overall survival[6] has been reported. However, none of these studies investigated the potential association of CD20 + B-cells and LR-risk. Also, the effect observed in the present study was only present in the subgroup analysis for myxofibrosarcoma. Considering that the prognostic role of tumor-infiltrating CD20 + B-cells is still controversially discussed, with both positive and negative effects reported for different tumors, [40] our observations warrant further investigations. As to the authors knowledge, this is the first study reporting a potential association between macrophage levels and local recurrence in STS. Similar results have been only reported for cholangiocarcinoma[41] and colorectal cancer, [42] with high CD68+ macrophages being associated with early LR.

Considering that STS with complex karyotypes appear to more likely present high amounts of tumor-infiltrating CD68 + macrophages, anti-tumor agents targeting macrophages (e.g. trabectedin) may be applied in these tumors as an adjuvant treatment to improve local control. Yet, to better understand the role of macrophages in STS, further in-depth research, focusing on patterns of immune cell infiltration, mechanistic pathways, confirmation of the generated hypothesis on an independent dataset, and correlation of findings with additional methods as flow-cytometry and sequencing, is warranted.

The current study has several limitations that should be considered when interpreting the results. First, the combination of immunohistochemical markers used to identify the immune cell phenotypes may not identify all relevant cells. However, since standardized, uniformly available assays for immunophenotyping are still pending, [43,44] this study focused on established markers of B-cells, T-cells and macrophages with a multiplex IHC staining platform. Second, we used CD68 as a general marker for macrophages staining for both M1- and M2-like subtypes, [31,45,46] rather than further differentiating between these subtypes by applying additional markers. However, taking into consideration that either phenotype of tumor-associated macrophages is susceptible to DNA-binding anti-tumor agents (as e.g. trabectedin) approved for specific STS subtypes, [37] the additional value of differentiating into M1- or M2-like macrophages remains to be questioned. Third, the overall abundance of tumor-infiltrating immune cells was generally low, an issue related to the relatively poor immunogenicity of STS in general. Nonetheless, by using computer-assisted scoring for simultaneously stained markers, a standardized and un-biased evaluation is possible. Fourth, the limited sample size – particularly regarding different histological subtypes – may have led to model overfitting. On the other hand, C-indices for competing risk regression and Cox-regression models were all near to or above 0.70, indicating good model calibration. Yet, C-index for the multivariate model performed on myxofibrosarcomas only was already lower at 0.642, indicating poorer model calibration. Thus, results have to be seen as preliminary, warranting validation on independent sample sets. Fifth, owing to the methodological approach chosen, we

did not differentiate between central or peripheral immune cell infiltrates, high and/or low-level patterns but rather focused on the TME holistically. Furthermore, due to the limited clinical information available in the TCGA-SARC database, we could only validate IHC findings with gene expression data on selected clinical characteristics and OS as the primary endpoint. Yet, gene expression data largely confirmed results obtained with IHC, particularly regarding demographic variable age and histological subtypes, although it should be noted that correlation between mRNA expression- and protein expression-levels is generally weak, owing to regulatory processes taking place between protein transcription and formation.[47–50] Thus, the current preliminary results generated a hypothesis that may provide a basis for future research on the role of TAMs in STS.

To the author's knowledge, this is the first study identifying high amounts of tumor-infiltrating CD68+ macrophages as a poor prognostic factor for local recurrence, irrespective of margin status, CD20 + B-cell infiltration, or patient age. Based on our observations, it can be hypothesized that anti-tumor agents targeting macrophages may be applied more frequently in patients with tumors showing high levels of CD68+ macrophages. However, to better understand the role of macrophages in STS, further in-depth research is warranted.

Acknowledgement

The current study was supported by the K1 COMET Competence Center CBmed (Center for Biomarker Research in Medicine), which is funded by the Federal Ministry of Transport, Innovation and Technology (BMVIT), Land Steiermark (Department 12, Business and Innovation), the Federal Ministry of Science, Research and Economy (BMWFW), the Styrian Business Promotion Agency (SFG), and the Vienna Business Agency. The COMET program is executed by the Austrian Research Promotion Agency (FFG). Co-author L.H. was supported by CBmed via the PhD program Advanced Medical Biomarker Research (AMBRA). Furthermore, co-author J.F. was supported by the Austrian Science Fund (FWF).

Disclosure statement

No potential conflict of interest was reported by the authors.

Disclosures of Interest

The authors report no conflicts of interest.

Funding

The current study was funded by PharmaMar (Madrid, Spain) under GrantPM-ID 8048. The funding source had no impact on the design of the study, its conduction, analysis, presentation of results or interpretation.

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