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Title:
Characterising the immune microenvironment in liposarcoma, its impact on prognosis and the impact of radiotherapy

Date:
2021-01-01

Citation:
Snow, H., Mitchell, C., Hendry, S., McKinley, M., Byrne, D., Ngan, S., Chander, S., Chu, J., Desai, J., Bae, S., Henderson, M., Choong, P. & Gyorki, D. (2021). Characterising the immune microenvironment in liposarcoma, its impact on prognosis and the impact of radiotherapy. Journal of Surgical Oncology, 123 (1), pp.117-126. https://doi.org/10.1002/jso.26261.

Persistent Link:
https://hdl.handle.net/11343/276458

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Title Page

Title:

Characterising the immune microenvironment in liposarcoma, its impact on prognosis and the impact of radiotherapy

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Short Title:

Immune microenvironment in liposarcoma

Synopsis:

This study examines the impact of radiotherapy on the immune microenvironment in liposarcoma by comparing the immune infiltrate in 39 paired tissue samples pre and post radiotherapy. By scoring these liposarcoma specimens using established immune-infiltrate scoring systems described for other cancer types, it also attempts to establish the optimal immune-infiltrate scoring system for liposarcoma.

Keywords:

Liposarcoma

Lymphocytes, Tumour-Infiltrating

Tumor Microenvironment

Financial Support:

Australia and New Zealand Sarcoma Association, “Hannah’s Chance Foundation, Sarcoma Research Grant”, 2017. Grant ID: 35, $AUD 30,000.

Abstract

Background and Objectives:

Limited literature exists examining the immune microenvironment in liposarcoma, particularly with regard to the impact of radiotherapy. A major problem is the lack of scoring system for the tumour-infiltrating lymphocytes (TILs) in sarcoma. This study aims to describe the immune environment pre and post-radiotherapy and identify the optimal immune infiltrate scoring system for sarcoma.

Methods:

39 paired tissue samples (pre and post-radiotherapy) from patients with liposarcoma were scored by two pathologists for TILs using pre-existing systems (for breast cancer and melanoma) and compared for interobserver reliability. Immunohistochemical staining was performed for various immune markers.

Results:

The TIL scoring system for breast cancer yielded perfect agreement (κ =1.000). 21% of patients had increased TILs after radiotherapy, 87.5% of whom had dedifferentiated liposarcoma. Immune suppressor expression was increased frequently after radiotherapy (CD68 increased in 59.4%, PD-L1 increased in 25%). Immune effector expression (CD8) was unchanged in 84.4%.

Conclusions:

Breast cancer TIL scoring is reproducible in liposarcoma and has high interobserver reliability. Radiotherapy was observed to have limited impact on immune effectors but seemed to have more impact in upregulating immune suppressors, suggesting radiotherapy may contribute to disease control through immunomodulatory effects. Dedifferentiated liposarcoma represents a uniquely responsive subtype.

Introduction

The tumour immune microenvironment is being increasingly recognised as a key factor in cancer development and progression. The presence of tumour-infiltrating lymphocytes (TILs), tumour-associated macrophages (TAMs) and other cell types reflect immune engagement [1, 2]. However, this interaction between tumour and immune system is complex and only partially understood. It is generally considered that an inflammatory environment reflects immune recognition, leading to improved outcomes, and an absence of an immune infiltration reflects a lack of immune engagement associated with poor outcomes. In reality, there is no consensus as to exactly which immune cells in the tumour stroma are most strongly correlated with outcome and vast heterogeneity exists between tumour types. Broadly, tumours with a higher mutation burden are more likely to express foreign epitopes and therefore to elicit an immune response. In most sarcomas, the mutational burden is very low[3], with some exceptions, for example cutaneous angiosarcoma[4, 5]. However, the immune response and its relationship to mutational burden in sarcoma is less well described. The mainstay of treatment for patients with primary liposarcoma is surgery with or without radiotherapy, with responses to systemic therapy, particularly for well-differentiated/de-differentiated liposarcoma (WD/DDLPS), generally poor[6]. As such, improved understanding of the immune microenvironment in liposarcoma may create new avenues for more effective treatments. Despite some studies into the immune environment in sarcoma[7-11], knowledge remains limited. Furthermore, with radiotherapy known to reduce recurrence rates in patients with soft tissue sarcoma[12, 13], it is of interest to understand the effect of radiotherapy on the immune microenvironment, and whether this may effect patient outcomes.

One of the major problems in attempting to characterise the immune microenvironment in sarcoma is the lack of accepted reporting methods and scoring systems to categorise the immune infiltrate. Different classifications exist for breast[14], melanoma[15], colorectal cancer[16] and others designed for all cancer types[17]; none of these have been evaluated in sarcoma.

The primary aim of this study was to characterise the immune microenvironment in liposarcoma and to analyse for changes in the immune signature before and after radiotherapy. It also aimed to explore any prognostic implication of the radiotherapy -induced immune changes. By comparing two established TILs grading methods, it aims to identify the optimal TILs scoring system for sarcoma.

Materials and Methods

Patients and Setting

A prospectively collected, ethics-approved sarcoma database (including patients from 2008-2016) was searched for appropriate patients. Retrospective analysis was then performed using paired tissue samples from patients with liposarcoma undergoing surgery with curative intent after neoadjuvant radiotherapy. Archival pre-radiotherapy biopsy specimens and post-radiotherapy surgical resection specimens were retrieved from two pathology departments servicing a tertiary referral sarcoma centre. Given that sarcoma consists of many subtypes, liposarcoma, one of the most common subtypes, was chosen for increased homogeneity. Liposarcoma subtypes included were well-differentiated liposarcoma/atypical lipomatous tumour (WDLPS/ALT), de-differentiated liposarcoma (DDLPS), myxoid liposarcoma and pleomorphic liposarcoma.

Clinicopathological data was collected from medical files including radiotherapy data. Recurrence was defined as either local or distant. This study was approved by the institutional ethics committee and was performed in accordance with the NHMRC National Statement on Ethical Conduct in Human Research 2007 (and updates) and the World Medical Association Declaration of Helsinki 2013.

Surgery and radiotherapy

Radiotherapy was administered using the external beam technique. Doses ranged from 45.0 to 50.4Gy divided in 25-28 fractions. In all cases, surgery was performed between 6-8 weeks from the completion of radiotherapy by surgeons experienced in treating soft tissue sarcomas and achieved an R0/R1 resection in all cases. No patient received adjuvant chemotherapy.

Specimen preparation and immunohistochemistry

Formalin-fixed, paraffin-embedded tissue blocks from the paired specimens were retrieved from hospital archives. In addition to the existing haematoxylin and eosin (H&E)-stained slides, further 3μm sections were cut for immune marker staining. Immunohistochemistry was performed with the following antibodies: CD4, CD8, CD68, CD163, FoxP3 and PD-L1, using either the automated Ventana Benchmark Ultra (Roche, AZ, USA) or the Dako Autostainer Link 48 (Agilent, CA, USA). For more detailed protocol conditions, refer to Supplementary Table S1. For staining quality purposes, human tissue controls were added to all slides.

Specimen analysis and scoring

All specimens were assessed by two expert, independent sarcoma histopathologists, blinded to the other's assessment. Scores were assigned to each specimen, biopsy or resection, for a generic grade of inflammation: 0 (minimal), 1 (mild), 2 (moderate), 3 (severe). TIL scores were assigned according to two established scoring systems that were developed for breast cancer (Salgado)[14] and melanoma (Azimi)[15]. These methods were chosen due their simplicity and lack of requirement for any further stains, equipment or specialised training. The Salgado score involves assigning a visual estimate of the percentage of the tumour stromal area involved by TILs to the nearest 5%. The Azimi system uses a four-tier grading scheme (0 to 3) based on assessment of TIL density (mild, moderate, or marked) and distribution (focal, multifocal, or diffuse) within the tumour.

Immunohistochemistry sections were assigned scores for the various immune infiltrates using two methods. Firstly, sections were scored with a "hotspot" count, which is a count of immune cells reactive for that antibody in a 1mm2 area, scored where the immune infiltrate is highest. Second, an "overall" assessment was given, scored as 0 (none), 1 (mild), 2 (moderate), 3 (marked). To assign a final score for each of the above assessments, the two blinded scores were then averaged.

Statistical analysis

This study is descriptive in nature. Continuous valued quantities are described numerically by providing means, standard deviations, medians and ranges. Categorical variables are expressed as counts and proportions. TIL scores were tested for interobserver reliability using Cohen's kappa coefficient. Statistical analyses and graphical representation were generated using SPSS statistical software (Version 25, IBM, USA).

Results

A total of 39 patients were deemed suitable for enrolment and had both biopsy and resection specimens available for analysis. Immunohistochemical staining partially failed in seven, resulting in incomplete immunoprofiles. Therefore, 39 paired samples were available for TIL analysis but only 32 were available for both TIL and immune marker analysis. Patient and tumour characteristics can be seen in Table 1. Tumour size varied widely (range 50-450mm) owing to the cohort containing both large retroperitoneal (median size 195mm) and smaller extremity (median size 130mm) sarcomas. 16 patients had microscopically involved margins (R1); 12 of these (75%) were retroperitoneal sarcomas, where microscopically clear margins are difficult to achieve given the large size and location adjacent to vital structures.

Ten patients (25.6%) sustained recurrence; five local recurrences and five distant recurrences. Five patients with recurrence had retroperitoneal primary tumours and the other five were extremity locations. Of the five retroperitoneal tumours four were DDLPS and only one of the recurrences was distant with the other four being local recurrence. Microscopic margins were positive in four patients and three of these patients recurred locally. Four of the extremity recurrences were in myxoid liposarcomas with R0 resections and all of these were distant recurrences. The other was in a pleomorphic liposarcoma who had an R1 resection and the recurrence was local. All recurrences occurred within 3 years of surgery, at a median 18 months from surgery. Median follow-up was 54 months.

Interobserver reliability of TIL scores

Cohen's kappa coefficient (κ) for both the biopsy and resection specimens were calculated (Table 2). The Salgado TIL scoring system yielded perfect agreement (κ =1.000) between the assessing pathologists on the biopsy specimen and was superior to the Azimi system, which yielded only weak agreement (κ =0.455) on both biopsy and resection specimens. As a result of this finding, the Salgado score was used for all subsequent analyses in this study.

Impact of radiotherapy on TIL scores, immune marker expression and inflammation

TIL scores and grades of immune marker expression pre- and post-radiotherapy are shown in Table 3. In most patients, TIL scores remained unchanged following radiotherapy (74%), but in 8 patients (21%) TIL score increased. Seven of these patients had DDLPS and one had pleomorphic liposarcoma. All of these were either intermediate (3) or high-grade (5) tumours. Of all the DDLPS patients, 63.6% had increased TIL scores, whilst no myxoid or WDLPS/ALT patients had TIL score increases.

Inflammation grades and TIL score changes in different liposarcoma subtypes are represented in Fig 1, showing that the DDLPS group had a tendency towards higher inflammation grades compared to other liposarcoma subtypes. The inflammation grades were more often increased by radiotherapy in DDLPS (9/11 patients, 81.8%) compared with myxoid liposarcoma (4/12, 33.3%) and WDLPS/ALT (7/15, 46.7%).

There was no significant difference in the percentage of necrosis between patients showing increased TILs and those showing no change in TILs after radiotherapy (23.3% vs 36.0%, p=0.92).

Immunohistochemical stains for immune markers and changes induced by radiotherapy can be seen in Fig 2 and 3, and Table 3. Fig 2 graphically represents the "hotspot" count (as described above) for changes in immune marker expression, whilst Table 3 shows changes using the "overall" immunohistochemical grading (see above). Radiotherapy was observed to have minimal impact on immune effectors, with CD8 expression unchanged in the vast majority of cases (84.4%), increased in 9% and decreased in 6%. Conversely, immune suppressors showed upregulation in a larger proportion of patients. CD68 expression was increased in 59.4% after radiotherapy, with 34.4% unchanged and 6.3% decreased; and PD-L1 expression increased in 25% of patients with no patients showing decreased expression. Specifically, in the DDLPS group (only 9 of the 11 patients had complete immunohistochemistry profiles), 8 patients (88.9%) had increased CD68 expression and 7 patients (77.8%) had increased PD-L1 expression following radiotherapy.

Prognostic impact of radiotherapy-induced immune changes

The radiotherapy-induced changes in the immune infiltrates were compared between patients with and without disease recurrence (summarised in Table 3 and Fig 2). In patients whose TIL scores increased after radiotherapy, 50% recurred; while in patients with decreased or unchanged TIL scores after radiotherapy only 19% recurred. As above, most of the tumours with increased TILs were DDLPS; in these patients, of the seven with increased TILs after radiotherapy, three recurred, compared with one recurrence in the group whose TIL score was unchanged.

Expression of FoxP3 was downregulated by radiotherapy more commonly in patients without recurrence than those with recurrence (33.3% vs 0%), whilst PD-L1 expression showed the reverse trend, showing upregulation following radiotherapy more commonly in those patients without recurrence (29.2% vs 12.5%). CD163 and CD68 showed mixed responses depending on the method of analysis. Hotspot counts, as shown in Fig 2, seemed to show an upwards change in marker expression in patients without recurrence compared to those who recurred. However, when using the overall immune marker grading, as shown in Table 3, CD68 showed no difference in radiotherapy-induced changes, and CD163 expression was increased with radiotherapy in patients with recurrence compared to those without (37.5% vs 12.5%).

CD4 expression increased with radiotherapy in 62.5% of patients who recurred, compared with 25% in those without recurrence. However, the ratio of CD4:CD8 expression was no different between groups based on recurrence status. The CD8:FoxP3 ratio was seen to increase after radiotherapy more commonly in patients without recurrence compared to those who recurred (66.7% vs 50%).

Discussion

One of the major hurdles in analysing immune infiltrates in soft tissue sarcoma has been the absence of an accepted, standardised method for scoring the infiltrate. This study suggests that the TIL scoring system initially developed for breast cancer by Salgado[14] is reproducible in liposarcoma and provides high interobserver reliability. This is consistent with studies of this scoring system in breast cancer, which have also shown good interobserver agreement and high concordance[18].

Notably, whilst the Salgado score yielded perfect agreement in the biopsy specimens in this study, the concordance dropped to 60% in the post-radiation resection specimens. The likely explanation for this is the size and heterogeneity of sarcoma specimens. Pathologists assessing a biopsy specimen will necessarily be viewing and scoring the same small tissue sample. However, resection specimens are large and heterogenous, and pathologists may view different areas within the tumour when composing the score, leading to increased variability. This suggests a possible focus for refinement of the Salgado system, in order to ensure consistency in this method when used in sarcoma.

In fact, much further work has been performed to develop this TIL assessment method beyond breast cancer and broaden its applicability to a range of other solid tumours[19]. Whilst this has not been directly focussed towards soft tissue sarcoma, it is the breast TILs group authors' opinion that it should be able to be applied to all solid tumours, with some modifications for tumour architecture. For example, the breast system scores only stromal TILs, not intra-epithelial TILs. In sarcomas there are not separate stromal and intra-epithelial compartments, so this separation would not be applicable.

There exist many different TIL scoring methods that have been developed in several different tumour types. Whilst some of these are undoubtedly extremely informative, they can also be complex and resource intensive. For example, the Immunoscore, developed for colorectal carcinoma[20] has been shown to be strongly predictive for survival[21]. However, this method requires further immunohistochemical stains with digital quantitation image analysis, which requires specialised software and training. This is cumbersome and expensive which limits its use, particularly in less resource-rich locations, and furthermore it has been difficult to reproduce by other investigators. We have therefore chosen to compare two methods that are simple and require only routine H&E slides, making them broadly applicable and widely available. The Salgado method out-performed the Azimi method (developed for melanoma) and could be adopted for TIL assessment in sarcoma.

One of the unique challenges posed in sarcoma pathology assessment, in contrast to many other cancers, is that they are often large and heterogenous tumours. A biopsy into one part of a tumour may contain tissue with a very different appearance to another. For example, a biopsy from a well-differentiated area within a DDLPS may yield mature adipose tissue, while the resection specimen may contain high grade tumour with mitoses, atypia and necrosis. Likewise, the immune microenvironment in these areas may also be dissimilar. Nonetheless, some observations can be noted.

TIL scores were observed to increase following radiotherapy in a substantial number of patients (21%). However, in contrast to other cancers, where TILs seem to correlate with improved outcomes (breast, particularly triple-negative breast cancer[22], non-small cell lung cancer[23], colorectal[24], melanoma[15]), this current series has shown higher rates of TIL increase after radiotherapy in patients who have subsequently recurred (50% vs 19%). This is a somewhat surprising result, given the contrasting pattern to results seen in most other tumour types. It is difficult to be conclusive about the meaning of this result, particularly in this small, highly selected cohort without controlling for other prognostic variables.

One contributory factor seems to be the pattern of TIL change seen in different liposarcoma subtypes. Whilst there was a higher rate of increasing TIL in the group of patients who subsequently recurred, this pattern was seen almost entirely in intermediate to high-grade DDLPS, which reflects an inherently aggressive tumour with a high risk of recurrence. This must be taken into consideration when assessing the significance of the higher rate of recurrence in the group with increased TILs. Of all DDLPS patients in this cohort, approximately two-thirds had increased TILs after radiotherapy, while no WDLPS/ALT or myxoid liposarcoma had increased TIL scores. The reasons for this increase in the DDLPS group remain unclear and merits further exploration. It is an interesting parallel that in a phase 2 study of PD-1 inhibition in sarcoma (SARC028), DDLPS was one of the sarcoma subtypes that showed meaningful activity of PD-1 blockade[25]. Perhaps the robust immune infiltrates seen here in DDLPS also underpins the higher response to immunotherapy?

Another possible contributory factor considered was whether the degree of necrosis could contribute to TIL infiltration. TIL scores using the Salgado method explicitly exclude mononuclear immune cells in areas of necrosis from the overall score, but it was considered possible that immune infiltrates may exist within the tumour in response to nearby areas of tumour necrosis. However, we found no difference in the degree of tumour necrosis between those tumours with increased TILs and those with unchanged TIL levels.

The immune infiltrate is comprised of a combination of immune effector cells which can control tumour progression, and immune suppressor cells which can create an immunosuppressive environment in which the tumour can thrive[26]. It is the balance of these factors in the tumour microenvironment that contributes to tumour inhibition or promotion by the immune system.

In this study, we have seen that the immune effectors, particularly cytotoxic CD8+ T-cells, remained unaffected by radiotherapy, with the vast majority showing no change in CD8 expression. On the contrary, the cells that increased following radiotherapy were immune suppressors, namely CD68+ TAMs. Also seen to upregulate was expression of the PD-L1 protein on immune cells within the tumour. This binds to the inhibitory checkpoint PD-1 receptor on activated T-cells and represents another immunosuppressive mechanism by tumours to avoid attack by immune cells. Again, the DDLPS group has shown the most dramatic responses, with almost all DDLPS showing increased infiltration by both CD68 and PD-L1 expressing immune cells.

It is not possible to interpret prognostic information regarding the immune infiltrate in this group as the numbers are small and the groups are not controlled for other prognostic variables. In addition, we have grouped retroperitoneal and extremity liposarcomas together, which have vastly different recurrence patterns given the nature of their anatomic locations and ability to achieve wide resection margins. As a result, this study can only be descriptive regarding the effect of the radiotherapy-induced immune microenvironment changes on prognosis. However, some patterns have still emerged. Immunosuppressive FoxP3+ TILs were more likely to decrease following radiotherapy in tumours that did not recur; however conversely, PD-L1, which is also immunosuppressive, was more likely to increase in tumours that did not recur. Combined with the observations in the previous paragraph, these findings suggest that radiotherapy may contribute to disease control through immunomodulatory effects. However, the exact mechanisms remain imprecisely understood. A previous study by Sharma et al, suggested that radiotherapy caused upregulation of multiple immune effectors (including cytotoxic CD8+ T-cells) and downregulation of immune suppressors[27]. Whilst our data concur with the increased expression of CD4+ T-cells, findings related to the effect on immune suppressors are conflicting and warrant further study.

The finding of increased PD-L1 expression in patients who did not recur is notable and deserves further discussion. PD-L1 expression is generally considered immunosuppressive and has been correlated with poor prognosis in several cancer types[28-31]. However, consistent with our findings, there are also examples of cancers in which PD-L1 expression is associated with improved prognosis[32-34]. Consistent with our data, radiotherapy has previously been shown to increase PD-L1 expression in soft tissue sarcoma and is known to reduce rates of local recurrence[35]. This phenomenon is not unique to sarcoma, with lung cancer (and others) also shown to increase PD-L1 in response to radiotherapy[36]. Other studies have shown that immunohistochemical assessment of PD-L1 expression does not comprehensively predict response to immunotherapeutic inhibition of the PD-1/PD-L1 axis in different tumour types[37]. All of these facts contradict the cancer-promoting nature of PD-L1 and highlight that further research is needed to fully understand the role of PD-L1 in immune escape.

CD4+ T-cells can represent either effector T-cells or regulatory (suppressor) T-cells and routine immunohistochemistry is unable to differentiate these subtypes[38].

FoxP3 expression can be used as a surrogate marker and better reflects the immunosuppressive cells, but is still not an ideal marker[39]. To better reflect the balance of cytotoxic T-cells to regulatory T-cells, several authors have studied ratios of CD8+ to either CD4+ or FoxP3+ T-cells, with some finding positive correlations for outcome[40, 41] and even predicting for pathologic complete response following neoadjuvant chemotherapy in breast cancer[42]. In the current series, the CD8:FoxP3 ratio is increased more often in patients who do not recur than in those who do. This is perhaps further evidence that radiotherapy exerts some of its impact through immunomodulatory effects.

In this study, due to small numbers of recurrences, we have grouped local recurrences and distant recurrences together. Ultimately, it may be that distant recurrence alone is more reflective of the role of immune engagement in tumour control, given that the immune system is activated systemically. This is a limitation of this study and merits further consideration. Testing this hypothesis, the SARC032 trial is combining PD-1 inhibition (pembrolizumab) with radiotherapy and will specifically test extremity sarcomas at high risk of systemic disease[43]; these results are eagerly awaited. The SARC032 trial will include dedifferentiated liposarcoma in its enrolment (along with undifferentiated pleomorphic sarcoma) due to preliminary data from the SARC028 study suggesting increased response to PD-1 inhibition. As outlined above, this this observation may be reinforced by our data showing a robust immune response of DDLPS to radiotherapy.

This study is limited by its small size and retrospective nature. Also, due to small numbers we were unable to perform meaningful multivariate analyses. Therefore, we have not controlled for known prognostic factors and conclusions have to be made with care. Finally, when assessing immune infiltrates by immunohistochemistry in sarcoma, it is unclear whether hotspot or overall measurements provide more accurate information. This has the potential to produce misleading analyses.

Conclusions

The TIL scoring system developed for breast cancer by Salgado is reproducible in sarcoma and has high interobserver reliability. The prognostic implication of a high TIL score in liposarcoma is unclear but may not be associated with improved prognosis (in contrast to other cancers), although there are certain to be other contributing factors like tumour subtype and grade. In this cohort, radiotherapy was observed to have limited impact on immune effectors but seemed to have more impact in upregulating immune suppressors. Changes in the immune infiltrate suggest that radiotherapy may contribute to disease control through immunomodulatory effects. DDLPS seems to represent a uniquely responsive subtype, displaying the most profound changes in TIL scores and immune marker expression. Future studies in this space need to be large, likely requiring collaborative efforts, given the number of confounding clinical and pathological factors and tumour heterogeneity.

Acknowledgements:

The authors would like to acknowledge the assistance of the Australia and New Zealand Sarcoma Association, and in particular Jasmine Mar, for the maintenance of the ACCORD sarcoma database, and also the Hannah’s Chance Foundation for financial support.

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Data availability statement:

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Figure legends:

Fig 1. TIL score (by Salgado method) and inflammation grades before and after radiotherapy, by liposarcoma subtype. Black solid lines – no recurrence; red dotted lines – recurrence. A, Tumour-infiltrating lymphocyte (TIL) score; B, all liposarcoma (LPS); C, Well-differentiated liposarcoma/atypical lipomatous tumour (WDLPS/ALT); D, De-differentiated liposarcoma (DDLPS); E, myxoid liposarcoma (LPS)

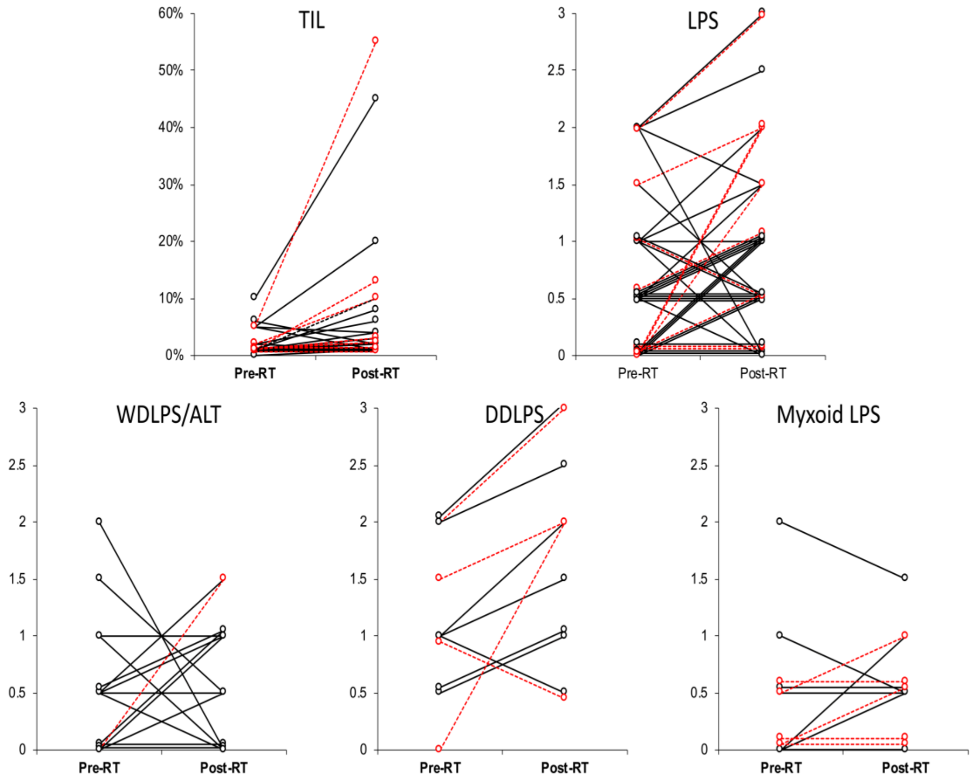

Fig 2. Immune marker counts before and after radiotherapy, by recurrence status

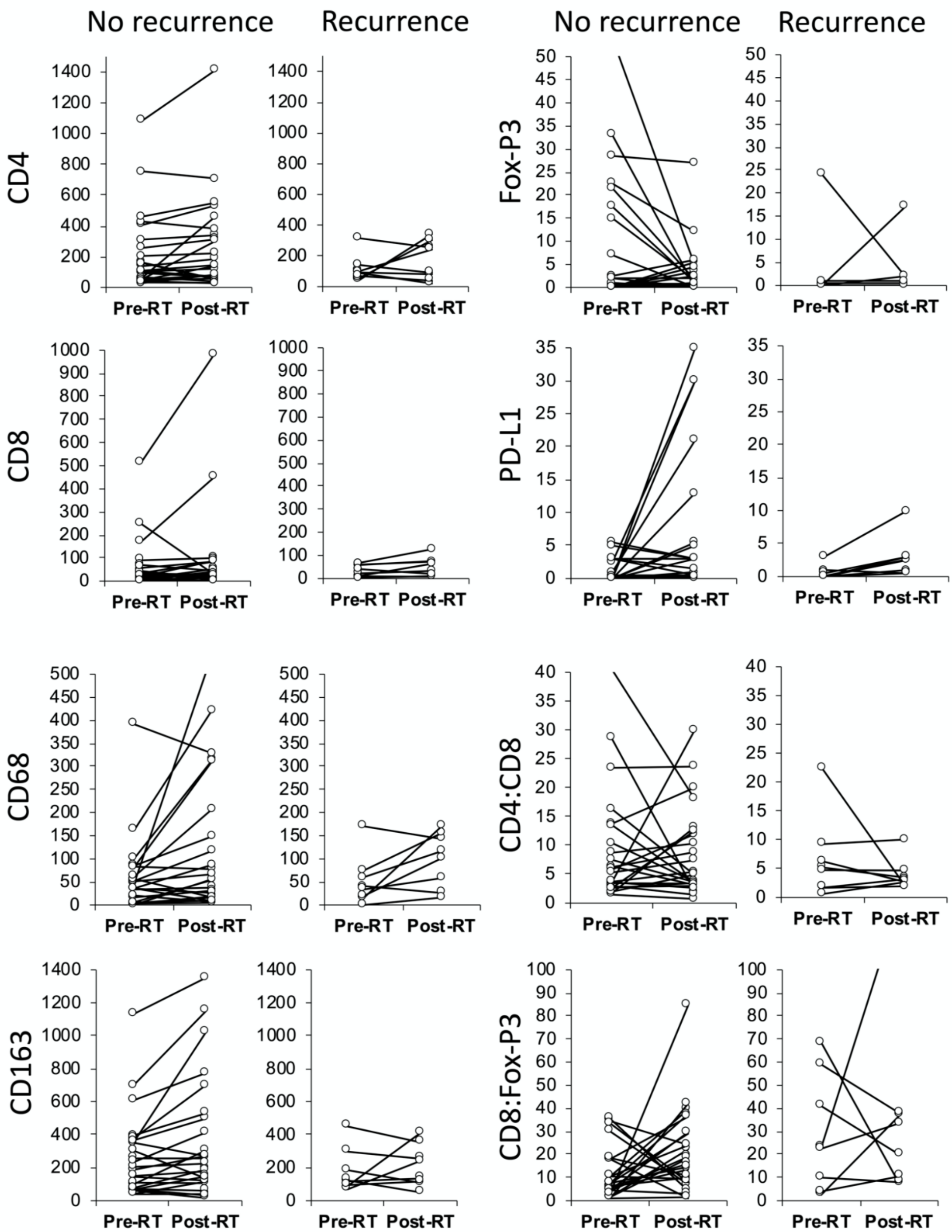

Fig 3. Immune marker immunohistochemistry, before and after radiotherapy

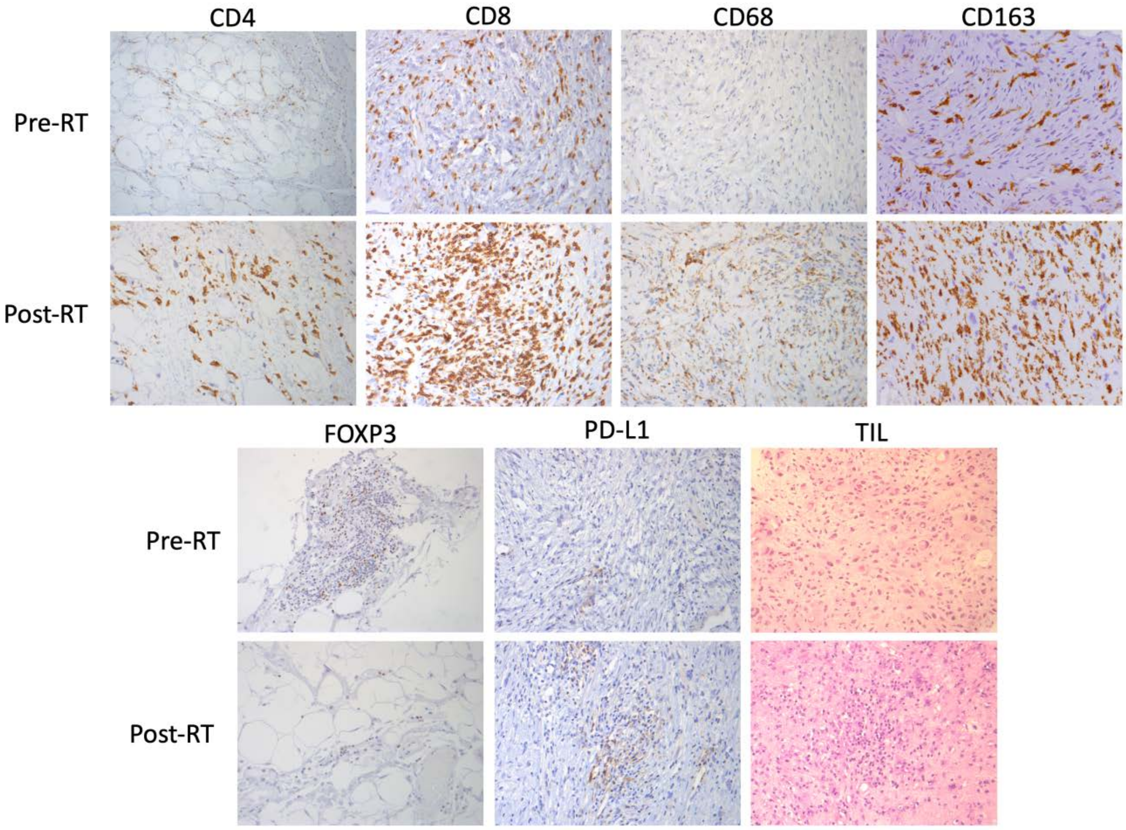

Table 1. Patient and tumour characteristics. WDLPS/ALT (well-differentiatied liposarcoma/atypical lipomatous tumour); DDLPS (dedifferentiated liposarcoma).

Characteristic	**Patients (n=39)**
Age, median (range)	55 (17-78)
Sex	
- male	22 (56.4%)
- female	17 (43.6%)
Tumour site	
- retroperitoneum	16 (41.0%)
- extremity/body wall	23 (59.0%)
Tumour size (mm), median (range)	150 (50-450)
Grade	
- 1	21 (53.8.0%)
- 2	11 (28.2%)
- 3	7 (17.9%)
Histology	
- WDLPS/ALT	15 (38.5%)
- DDLPS	11 (28.2%)
- Myxoid liposarcoma	12 (30.8%)
- Pleomorphic liposarcoma	1 (2.6%)

% necrosis following radiotherapy - 0-25% - 26-50% - 51-75% - 76-100% - not recorded	 19 (48.7%) 3 (7.7%) 2 (5.1%) 9 (23.1%) 6 (15.4%)
Resection margin status - R0 - R1	 23 (59.0%) 16 (41.0%)
Recurrence status - Recurrence-free - Local recurrence - Distant recurrence	 29 (74.4%) 5 (12.8%) 5 (12.8%)

Table 2. Testing TIL scores for interobserver reliability

TIL scoring method	**Specimen type**	**Cohen's kappa coefficient (κ) for interobserver reliability**
Salgado (2015) – developed for breast cancer [14]	Biopsy	1.000 (perfect agreement)
	Resection	0.601 (moderate agreement)
Azimi (2012) – developed for melanoma [15]	Biopsy	0.455 (weak agreement)

	Resection	0.394 (weak agreement)

Table 3. Impact of radiotherapy on tumour infiltrating lymphocytes (TILs) and immune markers

	No recurrence (n=29)			**Recurrence (n=10)**			**Overall (n=39)**		
	Decreased	Unchanged	Increased	Decreased	Unchanged	Increased	Decreased	Unchanged	Increased
TIL score	2 (6.9)	23 (79.3)	4 (13.8)	0 (0)	6 (60)	4 (40)	2 (5.1)	29 (74.4)	8 (20.5)
Immune marker:	(n=24; IHC incomplete in 5)			(n=8; IHC incomplete in 2)			(n=32)		
CD4	5 (20.8)	13 (54.2)	6 (25)	3 (37.5)	0 (0)	5 (62.5)	8 (25)	13 (40.6)	11 (34.4)
CD8	2 (8.3)	20 (83.3)	2 (8.3)	0 (0)	7 (87.5)	1 (12.5)	2 (6.3)	27 (84.4)	3 (9.4)
CD68	1 (4.2)	9 (37.5)	14 (58.3)	1 (12.5)	2 (25)	5 (62.5)	2 (6.3)	11 (34.4)	19 (59.4)
CD163	7 (29.2)	14 (58.3)	3 (12.5)	1 (12.5)	4 (50)	3 (37.5)	8 (25)	18 (56.3)	6 (18.8)
FoxP3	8	10	6 (25)	0 (0)	6 (75)	2 (25)	8 (25)	16 (50)	8 (25)

	(33.3)	(41.7)							
PD-L1	0 (0)	17 (70.8)	7 (29.2)	0 (0)	7 (87.5)	1 (12.5)	0 (0)	24 (75)	8 (25)
CD4:CD8	13 (54.2)	0 (0)	11 (45.8)	4 (50)	0 (0)	4 (50)	17 (53.1)	0 (0)	15 (46.9)
CD8:FoxP3	8 (33.3)	0 (0)	16 (66.7)	4 (50)	0 (0)	4 (50)	12 (37.5)	0 (0)	20 (62.5)