**Expression of cannabinoid receptors CB1 and CB2 in canine cutaneous mast cell tumours**

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**Abstract**

Cannabinoid receptors (CB1 and CB2) belong to endocannabinoid system (ECS), which is also composed from endocannabinoids and the enzymatic systems involved in their biosynthesis and degradation. The expression of CB1 and CB2 have been previously identified in normal canine mast cell and in atopic dermatitis. Canine cutaneous mast cell tumours (cMCTs) are among the most common cutaneous neoplasms in dogs and have a highly variable clinical behaviour. Expression of CB1-CB2 was assessed by means of immunohistochemistry in thirty-seven dogs (from 2019 to 2021) with proven histological diagnosis of cMCT. Dogs were divided in two groups according to the Kiupel’s grading system: high-grade (HG) cMCT and low-grade (LG) cMCT. A semiquantitative (score 0-3) and quantitative assessment of immunoreactivity (IR) was performed for each case. Our results show that there CB1 and CB2 are highly expressed in LG- cMCT, in contrast to HG- cMCT.

**Keywords**

Cannabinoid; Dog; Mast cell tumor; Histopathology; Immunohistochemistry

**1. Introduction**

The Endocannabinoid System (ECS) is composed of CB1 and CB2 cannabinoid receptors, the endocannabinoids (N-arachidonoylethanolamide, anandamide [AEA] and 2-arachidonoylglycerol [2-AG]), and the enzymatic systems involved in their biosynthesis, and degradation (fatty acid amine hydrolase [FAAH]; monoacylglycerol lipase [MAGL]).

CB1 and CB2 receptors have been shown to regulate many intracellular signal transduction pathways with transcriptional targets such as protein kinase B (PKB) and mitogen-activated protein kinases (MAPKs) (Samson et al., 2003). The nucleotide sequences of CB1 and CB2 receptors, despite their different anatomical distribution between humans and animals (Silver, 2019) are strongly conserved in mammals and belong to the G-protein-coupled receptor (GPCRs) superfamily (Anday and Mercier, 2005). The ECS has been largely validated as a therapeutic target for a variety of neurological, metabolic, immune, and neoplastic diseases (Alenabi and Malekinejad, 2021; Lu and Mackie, 2016; Shah et al., 2021). Many *in vitro* and *in vivo* studies have been carried out on the ECS in humans (Pisanti et al., 2009; Shah et al., 2021; Xu et al., 2006), whereas in small animals research, knowledge is limited. There are only few recent studies on the signalling of endogenous ligand in canine inflammatory disease (Febo et al., 2021) and in neoplastic disease (Hay et al., 2022) as well as on CB1 and CB2 expression in non-neoplastic conditions (Campora et al., 2012; Galiazzo et al., 2018; Polidoro et al., 2021; Stanzani et al., 2020). Among these, a study has described the presence of cannabinoid receptors on canine mast cells (MC) present in healthy skin and recruited in atopic dermatitis (Campora et al., 2012), setting the rational for their investigation in canine mast cell tumours (MCT).

Cutaneous MCT (cMCT) is the most common cutaneous neoplasia in dogs, making up to 21% of all skin tumours (Bostock, 1986) and has a wide degree of biological behaviours, ranging from benign to aggressive clinical presentations, characterised by widespread metastases and high rate of tumour related death (Blackwood et al., 2012). The histological grade (Patnaik I-III and Kiupel Low-High) represents one of the most robust prognostic factors (Blackwood et al., 2012; Kiupel et al., 2011; Murphy et al., 2004; Patnaik et al., 1984), even if this has to be associated to a thorough clinical staging work-up in order to refine prognosis, and to identify the most appropriate treatment approach (Blackwood et al., 2012; Warland et al., 2014).

The primary objective of this study was to evaluate the expression of CB1 and CB2 in cMCT by means of immunohistochemistry (IHC); as a secondary objective we aimed at investigating its correlation with tumour’s grading, to determine whether the ECS may represent a possible target for future clinical trials.

**2. Materials and Methods**

**2.1 Inclusion Criteria**

Medical records of client-owned dogs (from 2019 to 2021) with diagnosed with cMCT were retrieved by computerised database search from the Small Animal Teaching Hospital of Liverpool University (Neston, United Kingdom) and the Veterinary Teaching Hospital of Teramo University (Piano D'Accio, Teramo, Italy). To be eligible for recruitment, 1) dogs had to had surgical excision of the primary cMCT, 2) required a histological diagnosis of cMCT, detailed according to the Patnaik and Kiupel system, 3) and available formalin fixed paraffin embedded (FFPE) tissues. Patient’s signalment and clinical data were retrieved from the patients’ records. Information on clinical stage, when available, were obtained by means of hematological and biochemical analysis, cytological evaluation of the cMCT and regional lymph node, thoracic radiographs, abdominal ultrasound, and cytology of liver and spleen. Clinical stage was classified according to World Health Organization (WHO) (Owen,1980). Two groups were obtained: Low-grade cMCT (LG-cMCT) including histologic diagnosis of Patnaik I-II grade and Low-Kiupel grade and High-grade cMCT (HG-cMCT) including histologic diagnosis of Patnaik II-III grade and High-grade Kiupel. Dogs were excluded from the study if they were known to have received steroids, nonsteroidal anti-inflammatory drugs, and/or antineoplastic chemotherapy prior to the tissue collection.

**2.2 Histopathology & Immunohistochemistry**

The slides were originally prepared from FFPE tissues fixed in 10% neutral buffered formalin, routinely stained with haematoxylin and eosin (HE) and observed under a bright field upright microscope. All diagnoses were performed by board certified pathologists from commercial and University veterinary pathology laboratories and graded according to the Patnaik and Kiupel systems (Kiupel et al., 2011; Patnaik et al., 1984). Representative sections of the lesions were selected for immunohistochemistry (IHC). All sections were deparaffinized in xylene and hydrated with graded ethanol concentration up to distilled water. Tissue sections were heat-treated for antigen retrieval (3 X 5 min in microwave oven at 600 W, dipped in citrate buffer 0.01M pH 6.0) and then incubated overnight at 4°C with the following primary antibodies, which had been previously tested in the canine species (Campora et al., 2012): rabbit polyclonal antibody anti-CB1 (rabbit polyclonal, dilution 1:100, Abcam ab23703, UK) or rabbit polyclonal antibody anti-CB2 (rabbit polyclonal, dilution 1:100, Abcam ab45942, UK). Immune reactions were revealed by means of an avidin-biotin-peroxidase kit (Vectastain Elite ABC kit, Vector, Burlingame, CA, USA) and visualized using 3,3'-diaminobenzidine as a chromogen, Sigma-Aldrich, St Louis, MO, USA). As positive controls we used canine normal hippocampus for CB1 and lymph nodes for CB2 as previously described (Campora et al., 2012). Negative controls were obtained by omitting the primary antibody and by replacing it with an unrelated rabbit polyclonal antibody (anti-Von Willebrand factor, dilution 1:700, A0082, Dakocytomaton, Glostrup, Denmark). For each IHC marker the immune reactivity (IR) was semi-quantitatively scored as follows: 0, negative; 1, < 25% IR-neoplastic MC; 2, 26-50% IR-neoplastic MC; 3, >51% IR-neoplastic MC. Each slide was evaluated by two pathologists. To minimize the subjectivity of IHC evaluation, the IR was quantitatively scored using and *ad hoc* software (*ImageJ*, National Institutes of Health, Bethesda, USA) (Schneider et al., 2012) by taking 5 representative pictures of randomly selected, not overlapping fields, from each slide (Nikon Eclipse E600, Digital Camera DXM1200), at the final magnification X 400, avoiding the edges of tissue sections. The quote of immunoreactive cells was quantified as percentage of the entire microscopic field (IR percentage, IRp).

The study was approved by the University of Liverpool Veterinary Research Ethics Committee (VREC1066).

**2.3 Statistical analysis**

Statistical association between CB1 and CB2 expression and tumours’ histological low and high grade was investigated. The Shapiro-Wilk test was used to verify if the data were normally distributed. Therefore, data were analysed by non-parametric Mann-Whitney using SPSS (version 12.0; SPSS Inc., Chicago, Illinois, USA) to compare differences between the groups. The level for accepted statistical significance was p<0.05

**3. Results**

**3.1 Patient population**

Thirty-seven dogs satisfied the inclusion criteria. There were 9 cross breed, 4 Labrador Retriever, 3 for each of the following breeds: Boxer, Jack Russel terrier; 2 for each the following breeds: Pitt bull, Shar-Pei, Vizla, Staffordshire bull terrier; 1 for each of the following breeds: Yorkshire terrier, Beagle, Dogo Argentino, French bulldog, English setter, American Staffordshire terrier, Pug, Australian cattle dog, Maltese, English springer spaniel. There were 20 females (14 spayed) and 17 males (12 neutered). The median age was 9 years (range 1 – 16). Clinical data are summarized in Table 1.

**3.2 Histological grading and clinical staging**

The results of histological grading and clinical stage are shown in Table 1. Based on gradings’ distribution, the study population was divided in two groups, according to the Kiupel’s grading system: HG-cMCT (17 cMCT of grade II-III Patnaik/high-grade Kiupel) and LG-cMCT (20 cMCT of grade I-II Patnaik/Low-grade Kiupel).There were 23 cases in clinical stage I WHO, 13 patients in clinical stage II WHO, and 1 dog in clinical stage IV WHO (Table 1), due to the low number of LG and HG per each WHO group, analytic statistic was not run.

**3.3 Immunohistochemistry for CB1**

Data are summarized in Table 2; where discrepancy was identified, agreement was reached upon observation at multi-head microscope. In the LG-cMCT group, in 13 out of 20 samples neoplastic MC showed a strong and diffuse cytoplasmic immune reactivity (IR) for CB1 (score 3); IRr ranged between 16.9% and 37.05% (Figure 1). Intermediate IR for CB1 (score 2) was observed in 4 cases, with IRp ranging between 4,83% and 14.19%, while a low IR was detected in 3 cMCT (score 1), with IRp ranging between 0.63% and 1.43%.

In the HG-cMCT group IR was low in most of the samples (Figure 1) with 14 out of 17 cases classified as score 1 (mean IRp ranging between 0.23% and 3.23%). Two cases had an IR score 2 (mean IRp ranging between 3.56% and 7.11%), while 1 case was negative for CB1 (following both quantitative and semiquantitative evaluation). When CB1 IR and IRp were compared between LG- and HC-cMCT groups, these were significantly higher in the LG-cMCT group (p<0.05).

**3.4 Immunohistochemistry for CB2**

Data are summarized in Table 3; where discrepancy was identified, agreement was reached upon observation at multi-head microscope. Concerning the LG-cMCT group, in 9 out of 20 cases, neoplastic MC showed a strong and diffuse cytoplasmic IR for CB2 (score 3); IRp ranged between 10.67% and 21.34% (Figure 1). Four LG-cMCT demonstrated an IR score 2 with an IRp ranging between 3.94% and 7.86%, while the remaining 2 cases showed an IR score 1 with an IRp ranging between 0.13% and 2.26%). Five LG-cMCT were negative for CB2 (following both quantitative and semiquantitative evaluation).

In the HG-cMCT group, 13 out of 17 were negative for CB2 (following both quantitative and semiquantitative evaluation). CB2 IR was classified as score 1 in the remaining 4 cases with a IRp ranging between 0.4% and 2.5% (Figure 1). When CB2 IR and IRp were compared between LG- and HG-cMCT groups, these were significantly higher in the LG-cMCT group (p<0.05).

**4. Discussion**

To the best of our knowledge, there are not previous veterinary studies focusing on the expression of cannabinoids receptors in spontaneous tumours of dogs and cats; this differs from human and experimental medicines where the correlation between ECS and neoplastic diseases has been more thoroughly explored (Shah et al., 2021).

CB1 and CB2 receptors have a polypeptide structure spans the plasma membrane (Felder and Glass, 1998), but the immunolocalization has been observed at a cytoplasmic level, which occurs because the protein permanently and constitutively cycles between the plasma membrane and endosomes (Leterrier et al., 2004). This mechanism results in a predominantly intracellular localization of the receptor, leading to a steady state between exposed and removed CB1 membrane receptors (Mercati et al., 2012). Our results agree with previous literature on human normal dermal MC and mastocytosis, canine normal dermal MC and atopic dermatitis, confirming that cytoplasmic immunolocalization of CB1 and CB2 is also conserved in neoplastic MC. In our study Western Blot (WB) for anti-CB2 was not performed, whereas the anti-CB1 had been validated in the canine specie by the manufacturer. Although we cannot guarantee on anti-CB2 specificity, the positive control demonstrated cell membrane immune localization and preferential expression in cells of the immune system with higher expression in B cells; this would suggest adequate specificity and that lack of WB does not invalidate our results.

Downstream effects of CB1 and CB2 may vary based on the cell type, however, these include suppression of adenylate cyclase (and hence inhibition of cAMP-dependent pathways), including several ion channels together with impact on downstream signalling of PKB, Raf-1, MAPKs, JNK, p38, c-fos, c-jun among many more (Demuth and Molleman, 2006; Samson et al., 2003). A study conducted on murine MC demonstrated, co-expression of CB1 and CB2 on normal MC and that CB2 was the predominant mediator of signalling to PKB and MAPKs (and presumably, to their downstream transcriptional targets). CB1 ligation suppressed high-affinity IgE receptor (FcεRI)-induced serotonin release, and cannabinoid application did exert a suppressive effect on mediator release from MC (Samson et al., 2003).

The results of our study confirmed a positive IR for CB1 and CB2 in canine cMCT. Both the semiquantitative and quantitative evaluation of expression were similar between CB1 and CB2, highlighting the presence of a significantly higher IR in LG-MCT cases when compared to the HG-MCT group. This suggest a negative correlation between CB1 and CB2 IR and cMCT aggressiveness. It is plausible that the conserved expression of cannabinoid receptors in low-grade tumours is related to the phenotype of neoplastic MC that often reminds that of normal MC, and MC recruited in inflammatory responses (Campora et al., 2012). On the contrary, receptors’ IR is mainly lost in high-grade cMCT where tumours generally present with more aggressive biological features and higher degree of cellular atypia (Blackwood et al., 2012; Kiupel et al., 2011). Conversely it would be speculative to comment on the downstream effect of CB1 and CB2 in cMCT and the consequences that loss of IR may bring in high-grade diseases.

CB1 and CB2 expression is very variable in human tumours. In a 2003 study (Casanova et al., 2003), the immunoreactivity of CB1 and CB2 was investigated in both murine and human non-melanoma skin cancer. Authors reported that CB1 and CB2 receptors are expressed in both normal and tumours skin. In particular, cannabinoid receptors were expressed in normal murine skin, as well as in benign (papilloma) and malignant (squamous cell carcinoma) neoplasm. *In vitro*, pharmacological activation of cannabinoid receptors induced apoptosis in cancer cells while the viability of non-tumorigenic epidermal cells remained unaffected. In a more recent study on squamous cell carcinoma of the tongue, the increased expression of CB1 was considered a positive prognostic marker (Theocharis et al., 2016). In addition, Xu and colleagues (Xu et al., 2006) reported the expression of CB1 and CB2 in human liver cancer cells, correlating receptor overexpression to a better prognosis. In particular, they highlighted that in patients with greater CB1 and CB2 IR, negative prognostic factors such as poor cell differentiation and portal vein invasion were a less frequent phenomena. In pancreatic cancer, expression of both CB1 and CB2 receptors was increased compared to the normal pancreas (Carracedo et al., 2006), and an increased in CB1 expression has been related to a worse prognosis (Michalski et al., 2008). Similarly, in prostate cancer, the increase in CB1 expression coincided with a greater tumour’s aggressiveness (Chung et al., 2009), considering this as a negative prognostic marker (Cipriano et al., 2013). The CB1 receptor has also been evaluated in tumours of the nervous system, in which its physiological presence has been well documented (Hu and Mackie., 2015); CB1 overexpression in neoplastic nervous tissue has been associated with tumour regression in glioblastoma and paediatric gliomas (Schley et al., 2009; Sredni et al., 2016).

CB2 receptor has been shown to have a negative prognostic role in breast tumours in women, where overexpression of CB2 has been detected in more than 90% of Human Epidermal Growth Factor Receptor-2 (HER-2) positive tumours. On the contrary, in oestrogen-dependent and oestrogen-independent breast tumours, the high receptor affinity was considered a positive prognostic marker (Caffarel et al., 2010; Pérez-Gómez et al., 2015). Elbaz et al, (Elbaz et al., 2017) reported that the expression of these receptors in oestrogen-receptor-positive and oestrogen-receptor-negative mammary tumours was related to a better prognosis. The high degree of variability observed among studies in terms of association between CB1 and CB2 IR, tumour histotype and prognosis highlight the complexity of the ECS and the potential to impact different downstream signalling pathways based on the tissue’s origin (Demuth and Molleman, 2006).

The correlation between ECS and neoplastic diseases is a topic of interest for many scientists, focusing particularly on the use of cannabinoids receptors as a target for new therapeutic strategies. In human lung cancer the use of cannabidiol (CBD), non- psychotropic cannabinoids, increased the expression of intercellular adhesion molecule (ICAM), which are known for preventing the development of metastases (Haustein et al., 2014). Some studies indicate an impact of cannabinoids on the expression of vascular endothelial growth factor (VEGF), which is one of the main drivers of tumours’ angiogenesis (Vaccani et al., 2005). In gliomas, for instance, CBD reduces the expression of pro-angiogenetic factors (Vaccani et al., 2005). Furthermore Delta-9-tetrahydrocannabinol (THC), administrated with CBD, inhibited the proliferation of human glioblastoma cells (Marcu et al., 2010). Specifically in epithelial skin tumours, the activation of the cannabinoid receptors results in interference with inhibition of growth impaired vascularization (Bowles et al., 2012) and induction of apoptosis in tumorigenic epidermal cells (Kupczyk et al., 2009; Pisanti et al., 2009). CB receptors are expressed *in vitro* in murine and human melanoma cells (Blázquez et al., 2006) and their proliferation is inhibited by synthetic cannabinoid, which instead have no effect on normal melanocytes. A study shows that a drug composed by equal amounts of THC and CBD in the mice bearing BRAF wild-type melanoma xenografts substantially inhibited melanoma viability, proliferation, and tumour growth paralleled by an increase in autophagy and apoptosis compared with standard single-agent temozolomide (Armstrong et al., 2015). In two studies, Soliman et al. (Soliman et al., 2016; Soliman and Van Dross, 2016) examined specifically anandamide (AEA), a natural ligand for both CB1 and CB2. This ligand is metabolized by cyclooxygenase-2 (COX-2) to a novel metabolite, whose production was required for AEA cell death. In these studies it was reported that AEA was selected for cancer cells because the endogenous level of COX-2 are low in non-tumorigenic cells. The apoptosis induced by AEA, in non-melanoma skin cancer, was mediated by oxidative and endoplasmatic reticulum (ER) stress.

Chemotherapy is administered as a neo adjunctive treatment to facilitate surgical resection of certain MCTs, as an adjuvant treatment for MCT with a high risk of developing metastases, in case of metastatic disease and in cases where complete surgical excision has not been achieved (Warland et al., 2014). In cases of multiple low-grade MCTs or frequent development of *de novo* MCTs there is no consensus among veterinary oncologists, however, surgery may not be considered a feasible option. In the study by Taylor et al. in 2009 (Taylor et al., 2009) the approach was more conservative with an immunosuppressive/cytostatic therapy administered orally based on chlorambucil and steroids. This has been shown to induce few side effects in the face of a median survival time of nearly 5 months.

In dogs, the first therapeutic choice for low-grade mast cell tumours is wide-margin surgery, which in patients with negative staging is often resolutive. Today, the administration of cannabinoids and cannabimimetic compounds (e.g. PEA) reduces skin inflammation (Endocannabinoid Research Group et al., 2010), pain and itching (Kupczyk et al., 2009) in mice. Similarly considering these preliminary data, it could be hypothesized that the neoadjuvant or adjuvant use of cannabinoids as supportive therapy could be explored in clinical trials for patients with multiple low-grade mast cell tumours (also described as WHO stage III cMCT). Limitations of this study are the low number of patients in each WHO stage and lack of follow-up, which could have resulted in underestimating the association between CB1 and CB2 expression and prognosis in canine MCTs. Lastly, due to the retrospective nature of the study, CB1 and CB2 expression was not followed by the measuring of circulating endocannabinoids; however, this is the object of future investigations by our group.

**Declaration of competing interest**

The authors declare no conflicts of interest.

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**Picture Legend**

**Figure 1:**

1. - Diffuse and intense cytoplasmatic CB1 immunoreactivity in Low-grade cutaneous mast cell tumor.Bar = 100µm, low magnification. (b) - Diffuse and intense cytoplasmatic CB1 immunoreactivity in Low-grade cutaneous mast cell tumor. Bar = 25µm, high magnification. (c) - Diffuse and intense cytoplasmatic CB2 immunoreactivity in Low-grade cutaneous mast cell tumor - Low magnification. (d) - Diffuse and intense cytoplasmatic CB2 immunoreactivity in Low-grade cutaneous mast cell tumor. High magnification. (e) - Weak cytoplasmatic CB1 immunoreactivity in tumor cells of High-grade cutaneous mast cell tumor, low magnification. (f) - Weak cytoplasmatic CB1 immunoreactivity in mast cell tumor of High-grade cutaneous mast cell tumor, high magnification. (g) - Moderate to weak cytoplasmatic CB2 immunoreactivity in scattered High-grade cutaneous mast cell tumor. Low magnification. (h) - Moderate to weak cytoplasmatic CB2 immunoreactivity in scattered High-grade cutaneous mast cell tumor. High magnification.

**Figure 2**:

Positive and negative controls for the immunohistochemical experiments. a) CB1 immunoreactivity in canine brain: Positive cytoplasmic staining in the neurons of hippocampus; b). CB2 immunoreactivity in canine lymph node: Positive B lymphocytes. c). Negative control with an unrelated polyclonal antibody against Factor VIII: Canine low-grade MCT, negative tumour cells and cytoplasmic immunostaining of endothelial cells of blood vessels within the tumour mass.