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Prevalence of FoxP3<sup>+</sup> cells in canine tumours and lymph nodes positively correlates with glucose transporter 1 expression

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#### 1 Summary

2 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 3 4 two factors in canine neoplasia. Samples of 57 canine tumours and 29 canine lymph nodes 5 categorised as metastatic, draining or reactive were obtained. Sequential sections were 6 labelled by immunohistochemistry for glucose transporter 1 (Glut1) and FoxP3 as markers of 7 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 8 9 the number of FoxP3<sup>+</sup> cells at each ROI was counted. A generalised estimating equation with 10 negative binomial log link function was used to determine an association between Glut1 expression and FoxP3<sup>+</sup> cell count. Higher Glut1 immunoreactivity was correlated with 11 12 significantly higher numbers of FoxP3<sup>+</sup> cells in the total tumour sample pool and total lymph node sample pool. Analysis on various sub-categories of tumours and lymph nodes showed 13 this correlation was also present within samples characterised as malignant, round cell 14 tumours, mesenchymal tumours, epithelial tumours, lymphoma, metastatic lymph nodes and 15 reactive lymph nodes. These results indicate that hypoxia in canine tumours may result in an 16 17 increased infiltration by Tregs.

## 18 Keywords

19 Glut1, FoxP3, cancer, canine

## 20 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,

25 Freyer 1994, Konerding et al 1995, Secomb et al 1995). Hypoxia can promote malignant 26 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 27 28 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 29 prognostic indicator and can result in resistance to radiotherapy and some forms of 30 31 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 32 33 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 34 (Eltzschig and Carmeliet 2011, Mantovani et al 2008). Hypoxia has effects on the activity 35 36 and survival of several immune cells whose ability to adapt to hypoxia is crucial for an 37 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 38 supressing or promoting the transcription of certain genes (Hansen et al 2011, Kallio et al 39 1999, Snyder et al 2008). These HIF-induced transcriptomic changes adapt the cell to the 40 41 hypoxic environment, and some of the up-regulated proteins such as glucose transporter 1 42 (Glut1) and carbonic anhydrase IX have been shown to be biomarkers of hypoxia in certain

43 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

50 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, 51 Nishikawa and Sakaguchi 2010). To this end, high numbers of Tregs are found in a variety of 52 53 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, 54 Liyanage et al 2006, Nishikawa and Sakaguchi 2010, Oh et al 2014, Pinheiro et al 2014). The 55 56 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 57 58 and Burgering 2004).

59 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 60 61 proliferate faster under hypoxic conditions in vitro and in vivo (Clambey et al 2012, Neildez-Nguyen et al 2015) and HIF1 $\alpha$  induction increases their suppressive function in humans and 62 mice (Ben-Shoshan et al 2008, Noman et al 2015). Tregs and hypoxia are positively 63 correlated in a variety human tumours (Deng et al 2013, Duechler et al 2014, Facciabene et al 64 2011, Noman et al 2015, Yan et al 2011). All of these observations suggest a link between the 65 66 two factors in promoting tumour survival, but as far as the authors are aware no such 67 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.

74

# 75 Materials and Methods

76 *Samples* 

77	The study included formalin-fixed and paraffin-embedded biopsy and necropsy samples of
78	57 tumours and 29 lymph nodes from the archives of the Royal Veterinary College
79	Diagnostic Laboratory, all collected by licensed veterinarians for diagnostic purposes under
80	the Veterinary Surgeons Act (1966) following written informed consent by the owners of the
81	dogs. Benign and malignant tumour samples were as follows: 21 samples of epithelial cell
82	origin [adenocarcinoma (n=2), anal sac adenocarcinoma (n=1), apocrine gland carcinoma
83	(n=1), colonic papilloma (n=1), cutaneous papilloma (n=6), cystic trichoepithelioma (n=1),
84	mammary adenocarcinoma (n=1), mammary adenoma (n=1), meibomian gland epithelioma
85	(n=1), squamous cell carcinoma (n=6)], 14 samples of mesenchymal cell origin
86	[fibrosarcoma (n=4), haemangioma (n=4), haemangiosarcoma (n=2), myopericytoma (n=1),
87	soft tissue sarcoma (n=3)], and 22 samples of round cell origin [histiocytoma (n=13),
88	lymphoma (n=5), mast cell tumour (n=4)]. The lymph node samples were categorised as
89	being metastatic, i.e. containing neoplastic cells (MLNs; n=10), tumour draining, i.e. sampled
90	alongside a confirmed neoplasm in their drainage area (TDLNs; n=7), and reactive, i.e.
91	draining sites of inflammation without neoplasia (RLNs; n=12). Sequential sections were
92	taken from each sample.

93

# 94 Immunohistochemistry (IHC)

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

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

Antigen retrieval was conducted by immersing slides in a solution of 10mM citratebuffered saline at pH 6 with 0.05% v/v Tween<sup>®</sup>-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 103 104 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 105 with 0.05% Tween<sup>®</sup>-20 (PBST). The tissues on the slides were covered with 10% normal 106 107 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 108 diluted in the serum blocking solution, or with the relevant isotype controls or no-primary 109 controls incubated with only serum blocking solution (Supplementary Table 1). The slides 110 were incubated overnight at 4°C in a humid atmosphere. 111

The slides were rinsed in distilled water and PBST, then covered with appropriate 112 secondary antibody (Supplementary Table 1) and incubated for 30 minutes at room 113 temperature in a humid atmosphere, before rinsing again in distilled water and PBST. Tissues 114 were then covered with DAB+ Substrate Chromogen Solution® (Dako K3467) for 5 minutes 115 at room temperature and then rinsed in distilled water and PBST. Tissues were then covered 116 with haematoxylin for 30 seconds to counterstain, then rinsed with distilled water. Samples 117 were dehydrated by immersing for 30 seconds in 70% ethanol, 30 seconds in 90% ethanol, 118 two 2 minute changes and a 3 minute change of 100% ethanol then three 5 minute changes of 119 120 xylene. The samples were then mounted with DPX (Fisher, 12658646).

#### 121 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 129 et al 2013, Petty et al 2008). Images of the Glut1-labelled slides were first given a score 130 based on the estimated proportion of cells showing positive Glut1 expression, with <1% 131 given a score of 0, 2-50% given a score of 1, and 51-100% given a score of 2. A macro was 132 then developed using Volocity<sup>®</sup> software (PerkinElmer Inc, Massachusetts) to highlight areas 133 that had an intensity of labelling above a threshold we had arbitrarily designated as "strong". 134 The criteria for this designation were: hue from 201 to 37, saturation above 80 and intensity 135 above 14. Areas smaller than 10 pixels were discarded as noise. ROIs were given an intensity 136 score of 1 or 2 predicated on whether this strong expression was estimated to be present in 137 less than or greater than 50% of the labelled cells respectively. Both of these measurements 138 were made by a single observer, and the product of these two scores was then taken to give 139 the ROI a final Glut1 immuno-reactivity score of either 0, 1, 2 or 4. Examples of these scores 140 are given in Supplemental Figures 2-6. 141

142 The numbers of FoxP3<sup>+</sup> cells present within each ROI were then quantified. Each image 143 was first assessed for regions of exclusion, defined as areas of vasculature, dead space and 144 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<sup>®</sup>. 145 The images were then considered for presence of endogenous pigments including melanin, 146 haemosiderin and lipofuscin. If these pigments constituted <1% area of the ROI a macro 147 developed in Volocity<sup>®</sup> was applied to determine the Treg count, an example of which is 148 given in Figure 6. If these pigments constituted >1% area of an ROI, Tregs were manually 149 counted with the help of the counter function of Volocity<sup>®</sup>. Manual and automated counts 150 gave similar results when compared on non-pigmented samples. The area taken up by regions 151 of exclusion in each image as a proportion of the total image area was used to standardize the 152 FoxP3<sup>+</sup> cell number per ROI to an equal area of viable tissue between ROIs. 153

## 154 *Statistical testing*

Statistical analysis was conducted using SPSS software (IBM). A generalized estimating 155 equation (GEE) with ordinal logistic link function was used to assess association between 156 Glut1 immunoreactivity score and tumour origins, histotypes and malignancy. Exchangeable 157 correlation matrix was used to account for repeated measurements from the same tumour 158 sample. Odds ratio (OR) and its 95% confidence intervals (CI) were reported. Similarly, GEE 159 with a negative binomial log link function was used to assess the association between Foxp3<sup>+</sup> 160 cell counts and several factors. The factors tested for correlation with FoxP3<sup>+</sup> cells count in 161 tumour samples were: Glut1 immunoreactivity score, whether the tumour was round cell, 162 epithelial or mesenchymal in origin, and whether the tumour was benign or malignant. In 163 lymph node samples FoxP3<sup>+</sup> cell numbers were correlated with Glut1 immunoreactivity score 164 and whether the sample was a MLN, TDLN or RLR. The correlation between Glut1 165 immunoreactivity score and Foxp3<sup>+</sup> cell numbers was also assessed within the individual 166 tumour and lymph node categories. Rate ratio (RR) and its 95% CI were reported. Graphs 167 were made using R software (R Foundation for Statistical Computing). 168

#### 169 **Results**

#### 170 *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).

#### 178 FoxP3 expression

FoxP3<sup>+</sup> cells were identified in all lymph node samples and 73.7% of the tumour samples 179 (n=42). Their numbers varied greatly both between samples and between different ROIs 180 within samples (Figures 1b-3b), with the maximum number of FoxP3<sup>+</sup> cells in a ROI being 181 1872 while in some other ROI they were completely absent. The distribution pattern of 182 FoxP3<sup>+</sup> cells also varied between sample types. In round cell tumours they tended to be 183 184 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 185 areas of lymphocytic infiltration, although some had infiltrated between the neoplastic cells 186 187 themselves.

Mesenchymal tumours have less FoxP3<sup>+</sup> 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,</li>
95% CI: 0.041-0.528, p=0.003), although no difference was observed between the other two

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

195 *Prevalence of FoxP3*<sup>+</sup> *cells correlates with Glut1 expression in tumours in...* 

196 *<u>Total tumour sample pool</u>* 

197 In the overall sample pool ROIs with the higher Glut1 immunoreactivity scores of 2 and 4

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-

3.018, p<0.001), although differences could not be demonstrated within these respective pairs</li>
of groups (Figure 7a).

#### 203 <u>Malignant tumours</u>

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

212 <u>Round cell tumours</u>

Samples of round cell origin also displayed the same correlation between Glut1

immunoreactivity score and FoxP3<sup>+</sup> cell count as the overall tumour sample pool (Figure 7d).

ROIs with scores of 4 had significantly higher  $FoxP3^+$  cell counts than those with scores of 0

216	(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).
217	as did scores of 2 (respectively RR=3.71, 95% CI: 2.121-6.475, p<0.001, and RR=6.253,

1 (DD = 0.00 0 00 0 10 00 10 00

0.001

218 95% CI: 2.898-13.49, p<0.001). No differences were found between scores of 2 and 4 or

0.011

between 0 and 1.

## 220 <u>Mesenchymal tumours</u>

Another correlation between Glut1 immunoreactivity score and FoxP3<sup>+</sup> cell count was

found within mesenchymal tumours (Figure 7e). ROIs with immunoreactivity scores of 4 had

significantly higher FoxP3<sup>+</sup> cell counts than those with scores of 0 (RR=7.207, 95% CI:

224 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<sup>+</sup> 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.

0 177 050/ OT 1 007 7 700

#### 228 <u>Epithelial tumours</u>

In epithelial tumours (Figure 7f) ROIs with an immunoreactivity score of 2 had

significantly higher FoxP3<sup>+</sup> 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.

## 234 <u>Lymphoma</u>

235 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

238 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-

- 240 37.713, p<0.001). ROIs with scores of 0 also had significantly higher counts than those
- 241 scoring 1 (RR=2.159, 95% CI: 1.642-2.838, p<0.001).
- 242 *Prevalence of FoxP3*<sup>+</sup> *cells did not differ between lymph node categories*
- 243 No significant difference was found between the different categories of lymph node
  244 (p=0.085, figure 8).
- 245 *Prevalence of FoxP3*<sup>+</sup> *cells correlates with Glut1 expression in lymph nodes in...*
- 246 <u>Total lymph node sample pool</u>
- In the overall pool of lymph node samples a higher number of FoxP3<sup>+</sup> cells correlated with
- a higher Glut1 immunoreactivity score (Figure 9a), with ROIs scoring 4 having significantly
- 249 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,
- 251 p<0.001).
- 252 <u>MLNs</u>

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<sup>+</sup> 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).

258 <u>*RLNs*</u>

A weaker correlation was between Glut1 immunoreactivity score and FoxP3<sup>+</sup> 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) 263 No difference could be found between Glut1 immunoreactivity scores in TDLNs (Figure264 9d).

## 265 Discussion

This study set out to explore the hypothesis that there is a relationship between the 266 presence of hypoxia and Tregs in canine tumours and lymph nodes, using Glut1 and FoxP3 as 267 their respective markers in IHC. Our data demonstrated a positive correlation between Glut1 268 expression and the prevalence of FoxP3<sup>+</sup> cells in both tumours and lymph nodes, which to the 269 authors' knowledge is a novel observation in canines. This relationship was present 270 individually within all three tumour histotypes, as well as specifically within lymphoma cases 271 and both reactive and metastatic lymph nodes. The finding that there was a positive 272 correlation within malignant but not benign tumours was most likely because hypoxia is a 273 274 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 275 276 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). 277

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 287 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 288 glucose uptake in epithelial tissues, possibly as a result of greater metabolic demand due to 289 290 secretory functions in many cases. This shows that while Glut1 can be indicative of hypoxia, 291 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 292 293 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 294 295 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 296 297 single types of cancer, whereas we compared a variety of different tumour types. Since, as 298 mentioned previously, Glut1 expression varies between tissue types our finding that benign 299 tumours had higher expression is most likely due to the weight of different tumour types in the sample pool. 300

As Glut1 is a membrane transport molecule it is unlikely that Tregs are directly attracted 301 to the molecule itself. Since hypoxia is known to influence Glut1 expression we speculate 302 303 that hypoxia is the factor linking these observations, according with a number of studies that 304 have shown a relationship between Tregs and hypoxia in species other than the dog (Airley et 305 al 2003, Bussink et al 2003, Clambey et al 2012, Deng et al 2013, Duechler et al 2014, 306 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 307 308 by HIF, similar to that seen in mucosal inflammatory hypoxia (Clambey et al 2012) or due to 309 stimulation by other hypoxia-induced molecules in the environment, such as transforming 310 growth factor  $\beta$  which is synthesised by some hypoxic cells and implicated in Treg differentiation (Falanga et al 1991, Fu et al 2004). 311

In contrast to previous studies on both canine and human tumours, our results failed to 312 detect a correlation between malignancy and numbers of FoxP3<sup>+</sup> cells (Kim et al 2012, Oh et 313 al 2014, Ozgur et al 2014, Wang et al 2015, Yan et al 2011). We also failed to find a 314 significant difference between the numbers of FoxP3<sup>+</sup> cells present in different categories of 315 lymph nodes, where previous studies had found higher numbers of Tregs in tumour draining 316 lymph nodes (Nishikawa and Sakaguchi 2010). This could have reflected the diverse range of 317 tumours in the current study, categorized broadly as benign or malignant, making it less 318 sensitive in this respect compared to previous studies that have sought to correlate Treg 319 320 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. 321

322 There were several limitations to this study. FoxP3 can be transiently expressed by non-323 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 324 expression is influenced by factors other than hypoxia (Ciaraldi et al 1995, Haney 2001, 325 Sakoda et al 2000). Further research using a greater number of samples of a particular tumour 326 type and using additional markers would help address these issues; however, despite their 327 328 limitations both FoxP3 and Glut1 are commonly used as biomarkers for Tregs and hypoxia 329 respectively, and FoxP3 in particular is commonly accepted as the best single marker for 330 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.

## 336 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.

343

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349

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# 525 Figure legends

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



529

- 531 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.

549 Supplementary Fig. 6. Dog, immunohistochemistry for glucose transporter 1 (Glut1). (a)

550 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,
- labelled for Forkhead Box P3 (FoxP3). **Figure 7b.** Regions of exclusion highlighted in green;
- 556 FoxP3<sup>+</sup> cells counted by Volocity<sup>®</sup> macro highlighted in contrasting colours.



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

Figure 2. Squamous cell carcinoma, unknown site. Strong Glut1 expression in the nests of
neoplastic cells, particularly towards the periphery. Connective tissue and infiltrating
lymphocytes between nests display lower expression. Most FoxP3<sup>+</sup> cells are present in the
connective tissue, with small numbers infiltrating between the neoplastic cells.

**Figure 3.** Soft tissue sarcoma, unknown site. Uneven labelling of neoplastic cells by Glut1.

567 Small number of FoxP3<sup>+</sup> cells are present infiltrating between the neoplastic cells.



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<sup>+</sup> cells per region of interest (ROI) in 574 tumours. Circles indicate individual ROIs. The median, 25<sup>th</sup> and 75<sup>th</sup> percentiles are indicated 575 by the box, and the whiskers indicate the highest and lowest values within 1.5 times the 576 length of the quartiles. Letters group results that were not significantly different, where 577 results do not share a letter a statistically significant difference (p<0.05) was present. Figure 578 579 5. Comparison between different tumour histotypes. Figure 6. Comparison between benign 580 and malignant tumours. Figure 7. Comparisons between different glucose transporter 1 (Glut1) immunoreactivity in different categories of tumours. Figure 7a. All tumour samples. 581 582 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. 583 Lymphoma samples. 584

**Figures 8 and 9.** Comparisons of the number of FoxP3<sup>+</sup> cells per region of interest (ROI) in lymph nodes. Circles indicate individual ROIs. The median, 25<sup>th</sup> and 75<sup>th</sup> 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</li>
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

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
Monoclona I anti- canine FoxP3	Ebioscienc e	14-4321- 85 / eBR2a	Rat	Reactive lymph node	Rat Ig isotype control (eBioscienc e 14-4321- 85, clone eBR2a)	ImmPRES S HRP Anti-Rat Ig® (Vector Labs MP- 7404)	5µg/ml