

T lymphocytes in histiocytic sarcomas of Flat-coated retriever dogs

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Abstract

Flat-coated retriever dogs are predisposed to the development of histiocytic sarcoma (HS), a poorly differentiated, highly malignant neoplasm. We have previously documented a significant lymphocytic infiltrate in such tumors. The objective of this study was to examine the presence and expression of regulatory T cells in HS tumor samples. Forty tumors were included in this study. All tumors were immunolabelled for CD3, CD79a, CD25, CD45RA, and FOXP3. The proportion of positive cells was compared between tumors presenting as a localized primary soft tissue mass (soft tissue origin HS) and disseminated HS affecting viscera, especially the spleen (splenic origin HS). By immunohistochemistry (IHC), 95% of infiltrating T cells were positive for FOXP3 in all sections suggesting the presence of regulatory T cells. The proportion of cells positive for FOXP3 was higher in the tumors arising in soft tissues whilst the proportion of CD45RA-positive cells was higher in the splenic origin HS. Canine HS has an aggressive clinical behavior and is uniformly fatal. The difference in proportion of tumor-infiltrating lymphocytes positive for these two markers in the two locations may represent differences in tumor microenvironment between the two sites.

Key words: Histiocytic sarcoma, tumor-infiltrating lymphocytes, regulatory T cells, Flat-coated retriever, tumor microenvironment, cancer immunology, tumor-infiltrating lymphocytes, hematologic neoplasms.

Introduction

Histiocytic proliferative disorders are prevalent in dogs and represent a variety of clinical and pathologic presentations with recognized breed predispositions. We have previously documented that HS accounts for 25 % of all neoplasms affecting the Flat-coated retriever and up to 50 % of malignant neoplasms in this breed,^{3,9} where it presents with predominantly localized lesions but with a high metastatic rate.^{13, 20, 21}

Histiocytic sarcomas are most often derived from cells with the phenotypic profile of interstitial dendritic cells (DCs). These cells occur almost in all tissues except the brain, and therefore HS arise in almost any part of the body. Interstitial DCs are identifiable in tissues by immunophenotyping and they are positive to CD1a, CD11c, CD11b, and MHC class II.^{9, 20, 21} Most reports of HS in the Flat-coated retriever describe a localized form, arising within the musculature or fascia (including periarticular) of limbs. A disseminated, visceral form often affecting the spleen also occurs in the breed but is less common.^{9, 20} In the Flat-coated retriever, localized HS are highly malignant neoplasms, metastasis is initially via lymphatics to the loco-regional lymph nodes. Distant metastases, involving spleen, lungs, liver, bone marrow, subcutaneous tissue, bone, skeletal muscle, kidney, and central nervous system have also been reported, which confounds distinction between the two forms of the disease.^{3, 14, 20} HS is not exclusive to Flat-coated retrievers; other breeds of dogs where HS has been reported include Bernese Mountain dogs, Rottweilers, and Golden retrievers. We have previously documented a 10-20 % lymphocytic infiltrate in localized and visceral HS of Flat-coated retrievers and shown these to be T lymphocytes, suggesting a cell-mediated immune response.^{9, 21}

The host's immune system reacts to tumors via adaptive responses directed mainly towards tumor associated antigens (TAA).¹² However, there are mechanisms enabling the tumor to escape from or evade immune surveillance, one of which is suppression of the T cell-mediated anti-tumor response. This may be mediated by induction of regulatory T cells (Tregs).²⁹ In most human tumors, the majority of tumor-infiltrating lymphocytes are CD3+ cells which include CD8+ cytotoxic cells, CD4+ helper cells, and CD4+ regulatory cells (previously known as suppressor T cells).²² The presence of tumor infiltrating lymphocytes expressing CD8 is usually associated with better prognosis, whereas infiltration of lymphocytes positive for regulatory markers is mostly associated with a worse prognosis.¹¹

The X chromosome-encoded, intracellular forkhead transcription factor, forkhead box P3 (FOXP3) transcription factor was identified as an essential factor for the suppressive phenotype of natural Tregs and is a highly specific marker of murine Tregs. FOXP3 is also stably up-regulated in human Tregs.^{15, 30}

There is an intense interest in the role of Tregs in human and animal cancer.^{6,7} Increased numbers of CD4+CD25+FOXP3+ Tregs have been observed in peripheral blood, lymph nodes, and various neoplasms

in human cancer patients and are usually associated with poor prognosis.^{5, 7, 10, 24, 28, 31} However some studies have found no prognostic value and in others the presence of FOXP3+Treg in neoplasms has been correlated with a more favourable prognosis.^{8, 17} The presence of Tregs in healthy and cancer-bearing dogs has been documented; dogs with melanoma, osteosarcoma, mammary gland adenocarcinoma, and lymphoma have been shown to have increased numbers of Tregs in their peripheral blood.^{7, 24, 28} The presence of Tregs has also been investigated in canine tumor tissue.^{19, 23} The number of Tregs within canine neoplasms is significantly increased compared with Tregs in peripheral blood of dogs with cancer, suggesting that the presence of neoplastic cells induces either local proliferation or selective migration of Tregs to tumor-infiltrated sites.²³ Canine CD4+FOXP3+ Tregs show increased production of IL-10 and TGF-beta, which confirms their immunosuppressive function but studies of the prognostic implications of canine Tregs are still limited.²

The purpose of this work was to establish what proportion of the previously documented lymphocytic infiltrate in soft tissue and splenic HS of Flat-coated retriever dogs comprise regulatory T cells, to serve as basis for future studies assessing correlation between Tregs and prognosis for Flat-coated retrievers with HS.

Materials and Methods

The tumor sections selected for this study were from the forty HS samples previously reported by Constantino-Casas et al. (2011).⁹ For IHC, 3 µm serial sections were cut from the appropriate paraffin blocks and mounted on positively charged slides (Snowcoat; Surgipath Europe Ltd, UK). Sections were dried overnight at 50 °C before automated processing using the PT link instrument Envision Flex antigen retrieval solution, high pH (Dako Carpinteria, CA, USA). This allowed for deparaffinization, rehydration, and antigen retrieval in a combined 3-in-1 procedure. Sections were immersed in the pre-heated working solution (pH 9.0). Once the temperature had reached 97 °C, sections were incubated for 20 minutes, left to cool to 65 °C and immediately rinsed in buffer (Dako Envision wash buffer) at room temperature. An automated IHC system (Dako Autostainer, Dako, Carpinteria, CA) was used to process the prepared tissues. Endogenous peroxidase activity was inhibited using Dako REAL peroxidase-blocking solution. The immunohistochemical antibodies were anti-CD3 (mouse anti-human monoclonal, 1:150, clone F7-2-38 Dako, UK), anti-CD79a (mouse anti-human monoclonal, 1:400, clone HM57, Dako, UK), anti-CD25 (mouse anti-human monoclonal, 1:100, clone NCL-CD25-305, Leica/NOVACAstra, UK), anti-CD45RA (mouse anti-canine monoclonal, 1:50, clone CA21-4B3-IgG1, US Davis, USA), anti-FOXP3 (rabbit anti-human polyclonal, 1:500, ref. Rb anti-foxP3, lot 121220CF Insight Biotech, UK). Peroxidase activity was demonstrated using 3,3'-diaminobenzidine (DAB) solution for 10 minutes, and slides were counterlabelled with Gill's hematoxylin for 2 minutes before rinsing, dehydrating, clearing, and mounting with coverslips. Labelling

with antibodies anti-CD25 and anti-FOXP3 were performed on consecutive slides. Double IHC was not performed however consecutive slides were used and the same areas of each section were examined. A semi-quantitative analysis of immunolabelling was performed as previously described by Constantino-Casas et al.⁹ Positive control tissues included reactive dog lymph node or dog tonsil. Antibody diluent (Dako) was used in place of the primary antibody to act as a negative control in each immunolabelling procedure. In addition many of the tumor sections acted as internal positive and negative controls for the antibodies. Immunolabelling within tumors was assessed as positive or negative. Six slides per case were examined. Slides were initially examined at low magnification (x20) to confirm the presence of positive immunolabelling of lymphocytes within the neoplastic tissue. Detailed examination of selected regions was then carried out using forty times magnification and followed the pattern: left (field 2), left (field 3), down (field 4), and right (field 5) equating 5 fields per slide. The periphery of the section was excluded to avoid cutting artefacts and precipitated DAB. If necrosis, haemorrhage or artefacts were present in over 10 % of the field, the cells were not counted, and the field was moved to the left. If this continued or the edge of the slide was reached, it was moved down. This was repeated until a full, countable field was reached. The percentage of labelled lymphocytes was obtained by counting the number of immunolabelled cells and number of lymphocyte nuclei (as a measure of total cells) per field and calculating the percentage of labelled in total cells. The average was calculated for percentage of labelling for each slide.

Statistical analysis was performed using SPSS -Version 20.0 (Armonk, NY). Data were assessed for normality by use of the Shapiro-Wilk test, which demonstrated that data for all markers assessed was not normally distributed ($P > 0.05$). Median, 25th and 75th percentiles of cells that were positive for the various markers in each group were calculated. The Mann Whitney U test was used to compare the percentage of positive cells between the two groups. A p value of < 0.05 was considered statistically significant for this analysis. Data are presented as median [25th, 75th percentile].

Results

As previously described,⁹ of the 40 HS from Flat-coated retrievers, 20 were of splenic origin and 20 were from primary soft tissue neoplasms. There were 22 females and 18 males and the median age of affected dogs was 8.4 years (range from 5 to 11.8 years old). There was no difference in age or sex distribution between the soft tissue and splenic origin HS. Clinical-pathological data was available for all dogs, with follow up information available for 32/40 of the cases (80 %); 8 dogs (20 %; 3 from the splenic group and 5 from the soft tissue group) were lost to follow up (Supplemental Material).

The main presenting sign in the 20 dogs with splenic HS was a palpable abdominal mass (11), lethargy (9), severe anaemia (8), weight loss (6), and exercise intolerance (4). Less common findings recorded were: inappetence (3), pyrexia (2), vomiting (2), hypoalbuminaemia (2), polydipsia/polyuria (2), regurgitation (1), melena (1), neutropenia (1), thrombocytopenia (1), elevation in serum levels of liver enzymes (1), collapse (1), and pallor (1). None of these dogs presented with a detectable peripheral mass, primary limb tumour or history of lameness. Out of 20 dogs with splenic HS, 13 had exploratory laparotomy and of these, 6 dogs had documented splenectomy, two were treated with chemotherapy (1 with vincristine, the other with lomustine). One dog received a whole blood transfusion due to severe anaemia. Information regarding treatment was not available for one dog (lost to follow up). All of the dogs from this group were euthanized either at the time of diagnosis (6/20), at the time of exploratory laparotomy (4/20) or within 120 days of diagnosis (7/20). Three dogs were lost to follow up. One dog that was diagnosed with an abdominal mass during routine consultation underwent splenectomy and lived for 14 months, after which time he developed a quadriplegia and was suspected to have spinal metastases.

All 20 dogs with soft tissue HS presented with a solitary mass, 9/20 dogs presented with lameness. Twelve tumors presented in the front limb, 6 in the rear leg, one dog presented with a mass in the neck and one on the flank. Eight of the 20 dogs were euthanized at the time of the diagnosis. Six dogs (6) were euthanized between 5-730 days after diagnosis, six dogs (6) were lost to follow up. Of the 20 dogs, 7 were treated with surgical excision/cytoreductive surgery, 10 were not treated, and 2 received a course of hypofractionated radiotherapy (4x850cGy). One of these dogs was also treated with one dose of adjuvant chemotherapy (lomustine) and the other dog underwent surgical excision of the tumor (this dog was one of the seven dogs undergoing a surgical procedure). Treatment was not recorded for one dog which was one of the dogs lost to follow up. Biopsy samples were obtained from 4 of 20 dogs with the remaining 16 dogs having their samples collected at post mortem examination. Four dogs (3 front limb, 1 hind limb HS) had an apparent longer survival (365-1095 days) compared to the remaining dogs in this group. Three of them (two fore and one hind limb HS) were treated with surgery (limb amputation), one dog was treated with radiation therapy and lomustine.

For all antibodies, immunopositive labelling was present in the positive control sections but not detected in the negative controls. Despite the variable neoplasm morphology and different locations, all forty tumors contained lymphocytes expressing CD3 and CD45RA scattered through the neoplastic tissues, including the periphery of the neoplasm. CD45RA labelling was membranous and CD3 labelling was both membranous and cytoplasmic (Figures 1 & 2). FOXP3 labelling was nuclear. In all tumor sections examined, FOXP3 positive cells were present and scattered between the neoplastic cells, with over 95 % of CD3-positive cells being FOXP3+ (Figures 3 & 4). IHC data for CD3, CD25, CD45RA, CD79a, and FOXP3 is summarized in Table 1.

CD25 labelling was membranous and the location of CD25-positive cells was similar to FOXP3-positive cells (data not shown).

When the proportion of cells positive for CD3, CD79a, CD25, CD45RA, and FOXP3 were compared between neoplasms at the different sites, significant differences were identified in the proportion of FOXP3- and CD45RA-positive cells between the sites. The proportion of cells positive for CD45RA was significantly higher in splenic HS compared with soft tissue origin HS (7 % vs. 0 %; $p < 0.001$) (Figure 5) whereas the proportion of cells positive for FOXP3 was significantly higher in soft tissue tumors compared with splenic HS (19 % vs. 1 %; $p = 0.019$) (Figure 6). There were no differences in the expression levels of the other markers (CD3, CD25, CD79a) between the two sites (Table 1).

Discussion

The purpose of this study was to establish what proportion of the previously documented lymphocytic infiltrate in soft tissue and splenic HS of Flat-coated retriever dogs comprise regulatory T cells, to serve as basis for future studies to assess correlation between Tregs and prognosis for Flat-coated retrievers with HS.

The tumor samples selected for this study represented both the soft tissue ($n = 20$) and splenic ($n = 20$) variants of HS from Flat-coated retrievers. The splenic neoplasms were frequently associated with severe systemic signs, particularly anaemia (8/20 cases). It has been documented¹³ that anaemia in affected dogs, may be due to an haemophagocytic variant of HS, often associated with a paraneoplastic hypoalbuminaemia.²⁷ The severity of the clinical signs and / or presence of widespread visceral metastases at the time of diagnosis (often documented at the time of exploratory laparotomy), led to most of these dogs being euthanized immediately following diagnosis. Hence the prognosis for this group of dogs was universally poor. In only one dog was a splenic mass noted during routine examination; this dog underwent splenectomy and survived a further 14 months. This may provide some justification for routine screening of dogs over 7 years of age.

The dogs with the soft tissue form of the disease, all of which presented with a solitary mass, had a marginally better prognosis than those with splenic neoplasms. However, even in this group survival times were poor with 13/20 being euthanized at or within 6 months of diagnosis due to the extent of the primary neoplasms and its rapid and often painful progression. In those dogs where treatment was attempted, survival was still poor and did not exceed 6 months in most cases. This is consistent with previously reported survival times (median 167 days) for dogs receiving any kind of chemotherapy,

radiotherapy, surgery or any combination.¹⁴ However, there were 4 long-term survivors in this group living up to 1095 days, which supports the suggestion that the localised form of HS may have a better prognosis than the splenic / disseminated form. Two reports have recently suggested that dogs with the localized form of HS, with no evidence of metastasis at presentation, treated with aggressive local therapy and/or chemotherapy, can achieve longer survival times: 980 days and 568 days, respectively.^{18, 26} However only 2/19 reported by Klahn et al.¹⁸ and none of the 16 dogs reported in Skorupski et al.²⁶ were Flat-coated retrievers.

We have previously documented a high proportion (10 – 20 %) of infiltrating T cells in both splenic and soft tissue HS from Flat-coated retrievers. The present study has demonstrated that the majority of these tumor-infiltrating lymphocytes are FOXP3-positive suggesting them to be regulatory T cells.⁹

In dogs, HSs arise from interstitial DCs that occur in most tissues of the body (except the brain) and these cells are potent antigen presenting cells.

It is known that dendritic cells play a pivotal role in determining tolerance versus immunity, so determining the nature of the infiltrating T cells seemed a key step in better understanding the relationship between the neoplasm, its microenvironment, and the immune system. The finding that the majority of infiltrating T cells expressed FOXP3, suggesting them to be regulatory T cells, raises interesting questions of cause and effect, and whether there is a correlation between high numbers of FOXP3 positive cells and prognosis, which lie beyond the remit of the present study, but could direct future studies.

The proportion of cells labelling positive for FOXP3 was higher in the soft tissue form compared to the splenic form of HS. This may reflect the different microenvironment provided by a soft tissue versus splenic site of origin. A recent study has suggested that hypoxia in canine neoplasms may result in an increased infiltration by Tregs.¹⁶ We have previously demonstrated that HSs show higher Glut-1 immunoreactivity and angiogenesis than low grade soft tissue sarcomas, suggesting that hypoxia plays an important role in the biology of these neoplasms.¹

Whilst it has been well documented that an increased number of regulatory T cells in human patients with diverse solid neoplasms and haematological malignancies can be a positive prognostic factor, studies on Tregs and FOXP3-positive cells in canine solid neoplasms, although limited, all report increased numbers of Tregs as a negative prognostic factor.^{4,9}

In the present study, the soft tissue form of HS showed a higher proportion of FOXP3 cells, which is interesting as this form of HS has been demonstrated to carry a marginally improved prognosis when compared to the disseminated form, even though both variants are highly malignant.³ Too few cases in this study received any form of treatment to allow a meaningful assessment of the prognostic significance of the proportion of cells labelling positive for FOXP3 and further studies are warranted.

The proportion of FOXP3 and CD45RA positive cells differed between splenic and soft tissue neoplasms. CD45RA is a marker for naïve T cells but is also a common marker for haematopoietic cells except erythrocytes and platelets.²⁵ It detects basophils, granulocytes, lymphocytes, macrophages/histiocytes, mast cells, monocytes, and plasma cells.²⁵ The higher proportion of cells positive for CD45RA in splenic compared to soft tissue HS can be explained by a more heterogeneous haematopoietic cell population in the canine spleen where erythrocytes, granulocytes, and circulating mononuclear cells are commonly seen.

A limitation to this work is that some animals were lost to follow up and some information was not available. The sample size for both groups was relatively small, which limited the statistical power of the study. The use of formalin-fixed paraffin-embedded tissues limited the range of antibodies available for IHC, particularly with respect to CD4 and CD8.

This work demonstrated the presence of FOXP3-positive cells which are most likely regulatory T cells in the localized and disseminated forms of HSs of Flat-coated retrievers, and showed a lower percentage of FOXP3 and higher percentage of CD45RA expressing cells in splenic compared to soft tissue HS of Flat coated retriever dogs. These findings are a first step in the evaluation of tumor-infiltrating lymphocytes in the microenvironment of HS.

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Figure legends

Figures 1-4. Histiocytic sarcomas: spleen (Figure 1), subcutaneous tissue (Figure 2-4), dog, immunohistochemistry; 3,3'-diaminobenzidine chromogen. Figure 1. IHC for CD45RA-positive cells characterized by their membranous labelling. Figure 2. IHC for CD3-positive cells characterized by their membranous and cytoplasmic labelling. Figure 3. IHC demonstrating a strong FOXP3-positive nuclear labelling of cells. Figure 4. IHC demonstrating a weak FOXP3-positive nuclear labelling of cells.

Figure 5. Scatter plot showing the percentage of CD45RA-positive lymphocytes is higher in visceral compared to soft tissue HS. The line represents the median value for each group **: $p < 0.001$.

Figure 6. Scatter plot showing the percentage of FOXP3-positive lymphocytes is lower in visceral compared to soft tissue HS. The line represents the median value for each group. *: $p = 0.019$.

Table 1. Comparison of immunohistochemical findings in 20 visceral and 20 soft tissue histiocytic sarcomas of dogs. The data show the median percentage (and 25th, 75th percentiles) of cells that are labelled for each marker.

Marker	% positive cells seen Median [25th, 75th percentiles]	% positive cells in visceral tumours Median [25th, 75th percentiles]	% positive cells in soft tissue tumours Median [25th, 75th percentiles]	P value
CD3	16 [10, 28]	18 [9, 35]	16 [10, 23]	0.523
CD25	5 [1, 24]	3 [0, 26]	6 [2, 25]	0.382
CD45RA	3 [0, 15]	7 [3, 20]	0 [0, 3]	<0.001
CD79a	0 [0, 8]	1 [0, 10]	0 [0, 3]	0.498
FOXP3	7 [1, 20]	1 [0, 10]	19 [5, 30]	0.019

Figure 1-4

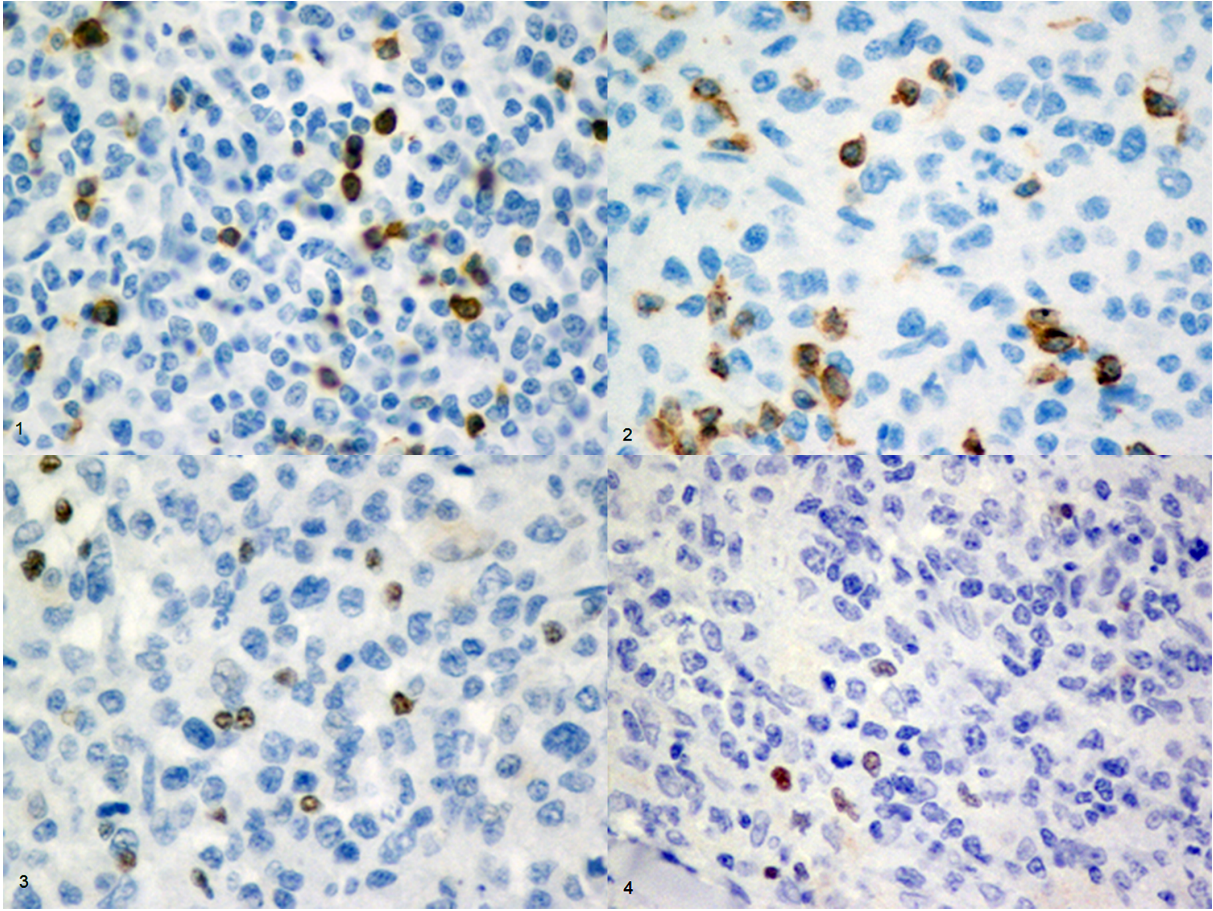


Figure 5

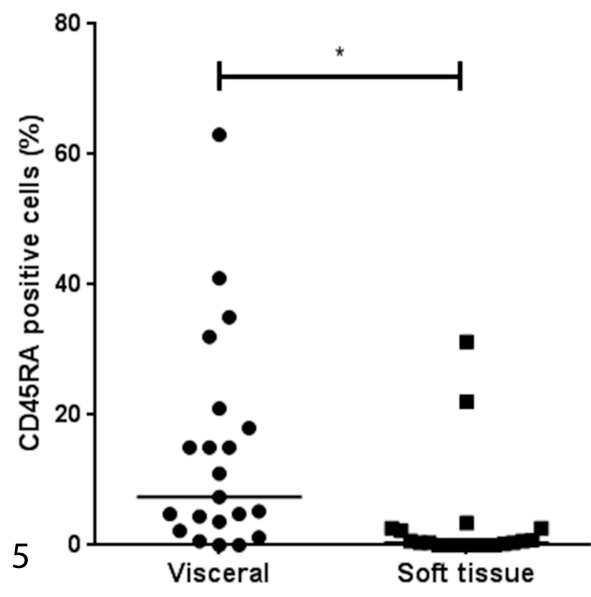


Figure 6

