Prognostic Factors for Canine Cutaneous Mast Cell Tumors including Ki67, MAC387, Factor 8 and MMP-9 immunohistochemistry

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Objective– To evaluate the prognosis of canine cutaneous mast cell tumors using immunohistochemical detection of Ki67, MAC387, Factor 8 and MMP-9 and using Patnaik and Kiupel grades and histological information concerning vessel invasion, infiltration of the surrounding tissue, amount of collagen, presence of lymphocytes, amount of edema, ulceration of the skin, location of the tumor, necrosis and flame figures.
Samples–33 mast cell tumors (MCTs) of different grades from 33 dogs submitted from different Dutch veterinary clinics from 2008-2010 to the pathology department of the Faculty of Veterinary Medicine of Utrecht University, The Netherlands. The population consisted of 14 male and 19 female dogs, representing a variety of breeds.
Procedures–The mast cell tumors were histologically graded with Patnaik and Kiupel grading schemes. Additional histologic information was noted concerning vessel invasion, infiltration of the surrounding tissue, amount of collagen, presence of lymphocytes, amount of edema, ulceration of the skin, location of the tumor within the skin, necrosis and flame figures.
Ki67, MAC387, Factor 8 and MMP-9 antibodies were used for immunohistochemistry. The relationships between the immunohistochemistry, the histology and the clinical follow-up data were investigated. The clinical data was used to compute overall survival time(OST) and the progression free survival(PFS).
Results– Parameters significantly correlated to the Patnaik and Kiupel grades are: tumor size, degree of tumor excision, vessel invasion, infiltration of the surrounding tissue, lymphocytes, ulceration of the skin, necrosis, flame figures, KI67 and Factor 8(blocks).
Significant relationships between the parameters and OST/PFS with the use of the Kaplan-Meier test were found with the parameters: tumor size, vessel invasion, lymphocytes, ulceration, necrosis, flame figures, KI67 and Factor 8(blocks).
Conclusions and clinical relevance– The parameters of tumor size, the amount of excision of the tumor and the presence of vessel invasion, lymphocytes, ulceration and necrosis should be evaluated when histologically examining MCTs. For further evaluation, extra immunohistochemistry for Ki67 and Factor 8 are useful for determining
the prognosis for dogs with MCTs.

Introduction

Mast cell tumors (MCTs) are frequently occurring skin tumors in dogs of any age (average age: 8-10 years old)[1],representing 20-25% of all skin tumors.[2] Besides the cutaneous form, there are also visceral and leukemic forms.[1,2] The predilection sites for cutaneous MCTs are the limbs, the ventral abdomen and the thorax.[1] Breeds such as Boxers, Pugs, Rhodesian Ridgebacks and Boston Terriers seem to have a predisposition for the development of MCTs.[1] Most dogs suffer from a single MCT, but about 10% of the dogs have multiple MCTs.[1]

The size and shape of MCTs vary, but the tumors most commonly present themselves as raised, nodular masses.[1] The clinical appearance alone is usually not sufficient for a diagnosis.[1,2]

Histologically, the tumors consist of a highly cellular center with smaller numbers of peripheral mast cells that can palpate as normal skin.[1]

Diagnosis of MCTs can be made with thin needle aspiration biopsy and a Wright-Giemsa stain.[1] However, for histology, an excisional biopsy is required.[3] Based on the histological appearance, the tumor can be graded with two commonly used grading systems, the Patnaik grading system[4] and the Kiupel (or 2-tier) grading system.[5] The histologic grading is prognostic for the clinical behavior of MCTs.[4,6,7] Grade 1 tumors have a metastatic rate of <10%, grade 2 tumors pose a low to moderate risk of metastasizing and grade 3 tumors have a metastatic rate of 55-95%.[8] When examining MCTs histologically, bizarre mitotic figures, like shown in figure 1, can be found. When there are more of these bizarre nuclei (more than three in 10 high power fields (HPF), the tumor automatically classifies as a Kiupel high grade tumor.[5]

Although histologic grading is prognostic for the behavior of MCTs, a study[9] has shown that there is variation between pathologists in the histologic grading of MCTs with the Patnaik grading system. This interobserver variation can cause the level of agreement between pathologists to become as low as 50%.[10]

In addition, the prognosis for grade 2 MCTs is difficult to predict. The tumor might behave like a grade 1 tumor and remain small and non-invasive or it might behave more like a grade 3 tumor and behave in a more aggressive way.[10,11]

The ambiguity of grade 2 MCTs and the degree of interobserver variation have led to questioning if the methods for determining a proper prognosis for canine MCTs are sufficient. The aim of this current study is thus to improve the determination of the prognosis for canine MCTs using immunohistochemistry, including Ki67, MAC387, Factor 8 and Matrix Metalloproteinase 9 (MMP-9) and additional histologic information including vessel invasion, infiltration of the surrounding tissue, collagen, lymphocytes, edema, ulceration of the skin, location of the tumor, necrosis and flame figures.

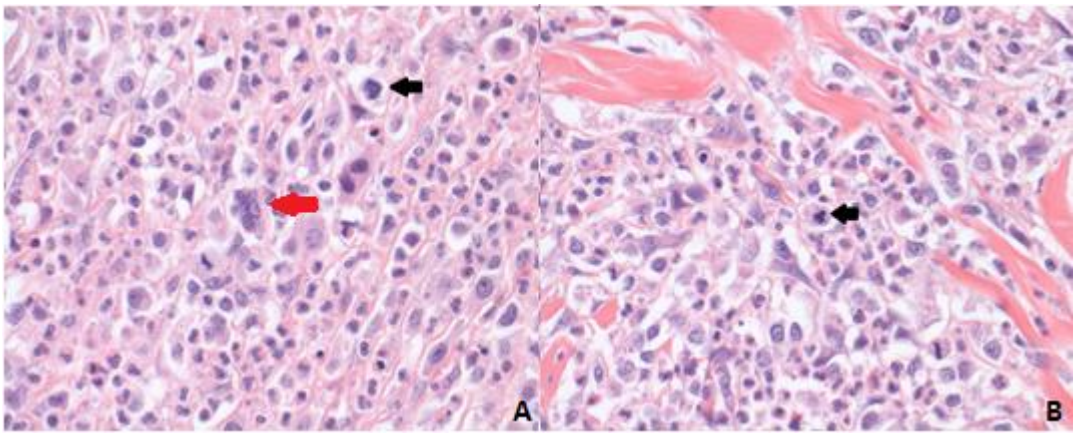

Figure 1 Heamatoxylin & Eosin (HE) staining. **A.** Bizarre mitotic figures (arrow) and a multinucleated cell (red arrow), 400x. **B**. Bizarre mitotic figures (arrow), 400x.

Ki67

The Ki67 protein is a cellular marker for proliferation. The protein is present during the active phases (G_1,S,G_2 and mitosis) and is absent during the G0 phase of the cell cycle.[12,13]

The propensity to proliferate uncontrollably is characteristic for cancer. A cancer with a high-proliferation index is considered more malignant. Cellular proliferation is thus useful for prognostication of neoplastic diseases.[10]

One of the most commonly used methods to evaluate the cellular proliferation of a certain tissue, is Ki67 immunohistochemistry.[14,15] The relative number of cells that are positive for the Ki67 staining in a certain tissue can provide an estimate of the total number of dividing cells.[12,13,14,15]

One of the aims of this study is to determine if the proliferation rate is correlated to grade and the prognosis of MCTs as reported in previous studies.[10,16,17]

MAC387

This anti-macrophage antibody recognizes the L1 or Calprotectin molecule, which is expressed by granulocytes, monocytes and tissue macrophages in the cytoplasm.[18]

Studies[19,20] in human tumors have shown that tumor associated macrophages (TAMs) can be indicative for prognosis. Macrophages are found in many solid tumors and the extent of their presence seem to correlate with increased micro vessel density (MVD) and a poorer prognosis.[20] TAMs have been associated with production of growth factors that lead to angiogenesis, remodeling of the extra cellular matrix (ECM) by enzymes and suppression of the host immune system allowing tumor cells to evade the normal regulatory pathways. Studies[19,20] have also shown that the existence and number of TAMs correlate directly with the prognosis of the cancer. Increased number of TAMs is associated with worse prognosis.[19,20]

One of the aims of this study is to determine if the number of macrophages within the tumor is correlated to grade, MVD and the prognosis of MCTs.

Factor 8

The anti-Factor 8 antibody reacts with factor 8 antigen in endothelial cells, megakaryocytes and platelets.[21]

Essential to tumor growth and metastasis is neoangiogenesis.[22,23,24,25,26] Without proper blood supply, a tumor can only grow to 10^6 cells, which corresponds roughly with a tumor of 1-2 mm in diameter.[27] When exceeding 10^6 cells, hypoxia occurs, which is one of the key drivers of angiogenesis.[20]

With the use of the Factor 8 immunohistochemistry, endothelial cells can be stained. Studies in other tumors[25,28,29,30] have shown that high levels of Factor 8 are significantly associated with short cancer-specific survival. With factor 8 immunohistochemistry, blood vessels are stained. Because of this staining, the microvessel density (MVD)can be determined. The MVD may be an important prognostic factor.[29]

A study of MCTs[25] has shown that MVD is correlated to grade.

As said before, the extent of the presence of TAMs seem to correlate with increased micro vessel density.[20,25] Study[25] also shows that leukocytes play a role in neoangionesis. Leukocytes produce angiogenic growth factors which induce neoangiogenesis. Mast cells may also act as a reservoir for angiogenic growth factors. This suggests that there might be a correlation between the number of mast cells and MVD within a tumor.[25]

One of the aims of this study is to determine if the MVD is correlated to grade, number of lymphocytes, number of TAMs and the prognosis of MCTs.

Matrix Metalloproteinase-9 (MMP-9)

The MMP-9 antibody binds to the C-terminus of the MMP-9 protein.[31] The MMP family is capable of degrading all components of the extracellular matrix (ECM).[32] Degradation of ECM is a key factor, together with vascular angiogenesis, for growth and progression of tumors.[22,23,24] Tumor invasion relies on proteolysis of the ECM. This process is largely mediated by MMPs that are produced by neoplastic cells.[33] Many studies have shown that MMPs are overexpressed in other tumors and human cancers.[11,19,22,33,34,35,36,37,38,39]

MMPs, including MMP-9, have been studied in canine mast cell tumors.[11,39] Leibman et al[40] and Giantin et al[11] have found that MMP-9 is present in canine MCTs and that it seems to be correlated to histologic grade. Giantin et al[11] suggests that MMP-9 markers are possible indicators of malignancy in canine MCTs.
One of the aims of this study is to determine if the intensity of the MMP-9 staining and the percentage of positive cells are correlated to grade and the prognosis of MCTs.

Materials and methods

Samples

33 cases of grade 1, 2 and 3 canine mast cell tumors were selected from the archive of the pathology department of the Faculty of Veterinary Medicine of Utrecht University, The Netherlands.
Inclusion criteria are: canine cutaneous mast cell tumors submitted to the pathology department between 2008 and 2010 from dogs with a single MCT over their lifetime. Animals were excluded based on the following exclusion criteria: The dogs suffered from multiple mast cell tumors at the time of diagnosis. This was chosen as an exclusion criteria, because otherwise, it would be impossible to determine which tumor caused metastasis, recurrence or death. The other exclusion criteria is that the dogs had to have a follow up available of at least three years. This was chosen as an exclusion criteria, because veterinary clinics had to be called to retrieve the dogs patient files. If the patient file could not be retrieved, and thus the overall survival and disease free period could not be calculated, the cases were marked as 'loss to follow up'.
Of each case, there was at least one formalin fixed, paraffin embedded tumor sample available in the archive of the pathology department.

Protocols

There are two tables (table 1 and table 2) that specify how the immunohistochemistry was performed. Below each diagram, there is a specification of the exact procedures.

Each step was performed at room temperature, unless otherwise specified.
For Ki67, MAC387 and Factor 8 antibodies, PBS (0.01M, pH 7.4) was used for rinsing and for dilution of the antibodies. For the MMP-9 antibody, TBS (0.01M, pH 7.6) was used for rinsing and for dilution of the antibodies.
With each stain, there was also a positive and negative control incorporated. The negative control was always performed by incubating the slides overnight with PBS(or TBS in the case of MMP-9) instead of the primary antibody.
The following tissues were used as positive control:

- Ki67: intestine (pig)
- MAC387: spleen (canine)
- Factor 8: liver (canine)
- MMP-9: skin (canine)

Deparaffinize & dehydrate: Formalin-fixed, paraffin-embedded tissue was cut and placed on glass slides [e]. The sections were deparaffinized in xylene and rehydrated in alcohol(100%,96%,70%) and aquadest.
Citrate: Antigen retrieval was performed using a preheated (10 min at 100% in an 1100W microwave) 10mM citrate buffer (pH 6) for 15 min at 70% in an 1100W microwave. The slides were then left to cool down at room temperature for 30 minutes.
Pronase: Antigen retrieval was performed using 0.1% pronase in MQ for 15 minutes.
1% H_2O_2: The endogenous peroxidase activity was then blocked by using a 1% H_2O_2 in methanol solution for 30 minutes.
Normal serum: Ki67;MAC387;factor 8: 1:10 in PBS for 30 minutes. MMP-9: 1:10 in TBS for 20 minutes.
Primary antibody: overnight at 4°C.
Secondary antibody:

- E: Goat anti Rabbit [f], 1:250 in PBS, for 30 minutes.
- F: Horse anti Mouse [f], 1:125 in PBS, for 15 minutes.
- G: Rabbit anti Goat [f], 1:100 in TBS, for 30 minutes.

ABC/PO-complex: The slides were incubated for 30 minutes with ABC/PO-complex [g] in PBS(240 PBS+10A, 240PBS+10B).

DAB: The color was developed using a DAB solution (45ml Tris buffer (0.05M, pH 7.8)+ 5µl H_2O_2 + 5ml DAB) for 15 minutes.

AEC: The color was developed using an AEC ready to use [h] solution for 15 minutes.

Table 1, Day 1 of the Ki67, MAC387, Factor 8 and MMP-9 protocols

	Dewax & rehydrate	**Antigen retrieval** *Citrate*	*Pronase*	*Nothing*	**1% H_2O_2**	**Normal serum** *Goat*	*Horse*	*Rabbit*	**Primary antibody**
Rabbit anti Ki67[a]	X	X			X	X			1:75 in PBS
Mouse anti MAC387[b]	X	X			X		X		1:500 in PBS
Rabbit anti Factor 8[c]	X		X		X	X			1:500 in PBS
Goat anti MMP-9[d]	X			X	X			X	1:100 in TBS

Table 2, Day 2 of the Ki67, MAC387, Factor 8 and MMP-9 protocols

	Secondary antibody	**ABC/PO-complex**	**Color development** *DAB*	*AEC*	**Haematoxylin**	**Mounting** *Dehydrating & Eukitt*	*Aquatex*
Rabbit anti Ki67	E	X	X		X	X	
Mouse anti MAC387	F	X		X	X		X
Rabbit anti Factor 8	E	X		X	X		X
Goat anti MMP-9	G	X	X		X	X	

Evaluation of the slides

Extra information about the H&E slides was obtained. Regarding the Ki67, MAC387, Factor 8 and MMP-9 immunohistochemistry, the positive cells(or blood vessels regarding the Factor 8 staining) were counted and with the use of statistical analysis, the correlation between the different stainings and the prognosis of MCTs was calculated. If there was correlation, a cut-off point was established.

Haematoxylin & Eosin

- The mast cell tumors were histologically regraded by D.J. Beekman and R.I. Keesler. This regrading was done according to the Patnaik[4] and the Kiupel[5] grading systems. The criteria are as followed:
  - Patnaik:
    - Grade 1: rows/clusters of monomorphic, well differentiated mast cells. The mast cells have round nuclei and medium-sized cytoplasmic granules. There are no mitotic figures present, minimal stromal reaction and minimal necrosis.[4,5]
    - Grade 2: moderate-high cellularity. The mast cells are more pleomorphic and have round-indented nuclei. The granules are less distinct than the granules of the grade 1 MCTs. There are few mitotic figures present. The MCT may contain some edema en necrosis.[4,5]

    - Grade 3: high cellularity, a lot of pleomorphic mast cells with indented-round vesicular nuclei and 1 or more multiple prominent nucleoli. There are a number of mitotic figures present per HPF. The MCT may contain areas of hemorrhage, edema and necrosis.[4,5]
- Kiupel: A MCT is considered high grade when at least one of the following criteria is present. When the tumor has none of these criteria, it is considered low grade.[5]
    - At least seven mitotic figures in 10 HPF
    - At least three multinucleated (three or more) cells in 10 HPF
    - At least three bizarre nuclei in 10 HPF
    - Karyomegaly: The nuclear diameters of at least 10% of the neoplastic mast cells vary by at least two-fold between each other

Extra histological information about presence or absence of vessel invasion, infiltration of the surrounding tissue, collagen, lymphocytes, edema, ulceration of the skin, necrosis and flame figures was also obtained. There was also information obtained regarding whether the tumor was in the dermis only or located in both the dermis and subcutis.

- Ki67: The number of positive cells were counted, with the use of a 10x10 grid, in five HPF at a 400x magnification. The average number of positive cells per HPF was then established.[10]
- MAC387**:** The number of positive macrophages were counted, with the use of a 10x10 grid, in twelve HPFs at a 400x magnification.[39] When scoring the macrophages, areas of ulceration were avoided, because of positive inflammatory cells in these areas.
- Factor 8: The number of separate blood vessels were counted with the use of a 10x10 grid, in five HPF at a 400x magnification.
  The number of blocks within the grid that had whole or partial blood vessels were counted separately. Blocks were only counted once, no matter how many blood vessels were present within one block.
  The average number of blood vessels and the average number of positive blocks in the grid per HPF were then established. The coverage of the blood vessels per HPF has a maximum of 100, since that is the size of the grid (10x10 blocks).
- MMP-9**:** The number of positive cells were counted, with the use of a 10x10 grid, in ten HPF at a 400x magnification.
  The average percentage of positive cells and average intensity of the staining were determined.
  The average percentage of positive cells was classified as 0=0%, 1=1-10%, 2=11-25%, 3=26-50%, 4=51-75% and 5=76-100% stained cells. The average intensity of the staining was separated in 0=not stained, 1=mild-moderate, 2=moderate-strong and 3=strong staining.[11]

Apart from the information that was obtained from the immunohistochemistry, there was also information obtained from patient files. Information including sex, weight, location of the tumor and the degree of excision (incomplete, marginal, complete) of the tumor was obtained. Originally, information was also obtained about treatment performed after the surgery and about clinical staging. Only two dogs received extra treatment and in twelve dogs staging was performed, with two cases having metastasis. Therefore, there was not enough data to perform statistical analysis on these parameters.

Statistical analysis is performed on: age, infiltration, ulceration, location(skin), sex, weight, location (body), tumor size, excision of the tumor, vessel invasion, collagen, lymphocytes, edema, necrosis, flame figures, Ki67, MAC387, factor 8 (amount of vessels), factor 8 (blocks), MMP-9 (positive cells), MMP-9 (intensity of the staining). When analyzing previous mentioned parameters, the Patnaik and Kiupel grades and the OST and PFS data will also be taken into account. The

statistical analysis will be performed with the use of SPSS (version 22) and a p value ≤ .05 is significant.

Results

Thirty-three dogs of eighteen different breeds were represented in this study, including 4 Jack Russell terriers, 2 French bulldogs, 2 boxers, 2 Rhodesian ridgebacks, 7 Labrador retrievers, 3 golden retrievers, 2 Staffordshire bullterriers and 11 additional breeds that were represented by single dogs. The mean (± sd) age of the dogs at the time of the diagnosis of MCT was 7.5 (±3.4) years and ranged from 1.6 to 14 years of age. The age of one dog was unknown. Nineteen females (six intact) and fourteen males (six intact) were included in this study. Seven of the submitted MCTs were Patnaik histologic grade 1, nineteen were grade 2 and seven were grade 3. Out of the submitted MCTs, 24 were Kiupel low grade and nine were high grade.

Overall, the dogs had an average PFS of 1424 days with a standard error of 137. The range was 6-1870 days. The median was not reached at the end of the study. After the first year, 81% (n=27) of the dogs had no recurrence and after the second year, 74% (n=25) of the dogs had no recurrence. The last recurrence of the MCT was at 539 days.
The results are displayed in figure 2.

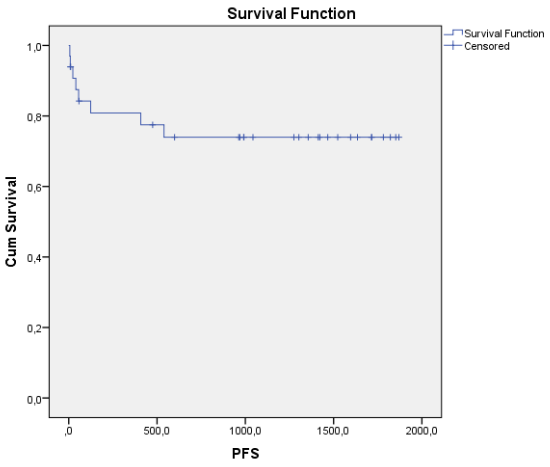

Figure 2, Survival function of the PFS

All dogs had an average OST of 1428.3 days with a standard error of 135.4 and an upper limit of the 95% confidence interval of 1693.7 days. The range was 6-1870 days. The median was not reached at the end of the study. After the first year, 81% of the dogs were still alive and after the second year, 74% of the dogs were alive with the last death at 686 days. During the study, eight dogs in total died. The results are displayed in figure 3.

Of the dogs that had a Patnaik grade 1 tumor, none of the dogs had recurrence at the end of the study. Of the dogs that had a grade 2 tumor, 1 dog had a recurrence during the course of the study after 539 days. At the end of the study, 6% (n=1) of the dogs that had a grade 2 tumor had recurrence of the tumor. After two years, 100%(n=7) of the dogs that had a grade 3 tumor had a recurrence of the tumor, the last dog in the grade 3 pool had a recurrence after 408 days.
Of the dogs that had a grade 1 tumor, 100%(n=7) of the dogs were still alive at the end of the study. Of the dogs that had a grade 2 tumor, 1 dog died during the course of the study after 539 days. At the end of the study, 94%(n=18) of the dogs that had a grade 2 tumor were still alive. For the dogs that had a grade 3 tumor, only 1 dog (14%) survived the first year and after two years, 100%(n=7)of the dogs that had a grade 3 tumor died, the last dog in the grade 3 pool died after 408 days. The results are displayed in figure 4 Only the OST graphics are displayed, since the OST and PFS graphics are similar.

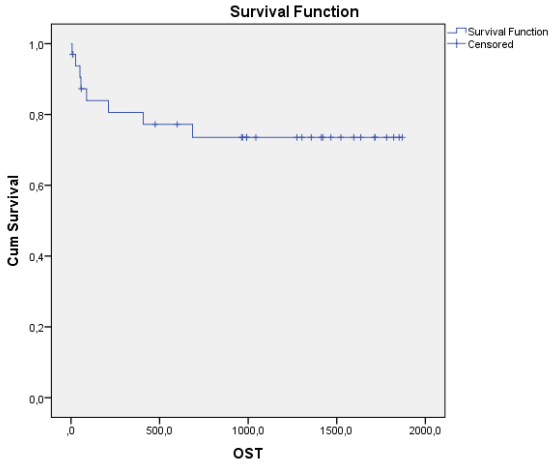

Figure 3, Survival function of the OST

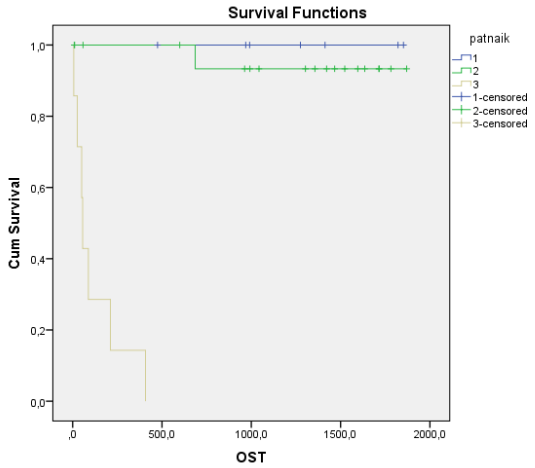

Figure 4, Kaplan-Meier survival curve for Patnaik gradation compared to OST
Log Rank (Mantel-Cox): p=.000

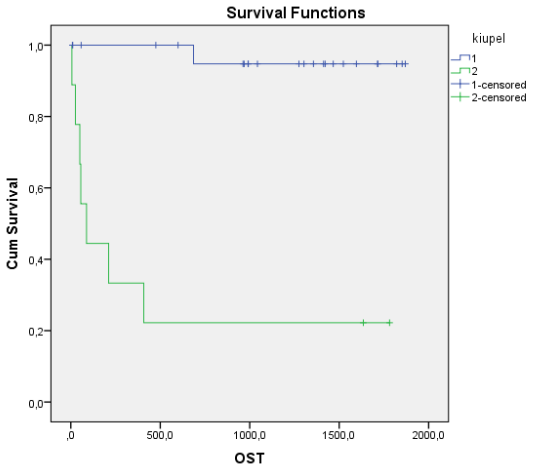

Figure 5, Kaplan-Meier survival curve for Kiupel gradation compared to OST
Log Rank (Mantel-Cox): p=.000

Of the dogs that had a Kiupel low grade tumor, 1 dog had a recurrence after 539 days, 95%(n=23) were still alive at the end of the study. For the dogs that had a high grade tumor, 67%(n=6) had recurrence of the tumor after the first year. In the second year, one more dog had a recurrence. After the second year, no more dogs developed a recurrence of the tumor and 22%(n=2) of the dogs that had a high grade tumor were still recurrence free at the end of the study.
Of the dogs that had a low grade tumor, 1 dog died in the second year of the follow up after 539 days, 95% was still alive at the end of the study. Only 33% (n=3) of the dogs with high grade tumors were alive after the first year. In the second year, one more dog died. After the second year, no more dogs died and 22% of the dogs that had a high grade tumor were still alive at the end of the study. The results are displayed in figure 5.

The weight of the dogs had a bimodal distribution, divided into a group of small dogs and a group of large dogs. The weight ranged from 6.8-51 kilograms, had a median of 29.5 kg and a mean(±sd) of 26.0 kg (±13.6).

Regarding the location of the tumor, 5 dogs (15.2%) had a MCT in the head and neck region, 14 dogs (42.4%) on the torso, 11 dogs (33.3 on the legs, 1 dog (3%) on the toe, 1 dog (3%) on the vulva and 1 dog (3%) on the preputium. The location of the tumor was not correlated to the Patnaik nor Kiupel grade (p=.227; p=.305 respectively)

The range of the tumor size was 0.4-6 centimeters, with a median of 1 and a mean(±sd) of 1.7 cm (±1.4) and these measurements were not normally distributed. The measurements were divided into a group that had a tumor size that was smaller than 3 cm and a group that had a tumor size of 3 cm or bigger.[41] 25 dogs (76%) had a tumor size that was smaller than 3 cm and eight dogs (24%) had a tumor size of 3 cm or larger. This division in tumor size is correlated to the Patnaik (r=.434, p=.012) and Kiupel (r=.447, p=.009) grades. The tumor size is also related to the Overall Survival Time (OST) (p=.012; Exp(B)=6.3) as well as the Progression Free Survival (PFS) (p=.023, Exp(B)=5.2) using a Cox regression test. The Kaplan-Meier curve is in figure 6.

Regarding excision of the tumor, 20 dogs (60.6%) had complete excision, 8 (24.2%) dogs had marginal excision and 5 dogs (15.2%) had incomplete excision. The degree of excision of the tumor is correlated to Patnaik (r=.376, p=.031) and Kiupel (r=.467, p=.006) grades. The amount of excision of the tumor is also related to the OST (p=.027; Exp(B)=2.5) as well as the PFS (p=.029, Exp(B)=2.5) using a Cox regression test. The Kaplan-Meier curve is displayed in figure 7. Only the OST graphics

are displayed, since the OST and PFS graphics are similar.
Even though there seems to be a clear distinction between the categories, the Log Rank (Mantel-Cox) is not significant (p=.054).

The Ki67 counts had a range from 0.4 to 83.2 positive cells per HPF, with a median of 5.2 and a mean (±sd) of 11.9 (±3.0). The counts were not normally distributed. The tumors were then divided in two categories at a cut-off value of 23 positive cells per HPF, which was established by previous study.[10]
When devided, 29 cases (88%) had a Ki67 count below 23 and 4 cases (12%) had a count of 23 or higher.
The dogs with Ki67<23 had an average PFS of 1551 days with a standard error of 130. The median was not reached at the end of the study.

The dogs that had Ki67≥23 had an average PFS of 551 days with a standard error of 345. The estimated median is 56 days with a standard error of 193. The dogs with Ki67<23 had an average OST of 1555 days with a standard error of 128. The median was not reached at the end of the study. The dogs that had Ki67≥23 had a mean PFS of 552 days with a standard error of 344. The estimated median is 56 days with a standard error of 191.

The dogs with Ki67≥23 had an increased hazard rate (chance of death) of 5.9 compared to the dogs with Ki67<23. The dogs with Ki67≥23, had an increased hazard rate (chance of recurrence) of 5.5 compared to the dogs with Ki67<23.
Examples of the immunohistochemistry are shown in figure 8.

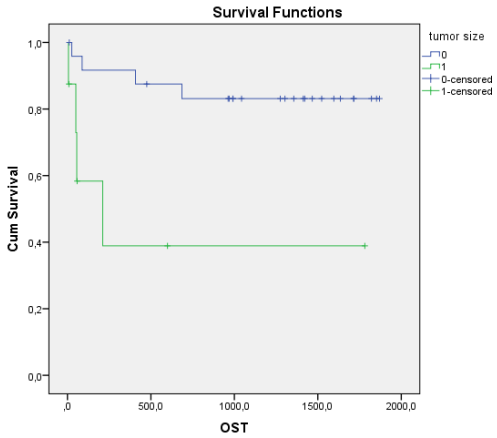

Figure 6, Kaplan-Meier survival curve for tumor size compared to OST.
Log Rank (Mantel-Cox): p=.005

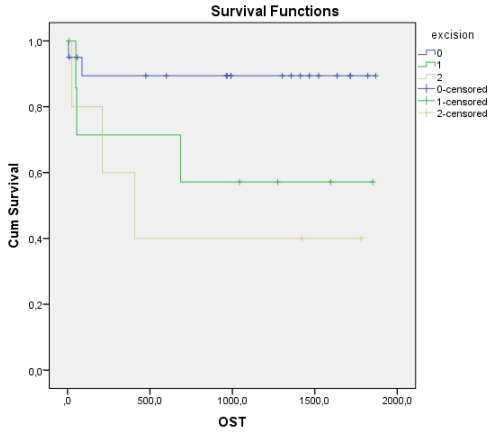

Figure 7, Kaplan-Meier survival curve for excision compared to OST
Log Rank (Mantel-Cox): p=.054

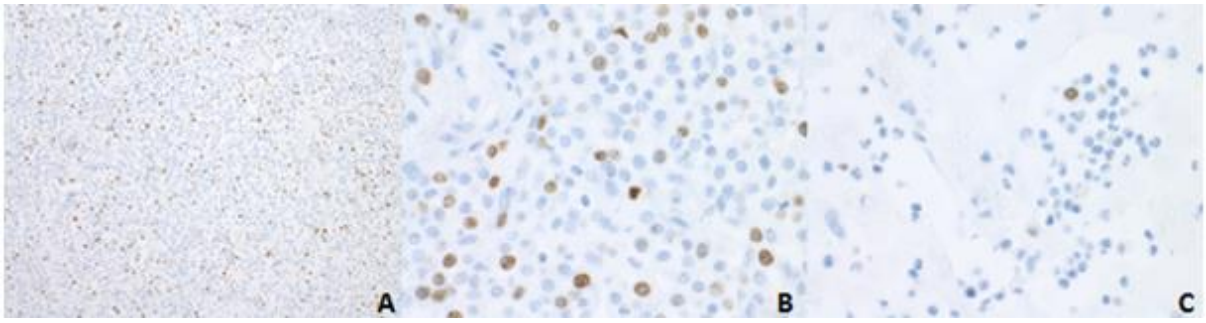

Figure 8. Immunohistochemistry with Ki67 antibody in cutaneous canine MCTs. **A.** High (≥23) counts of positive (brown) cells, 40x. **B.** High(≥23) counts of positive (brown) cells, 400x. **C.** Low (<23) count of positive (brown) cells, 400x.

Extra histological information

Extra information about vessel invasion, infiltration of the surrounding tissue (infiltration), collagen, lymphocytes, edema, ulceration of the skin (ulceration), location of the tumor (location), necrosis and flame figures was obtained. The results are displayed in table 3. Some examples are shown in figure 9.

In a few tumors, some of the parameters could not be evaluated because adjecent tissues were not present in the biosies. This was the case for infiltration, ulceration and location**.**

The correlation of these parameters with Patnaik and Kiupel grades was determined. The results are shown in Table 4.

Table 3, Numbers and percentages of tumors that did not have a parameter (0) or did have that parameter (1) on HE. For location, 0 indicates located in the dermis only and 1 indicates located in dermis and subcutis.

	N (%) 0	N (%) 1
Vessel invasion	27 (82)	6 (18)
Infiltration	22 (71)	9 (29)
Collagen	27 (82)	6 (18)
Lymphocytes	16 (48)	17 (52)
Edema	8 (24)	25 (76)
Ulceration	22 (73)	8 (27)
Location	11 (38)	18 (62)
Necrosis	26 (79)	7 (21)
Flame figures	26 (79)	7 (21)

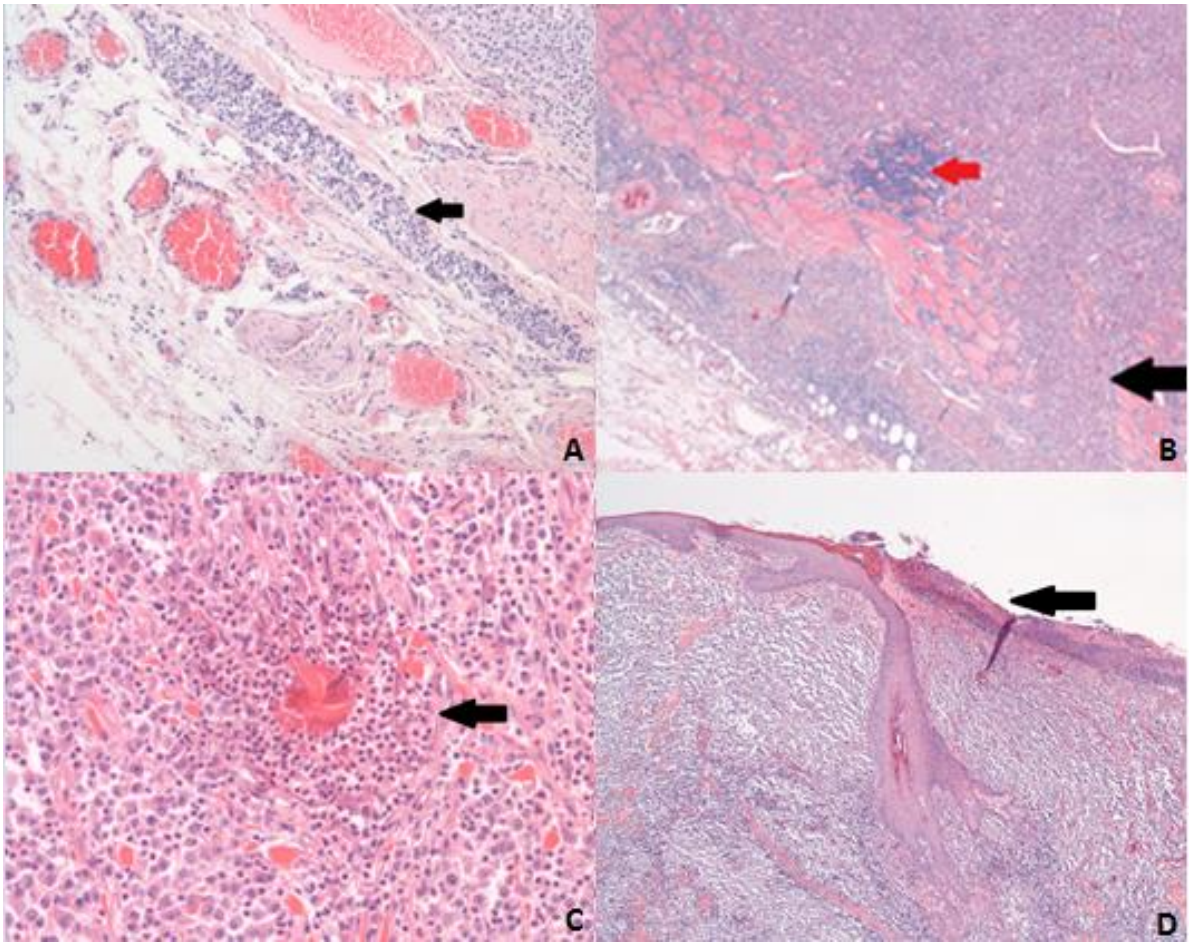

Figure 9. HE. **A.** Lymphatic vessel invasion (arrow), 100x. **B.** Infiltration of the surrounding tissue (arrow) and a cluster of lymphocytes (red arrow), 40x. **C.**Flame figure (arrow), 100x. **D.** Ulceration (arrow), 40x.

Table 4, correlations of the extra information obtained from the HE slides with Patnaik and Kiupel grades.

	Kiupel R (p value)	Patnaik R (p value)
Vessel invasion	.593 (.000)	.603 (.000)
Infiltration	.435 (.014)	.362 (.046)
Collagen	-.289 (.103)	.000 (1.000)
Lymphocytes	.458 (.007)	.559 (.001)
Edema	.188 (.296)	.326 (.064)
Ulceration	.380 (.038)	.380 (.008)
Location	.224 (.243)	.471 (.010)
Necrosis	.514 (.002)	.569 (.001)
Flame figures	.348 (.047)	.455 (.008)

As shown in table 4, vessel invasion, infiltration, lymphocytes, ulceration, necrosis and flame figures had a correlation with Kiupel and Patnaik grades. The parameter location only had a correlation with the Patnaik grades. A Kaplan-Meier analysis was then performed on the parameters. Only vessel invasion, lymphocytes, ulceration, necrosis and flame figures had significant results. The parameter infiltration had no significant result in the Kaplan-Meier analysis although this parameter is correlated to Patnaik and Kiupel grades. The results of the Kaplan-Meier survival analysis are shown in figures 10-14. Only the OST graphics are displayed, since the OST and PFS graphics are similar.

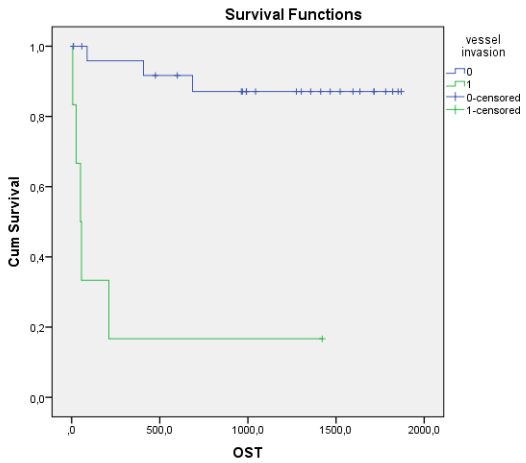

Figure 10, Kaplan-Meier curve of vessel invasion compared to OST.
Log Rank (Mantel-Cox): p=.000

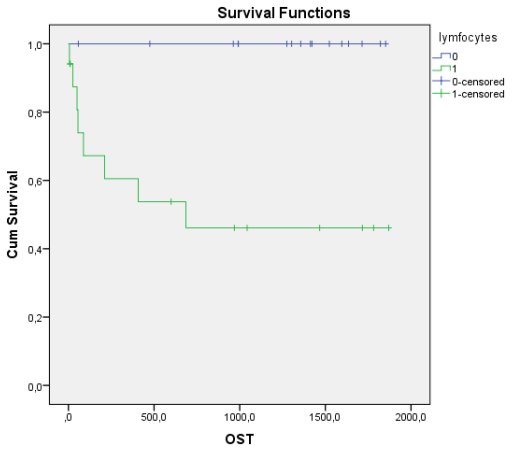

Figure 11, Kaplan-Meier curve of lymphocytes compared to OST.
Log Rank (Mantel-Cox): p=.001

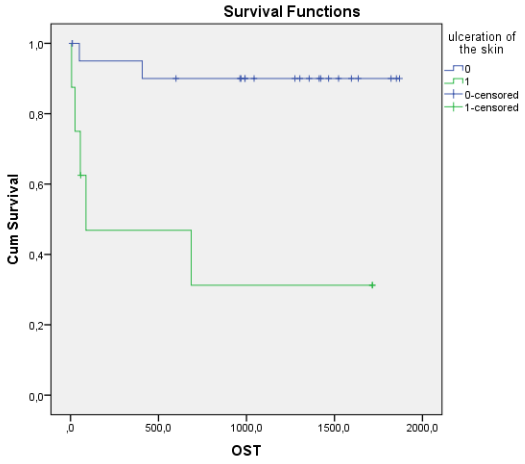

Figure 12, Kaplan-Meier curve of ulceration compared to OST.
Log Rank (Mantel-Cox): p=.001

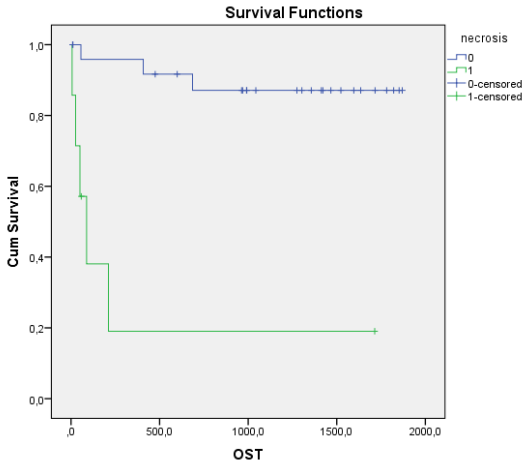

Figure 13, Kaplan-Meier curve of necrosis compared to OST.
Log Rank (Mantel-Cox): p=.000

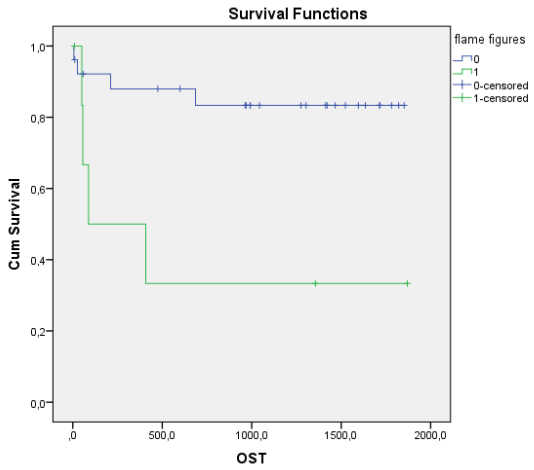

Figure 14, Kaplan-Meier curve of flame figures compared to OST.
Log Rank (Mantel-Cox): p=.009

Factor 8

For Factor 8, the number of the blood vessels (Factor 8,(vessels)) and the coverage of the blood vessels (Factor 8(blocks)) per HPF was analyzed. The distributions of both parameters are shown in figure 15 and 16. The range, median, mean and sd were calculated, the results are displayed in table 5. The counts were normally distributed. Examples of the staining are shown in figure 17.

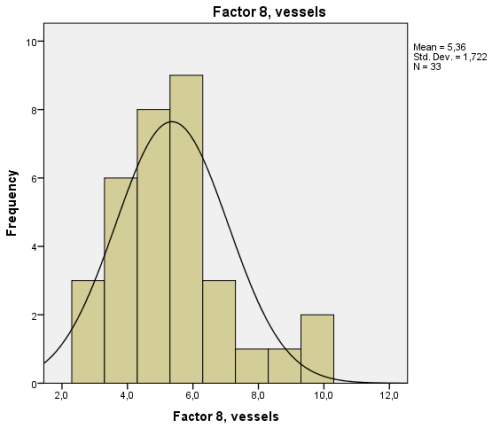

Figure 15, histogram of the distribution of Factor 8(vessels)

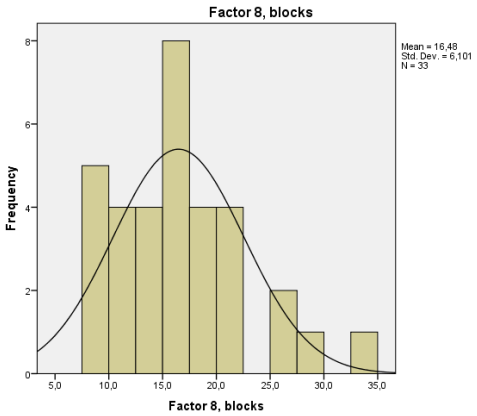

Figure 16, histogram of the distribution of Factor 8(blocks)

Table 5, range, median, mean and standard of the counts for Factor 8

	range	median	mean	Standard deviation
Factor 8(vessels)	2.8-9.6 per HPF	5.2	5.4	1.7
Factor 8(blocks)	7.6-33.4 per HPF	16.0	16.5	6.1

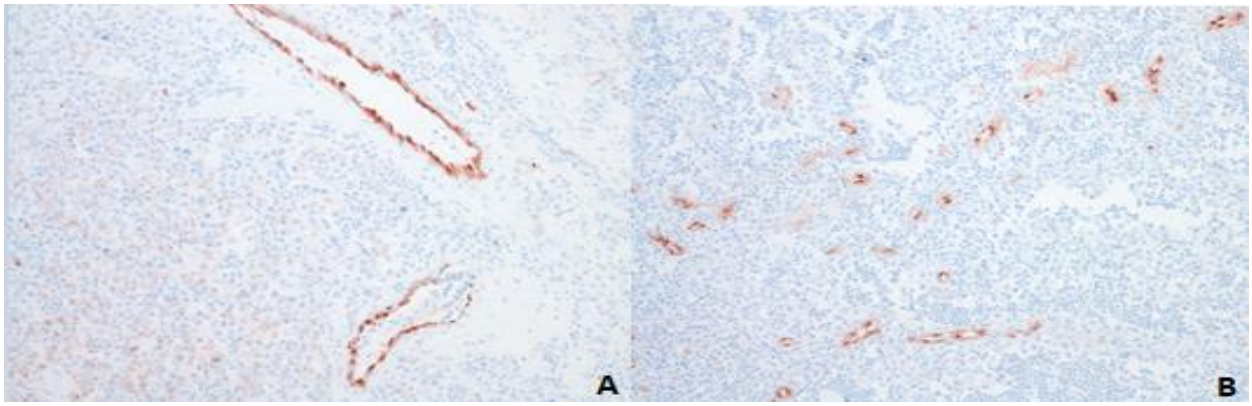

Figure 17. Immunohistochemistry for Factor 8 in cutaneous canine MCTs. **A.** Positive large blood vessels (red), 100x. **B.** Positive small blood vessels (red), 100x.

Only Factor 8(blocks) had a significant correlation with the Patnaik(p=0.036) and the Kiupel(p=0.035) grades. The results are displayed in table 6.
Even though there is a correlation between the Factor 8, blocks parameter and the grading schemes, when statistically comparing these results to the OST/PFS, it is not significant.

Table 6, correlation of Factor 8 parameters with Patnaik and Kiupel grades.

	Kiupel R (p value)	Patnaik R (p value)
Factor 8, vessels	-.081 (.654)	.132 (.465)
Factor 8, blocks	.368 (.035)	.366 (.036)

MAC 387
For MAC387, the total number of positive cells in 12 HPF was counted. The results were not normally distributed. The distribution of the results are shown in figure 20.

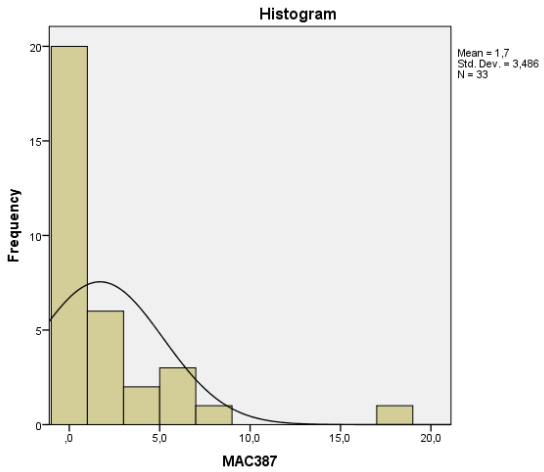

Figure 20, histogram of the distribution of number of MAC387 positive cells in 12 HPF.

Twenty of 33 samples (61%) were negative (zero positive cell count).

The range, median, mean and sd are displayed in table 7. The counts were normally distributed. Examples of the staining are shown in figure 21.

Table 7, range, median, mean and standard deviation of the MAC387 counts

	range	median	mean	Standard deviation
MAC387, positive cells	0-17 in 12 HPF	0	1.7	3.5

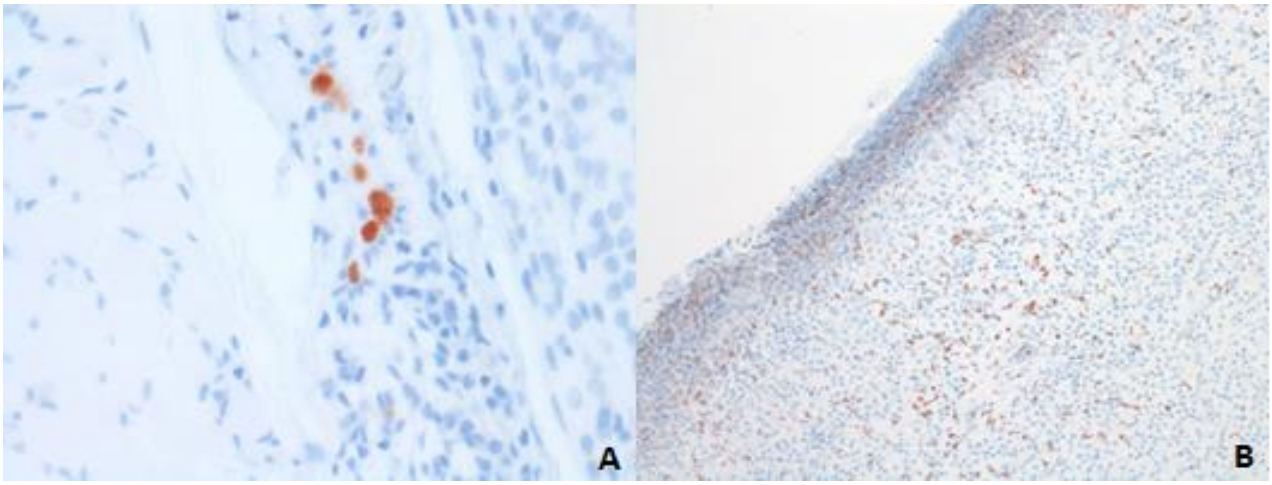

Figure 21, Immunohistochemistry for MAC387 in cutaneous canine MCTs. **A**. Example of positive macrophages (red), 400x. **B**. Example of high counts of positive inflammatory cells near an ulcer, 100x.

There was no significant correlation between MAC387 positive cells or grade (MAC387*Patnaik: p=.366; MAC387*Kiupel: p=.336). If the results are grouped (MAC387gr) into tumors without macrophages and tumors with macrophages, there was no significant correlation (MAC387gr*Patnaik: p=.598; MAC387gr*Kiupel: p=.674)
When using a Cox regression test to compare the MAC387 counts with PFS and OST, the results are not significant (MAC387*PFS: p=.287; MAC387*OST: p=.296). If the groups were compared to PFS and OST, both were insignificant (MAC387gr*PFS: p=.825; MAC387gr*OST: p=.795)
It should be noted that with the staining of MAC387, eosinophils also stained with the MAC387 staining, making the counting of the macrophages more difficult.

MMP-9

For MMP-9, the counts were separated into the average amount of stained cells and the average intensity of the staining. The results were put in categories as described by Giantin et all.[11] The distribution is displayed below in table 8 and figures 22 and 23. Examples of the staining are given in figure 24.

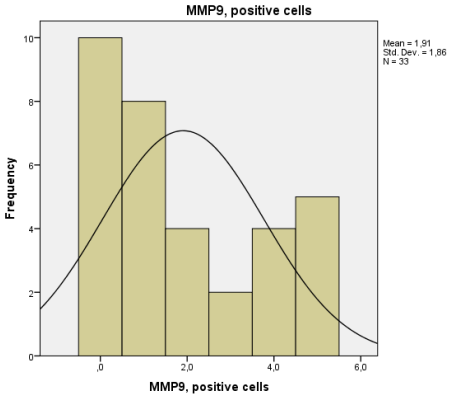

Figure 22, distribution of MMP-9, number of positive cells

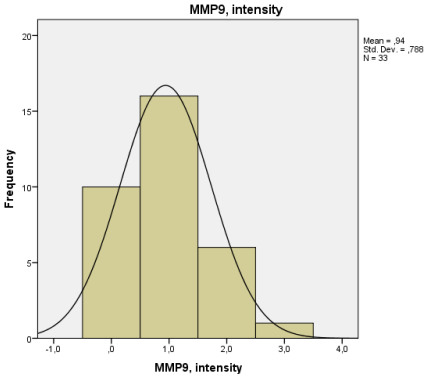

Figure 23, distribution of MMP-9, intensity of the staining

Table 8, Numbers and percentages of tumors that had a score of 0=0%, 1=1-10%, 2=11-25%, 3=26-50%, 4=51-75% and 5=76-100% for the amount of positive cells per HPF or a 0=not stained, 1=mild-moderate, 2=moderate-strong and 3=strong staining for the average intensity of the staining.

	Amount of positive cells per HPF						**Intensity of the staining**			
	0	*1*	*2*	*3*	*4*	*5*	*0*	*1*	*2*	*3*
N (%)	10 (31)	8 (24)	4 (12)	2 (6)	4 (12)	5 (15)	10 (30)	16 (49)	6 (18)	1 (3)

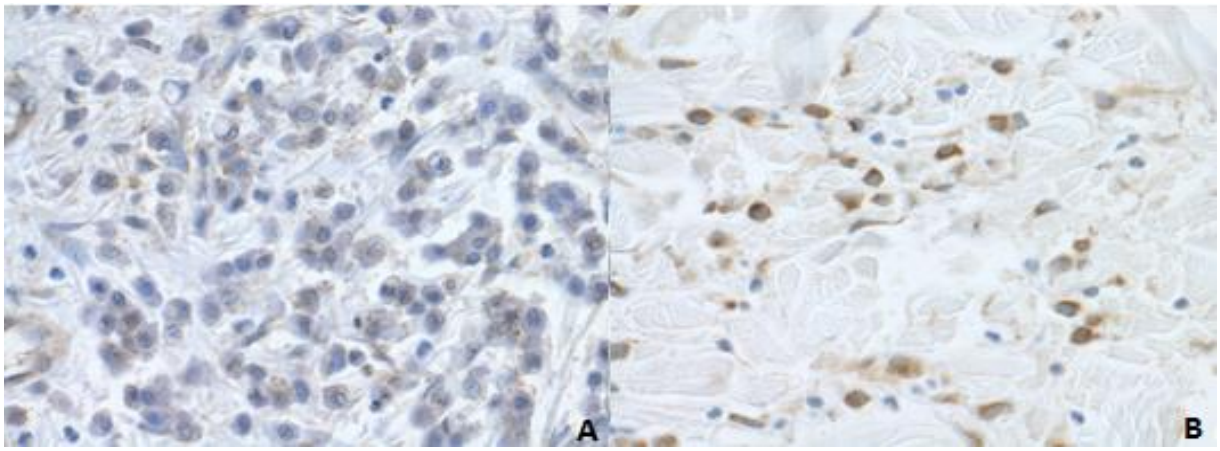

Figure 24. Immunohistochemistry for MMP-9 in cutaneous canine MCTs. **A.** Example of high percentage of positive cells, but low intensity of the staining(brown), 400x. **B.** Example of low percentage of positive cells, but high intensity of the staining (brown), 400x.

There was no significant correlation between MMP-9 positive cells or intensity with grade (MMP-9, positive cells*Patnaik: p=.673; MMP-9, positive cells *Kiupel: p=.654; MMP-9, intensity* Patnaik: p=.317; MMP-9, intensity*Kiupel: p=.452).
When using a Cox regression test to compare the MMP-9, positive cells and MMP-9, intensity with PFS and OST, the results were insignificant (MMP-9, positive cells*PFS: p=.739; MMP-9, positive cells *OST: p=.775; MMP-9, intensity* PFS: p=.633; MMP-9, intensity*OST: p=.608).

Discussion

The results of this study indicate that tumor size, degree of excision, presence of vessel invasion, lymphocytes, ulceration, necrosis and flame figures and the Ki67 and the Factor 8(amount of covered blocks) immunohistochemistry are significantly correlated with the Patnaik and Kiupel grades and are indicative for the OST and PFS. The how and why of these correlations will be given below.

This study has shown that a tumor size of 3 cm or bigger has a shorter OST/PFS and is correlated to a higher grade. Monteiro et all[42] reported that increased tumor size is related to increased risk of incomplete excision. This incomplete excision results in a shorter OST/PFS, as shown by this study.
Another possible explanation of this result might be that when the tumor is capable of growing to that size, the tumor is either growing quickly or has been present for a longer time. Both options pose a greater risk of metastasis. When a tumor is growing quickly, it is likely that there are more mitotic figures present when histologically examining the tumor, which puts the tumor in a higher grade. The other option, when the tumor is there for a longer time, poses a higher risk for metastasis as well. The risk of metastasis becomes higher the longer the tumor gets the chance to develop the methods to metastasize.

This study has shown that tumors that are incompletely removed, have a shorter OST/PFS and are correlated with a higher grade. The shorter OST/PFS can be explained due to the fact that when the tumor is not completely removed, it is easier for the tumor to recur. The correlation between the higher grade and the amount of excision is fairly logical. A high grade MCT is more aggressive/invasive and is more difficult to completely remove.
As mentioned in the results, the Log Rank (Mantel-Cox) is not significant (p=.054), even though there seems to be a clear distinction between the categories. This might be due to the low number of tumors in this study.

This study has shown that the OST/PFS is shorter when the tumor invades vessels. This can be explained because when there is vessel invasion, the tumor is more invasive/aggressive and there is a higher chance of metastasis. This poses a higher chance of shortening of the OST/PFS. Vessel invasion is also correlated with a higher grade. When a tumor is capable of invading vessels, it is considered malignant, since the tumor creates an opportunity for metastasis. When the tumor is more malignant, other signs of malignancy might also be present, such as high mitotic rate, which gives the tumor a higher grade. The shorter OST/PFS may be caused by the malignancy of the tumor and the tumors capability to metastasize.

This study has shown that when lymphocytes are present, the OST/PFS is shorter and is correlated with a higher grade. Lymphocytes belong to the adaptive defense system of the body. So if there are lymphocytes present in the tumor, it might seem that the body is specifically attacking the tumor, but the results of this study indicate otherwise. When there are lymphocytes present, the dog has a worse prognosis then when there are no lymphocytes present. A possible explanation might be that when a tumor is fast growing and invasive, which are characteristic of grade 3 or high grade MCTs, it might stimulate an immune response. Apparently, the body's reaction is insufficient and the body is not able to do something about the tumor.

This study has shown that when ulceration is present, the OST/PFS is shorter. Ulceration is also significantly associated with the higher grade MCTs. Occurrence of ulceration with higher grade MCTs can be due to the fact that higher grade tumors are usually growing faster than lower grade tumors. This fast growth might have the result that the blood supply will not be able to keep up with the growth. Because of the insufficient blood supply, an ulcer may arise.

This study has shown that if pieces of the tumor are necrotic, the OST/PFS is shorter. The presence of necrotic areas is also correlated with higher grade tumors. A possible explanation is that when the tumor grows too fast, the blood supply will not be able to keep up with the growth. Lack of blood supply causes hypoxia or ischemia and eventually necrosis. A higher grade MCT will more likely grow faster than lower grade tumors, which supports the results. More active/aggressive and higher grade tumors can also explain the shorter OST/PFS.

This study has shown that when flame figures are present, the OST/PFS is shorter. Flame figures are also mainly seen with higher grade MCTs. It is, however, unclear why these flame figures are present. Flame figures are the result of eosinophils that degranulate on collagen bundles. It thus cannot be explained why the OST/PFS will shorten when flame figures are present and why the presence of flame figures is mainly correlated with higher grade tumors.

This study has shown that dogs with Ki67≥23, have a shorter OST/PFS and is correlated to a higher grade MCT. This can be explained due to the fact that when the cells of the tumor divide more, there are more mitotic figures and according to the Patnaik[4] and Kiupel[5] scales, it should get a higher grade. Also, when the cells of a tumor are dividing more, the tumor is more active/aggressive. This could explain the shorter OST/PFS.

This study has shown that the grid coverage with Factor 8 immunohistochemistry is correlated with a higher grade. A possible explanation for these results is that when a tumor is capable of angiogenesis, the tumor will have more capabilities of progression. The more blood vessels the tumor is able to create, the more it is able to grow, since there are enough resources available. A more actively growing tumor is able to grow bigger and as said before, a bigger (>3cm) tumor is related to a shorter OST/PFS. A more active growing tumor is furthermore more likely to have more mitotic figures showing when histologically examined. More mitotic figures will place the tumor is a higher grade.
The not significant correlation with OST/PFS could be due to the low number of samples incorporated in this study.

In this study, the Ki67 results matched with the data other studies have found and thus we can say that our samples are likely representative for the population.

In addition to grading, tumor size, the degree of excision and vessel invasion, lymphocytes, ulceration and necrosis should be noted when histologically examining MCTs. When the tumor is 3 cm or bigger, or not completely excised and when vessel invasion, lymphocytes, ulceration or necrosis are found, the dog has a significantly worse prognosis. Pathologists should also be aware of flame figures. This study has shown that when flame figures are present in a MCT, the prognosis for the dog is worse than when no flame figures are found, but it is unclear why the eosinophils degranulate in the MCTs. This might be something for further research, since this study indicates that there might be a connection between flame figures and prognosis.
For further evaluation, immunohistochemistry could be performed. Immunohistochemistry for Ki67 provide useful information for determining the prognosis for dogs suffering from MCTs. When a positive cell count of 23 or more with the Ki67 staining the dog has a significantly worse prognosis.
Factor 8, blocks could be an interesting parameter for further evaluations, since there seems to be a correlation between the number of covered blocks found and the Patnaik and Kiupel grades.

For further study
The parameter lymphocytes could be an interesting parameter for further evaluation. It might be interesting to determine which types of lymphocytes are infiltrating the study. Determining which type of lymphocytes might help in better understanding why there is immune response to the tumor, but the prognosis is worse.
The MMP-9 parameter could also be a good candidate for further research. This because other studies[11,40] have shown that the MMP-9 parameter is correlated with gradation. If the MMP-9 parameter proves inadequate, MMP-2 might be another member of the MMP family that might be an interesting candidate for further research.
As said before, the factor 8 parameter could be an interesting parameter for further evaluation since there seems to be a correlation with the grading schemes.

References

Used products:
a: Lab vision corporation (Thermo Scientific), Fremet, CA
b: Abcam Cambridge, UK
c: Dako Cytomation Glostrup, Denmark
d: Santa Cruz Biotechnology Inc., MMP-9 (C-20): SC-6840
e: Klinipath, Duiven, KP-Sil-3061, adhesive slide
f: Vector laboratories Inc. Burlingame, CA
g: Vector Laboratories, ABC-kit, vectastain
h: Dako North America Inc. Carpinteria, Ca, AEC ready to use

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