Prevalence of FoxP3^{+} cells in canine tumours and lymph nodes positively correlates with glucose transporter 1 expression

Luca Fortuna*, Joshua Relf*, Yu-Mei Chang*, Henny M Martineau* and Oliver A Garden*

* The Royal Veterinary College, Royal College Street, London, NW1 0TU, United Kingdom

Corresponding author: Luca Fortuna, 25 Wansunt Road, Bexley, Kent, DA52DQ, United Kingdom, +447836466401, lfortuna2@rvc.ac.uk

Summary

The presence of hypoxia and regulatory T cells (Tregs) in tumours are both known to be negative prognostic factors in cancer, and this study demonstrated a correlation between the two factors in canine neoplasia. Samples of 57 canine tumours and 29 canine lymph nodes categorised as metastatic, draining or reactive were obtained. Sequential sections were labelled by immunohistochemistry for glucose transporter 1 (Glut1) and FoxP3 as markers of hypoxia and Tregs respectively. Up to 21 regions of interest were selected on each sample in a representative pattern and given a semi-quantitative score based on its Glut1 labelling, and the number of $FoxP3^+$ cells at each ROI was counted. A generalised estimating equation with negative binomial log link function was used to determine an association between Glut1 expression and $FoxP3^+$ cell count. Higher Glut1 immunoreactivity was correlated with significantly higher numbers of $FoxP3^+$ cells in the total tumour sample pool and total lymph node sample pool. Analysis on various sub-categories of tumours and lymph nodes showed this correlation was also present within samples characterised as malignant, round cell tumours, mesenchymal tumours, epithelial tumours, lymphoma, metastatic lymph nodes and reactive lymph nodes. These results indicate that hypoxia in canine tumours may result in an increased infiltration by Tregs.

Keywords

Glut1, FoxP3, cancer, canine

Introduction

Hypoxia, defined as a cellular oxygen tension below which biological functions are compromised, is a hallmark of solid tumours (Höckel and Vaupel 2001, Pinheiro et al 2014). Regions of hypoxia arise as a result of the unorganised and ineffective vasculature within tumours and their high metabolic demands (Brown and Giaccia 1998, Fenton et al 1999,

Freyer 1994, Konerding et al 1995, Secomb et al 1995). Hypoxia can promote malignant changes within a tumour by activating cellular survival responses, suppression of DNA repair, and providing a selection pressure in favour of more malignant clones (Brizel et al 1996, Hansen et al 2011, Höckel et al 1996, Kinoshita et al 2001, Luoto et al 2013, Pennacchietti et al 2003). Regions of hypoxia in tumours are widely accepted as a negative prognostic indicator and can result in resistance to radiotherapy and some forms of chemotherapy (Brown 2007, Comerford et al 2002, Grau and Overgaard 1992, Gray et al 1953, Koch et al 2003, Snyder 2008, Vaupel and Mayer 2007, Yan et al 2011). Hypoxia is also a common finding in inflammation, as cellular metabolic requirements are also increased in this context, and oxygen supply is disrupted by alteration of local tissue structure (Eltzschig and Carmeliet 2011, Mantovani et al 2008). Hypoxia has effects on the activity and survival of several immune cells whose ability to adapt to hypoxia is crucial for an effective immune response (Imtiyaz and Simon 2010, Scholz and Taylor 2013).

The response of cells to hypoxia is mediated by hypoxia inducible factor (HIF) molecules supressing or promoting the transcription of certain genes (Hansen et al 2011, Kallio et al 1999, Snyder et al 2008). These HIF-induced transcriptomic changes adapt the cell to the hypoxic environment, and some of the up-regulated proteins such as glucose transporter 1 (Glut1) and carbonic anhydrase IX have been shown to be biomarkers of hypoxia in certain tumours (Abbondati et al 2013, Airley et al 2003, Bussink et al 2003).

Regulatory T cells (Tregs) have long been a subject of interest in oncology. In the healthy animal their function is to prevent inappropriate immune responses, and their absence or lack of function may result in autoimmune disease (Brusko et al 2008). They carry out this function by suppressing other immune cells through the production of immunosuppressive cytokines such as interleukin-10 and through cell-cell contact (Campbell and Koch 2011, Josefowicz et al 2012, Thornton and Shevach 1998). However, this immunosuppressive

function can be to the body's detriment as Tregs are thought to be a mechanism by which tumours are able to suppress the body's anti-neoplastic immune response (Clarke et al 2006, Nishikawa and Sakaguchi 2010). To this end, high numbers of Tregs are found in a variety of different tumours and their draining lymph nodes, and in many cases higher frequencies correlate with poorer prognosis in man, rodent and dog (Deng et al 2010, Kim et al 2012, Liyanage et al 2006, Nishikawa and Sakaguchi 2010, Oh et al 2014, Pinheiro et al 2014). The principal marker for Tregs is the transcription factor Forkhead box P3 (FoxP3), which is crucial for Treg function and mutations in which result in severe autoimmune disease (Coffer and Burgering 2004).

While hypoxia and Tregs have each been shown to contribute individually to a poorer clinical outcome in cancer, their effects are likely to be complementary to each other. Tregs proliferate faster under hypoxic conditions in vitro and in vivo (Clambey et al 2012, Neildez-Nguyen et al 2015) and HIF1α induction increases their suppressive function in humans and mice (Ben-Shoshan et al 2008, Noman et al 2015). Tregs and hypoxia are positively correlated in a variety human tumours (Deng et al 2013, Duechler et al 2014, Facciabene et al 2011, Noman et al 2015, Yan et al 2011). All of these observations suggest a link between the two factors in promoting tumour survival, but as far as the authors are aware no such association has previously been demonstrated in dogs.

The purpose of this study was to further explore the link between Tregs and hypoxia within the context of canine tumours and lymph nodes. The molecules Glut1 and FoxP3 were used as biomarkers for hypoxia and Tregs respectively to investigate the hypothesis that **Tregs are more abundant in areas of tumours and lymph nodes displaying high Glut1 expression as a marker of hypoxia.**

Materials and Methods

Samples

The study included formalin-fixed and paraffin-embedded biopsy and necropsy samples of 57 tumours and 29 lymph nodes from the archives of the Royal Veterinary College Diagnostic Laboratory, all collected by licensed veterinarians for diagnostic purposes under the Veterinary Surgeons Act (1966) following written informed consent by the owners of the dogs. Benign and malignant tumour samples were as follows: 21 samples of epithelial cell origin [adenocarcinoma (n=2), anal sac adenocarcinoma (n=1), apocrine gland carcinoma (n=1), colonic papilloma (n=1), cutaneous papilloma (n=6), cystic trichoepithelioma (n=1), mammary adenocarcinoma (n=1), mammary adenoma (n=1), meibomian gland epithelioma (n=1), squamous cell carcinoma (n=6)], 14 samples of mesenchymal cell origin [fibrosarcoma (n=4), haemangioma (n=4), haemangiosarcoma (n=2), myopericytoma (n=1), soft tissue sarcoma (n=3)], and 22 samples of round cell origin [histiocytoma (n=13), lymphoma (n=5), mast cell tumour (n=4)]. The lymph node samples were categorised as being metastatic, i.e. containing neoplastic cells (MLNs; n=10), tumour draining, i.e. sampled alongside a confirmed neoplasm in their drainage area (TDLNs; n=7), and reactive, i.e. draining sites of inflammation without neoplasia (RLNs; n=12). Sequential sections were taken from each sample.

Immunohistochemistry (IHC)

Slides were incubated for 20 minutes at 55˚C to melt the wax, before deparaffinisation by immersion in Neoclear® for two 5 minute changes while still molten. The slides were then

hydrated by immersion in two 1 minute changes of 100% ethanol, then for 1 minute in 90% ethanol and 70% ethanol, before being placed into distilled water.

Antigen retrieval was conducted by immersing slides in a solution of 10mM citrate-buffered saline at pH 6 with 0.05% v/v Tween®-20 while heating in an autoclave to 126°C for 25 minutes. Slides were maintained at this temperature for five minutes for Glut1 and three minutes for FoxP3.

The slides were then immersed in a solution of 94% methanol and 6% hydrogen peroxide for 20 minutes to block endogenous peroxidases in the tissues; tap water was then slowly run into this solution for 5 minutes. Slides were rinsed with distilled water and then with PBS with 0.05% Tween®-20 (PBST). The tissues on the slides were covered with 10% normal goat serum (Vector Laboratories, S-1000) in PBST and incubated for 30 minutes at room temperature in a humid atmosphere. This blocking solution was replaced with stock antibody diluted in the serum blocking solution, or with the relevant isotype controls or no-primary controls incubated with only serum blocking solution (Supplementary Table 1). The slides were incubated overnight at 4˚C in a humid atmosphere.

The slides were rinsed in distilled water and PBST, then covered with appropriate secondary antibody (Supplementary Table 1) and incubated for 30 minutes at room temperature in a humid atmosphere, before rinsing again in distilled water and PBST. Tissues were then covered with DAB+ Substrate Chromogen Solution® (Dako K3467) for 5 minutes at room temperature and then rinsed in distilled water and PBST. Tissues were then covered with haematoxylin for 30 seconds to counterstain, then rinsed with distilled water. Samples were dehydrated by immersing for 30 seconds in 70% ethanol, 30 seconds in 90% ethanol, two 2 minute changes and a 3 minute change of 100% ethanol then three 5 minute changes of xylene. The samples were then mounted with DPX (Fisher, 12658646).

Image analysis

Each slide was visually inspected for antibody labelling, and an image was taken at regions of interest (ROIs) on each Glut1-labelled slide at a 20X magnification using a Leica DFC300 FX camera (Leica Microsystems UK LTD). Another image was taken at the corresponding sites on the FoxP3-labelled slide. Up to 21 ROI were selected within each sample following a standardized, radial pattern (Supplemental Figure 1) in order to be representative of the whole section, with size and shape differences necessitating variation of the pattern in some samples.

Each ROI was categorised for its Glut expression using an established method (Abbondati et al 2013, Petty et al 2008). Images of the Glut1-labelled slides were first given a score based on the estimated proportion of cells showing positive Glut1 expression, with <1% given a score of 0, 2-50% given a score of 1, and 51-100% given a score of 2. A macro was then developed using Volocity® software (PerkinElmer Inc, Massachusetts) to highlight areas that had an intensity of labelling above a threshold we had arbitrarily designated as "strong". The criteria for this designation were: hue from 201 to 37, saturation above 80 and intensity above 14. Areas smaller than 10 pixels were discarded as noise. ROIs were given an intensity score of 1 or 2 predicated on whether this strong expression was estimated to be present in less than or greater than 50% of the labelled cells respectively. Both of these measurements were made by a single observer, and the product of these two scores was then taken to give the ROI a final Glut1 immuno-reactivity score of either 0, 1, 2 or 4. Examples of these scores are given in Supplemental Figures 2-6.

The numbers of $FoxP3^+$ cells present within each ROI were then quantified. Each image was first assessed for regions of exclusion, defined as areas of vasculature, dead space and folded tissue due to antigen retrieval. If regions of exclusion were present they were covered

over in green using Paint.NET software (Figure 6) and their area measured using Volocity®. The images were then considered for presence of endogenous pigments including melanin, haemosiderin and lipofuscin. If these pigments constituted <1% area of the ROI a macro developed in Volocity® was applied to determine the Treg count, an example of which is given in Figure 6. If these pigments constituted >1% area of an ROI, Tregs were manually counted with the help of the counter function of Volocity®. Manual and automated counts gave similar results when compared on non-pigmented samples. The area taken up by regions of exclusion in each image as a proportion of the total image area was used to standardize the $FoxP3^+$ cell number per ROI to an equal area of viable tissue between ROIs.

Statistical testing

Statistical analysis was conducted using SPSS software (IBM). A generalized estimating equation (GEE) with ordinal logistic link function was used to assess association between Glut1 immunoreactivity score and tumour origins, histotypes and malignancy. Exchangeable correlation matrix was used to account for repeated measurements from the same tumour sample. Odds ratio (OR) and its 95% confidence intervals (CI) were reported. Similarly, GEE with a negative binomial log link function was used to assess the association between $Foxp3^+$ cell counts and several factors. The factors tested for correlation with $FoxP3^+$ cells count in tumour samples were: Glut1 immunoreactivity score, whether the tumour was round cell, epithelial or mesenchymal in origin, and whether the tumour was benign or malignant. In lymph node samples $FoxP3^+$ cell numbers were correlated with Glut1 immunoreactivity score and whether the sample was a MLN, TDLN or RLR. The correlation between Glut1 immunoreactivity score and $Foxp3^+$ cell numbers was also assessed within the individual tumour and lymph node categories. Rate ratio (RR) and its 95% CI were reported. Graphs were made using R software (R Foundation for Statistical Computing).

Results

Glut1 expression

There was moderate variation in Glut1 labelling between samples (Figures 1a-3a). No significant difference in Glut1 immunoreactivity was found between different categories of lymph nodes (Figure 4a), but malignant tumours had significantly lower expression than benign ones (Figure 4b, OR=0.160, 95% CI: 0.075-0.343, p<0.001). Mesenchymal tumours had significantly lower Glut1 immunoreactivity than epithelial tumours (OR=0.325, 95% CI: 0.124-0.851, p=0.022) although no other significant differences between histotypes could be observed (Figure 4c).

FoxP3 expression

$FoxP3^+$ cells were identified in all lymph node samples and 73.7% of the tumour samples (n=42). Their numbers varied greatly both between samples and between different ROIs within samples (Figures 1b-3b), with the maximum number of $FoxP3^+$ cells in a ROI being 1872 while in some other ROI they were completely absent. The distribution pattern of $FoxP3^+$ cells also varied between sample types. In round cell tumours they tended to be scattered throughout the tumour interspersed between the neoplastic cells, while in epithelial and mesenchymal tumours they were mostly found in the stroma between neoplastic cells or areas of lymphocytic infiltration, although some had infiltrated between the neoplastic cells themselves.

Mesenchymal tumours have less $FoxP3^+$ cell infiltration than those of epithelial or round cell origin

Samples of mesenchymal origin had significantly fewer FoxP3+ cells per ROI than the epithelial (RR=0.073, 95% CI: 0.018-0.297, p<0.001) and round cell samples (RR=0.147, 95% CI: 0.041-0.528, p=0.003), although no difference was observed between the other two

193 histotypes (Figure 5). No difference in FoxP3+ cell prevalence was found between benign
194 and malignant samples (p=0.094, Figure 6).

195 *Prevalence of FoxP3$^+$ cells correlates with Glut1 expression in tumours in…*

196 *Total tumour sample pool*

197 In the overall sample pool ROIs with the higher Glut1 immunoreactivity scores of 2 and 4
198 had significantly higher FoxP3$^+$ cell counts than those with scores of 0 (respectively
199 RR=1.912, 95% CI: 1.455-2.513, p<0.001, and RR=1.895, 95% CI: 1.118-2.277, p=0.010) or
200 1 (respectively RR=2.433, 95% CI: 1.872-3.161 p<0.001, and RR=2.030, 95% CI: 1.448-
201 3.018, p<0.001), although differences could not be demonstrated within these respective pairs
202 of groups (Figure 7a).

203 *Malignant tumours*

204 When the analysis was conducted on only the malignant tumour samples the same
205 relationship was shown to be present as in the total tumour sample pool (Figure 7b).
206 Immunoreactivity scores of 4 were associated with significantly higher FoxP3$^+$ cell counts
207 than regions scoring 0 (RR=2.565, 95% CI: 1.091-6.032, p=0.031) or 1 (RR=3.367, 95% CI:
208 1.334-8.499, p=0.010), and ROIs with scores of 2 also had significantly higher FoxP3$^+$ cell
209 counts than those scoring 0 (RR=2.337, 95% CI: 1.445-3.785, p=0.001) or 1 (RR=3.068, 95%
210 CI: 1.589-5.930, p=0.001). No differences were found between scores of 2 and 4 or between
211 0 and 1. Benign samples did not show the same pattern of correlation (Figure 7c).

212 *Round cell tumours*

213 Samples of round cell origin also displayed the same correlation between Glut1
214 immunoreactivity score and FoxP3$^+$ cell count as the overall tumour sample pool (Figure 7d).
215 ROIs with scores of 4 had significantly higher FoxP3$^+$ cell counts than those with scores of 0

(RR=3.177, 95% CI: 1.297-7.783, p=0.011) or 1 (RR=5.360, 95% CI: 2.170-13.22, p<0.001), as did scores of 2 (respectively RR=3.71, 95% CI: 2.121-6.475, p<0.001, and RR=6.253, 95% CI: 2.898-13.49, p<0.001). No differences were found between scores of 2 and 4 or between 0 and 1.

Mesenchymal tumours

Another correlation between Glut1 immunoreactivity score and $FoxP3^+$ cell count was found within mesenchymal tumours (Figure 7e). ROIs with immunoreactivity scores of 4 had significantly higher $FoxP3^+$ cell counts than those with scores of 0 (RR=7.207, 95% CI: 3.353-15.50, p<0.001), 1 (RR=9.300, 95% CI: 4.855-17.80, p<0.001) or 2 (RR=6.184, 95% CI: 3.17-12.06, p<0.001), and ROIs with scores of 2 had significantly higher $FoxP3^+$ cell counts than those with scores of 1 (RR=1.502, 95% CI: 1.226-1.842, p<0.001). No other significant differences between scores was found.

Epithelial tumours

In epithelial tumours (Figure 7f) ROIs with an immunoreactivity score of 2 had significantly higher $FoxP3^+$ cell counts than those with scores of 1 (RR=1.891, 95% CI: 1.302-2.748, p=0.001). The analysis also reported ROIs with scores of 0 as having higher counts than those with scores of 1 or 4, but this is thought to be an artefact from a single anomalous sample.

Lymphoma

Lymphoma samples also demonstrated a correlation between Glut1 immunoreactivity score and FoxP3+ cell count (Figure 7g). ROIs with scores of 2 and 4 could not be differentiated, but they each had significantly higher counts than those with scores of 0 (respectively RR=4.380, 95% CI: 2.489-7.706, p<0.001, and RR=8.331, 95% CI: 3.702-18.73, p<0.001) and 1 (respectively RR=9.459, 95% CI: 6.153-14.54, p<0.001; RR=17.975, 95% CI: 8.568-

37.713, p<0.001). ROIs with scores of 0 also had significantly higher counts than those scoring 1 (RR=2.159, 95% CI: 1.642-2.838, p<0.001).

Prevalence of FoxP3$^+$ cells did not differ between lymph node categories

No significant difference was found between the different categories of lymph node (p=0.085, figure 8).

Prevalence of FoxP3$^+$ cells correlates with Glut1 expression in lymph nodes in…

Total lymph node sample pool

In the overall pool of lymph node samples a higher number of FoxP3$^+$ cells correlated with a higher Glut1 immunoreactivity score (Figure 9a), with ROIs scoring 4 having significantly higher counts than those scoring 2 (RR=1.218, 95% CI: 1.033-1.436, p=0.019), and those scoring 2 having higher counts than those scoring 1 (RR=3.183, 95% CI: 2.678-3.781, p<0.001).

MLNs

When the analysis was conducted on MLNs (Figure 9b) the same correlation was found as that in the overall lymph node pool, with ROIs scoring 4 having significantly higher FoxP3$^+$ cell counts than those scoring 2 (RR=1.343, 95% CI: 1.259-1.763, p=0.034), and ROIs scoring 2 in turn having significantly higher counts than those scoring 1 (RR=6.666, 95% CI: 6.172-7.199, p<0.001).

RLNs

A weaker correlation was between Glut1 immunoreactivity score and FoxP3$^+$ cell count was found in RLNs (Figure 9c), with ROIs scoring 4 or 2 having significantly higher counts than those scoring 1 (respectively RR=3.330, 95% CI: 2.340-4.735, p<0.001, and RR=2.855, 95% CI: 2.125-3.838, p<0.001)

No difference could be found between Glut1 immunoreactivity scores in TDLNs (Figure 9d).

Discussion

This study set out to explore the hypothesis that there is a relationship between the presence of hypoxia and Tregs in canine tumours and lymph nodes, using Glut1 and FoxP3 as their respective markers in IHC. Our data demonstrated a positive correlation between Glut1 expression and the prevalence of $FoxP3^+$ cells in both tumours and lymph nodes, which to the authors' knowledge is a novel observation in canines. This relationship was present individually within all three tumour histotypes, as well as specifically within lymphoma cases and both reactive and metastatic lymph nodes. The finding that there was a positive correlation within malignant but not benign tumours was most likely because hypoxia is a less significant determinant of Glut1 expression in benign tumours as they have less abnormal vasculature (Skinner et al 1995); many factors other than hypoxia are known to regulate Glut1 expression, such as glucose and insulin levels (Ciaraldi et al 1995), and the presence of various hormones (Ishida et al 1995, Medina and Owen 2002).

That fewer Tregs were observed in mesenchymal origin tumours may be due to differences in tissue structure resulting in a reduced ability of Tregs to infiltrate these tumours. In epithelial tumours Tregs were normally found within the stroma, which appeared to act as a scaffold to allow them to infiltrate from the vasculature; this facilitation appeared less commonly in mesenchymal samples.

Benign tumours were observed to have higher Glut1 expression than malignant samples and epithelial tumours to have higher expression than mesenchymal tumours, thought likely to reflect factors other than hypoxia. The latter finding is most likely explained by the fact that epithelial cells generally appeared to express Glut1 more strongly than other cell types

even in normal parts of the tissue, and so higher Glut1 expression in these tissues is unlikely to indicate higher levels of hypoxia. More likely this indicates a generally higher level of glucose uptake in epithelial tissues, possibly as a result of greater metabolic demand due to secretory functions in many cases. This shows that while Glut1 can be indicative of hypoxia, tissue differences mean the marker is limited for comparisons between tumour types. Since Glut1 expression has previously been correlated with malignancy our finding to the contrary is most likely due to the differences in method; previous studies correlated Glut1 with specific features of malignancy or with long term clinical outcome, whereas the current study was limited to broader histological classification (Haber et al 1998, Rudlowski et al 2003, Younes et al 1995). Previous studies also looked at different levels of malignancy within single types of cancer, whereas we compared a variety of different tumour types. Since, as mentioned previously, Glut1 expression varies between tissue types our finding that benign tumours had higher expression is most likely due to the weight of different tumour types in the sample pool.

As Glut1 is a membrane transport molecule it is unlikely that Tregs are directly attracted to the molecule itself. Since hypoxia is known to influence Glut1 expression we speculate that hypoxia is the factor linking these observations, according with a number of studies that have shown a relationship between Tregs and hypoxia in species other than the dog (Airley et al 2003, Bussink et al 2003, Clambey et al 2012, Deng et al 2013, Duechler et al 2014, Facciabene et al 2011, Neildez-Nguyen et al 2015). A hypoxia-mediated increase in Treg numbers could be attributed to direct stimulation of Treg induction, infiltration or expansion by HIF, similar to that seen in mucosal inflammatory hypoxia (Clambey et al 2012) or due to stimulation by other hypoxia-induced molecules in the environment, such as transforming growth factor β which is synthesised by some hypoxic cells and implicated in Treg differentiation (Falanga et al 1991, Fu et al 2004).

In contrast to previous studies on both canine and human tumours, our results failed to detect a correlation between malignancy and numbers of $FoxP3^+$ cells (Kim et al 2012, Oh et al 2014, Ozgur et al 2014, Wang et al 2015, Yan et al 2011). We also failed to find a significant difference between the numbers of $FoxP3^+$ cells present in different categories of lymph nodes, where previous studies had found higher numbers of Tregs in tumour draining lymph nodes (Nishikawa and Sakaguchi 2010). This could have reflected the diverse range of tumours in the current study, categorized broadly as benign or malignant, making it less sensitive in this respect compared to previous studies that have sought to correlate Treg numbers with particular features of malignancy within specific tumour types, or it could be a result of differences in measurement due to the use of different techniques.

There were several limitations to this study. FoxP3 can be transiently expressed by non-regulatory T cells so positive expression in cells did not categorically identify them as Tregs nor did it differentiate the different Treg subclasses (Wang et al 2007); similarly Glut1 expression is influenced by factors other than hypoxia (Ciaraldi et al 1995, Haney 2001, Sakoda et al 2000). Further research using a greater number of samples of a particular tumour type and using additional markers would help address these issues; however, despite their limitations both FoxP3 and Glut1 are commonly used as biomarkers for Tregs and hypoxia respectively, and FoxP3 in particular is commonly accepted as the best single marker for Tregs.

The biggest strength of this study was the large number of repeated measurements taken per sample. By taking measurements at up to 21 locations in each tumour or lymph node it allowed our analysis to take into account both differences between samples and between different regions within samples, and allowed a large amount of data to be collected relative to the number of samples used.

Conclusion

This study revealed a significant relationship between Treg numbers and the levels of Glut1 expression that we speculate was driven by hypoxia. As far as we are aware this is a novel finding in dogs, and further research into this relationship is warranted as both hypoxia and Tregs are thought to be important factors in tumour prognosis and offer potential targets for novel therapies. A better understanding of their interactions could lead to more effective treatment protocols in future.

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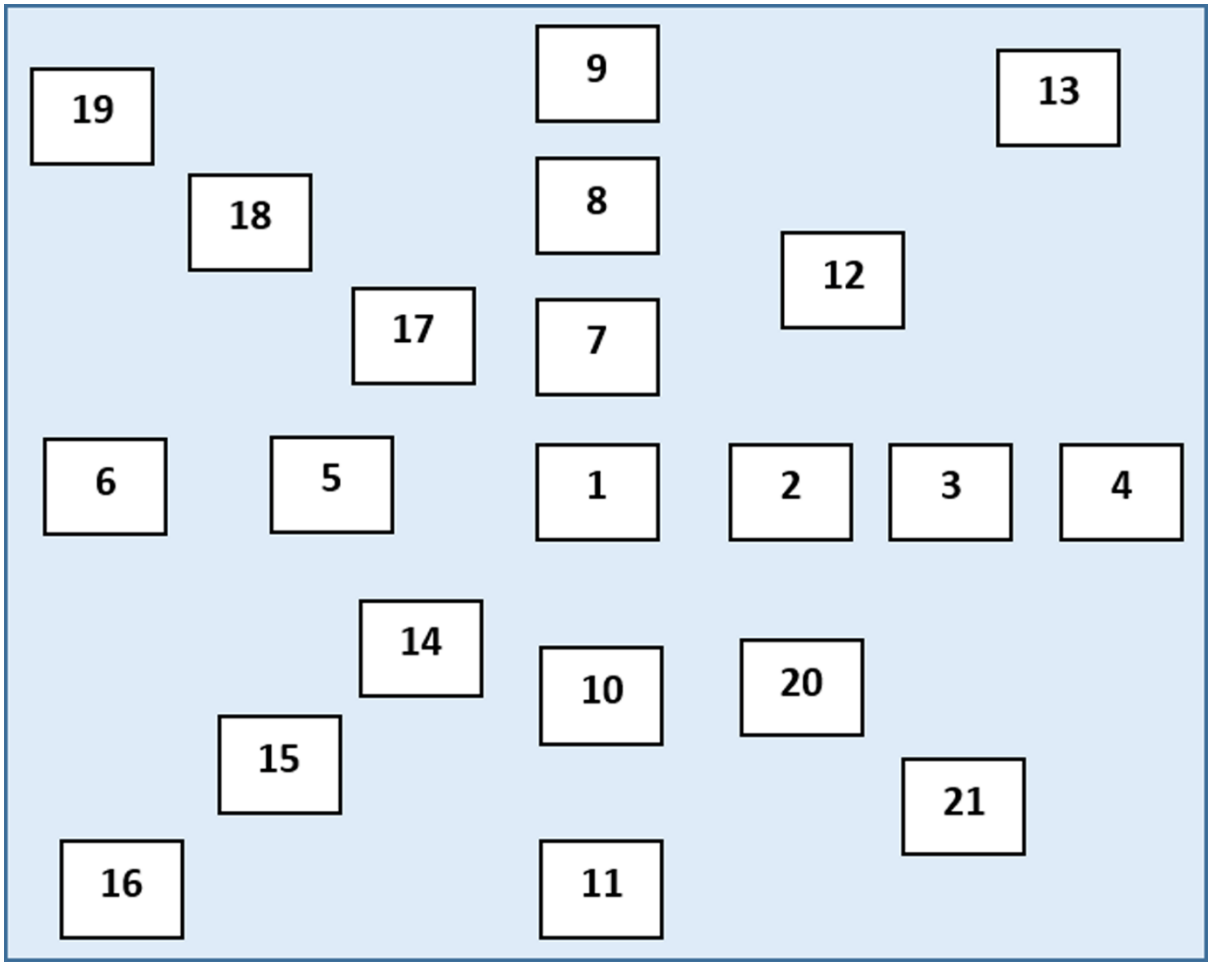
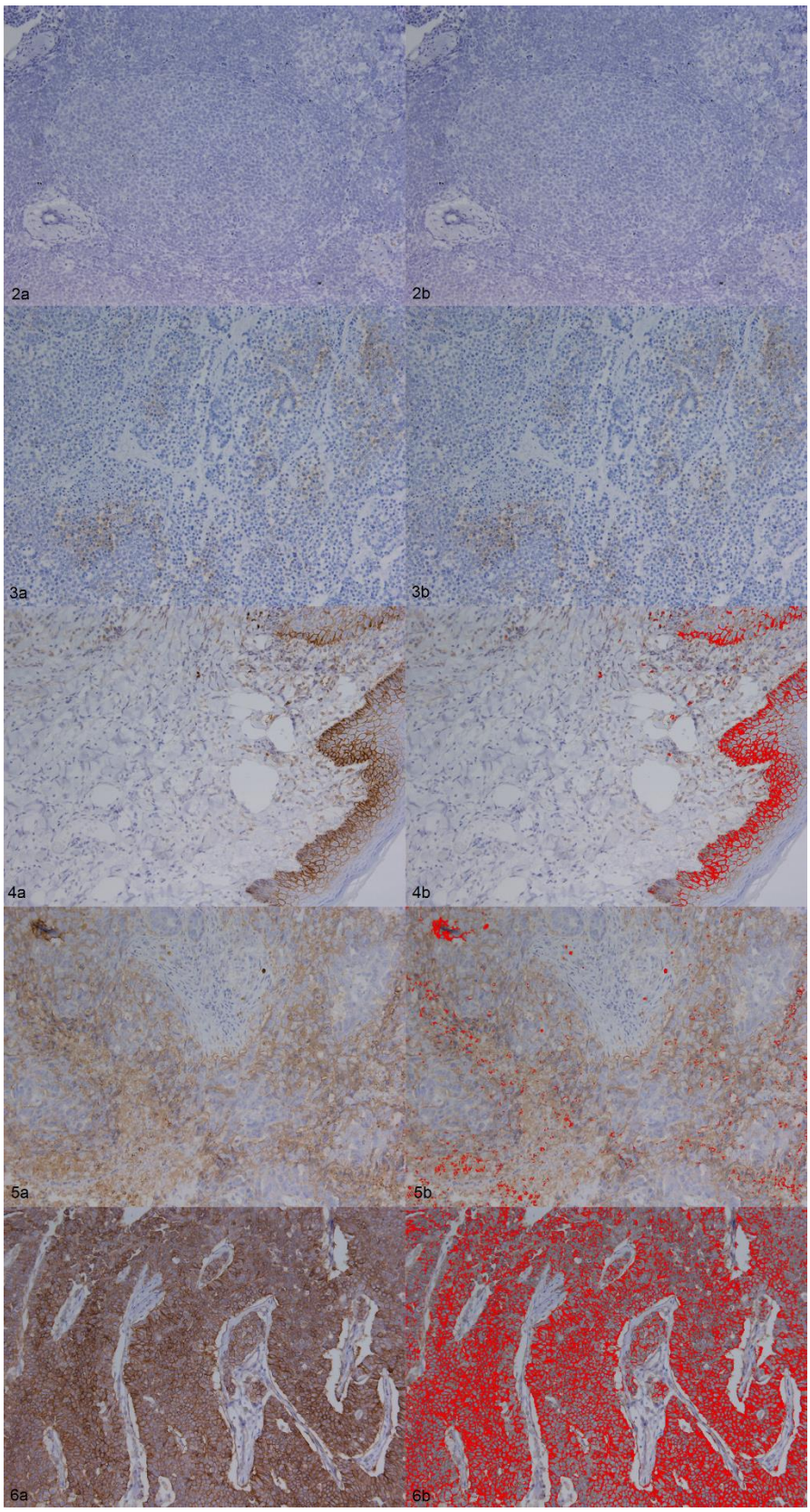
Figure legends

Supplemental Figure 1. General pattern of ROI (region of interest) selection. Blue box indicates tissue section on slide, numbered boxes indicate location of ROIs and the order they were selected.



Supplemental Figures 2-6. Dog, immunohistochemistry for glucose transporter 1 (Glut1), hematoxylin and eosin. Red highlighting in (b) images indicates regions of strong labelling.



Supplementary Fig. 2. Dog, immunohistochemistry for glucose transporter 1 (Glut1). Red highlighting in (b) images indicates regions of strong labelling. Lymphoma, lymph node. Absence of Glut1 labelling results in an immunoreactivity score of 0.

Supplementary Fig. 3. Dog, immunohistochemistry for glucose transporter 1 (Glut1). (a) Unaltered images of samples. Red highlighting in (b) images indicates regions of strong labelling. Adenocarcinoma metastasis, unknown site. Few cells weakly labelling with Glut1 results in a reactivity score of 1.

Supplementary Fig. 4. Dog, immunohistochemistry for glucose transporter 1 (Glut1). (a) Unaltered images of samples. Red highlighting in (b) images indicates regions of strong labelling. Cutaneous papilloma. Low percentage of cells labelling for Glut 1, but the majority of positive cells label strongly, so the region has an immunoreactivity score of 2.

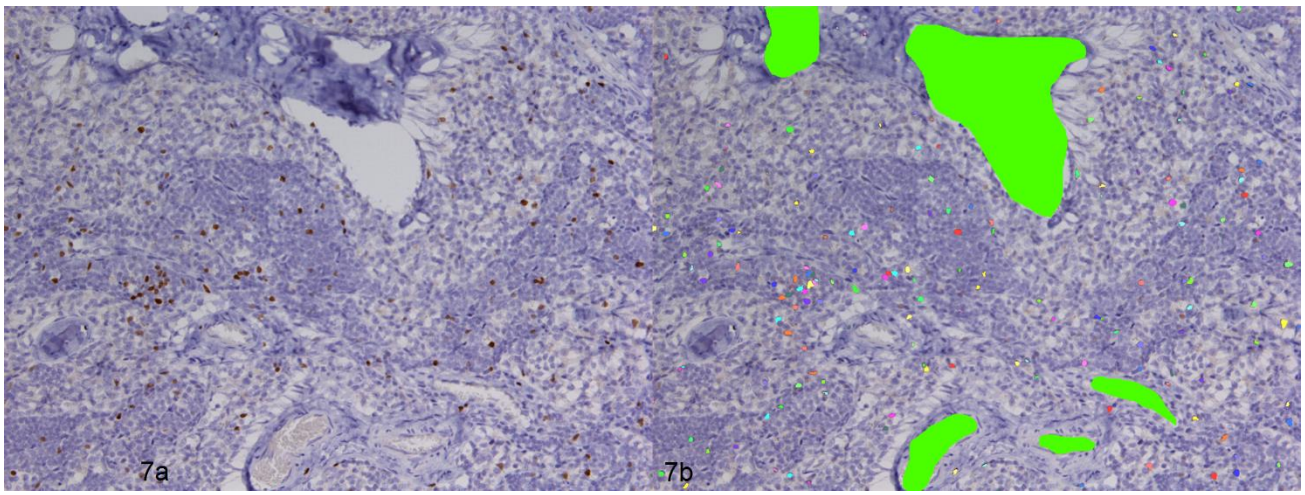
Supplementary Fig. 5. Dog, immunohistochemistry for glucose transporter 1 (Glut1). (a) Unaltered images of samples. Red highlighting in (b) images indicates regions of strong labelling. Squamous cell carcinoma metastasis, unknown site. The majority of cells label for Glut1, but few of these label strongly, yielding an immunoreactivity score of 2.

Supplementary Fig. 6. Dog, immunohistochemistry for glucose transporter 1 (Glut1). (a) Unaltered images of samples. Red highlighting in (b) images indicates regions of strong labelling. Anal sac adenocarcinoma. The majority of cells label for Glut1 and the majority of these label strongly, yielding an immunoreactivity score of 4.

554 **Supplemental Figure 7a.** Malignant melanoma metastasis to mandibular lymph node,

555 labelled for Forkhead Box P3 (FoxP3). **Figure 7b.** Regions of exclusion highlighted in green;

556 $FoxP3^{+}$ cells counted by Volocity® macro highlighted in contrasting colours.



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560 **Figure 1-3.** Dog, hematoxylin and eosin. (a) Images labelled by immunohistochemistry for
561 glucose transporter 1 (Glut1), (b) images labelled for Forkhead Box P3 (Foxp3).

562 **Figure 2.** Squamous cell carcinoma, unknown site. Strong Glut1 expression in the nests of
563 neoplastic cells, particularly towards the periphery. Connective tissue and infiltrating
564 lymphocytes between nests display lower expression. Most $FoxP3^+$ cells are present in the
565 connective tissue, with small numbers infiltrating between the neoplastic cells.

566 **Figure 3.** Soft tissue sarcoma, unknown site. Uneven labelling of neoplastic cells by Glut1.
567 Small number of $FoxP3^+$ cells are present infiltrating between the neoplastic cells.

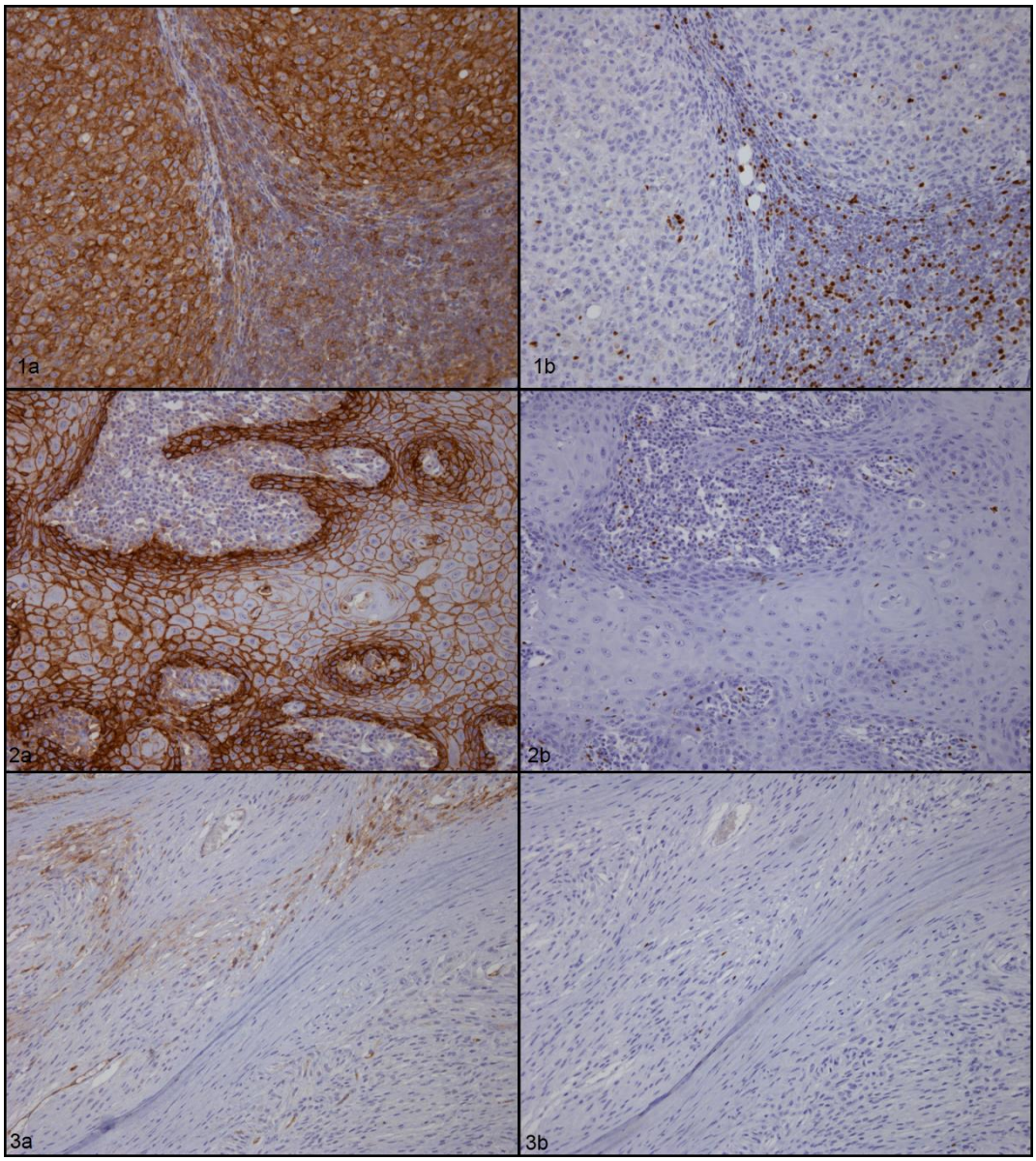

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Figure 4. Proportions of different glucose transporter 1 (Glut1) immunoreactivity scores compared between (a) different lymph node categories, (b) benign and malignant tumours, (c) different tumour histotypes.

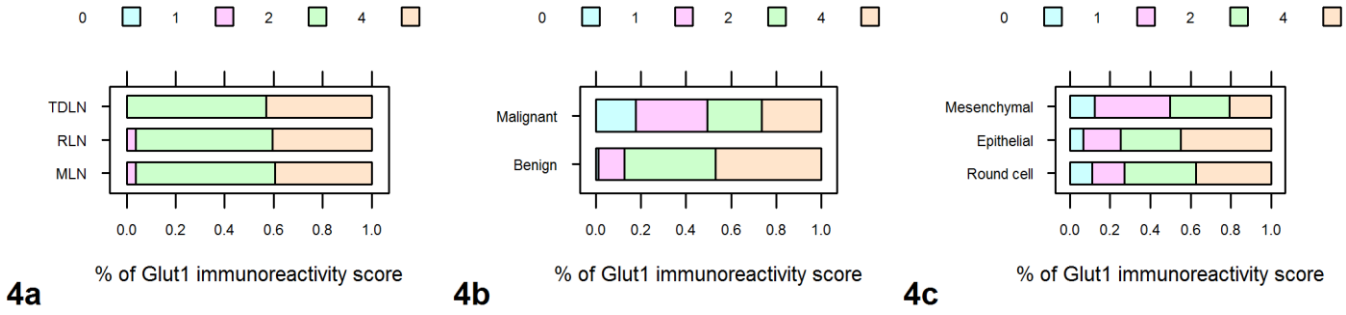
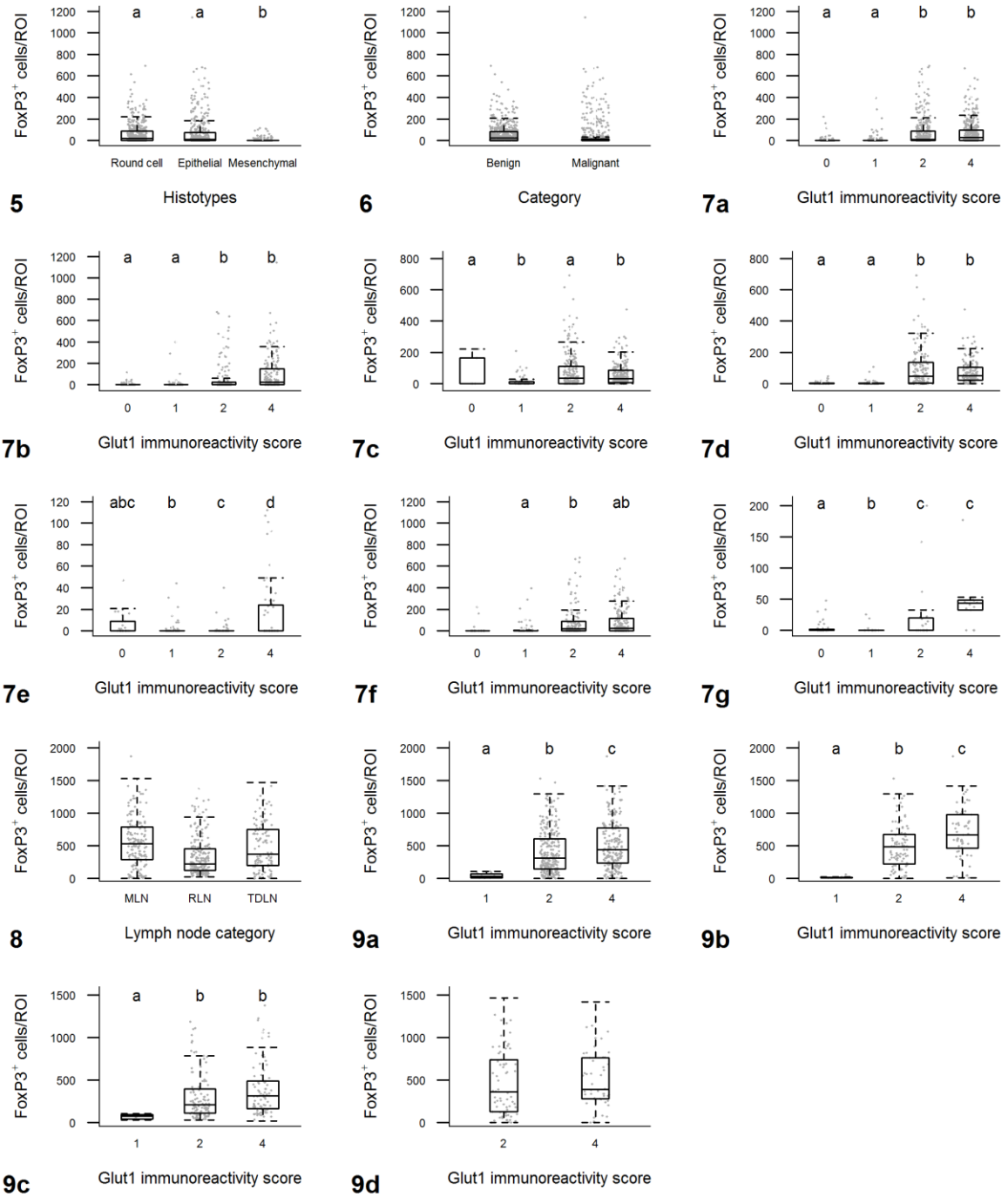


Figure 5-7. Comparisons of the number of FoxP3^{+} cells per region of interest (ROI) in tumours. Circles indicate individual ROIs. The median, 25th and 75th percentiles are indicated by the box, and the whiskers indicate the highest and lowest values within 1.5 times the length of the quartiles. Letters group results that were not significantly different, where results do not share a letter a statistically significant difference ($p<0.05$) was present. **Figure 5.** Comparison between different tumour histotypes. **Figure 6.** Comparison between benign and malignant tumours. **Figure 7.** Comparisons between different glucose transporter 1 (Glut1) immunoreactivity in different categories of tumours. **Figure 7a.** All tumour samples. **Figure 7b.** Malignant samples. **Figure 7c.** Benign samples. **Figure 7d.** Samples of round cell origin. **Figure 7e.** Mesenchymal cell origin. **Figure 7f.** Epithelial cell origin. **Figure 7g.** Lymphoma samples.

Figures 8 and 9. Comparisons of the number of FoxP3^{+} cells per region of interest (ROI) in lymph nodes. Circles indicate individual ROIs. The median, 25th and 75th percentiles are indicated by the box, and the whiskers indicate the highest and lowest values within 1.5 times the length of the quartiles. Letters group results that were not significantly different, where

results do not share a letter a statistically significant difference ($p<0.05$) was present. **Figure 8.** Comparison between lymph node categories. **Figure 9.** Comparison between Glut1 immunoreactivity scores in different lymph node categories **Figure 9a.** All lymph node samples. **Figure 9b.** Metastatic lymph nodes. **Figure 9c.** Reactive lymph nodes. **Figure 9d.** Tumour draining lymph nodes.



595 **Supplementary Table 1.** Antibodies used for immunohistochemistry with their respective

596 controls and concentrations

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Supplementary Table 1. Antibodies used for immunohistochemistry with their respective controls and concentrations							
Antibody	Supplier	Product code/clone number	Raised in	Positive control tissue	Negative control	Secondary antibody	Concentration
Polyclonal anti-canine glucose transporter 1 antibody	Abcam	ab15309	Rabbit	Healthy kidney	Normal rabbit IgG (Vector Labs I-1000)	Envision HRP® (Dako K4002)	2µg/ml
Monoclonal anti-canine FoxP3	Ebioscience	14-4321-85 / eBR2a	Rat	Reactive lymph node	Rat Ig isotype control (eBioscience 14-4321-85, clone eBR2a)	ImmPRESS HRP Anti-Rat Ig® (Vector Labs MP-7404)	5µg/ml