Prognostication of Canine Mammary Carcinomas Based on Histological and Immunohistochemical Subtypes

by

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Mammary tumours are the most common tumour in intact female dogs. Although the majority are benign, it can be difficult to determine which histologically malignant mammary tumours will actually metastasize or recur. To address this, I investigated several potential prognostic markers for canine mammary carcinomas in combination with histological and immunohistochemical subtypes. The presence of neoplastic cell emboli and overt metastasis, and invasion of neoplastic cells at the periphery of the tumour were the best prognostic markers for predicting poor survival. Of the most common histological subtypes, solid carcinomas were associated with the worst prognosis followed by ductal carcinomas and finally, complex carcinomas. With regards to immunohistochemical subtypes, ER&HER negative (similar to triple negative breast cancer in humans) and unclassified carcinomas had shorter survival and were associated with poor prognostic indicators when compared to luminal A carcinomas. Regardless of other factors, estrogen receptor (ER) negative carcinomas were associated with shorter survival than ER positive carcinomas. In summary, this study demonstrated that canine mammary carcinomas exhibited similar immunohistochemical subtypes to those in human breast carcinoma with similar patterns of prognosis between these subtypes. As well, we show that specific markers that can be used to better prognosticate canine mammary carcinomas, including specific histological subtypes and ER status.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CK 5/6</td>
<td>Cytokeratin 5/6</td>
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<tr>
<td>COX-2</td>
<td>Cyclooxygenase-2</td>
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<tr>
<td>DAB</td>
<td>3,3’-Diaminobenzidine</td>
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<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<td>ER</td>
<td>Estrogen receptor</td>
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<tr>
<td>FISH</td>
<td>Fluorescent in situ hybridization</td>
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<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
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<tr>
<td>HEPACAM</td>
<td>Hepatocyte cell adhesion molecules</td>
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<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
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<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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<td>OR</td>
<td>Odds ratio</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
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<tr>
<td>SCC</td>
<td>Squamous cell carcinoma</td>
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<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TMA</td>
<td>Tissue microarray</td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase plasminogen activator</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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STATEMENT OF WORK DONE

Chapter 2: All work in this chapter was done by me, with the exception that some of the survival data collection was done by Elizabeth Martineau; the rest was collected by me. As well, Dr. David Pearl helped with the statistical analysis.

Chapter 3: All work in this chapter was done by me, with the exception of sectioning of slides for immunohistochemistry, which was done by Barbara Jefferson. As well, Dr. David Pearl helped with the statistical analysis.

Chapter 4: All work in this chapter was done by me, with the exception of sectioning of slides for immunohistochemistry, which was done by Tami Harvey and Barbara Jefferson. As well, Dr. David Pearl helped with the statistical analysis.
INTRODUCTION

Mammary tumours are the most common type of tumour affecting intact female dogs. The common practice of spaying dogs prior to their first estrus has likely decreased the incidence of these tumours, but canine mammary tumours still remain a common diagnosis in veterinary medicine. Mammary carcinomas can be a challenge to prognosticate and over the years many different prognostic markers have been examined, including presence of metastases,\textsuperscript{1,2} peripheral invasion,\textsuperscript{3,4} size of the tumour,\textsuperscript{1,5-8} ulceration of the tumour,\textsuperscript{1,3,8} histological grade,\textsuperscript{9,10} and histological subtypes.\textsuperscript{3,4,11-13} With the advent of molecular techniques, subtypes of human breast carcinoma were discovered and found to correlate with prognosis and new treatment modalities. Few studies have investigated whether these subtypes exist in canine mammary carcinomas and if there are differences in prognosis.\textsuperscript{11,14,15}

Tissue microarray (TMA) technology allows for the analysis of many different tumours on a single slide. A tissue microarray is composed of one or more paraffin blocks with hundreds of paraffin-embedded tissue cores in each. This technology has been used on many different human and canine tumour types,\textsuperscript{16-26} but has not yet been performed on canine mammary tumours. Tissue microarrays have many advantages, the most important of which is consistency of the immunohistochemistry preparation as all the tumour cores are on a single slide and are exposed to the same antigen retrieval, washing, and primary and secondary incubation times. As well, both positive and negative controls can be placed throughout the block acting as internal controls for each antibody used.
The studies compiled in this thesis were designed to a) look at potential prognostic markers (presence of emboli/metastasis, peripheral invasion, and histological grade) and whether they correlated with survival data; b) identify histological and immunohistochemical subtypes and compare them to survival data and the aforementioned prognostic indicators; and c) determine whether TMA technology could be used on canine mammary carcinomas.

**Summary of Following Chapters**

Chapter 1 consists of a literature review discussing mammary cancer of dogs, breast cancer in humans, and tissue microarray technology.

Chapter 2 compares survival data to the prognostic markers (presence of emboli or metastasis, peripheral invasion, histological grade) and histological subtypes as well as comparing the histological subtypes to the prognostic markers. Results are discussed on the prognostication of the different histological subtypes of canine mammary carcinomas.

Chapter 3 validates the canine mammary carcinoma tissue microarray and assesses the heterogeneity of canine mammary carcinomas. Results are discussed on the use of tissue microarray technology with canine mammary carcinomas.

Chapter 4 compares the immunohistochemical subtypes (luminal A and B, ER&HER negative, HER2, and unclassified) and estrogen receptor status to survival data, prognostic markers (presence of emboli or metastasis, peripheral invasion, histological grade), and histological subtypes. Results are discussed on the prognostication of the different immunohistochemical subtypes of canine mammary carcinomas.
Chapter 5 provides a general discussion of the significance of the findings in terms of prognosticating canine mammary carcinomas, limitations of the study, and discusses ongoing and future work.
MAMMARY CANCER OF DOGS

Mammary cancer is the most common malignant neoplasm in dogs with an annual incidence estimated to be 198/100 000. However, that number has likely been reduced since the time when this study was published (1968) as it is common in North America to perform ovariohysterectomies at an early age. In the intact bitch, mammary tumours make up approximately 50% of all cancer diagnoses. Mammary cancer tends to be a disease of older animals with an average age at diagnosis of 10-11 years. It has been previously estimated that anywhere between 10-50% of all mammary tumours are malignant; however this is a difficult number to accurately assess as it is likely that small benign masses are not frequently biopsied by veterinarians so this may be an overestimation. In order to prognosticate mammary tumours, many different parameters have been examined, including size of the tumour, invasion of the tumour into surrounding tissue, lymphatic or vascular invasion, lymph node metastasis, histological grade, and histological classification, as well as many others.

Histological Classification

When a pathologist is presented with a slide of a mammary tumour, one of the first steps is to histologically classify the tumour as the overall phenotype of the tumour could provide clues on its biological behaviour. The basic types of mammary tumours in dogs include adenomas, papillomas, carcinomas, and sarcomas. These can be further
classified based on appearance and there have been several papers that have attempted to further subclassify mammary tumours.\textsuperscript{32-34} The most recent of these is by Goldschmidt et al.\textsuperscript{32} who created a classification system that separated these tumours into their own categories and considered neoplasms of the nipple as a separate entity from neoplasms of the mammary gland. This classification system includes eight main categories (malignant epithelial neoplasms, malignant epithelial neoplasms – special types, malignant mesenchymal neoplasms – sarcomas, carcinosarcoma – malignant mixed mammary tumour, benign neoplasms, hyperplasia/dysplasia, neoplasms of the nipple, and hyperplasia/dysplasia of the nipple).\textsuperscript{32} It is not clear from the article by Goldschmidt et al. what the prevalence of these subtypes is in dogs\textsuperscript{32} and to our knowledge only one study has compared these histological subtypes to prognostic markers.\textsuperscript{11} In the latter study, by far the most common carcinoma type was carcinoma arising in a complex adenoma/mixed tumour (41.8 \% of carcinomas), followed by complex carcinomas (12.1 \%) and then tubulopapillary (7.7 \%), ductal (5.9 \%), and tubular (5.6 \%) carcinomas in similar numbers.\textsuperscript{11} This appears to be in contrast to humans as by far the most common invasive subtype is ductal (not otherwise specified), which accounts for 55 \% to over 75 \% of invasive breast carcinomas.\textsuperscript{35,36}

Numerous studies have looked at prognostication based on histological type.\textsuperscript{3,4,12,13} Sarcomas have been found to have the worst prognosis.\textsuperscript{3,12} As for carcinomas, solid carcinomas have been shown to have a longer survival time than anaplastic carcinomas, but a shorter survival time than papillary or tubular carcinomas.\textsuperscript{4} Histological types associated with a better prognosis include complex carcinomas and carcinoma in situ.\textsuperscript{13} For the most recent classification system, Im et al. compared these
Histological subtypes to histological grade and presence of lymphatic invasion, but not survival.\textsuperscript{11} When Im et al. compared the histological subtypes to lymphatic invasion, two subtypes (anaplastic carcinoma and comedocarcinoma) stood out as all cases of each (five for each subtype) were associated with lymphatic invasion;\textsuperscript{11} this would indicate that these two particular subtypes have a poor prognosis. In comparison, of the three most common subtypes mentioned above, only one carcinoma arising in a complex adenoma/mixed tumour, one tubulopapillary carcinoma, and no complex carcinomas were associated with lymphatic invasion,\textsuperscript{11} which would indicate these three subtypes are associated with a better prognosis. These results indicate that certain histological subtypes are correlated with differences in prognosis, but there is still a need for more studies to specifically compare the histological subtypes proposed by Goldschmidt et al.\textsuperscript{32} to accurate prognostic indicators or ideally, to survival data.

**Histological Grading**

In order to determine clinical prognosis of mammary carcinomas, grading systems can be useful as different grades have previously been linked to differences in overall survival.\textsuperscript{9,10} Grading systems are based on both cellular and nuclear features of the neoplastic cells.\textsuperscript{37,38} In humans, the scheme created by Elston and Ellis is the most common histological grading system used.\textsuperscript{39} This grading system showed a strong prognostic relationship in a large number of patients with long-term follow-up.\textsuperscript{39} Karayannopoulou et al.\textsuperscript{9} as well as other studies\textsuperscript{10,31} developed a similar system in dogs based on the Elston and Ellis method\textsuperscript{39}, which was based on three variables (proportion of tubule formation, nuclear pleomorphism, and mitotic rate) (Table 1-1). The authors
found that survival was significantly shorter in dogs diagnosed with grade 3 carcinomas than dogs diagnosed with either grade 1 or grade 2 carcinomas; with up to 86.7% of dogs with grade 3 carcinomas having died or been euthanized within 2 years after mastectomy. Another result from this study was that 46.4% of dogs with a grade 2 carcinoma died within 2 years after mastectomy. This provides a major clinical concern as our chances of predicting clinical outcome based on a grade 2 tumour is hindered as 50% will survive greater than 2 years whereas the other half will die of cancer related illness within that same time period. However, a more recent study by Pena et al. looking at grading canine mammary carcinomas found that over 80% of dogs diagnosed with a grade 2 carcinoma were alive more than 3 years later. The reason for the difference in survival of grade 2 carcinomas between these two studies is unclear as the number of dogs involved in each study was similar (85 in Karayannopoulou et al.; 65 in Pena et al.), as well the selection criteria was almost identical except that Karayannopoulou et al. used exclusively infiltrating carcinomas, whereas Pena et al. appeared to use non-infiltrating carcinomas in their study as carcinoma in situ was one of the histological subtypes analyzed. Based on the original human grading system using only invasive adenocarcinomas, the Karayannopoulou et al. system followed it more closely with their use of infiltrating carcinomas. Although Pena et al. used noninvasive carcinomas in their analysis, their system may be more beneficial for grading canine mammary carcinomas as a percentage of these tumours are noninvasive.

One big difference in results between these studies was the percentage of animals that died from mammary carcinoma (45.9% in Karayannopoulou et al.; 20.0% in Pena et al.). Both studies had performed physical exams and thoracic radiographs, but it is
unclear if these were the only two diagnostic tests performed to determine whether metastasis had occurred or not. As well, neither study specifically states what information they used to conclude that an animal died or was euthanized as a result of the mammary carcinoma. Therefore, it is possible that the difference in the proportion of animals that died or were euthanized as a result of mammary carcinoma was a direct result of how each study determined this variable. As well, it could be that because the Karayannopoulou et al. study used only infiltrating carcinomas, their population may have been at a higher risk for metastasis as it has been shown in other studies that peripheral invasion of mammary carcinomas is correlated with a worse prognosis. Regardless of this, it does appear that, based on both histological grading studies, grade 3 carcinomas are associated with a shorter overall survival than both grade 1 and 2 carcinomas.

Prognostication

In an attempt to better prognosticate canine mammary tumours, researchers have looked into other clinical factors or molecular markers that may help in prognostication. Clinical factors that have been examined include age of the patient, ovariohysterectomy status, estrous duration, number of estrous cycles, lymph node involvement, size of the tumour, growth pattern of the tumour, and ulceration of the tumour. As discussed above, categorizing the tumour can help with prognosis in some cases. Of these clinical factors, the size of the tumour, ulceration of the tumour, infiltrative growth pattern, and lymph node involvement have been shown in multiple studies to be associated with prognosis. Tumour size has been shown to correlate with decreased
survival; however the size of tumour differs by study, with one showing a decreased
survival in tumours > 3 cm\textsuperscript{5} and the other with tumours > 5 cm.\textsuperscript{6} Both of these studies
actually split the tumours into three size categories (< 3 cm, 3-5 cm, and > 5 cm);
however, neither study specifically states why they chose to set their size cutoffs at those
points (3 vs 5 cm) so it is unclear as to what the exact cutoff should be.\textsuperscript{5,6} Regardless of
the exact size, it appears that larger tumours are associated with increased lymph node
metastasis and decreased survival.\textsuperscript{6} Ulcerated tumours have been shown to have a poorer
prognosis than non-ulcerated tumours\textsuperscript{3} and infiltrative growth has been shown to have a
poorer prognosis than tumours with a non-infiltrative growth pattern.\textsuperscript{3,4,40} Finally, cases
with lymph node metastasis have a shorter overall and disease free survival than cases
with no evidence of lymph node metastasis.\textsuperscript{2,3,13}

Molecular Pathology

In addition to the previously discussed clinical and histological parameters
molecular markers have also been examined to determine if they are prognostically
significant in canine mammary tumours. One group of markers that has been studied are
markers that are involved in the cell cycle. These cell cycle-related markers include Ki-
67 and proliferating cell nuclear antigen (PCNA). Both are involved in the cell cycle and
are present in proliferating cells; however, Ki-67 is no longer expressed in cells after
mitosis is finished whereas PCNA has a longer half-life and is also involved in DNA
repair and is expressed in non-cycling cells as well.\textsuperscript{8,41,42} When comparing these
markers, it was found that high levels of Ki-67 were associated with metastasis and death
from mammary neoplasia, but not recurrence; PCNA, on the other hand, failed to predict
metastases, recurrences, or cancer related death.\textsuperscript{1,8} Multiple studies have shown that Ki-67 is an independent prognostic factor in predicting metastases, disease free survival, and overall survival,\textsuperscript{1,8} however this only holds true when considering poorly differentiated areas of the tumour and counting both weakly and strongly staining cells as positive.\textsuperscript{8} These results are promising and it appears that Ki-67 could be used as a prognostic indicator; however, the study size in Pena et al. with survival data was only 45 dogs and they were only followed for a total of 18 months\textsuperscript{8} so it would be interesting to see if these results were confirmed in a larger study size and for a longer survival interval. These results may also not be practical for a diagnostic laboratory as it would be extremely time consuming for a pathologist to search out the most poorly differentiated areas of every tumour and then proceed to count every cell in 8-10 high-power fields (minimum 1,000 neoplastic cells) as was performed in the study by Pena et al.\textsuperscript{8} A simpler, less time consuming method would be required before this could be instituted as a routine prognostic test.

As canine mammary tumour development is suspected to be hormonally dependent,\textsuperscript{28,43,44} it makes sense to investigate steroid hormone receptors as prognostic markers. It has been shown that lower levels of estrogen receptor (ER) expression were observed in primary tumours that had given rise to distant metastases.\textsuperscript{1} Many studies have found that decreasing percentages of ER immunoreactivity correlates with increasing malignancy\textsuperscript{1,5,45-47} (definitions of malignancy were based on the histological malignancy grade\textsuperscript{1,45} or in some studies, not defined\textsuperscript{5,46,47}). In one study, ER status was found to be an independent prognosticator for disease free survival.\textsuperscript{1} Another study, that looked at both ER and progesterone receptor (PR) status, was unable to correlate steroid
receptor status as an independent prognosticator for survival; however, this study did not look at ER and PR separately so it is unclear if ER alone would have been more closely related to survival or not. PR presence was significantly decreased in large tumours and in malignant tumours; however, the authors didn’t actually define what constitutes a malignant versus a benign tumour so the significance of this is unclear. Although tumour size was linked to prognosis and PR presence; PR was not compared to overall survival so it is unclear if it has any prognostic value.

In humans, mutations in the BRCA1 gene have been associated with hereditary breast cancers. BRCA1 helps to maintain genome integrity as it is involved in repair of damaged DNA, is a checkpoint of the cell-cycle, and is a regulator of centrosome duplication. Loss of these functions secondary to mutation of BRCA1 leads to genetic instability as well as inactivation of p53, eventually leading to tumourigenesis. In dogs, loss of BRCA1 immunohistochemical (IHC) expression has not been significantly correlated with disease free or overall survival. On the other hand, mutations in BRCA1 were found to be correlated with histologically malignant canine mammary tumours when compared to histologically benign mammary tumours; however the correlation was not significant in this case. Another study found that in canine mammary tumours, low IHC expression of BRCA1 was significantly correlated with indirect markers of malignancy (poor tubular differentiation, high mitotic rate, high rate of nuclear pleomorphism, increased proliferation rate (elevated Ki-67 index), and absence of estrogen receptors). Another protein (topoisomerase IIβ binding protein 1 [TopBP1]) that is similar in both structure and function to BRCA1 has been investigated in dogs, but unlike BRCA1 it has not been investigated to determine if expression of TopBP1 is
correlated with differences in survival in canine mammary tumours. It was found that TopBP1 is expressed in a higher percentage of cells in histologically malignant than in histologically benign canine mammary tumours. As well, the cellular localization of staining changed from nuclear to both nuclear and cytoplasmic in more histologically malignant phenotypes. A similar change in immunolabelling location has been noted in BRCA1 with canine mammary tumours, but the significance of this is undetermined as it did not correlate with survival data or any prognostic parameters.

Mutations in the \( p53 \) gene are one of the most common genetic aberrations in human cancers and they have been detected in several canine cancers. The \( p53 \) gene product is one of the main checkpoints during the cell cycle and arrests cell division when there is DNA damage or a metabolic disturbance. During this arrest the cell can be repaired, but if that fails then the cell will undergo apoptosis mediated by \( p53 \). A mutation in \( p53 \) can lead to loss of this control and can enable malignant transformation of these damaged cells. Mutations in \( p53 \) have been found in a small percentage of canine mammary carcinomas. BRCA1 and \( p53 \) have been found to work cooperatively to induce apoptosis of cancer cells and an increase in \( p53 \) immunolabelling was found in canine mammary tumours with increased BRCA1 expression. Two studies have found an association between \( p53 \) IHC overexpression and both increased malignancy and metastasis of canine mammary carcinomas. There is disagreement in the literature as to the usefulness of \( p53 \) overexpression as a prognostic marker as some studies have found \( p53 \) overexpression to be an independent risk factor for increased recurrence and death from canine mammary tumours and with a significantly shortened survival time, whereas others have not found the same prognostic value.
many potential reasons for the differences in these results as the methods between these two studies differed significantly. The study by Wakui et al. that did not find a correlation between p53 overexpression and survival used a total of 69 dogs that were diagnosed histologically with mammary carcinomas,\textsuperscript{62} whereas Lee et al. only had 20 dogs enlisted in the study and had a mix of histologically benign (eight mixed and two adenomas) and malignant (five malignant mixed and five adenocarcinomas) mammary tumours.\textsuperscript{60} As well, Lee et al. do not actually state which tumours had overexpression of p53 aside from stating that 20\% of the histologically benign and 30\% of the histologically malignant tumours were positive.\textsuperscript{60} Due to this difference in case selection there is no way to directly compare the results of these studies as Wakui et al. were assessing the prognostic significance of p53 overexpression in canine mammary adenocarcinomas,\textsuperscript{62} whereas Lee et al. were looking at canine mammary tumours as a whole and unfortunately did not state which of the malignant tumours (mixed versus adenocarcinomas) were positive.\textsuperscript{60} Based on the explanation by Lee et al. it is difficult to determine what exactly they were measuring for survival time as they state that survival time was recorded as death due to progression of disease or last clinical assessment.\textsuperscript{60} Based on this it is unclear if cases that were lost to follow-up soon after diagnosis were included in the survival results or not, which potentially could skew the results. Because of these differences in results, more studies investigating the correlation between p53 overexpression and survival are required before definitive conclusions can be made.

The \textit{c-erbB-2/HER2} proto-oncogene is involved in pathways that lead to both normal cell growth and differentiation.\textsuperscript{63} Overexpression of HER2 (gene amplification) is present in a number of human tumours,\textsuperscript{64-66} including breast carcinoma.\textsuperscript{67-69} In dogs,
overexpression of HER2 has been significantly associated with increased mitotic index; however, no association was found with local invasion or regional metastasis and HER2 overexpression has not been shown to correlate with a decrease in survival. Expression of epidermal growth factor receptor (EGFR), a related receptor, in canine mammary carcinomas has not been shown to correlate significantly with survival, but was shown to correlate significantly with histological malignancy and was associated with a reduced disease-free and overall survival compared to the histologically benign mammary tumours; however, this association was not significant. Numerous other markers have been investigated in canine mammary tumours and the following have been shown to significantly correlate with a reduction in survival: E-cadherin (mediates cell-cell adhesion), urokinase plasminogen activator (uPA, involved in extracellular matrix and basement membrane degradation), cyclooxygenase (COX)-2 (affects cell proliferation and adhesion, apoptosis, immune-surveillance, and angiogenesis), and CK19 (intermediate filament involved in structural integrity of epithelial cells). The remaining markers have been correlated with indirect measures of malignancy (carcinomas compared to adenomas; vascular invasion; lymph node metastases), but have not been found to correlate with survival. These markers include caveolin-1 (suggested to be involved in tumour suppression and oncogenesis), Derlin-1 (involved in exportation of misfolded proteins and is an inhibitor of apoptosis), insulin receptor (involved in the inactivation of growth factors), CD44 (involved in cell-cell interactions, cell adhesion, migration, and angiogenesis), T-lymphocytes (involved in the inflammatory response), galectin-3 (involved in cell-cell and cell-extracellular
matrix interactions), and vascular endothelial growth factor (VEGF, involved in angiogenesis).

**Immunohistochemical Subtyping**

The investigation of human breast cancer has led to the discovery of molecular subtypes within human breast carcinomas, which have been classified using immunohistochemistry (Table 1-2) and will be discussed in more detail in a later section. Since then, a few studies have looked to see if similar IHC subtypes existed in canine mammary carcinomas. Only Gama et al. and Sassi et al. correlated their results with survival data so they will be discussed in more detail. Even though these studies were investigating the same problem, they went about it slightly differently (summarized in Table 1-3). The main subtypes in humans are the luminal A and B, basal-like/triple negative, HER2 overexpressing, and the negative/unclassified phenotype. As with the human studies, which found that ER and HER2 positivity were important in correlating the IHC subtypes with the molecular subtypes, the canine studies also first looked at ER and HER2 expression with the basal markers mainly differentiating the basal-like/triple negative subtype from the negative/unclassified subtype. One of the major differences between how these two studies determined subtypes was the markers they used to determine basal-like tumours. Gama et al. used CK5, P-cadherin, and p63 as markers based on the findings in Matos et al. in human basal-like breast carcinomas. Whereas Sassi et al. used CK5/6 and CK14 as their markers as was used by Cheang et al. for human breast carcinoma. Although there are differences in how each of these studies determined the basal-like
subtype, they were both based on human studies that correlated their data with the molecular subtypes.\textsuperscript{88,89} Both studies used similar criteria to determine the subtypes with estrogen receptor positive tumours indicating a luminal subtype, HER2 positivity determining the tumour to be luminal B and HER2 negativity luminal A, and basal marker positivity being inconsequential;\textsuperscript{14,15} however Sassi et al.\textsuperscript{15} also used progesterone receptor positivity to classify the tumours as a luminal subtype. Both studies also used estrogen receptor negativity (and progesterone receptor negativity in Sassi et al.)\textsuperscript{15} with HER2 positivity and inconsequential basal marker expression to indicate HER2 overexpressing tumours.\textsuperscript{14,15} Finally the basal-like tumours were similar to the HER2 overexpressing tumours except they were HER2 negative and basal marker positive,\textsuperscript{14,15} and the negative phenotype was negative for all antibodies.\textsuperscript{14,15}

When comparing the canine subtypes to those in humans, Gama et al.\textsuperscript{14} found that, similar to humans, the basal-like tumours were associated with lower survival rates than were the luminal subtypes. The HER2 overexpressing subtype was associated with poor prognostic indicators (large size, high histological grade, peripheral invasion, no myoepithelial proliferation, and high mitotic rate); however unlike humans these tumours tended to be associated with higher survival rates.\textsuperscript{14} One possible reason for this difference may have been that only 8 HER2 overexpressing tumours were diagnosed in this study so larger sample sizes may be required to better correlate this subtype with survival in dogs.\textsuperscript{14} Sassi et al.\textsuperscript{15} on the other hand failed to associate any of the subtypes with differences in survival. The likely reason for this is the difference in defining basal-like subtypes based on the use of different immunohistochemical markers.\textsuperscript{14,15} As well Sassi et al.\textsuperscript{15} failed to diagnose any HER2 overexpressing or null phenotype tumours
within their study. Based on these findings it is evident that further larger scale studies are required to determine if canine mammary carcinomas can be subdivided into categories and whether these subtypes correlate with prognosis as has been shown in humans.84-86

**Scoring in Immunohistochemical Studies**

Comparing immunohistochemical studies is difficult as scoring the immunolabelling is often not consistent between the studies. With the previously discussed immunohistochemical subtypes in canine mammary tumours, the difference in results may be partially determined by the differences in how the subtypes were determined. All three of these papers11,14,15 used a combination of hormone receptors (ER and/or PR), HER2, and myoepithelial markers (CK5/6, CK14, p63, P-cadherin); however how they determined positive results varied by the study. HER2 categorization was relatively consistent with all three studies using a positive cutoff of > 10 % complete membranous immunolabelling of neoplastic cells.11,14,15 There were slight differences in hormone receptor cutoffs with both Gama et al.14 and Im et al.11 using a positive cutoff of > 10 % immunolabelling of neoplastic cells and Sassi et al.15 using a positive cutoff of at least 5 % immunolabelling of neoplastic cells. The largest difference between these three studies is the positive cutoff use for the myoepithelial markers, which help differentiate basal-like tumours from negative/unclassified tumours. The three studies range from at least 1 % immunolabelling being considered positive15 up to greater than 50 % immunolabelling being considered positive14 with Im et al. closer to the former (5 %
immunolabelling).\textsuperscript{11} This could be one reason why Gama et al. were able to correlate the basal-like subtype with lower survival rates whereas Sassi et al. were not.\textsuperscript{14,15}

In order to try and avoid this problem of being unable to accurately compare between studies a group of researchers proposed a set of standardized procedures and immunohistochemical cutoffs for each of the three types of markers stated above (hormone receptors, HER2, and myoepithelial markers) for future studies in this field.\textsuperscript{90} One large problem with these recommendations is that they don’t appear to be correlated with survival data or even other prognostic information (grade, metastases, etc.), which makes their utilization in practice undetermined at this point.

The cutoffs that they do propose for the myoepithelial markers are at least 10 % of neoplastic cells exhibiting immunolabelling.\textsuperscript{90} The reasoning that the authors give for this is that most previous studies have used this number so it appears this would be the easiest cutoff point to stick with.\textsuperscript{90} Although this makes for convenient comparison between studies it is clear that more research is required to determine an appropriate cutoff point. In comparison, the authors suggest that for the HER2 antibody, canine mammary tumours should directly mirror what has been recommended in humans\textsuperscript{91} and use the HercepTest scoring system with only 3+ tumours being considered positive (at least 30 % of neoplastic cells exhibiting complete membranous immunolabelling of strong intensity).\textsuperscript{90} The human cutoff point is set that high as it is mainly used for determining which patients could benefit from anti-HER2 therapy.\textsuperscript{91} As well, in humans a HercepTest result of 2+ does not necessarily indicate that the test is negative. The standard protocol for a 2+ result is to run fluorescent in situ hybridization (FISH) to determine the HER2 gene amplification\textsuperscript{91} as it has been shown that up to 48 % of human
cases with a HercepTest score of 2+ have amplification of the gene. As far as we are aware, no anti-HER2 treatments have been attempted in dogs with mammary tumours so the proposal of setting cutoffs that mirror what is currently recommended in humans for treatment recommendations is unwarranted at this time.

The one set of markers that has the best recommendation associated with it is the hormone receptors. The authors recommend that the accepted human Allred scoring system is used and that instead of stating an arbitrary cutoff the authors also recommend that the Allred score is published for each tumour as not enough work has been done as of yet to assess what an accurate cutoff would be. At this point, more studies are required to correlate these markers with survival data to determine what the most accurate cutoffs should be. Ideally the best study would statistically correlate immunohistochemical expression of each antibody in question with survival data, or if survival data is not available then at the very least prognostic markers that have been correlated with survival data (presence of metastasis, peripheral invasion, histological grade), to determine appropriate cutoff points.

**Treatment**

Surgery can be curative in dogs with benign tumours as well as low histological grade malignant tumours; however dogs with higher histological grade tumours that have more potential for metastasis tend to perform worse following surgical removal and may benefit from additional therapy. Previously it was thought that the type of surgery performed did not affect outcome as a study comparing simple mastectomy to radical mastectomy found that there was no significant difference in recurrence rates or survival
times. However, a more recent study found that dogs that had a regional (or simple) mastectomy were significantly more likely to have a second tumour in the immediately adjacent gland and that histologically malignant tumours were more likely to recur. These new results suggest that radical mastectomy is indicated to prevent local recurrence of mammary tumours and that regional (or simple) mastectomy is not sufficient.

With intact female dogs there is always the question as to whether ovariohysterectomy at the time of surgery is beneficial or not. The results to date are unclear with Morris et al. finding that ovariohysterectomy at the time of tumour removal had no significant effect on tumour progression; however the authors did not investigate survival time. In comparison, Sorenmo et al. found that dogs spayed less than 2 years before tumour development or at the time of tumour surgery had a significantly longer survival time than did dogs spayed more than 2 years before tumour development or intact dogs (there was no significant difference in survival between dogs spayed less than 2 years prior to tumour removal and those spayed at the time of surgery so these dogs were combined into one group). One explanation for these results suggested by the authors is that a long interval between ovariohysterectomy and tumour development could be associated with a decrease in estrogen receptor expression by the tumour and thus surgical removal of the ovaries (and subsequent decrease in circulating estrogen) would have less benefit for these patients. One limitation with this study is that estrogen receptor expression of the mammary tumours was not determined, which makes it difficult to draw conclusions based on what affect ovariohysterectomy at or close to the time of tumour removal is actually having on the tumour itself.
Unlike in humans, there are no standards for adjuvant chemotherapy for mammary cancer treatment in dogs. Different chemotherapy protocols have been investigated in dogs with varying results. Post-operative chemotherapy with either doxorubicin or docetaxel did not lead to any increase in recurrence-free interval, time to metastasis, or overall survival.\(^9^8\) Although the researchers did not find significant differences in survival there was a tendency toward longer survival in the chemotherapy group.\(^9^8\) Even with a lack of significant differences in survival, there were a couple of potential issues with the study design that could have biased results. The first is that the patients were not truly randomized into different groups; the control group (surgery alone) was composed of dogs whose owners declined further therapy and the chemotherapy group was composed of dogs with owners who agreed to additional therapy. This creates a potential bias by placing owners who may be more likely to try more therapy or other options to keep the dog alive longer in the chemotherapy group. The other issue is that in the statistical analysis there was no attempt made to differentiate cancer related death from death unrelated to cancer. This again can create bias as these are older dogs that are more likely to suffer from other diseases that could result in euthanasia and death and again there could be a difference in the owners willingness to pursue treatment for these other conditions between the two groups (based on their willingness to agree to chemotherapy or not). In comparison, another study found a significantly improved survival time in dogs treated with 5-fluorouracil and cyclophosphamide given at the same time commencing one week post-surgery compared with dogs treated with surgery alone.\(^9^9\) However, this study had a similar design problem to the study by Simon et al. as the dogs were placed into two groups based on the owners’
willingness to pursue further treatment. As well, there were only 16 dogs enrolled in this study, which makes for a good pilot study size; however, it is clear that a larger study sample with dogs appropriately randomized into different treatment groups is required before definitive conclusions can be reached on this treatment protocol. Other therapeutic agents (tamoxifen, tyrosine kinase inhibitors, and paclitaxel) have been utilized to treat canine mammary tumours but there are few studies to make definitive conclusions about these therapies. If subtypes are discovered in canine mammary carcinomas this may lead to additional targeted treatment options as has been the case with human breast carcinoma.

BREAST CANCER IN HUMANS

In the past 15 years, advances have been made in the diagnosis and classification of breast cancer in humans. These advances have led to the development of new prognostic markers, especially molecular and immunohistochemical subtypes, which have in turn led to the development of new therapies. As dogs also develop spontaneous mammary tumours, it is interesting to determine if similar immunohistochemical subtypes exist in dogs and whether they correlate similarly with prognosis as they do in humans.

“In Canada, breast cancer is the most common cancer in women (excluding non-melanoma skin cancers) and is the second leading cause of death from cancer in Canadian women.” "In 2013, it is estimated that approximately 23,800 will be
diagnosed with breast cancer (26% of all new cancer cases in women in 2013) and approximately 5,000 women will die from breast cancer (14% of all cancer deaths in women in 2013). The 5-year relative survival is estimated to be 88%. It is estimated that 1 in 9 Canadian women will develop breast cancer during her lifetime and 1 in 29 will die from it.

Three types of epithelial cells make up normal breast tissue: (1) luminal or glandular cells, (2) basal or myoepithelial cells, (3) and stem cells. Using immunohistochemistry, the luminal and myoepithelial cells can be distinguished based on their cytokeratin expression patterns, with myoepithelial cells expressing both cytokeratin 5/6 and cytokeratin 17, while luminal cells express cytokeratins 8 and 18.

Similar to what has previously been discussed in dogs, human breast carcinomas are classified based on histological appearance. By far the most common invasive histological subtype is the ductal not otherwise specified, which accounts for anywhere between 55% up to over 75% of invasive breast carcinomas. The remaining invasive histological subtypes often account for less than 10-20% of the overall cases and include patterns, such as mixed tubular, lobular, and mixed ducto-lobular.

**Molecular Subtyping by Gene Expression**

Similar to dogs, many different parameters have been correlated with prognosis in breast cancer including tumour size, lymph node metastases, histological grade, expression of estrogen receptor, and overexpression of ERBB2/HER2. With the introduction of DNA microarray analysis the molecular make-up of breast tumours became better categorized. Perou et al. were able to identify four groups of tumours
with different molecular features (ER+/luminal-like, basal-like, ERBB2+/HER2, and normal breast). Sorlie et al.\textsuperscript{85} further characterized tumours into six distinct categories (basal-like, ERBB2+, normal breast-like, luminal A, luminal B, and luminal C). The basal-like, ERBB2+, and normal breast-like all had little to no gene expression of ER.\textsuperscript{85} The basal-like subtype had high expression of keratins 5 and 17, laminin, and fatty acid binding protein 7.\textsuperscript{85} In contrast, the ERBB2+ subtype had high expression of several genes in the \textit{ERBB2} amplicon (\textit{ERBB2} and \textit{GRB7}).\textsuperscript{85} Finally, the normal breast-like subtype had very high expression of genes, expressed by adipose tissue and other nonepithelial cell types, as well as expression of the basal epithelial genes and low expression of the luminal epithelial genes.\textsuperscript{85} The luminal/ER+ subgroup previously defined by Perou et al.\textsuperscript{84} was further separated into three distinct subgroups by Sorlie et al.\textsuperscript{85} Luminal subtype A had the highest expression of the \textit{ER\alpha} gene as well as genes encoding other proteins (GATA binding protein 3, etc.), whereas luminal subtypes B and C showed lower expression of the \textit{ER\alpha} gene and other luminal specific genes.\textsuperscript{85} Luminal subtype C could be differentiated from subtypes A and B by a high expression genes of which the function is unknown.\textsuperscript{85} Clinically these findings are important as they correlated with disease outcome.\textsuperscript{85,86} The basal-like and ERBB2+ subtypes were associated with the shortest survival times and disease-free survival.\textsuperscript{85} Luminal subtype A had the best clinical outcome and the luminal subgroups B and C had intermediate survival.\textsuperscript{86}
Immunohistochemical Subtyping

Molecular subtyping of breast carcinomas is the gold standard for determining subtypes as it most accurately determines how each carcinoma is related to another. One issue is that molecular subtyping is more involved, time consuming, and costly so more recent studies have looked into correlating immunohistochemical subtypes with the molecular subtypes as the use of immunohistochemistry (IHC) to subtype breast cancer would be more practical in a clinical setting. Nielsen et al. used IHC to categorize breast cancer in the following way: any tumour that is HER2 (ERBB2) positive is placed in the HER2 category; any tumour that is HER2 negative-low and ER positive is placed in the luminal category; any tumour that is both HER2 and ER negative but positive for at least one of cytokeratin 5/6 and/or HER1 is placed in the basal-like category; and finally any tumour that is negative for all four markers is placed in the negative category. This basal-like subtype categorized by IHC was found to correlate well with the basal-like subtype determined by gene expression and subsequently had a much worse prognosis than the luminal subtype. Carey et al. further subcategorized these IHC-defined subtypes as follows: basal-like (negative for ER, PR, and HER2; positive for cytokeratin 5/6 and/or HER1), HER2+/ER- (negative for ER and PR; positive for HER2), luminal A (positive for ER and/or PR, negative for HER2), and luminal B (positive for ER and/or PR, as well as HER2) (Table 1-2). Tumours that were found to be negative for all 5 IHC markers were considered unclassified. The HER2+/ER-subtype was found to have the worst prognosis followed by the basal-like; however, treatment data was not available to the researchers so they could not comment on whether differences in treatment protocol resulted in these differences in survival. These two
studies are slightly different in how they categorize the tumours particularly in how the HER2 overexpressing tumours are classified; with Nielsen et al.\textsuperscript{117} using only HER2 positivity as the only inclusion criterion, whereas Carey et al.\textsuperscript{87} using ER negativity and HER2 positivity to classify the HER2+ tumours and having a separate category for HER2+ and ER+ tumours (luminal B). Based on the original molecular studies the more accurate categorization appears to be that of Carey et al.\textsuperscript{87} as the original HER2 tumours were found to have low expression of ER and high expression of HER2, whereas the luminal B were found to have expression of ER as well as often having expression of HER2.\textsuperscript{85,86}

**Therapeutic Implications of Subtyping**

Breast cancer subtypes are important for more than just prognosis as targeted therapy can be used for these individual subtypes. Specifically, trastuzumab (a recombinant monoclonal antibody against HER2) is used to treat women with HER2+ breast carcinoma and has been shown, in combination with chemotherapy, to be associated with an increased time interval to disease progression and a longer duration of response time, as well as longer survival times in these women.\textsuperscript{104} Similarly, tamoxifen has been found to increase disease free survival and overall survival, as well as a reduction in contralateral breast cancer in women with ER-positive tumours.\textsuperscript{105} Rouzier et al.\textsuperscript{103} used molecular classification to subtype breast cancer and found that basal-like and HER2+/ER- subtypes of breast carcinoma had a higher rate of pathologic complete response (45 % for both) with paclitaxel- and doxorubicin-containing preoperative chemotherapy when compared with luminal (6 %) and normal-like (0 %) tumours. The
authors defined pathologic complete response as patients who had no evidence of residual invasive cancer in the breast or axillary lymph nodes or patients with residual in situ cancer. This was a very small study with only 82 patients and a range of 10-30 patients in each category so a larger patient pool would be ideal to confirm these results and percentages of pathologic complete response. Similarly, using IHC to subtype breast cancer, Carey et al. found that the basal-like (85 %) and HER2+/ER- (70 %) have higher rates of complete response or partial response to doxorubicin plus cyclophosphamid chemotherapy than did the luminal A (39 %) and B (58 %) subtypes; however, the HER2+/ER- and basal-like tumours still had a shorter overall survival than did the luminal tumours. Berry et al. found that doxorubicin/cyclophosphamide plus paclitaxel every two weeks for estrogen receptor negative breast cancer lowered the rate of recurrence and death to levels similar to patients with estrogen receptor positive tumours that are optimally treated. All of these results suggest that ER- tumours have a better response to primary chemotherapy than do the ER+ tumours. One potential reason for this difference in response is that the ER- subtypes (basal-like and HER2+/ER-) were characterized by high expression of a cluster of genes involved in proliferation. A recent study found that tumours that are characterized by these proliferation genes showed pathologic complete response (defined as the absence of invasive cancer in the breast; residual carcinoma in situ was allowed) to primary chemotherapy. This result supports the idea that there is a relationship between proliferation and chemotherapy sensitivity.
TISSUE MICROARRAYS

Tissue microarrays (TMAs) are a platform for high throughput analysis of tissue and a well-designed microarray can reduce the need to perform the same experiment multiple times. A tissue microarray is one or more paraffin blocks with hundreds of paraffin-embedded tissue cores of interest in each block. Advantages of TMAs are that all of the IHC steps (antigen retrieval, temperature, washing time, and reagent concentration) are the same for all of the samples examined. The size of the tissue core is one of the most important aspects when constructing a TMA. Most samples can be taken with a 0.6 mm diameter needle; however, certain tissues (bone) can be very difficult to array and best results are obtained with 1.0 mm needles. Another consideration, as with the use of immunohistochemistry in general, is to include normal/control tissue in the TMA. The use of appropriate controls allows the user to determine the quality of the staining and some investigators advocate placing control/normal tissue distributed throughout the array to ensure the entire slide is stained adequately. One of the major concerns with TMAs is that such small cores may not represent the tissue as a whole, especially in cases where the tissue is heterogeneous. The best way to sample heterogeneous tissues is to target the most representative areas of each tumour.

Validation is necessary when constructing a TMA from a new tissue type. Validation has previously been performed on many different human tumours including breast carcinoma, ovarian carcinoma, fibroblastic neoplasms, urinary bladder carcinoma, and lung tumours (carcinomas, carcinoids, neuroendocrine tumours, and
malignant melanomas). In dogs, TMAs have yet to be used extensively, but there are a few studies that have investigated different canine tumours using TMAs, including osteosarcoma, primary tumours of the central nervous system, lymphoma, mast cell tumours, and gliomas.

**Validation**

Validation involves determining how many tissue cores are required per tumour sample to adequately represent expression of any particular antigen. Torhorst et al. designed a TMA study to address the sampling of large, potentially heterogeneous tumours and found that the frequency of positivity was virtually the same between the small cores and the large sample, which has also been seen in other studies. Camp et al., as well as another group, have found that even with the variability of antigen expression between cores that analysis of just two disks significantly represented the overall tumour expression. One pitfall with this is that two readable disks are required and the number of readable disks can be affected most importantly by the individual constructing the array. In one study, one person was given the task of constructing the arrays and it was found that the number of useable disks increased with each array constructed indicating that proper training prior to constructing TMAs is essential. Other factors that will increase the number of useable disks is correctly identifying the tumour in question histologically and accurately sampling it, while avoiding the other cell types in section. The standard that Camp et al. employed was to take three separate cores from each tumour block, ensuring that they included the edge and the tumour centre. Other studies have suggested that selection of two to ten cores per tumour sample
can minimize sampling error by the operator as well as decrease the impact that loss of
tissue during processing can have.\textsuperscript{121}

\textbf{Antigen Degradation}

One of the major concerns with any retrospective immunohistochemical study is
whether the antigen will be expressed after prolonged periods in storage.\textsuperscript{16} Also, time to
tissue fixation and fixation time may effect antigen expression.\textsuperscript{125,126} Camp et al.\textsuperscript{16}
showed that prolonged periods of storage did not appear to affect antigen expression for
blocks kept up to 60 years; however cases from 68 years previously showed a
significantly decreased expression of estrogen receptor suggesting that antigen expression
may be affected for some antigens after greater than 60 years of storage. According to a
recent study performed by Pinhel et al.,\textsuperscript{125} expression of phosphorproteins (p-Akt, p-Erk)
was significantly lower for samples with longer time to fixation. This correlation of
decreased expression in phosphoproteins, which is secondary to rapid dephosphorylation
when fixation is delayed, validates data seen in earlier studies.\textsuperscript{127-129} In contrast the breast
cancer biomarkers, estrogen receptor, progesterone receptor, HER2, and ki-67, did not
differ significantly in tissues delayed until fixation when compared with tissues with
immediate fixation.\textsuperscript{125} Based on these results, the only concern would be in a study
examining a particular phosphoprotein. In studies like these the authors would have to
ensure that sample collection is standardized to minimize artifactual discrepancies in
protein expression, but in most other studies slight variations in sample collection are
unlikely to result in significant differences in protein expression.
Tissue Microarray Scoring

Another concern with TMAs is scoring the immunohistochemical staining of each core. The background and stromal staining as well as order in which the slide is examined can influence the subjective assessment. Semi-quantitative scoring methods have been developed to standardize this scoring. Recently commercial software has been developed to score TMAs based on the optical density of antigen labels, which results in a continuous scale based on objective scores. Multiple studies have compared manual scoring with an automated system. Wang et al. compared manual scoring with an automated system using fluorescence in situ hybridization as the standard and found that the automated system achieved higher accuracy than manual scoring. Similarly, Camp et al. found that an automated system was at least as accurate as manual scoring, but automated scoring had an obvious advantage in that it could be scored on a true continuous scale. For a practical research study it is likely that both methods of scoring would be required. The automated scoring would be more accurate and provide more precise data; however in a diagnostic pathology setting, manual scoring would ensure that the results could be used in practice.

SUMMARY

Mammary tumours are the most common type of neoplasia in intact female dogs, but prognosticating these tumours can be difficult. Numerous prognostic markers have been investigated previously, but none of these are used routinely in practice. Human breast carcinomas have been divided into multiple
molecular subtypes that seem to correlate with prognosis and they respond differently to

different chemotherapeutic agents.\textsuperscript{84-86} Identification of molecular subtypes in dogs is
important to further investigate targeted therapies and potentially provide better
prognostic markers that could be used in routine surgical pathology.
<table>
<thead>
<tr>
<th>Score</th>
<th>Tubule formation</th>
<th>Nuclear pleomorphism</th>
<th>Mitotic counts/10 HPF</th>
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</thead>
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<tr>
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<td>Small, regular uniform nuclei</td>
<td>0 to 9</td>
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<tr>
<td>2</td>
<td>10-75 % tubule formation</td>
<td>Moderate increase in nuclear size and variability</td>
<td>10-19</td>
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<tr>
<td>3</td>
<td>&lt;10 % tubule formation</td>
<td>Marked variation in nuclear size</td>
<td>20 or more</td>
</tr>
</tbody>
</table>

**Table 1-1. Histological grading of canine mammary tumours.** Grading scheme from Pena et al.\textsuperscript{10} based on the human grading scheme proposed by Elston and Ellis.\textsuperscript{39} Grade I = 3-5 points; Grade II = 6, 7 points; Grade III = 8, 9 points. HPF = high powered field.
<table>
<thead>
<tr>
<th>Subtype</th>
<th>ER and PR</th>
<th>HER2</th>
<th>Cytokeratin 5/6 and HER1</th>
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</thead>
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<tr>
<td>Luminal A</td>
<td>ER+ and/or PR+</td>
<td>HER2-</td>
<td>Staining inconsequential</td>
</tr>
<tr>
<td>Luminal B</td>
<td>ER+ and/or PR+</td>
<td>HER2+</td>
<td>Staining inconsequential</td>
</tr>
<tr>
<td>Basal-like</td>
<td>ER- and PR-</td>
<td>HER2-</td>
<td>Cytokeratin 5/6+ and/or HER1+</td>
</tr>
<tr>
<td>HER2+/ER-</td>
<td>ER- and PR-</td>
<td>HER2+</td>
<td>Staining inconsequential</td>
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<td>Unclassified</td>
<td>ER- and PR-</td>
<td>HER2-</td>
<td>Cytokeratin 5/6- and HER1-</td>
</tr>
</tbody>
</table>

Table 1-2. Immunohistochemical subtypes of human breast carcinoma. Subtype immunolabelling classification adapted from Carey et al.\textsuperscript{87} ER = estrogen receptor. PR = progesterone receptor. HER2 = human epidermal growth factor receptor 2. HER1 = human epidermal growth factor receptor 1.
### Table 1-3. Immunohistochemical subtypes in canine mammary carcinomas.

Differences between immunohistochemical categories of mammary carcinoma subtypes used by Gama et al.\textsuperscript{14} and Sassi et al.\textsuperscript{15} ER = estrogen receptor. HER2/ERB-B2 = human epidermal growth factor receptor 2. P-CD = P cadherin. CK = cytokeratin. PR = progesterone receptor.

<table>
<thead>
<tr>
<th>Subtypes</th>
<th><strong>Gama et al. classification\textsuperscript{14}</strong></th>
<th><strong>Sassi et al. classification\textsuperscript{15}</strong></th>
</tr>
</thead>
<tbody>
<tr>
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<td>Luminal B</td>
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<td>-</td>
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<td>HER2/ERB-B2-overexpressing</td>
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<td>+</td>
</tr>
<tr>
<td>Negative/ unclassified</td>
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</tbody>
</table>

Study Rationale

In the first study (chapter 2), prognostic indicators (presence of emboli or metastasis, peripheral invasion, and histological grade) and histological subtypes were compared to survival data collected. I hypothesize that the presence of emboli or metastasis, peripheral invasion, and worsening histological grade will all correlate with a shorter overall survival time. As well, I hypothesize that there will be certain histological subtypes (comedocarcinomas, invasive micropapillary carcinomas, anaplastic carcinomas, solid carcinomas) that correlate with an overall shorter survival time as well as with poor prognostic indicators.

The second study (chapter 3) tests (a) whether tissue microarray technology can be used in canine mammary carcinomas and (b) the heterogeneity of canine mammary carcinomas in regards to the immunohistochemical expression of the different antigens. I hypothesize that the tissue microarray cores will represent the tumour as a whole thus validating the use of tissue microarray technology in canine mammary tumours. I also hypothesize that some of the antigens investigated will be differentially expressed when comparing the histologically aggressive area of the tumour and the edge.

In the third study (chapter 4), immunohistochemical subtypes and estrogen receptor status were compared to survival data, the prognostic markers (presence of emboli or metastasis, peripheral invasion, and histological grade), and histological subtypes to determine if different immunohistochemical subtypes or estrogen receptor status correlates with differences in survival or correlate differently to the prognostic markers and histological subtypes. I hypothesize that immunohistochemical subtypes within canine mammary carcinomas will be found and they will be associated with a
shorter survival and correlate differently with poor prognostic indicators similar to those found in human breast carcinomas (HER2 and ER&HER negative linked to shorter survival and more poor prognostic indicators than luminal A and B carcinomas). I also hypothesize that estrogen receptor negative carcinomas will be associated with a shorter survival and more poor prognostic indicators than estrogen receptor positive carcinomas.
CHAPTER 2: PROGNOSTIC MARKERS OF CANINE MAMMARY CARCINOMAS AND THEIR RELATIONSHIP TO SURVIVAL

Abstract

Mammary tumours are the most common type of neoplasm in female dogs and although the majority are benign, determining which of the malignant tumours will result in distant metastasis and eventual death can be challenging. A total of 528 mammary carcinomas were examined and evaluated for presence of vascular/lymphatic emboli and distant metastasis, peripheral invasion, histological grade, and histological subtype. Survival data was analyzed for each of the above variables with a total of 53 cases having full survival data available and an additional 7 cases providing at least one year of data. Prognostic markers were analyzed for cancer related overall survival, but due to the low number of cases the markers were also compared to survival at six months and one and two years after diagnosis. When cancer related overall survival was examined, it was determined that the presence of emboli or metastasis at the time of diagnosis as well as peripheral invasion were significantly associated with a shorter cancer related overall survival (p < 0.001, p = 0.032 respectively). Many of the prognostic markers were associated with significantly lower survival at both the six month and one year time point (presence of emboli or metastasis, peripheral invasion, and histological grade of 3). However, by the two year time point fewer prognostic markers were associated with significantly lower survival (presence of emboli or metastasis and solid carcinomas versus complex carcinomas). When comparing histological subtypes to the prognostic markers it was discovered that both comedocarcinomas and invasive micropapillary...
carcinomas were significantly associated with the presence of emboli or metastasis. Both solid and tubulopapillary carcinomas were significantly more likely to be associated with poor prognostic markers (presence of emboli or metastasis, peripheral invasion, and high grade for solid but not tubulopapillary) than were ductal and complex carcinomas. Based on the survival analysis results, the best prognostic markers in descending order for predicting survival time are the presence of emboli or metastasis at the time of diagnosis, followed by peripheral invasion of the tumour, histological grade, and finally, certain histological subtypes.
Introduction

Canine mammary tumours are the most common type of neoplasm in intact female dogs, but prognosticating them can be challenging. Mammary tumours have been examined extensively, yet few studies correlate results with clinical outcome, or only involve a low number of cases.\cite{11,14,15,45,47,71} One of the best prognostic indicators in canine mammary tumours is the presence of local or distant metastasis at the time of diagnosis. Numerous studies show the presence of lymph node involvement correlates with a significant decrease in overall survival.\cite{2,3,6,9,142} Yamagami et al. also found that just the presence of lymphatic or vascular emboli also correlates with a significantly lower survival.\cite{30}

Peripheral invasion was one of the earliest prognostic indicators that was correlated with survival and multiple studies indicate it is significantly associated with shorter survival.\cite{4,30,143} More recently, grading of canine mammary carcinomas has been investigated. These grading studies found that in general the high grade tumours (grade 3) were found to correlate with a significantly shorter survival time than were both grade 1 and 2 tumours.\cite{9,10}

Histological classification of canine mammary tumours was standardized by the WHO classification system in 1974 and updated in 1999.\cite{33,34} This was subsequently modified by Goldschmidt et al.’s classification system, which also expanded on the original WHO system.\cite{32} While there is one study to the author’s knowledge that has compared these histological subtypes with prognostic markers,\cite{11} there has yet to be a study that correlated this system with survival. However, one of the subtypes (invasive
micropapillary) in this recent classification system has been examined and was found to correlate with a median survival time of only 120 days.\textsuperscript{144} Prior to this classification system, other more basic subtypes were correlated with survival and non-tubular carcinomas (likely similar to the solid subtype in Goldschmidt et al.\textsuperscript{32}) correlated with a significantly shorter survival time, whereas those with a proliferative myoepithelial cellular component (likely similar to the complex subtype in Goldschmidt et al.\textsuperscript{32}) correlated with a significantly longer survival time.\textsuperscript{30}

The goal of this study is to examine histological features of canine mammary carcinomas with survival data collected retrospectively, and to determine if any of the histological subtypes correlate with differences in survival time or with the aforementioned prognostic markers.
Materials and Methods

Histological Classification and Grading

Blocks with paraffin embedded tissue were collected from the Animal Health Laboratory (Ontario Veterinary College, University of Guelph), Histovet Surgical Pathology (Guelph, Ontario), and Yager-Best Histological and Cytological Services (Guelph, Ontario) by searching the databases for canine mammary carcinomas. Blocks and slides were retrieved by employees of Histovet from their collection, an employee from AHL from the Animal Health Laboratory collection, and by Brian Stevens from the Yager-Best collection. If slides were not available a new slide was sectioned and stained with H&E for examination. Blocks and slides from the following submitted time frames were collected: AHL from 2007-2013, Histovet from 2012-2013; and Yager-Best from 2002-2008. All cases from AHL and Histovet were examined. All Yager-Best cases from 2006-2008 were examined; as well cases with histological evidence of emboli or metastasis and cases with no evidence of metastasis to lymph nodes from 2002-2005 were examined in order to increase the number of cases with evidence of either emboli or metastasis to make comparisons to cases without.

All primary tumours were examined by a single pathologist (BAS) and classified using the canine mammary classification system proposed by Goldschmidt et al.32 Cases were diagnosed as mammary carcinomas if they were of epithelial origin and had evidence of peripheral invasion, areas of necrosis, high nuclear pleomorphism, or a high mitotic rate. Cases with a malignant mesenchymal component (carcinosarcoma, osteosarcoma) were excluded from the study as were those that were determined to be
benign (adenomas, benign mixed mammary tumours). Cases that only contained emboli or metastasis in the sections examined without a primary tumour were not classified histologically.

Tumours were examined for evidence of emboli (lymphatic or vascular) and metastasis. Tumours were also examined for peripheral invasion when possible. A case was determined to be invasive if it invaded into the surrounding tissue (for unencapsulated tumours) or if it invaded through the capsule (for encapsulated tumours). Encapsulated tumours with invasion into the capsule that failed to break through into the surrounding tissue were classified as well-circumscribed and thus non-invasive.

All mammary carcinomas with a primary tumour were graded using the system proposed by Pena et al.\textsuperscript{10} based on the Nottingham method developed by Elston and Ellis.\textsuperscript{39} Cases with only lymphatic emboli or metastasis without a primary tumour were not graded.

Case selection for Determination of Presence of Emboli Predicting Metastasis

Every case was examined histologically for evidence of lymphatic or vascular invasion (emboli), evidence of lymph node or distant metastasis, and evidence of lymph node without metastasis. Cases were then selected with the following characteristics: (1) tumours with both emboli and lymph node or distant metastasis (17 cases); (2) tumours with lymph node or distant metastasis (Figures 2-1E and 2-1F), but no evidence of emboli (17 cases); (3) tumours with emboli (Figures 2-1A and 2-1B), but no evidence of lymph node metastasis (3 cases); and (4) tumours with no evidence of either emboli nor lymph node or distant metastasis (Figures 2-1C and 2-1D) (31 cases).
Survival Data Collection

308 cases from 2006-2008 from the Yager-Best database were selected for survival data collection and referring clinics were contacted with a questionnaire inquiring about date of birth, date of diagnosis/removal, date of euthanasia/death, cause of euthanasia/death, as well as other inquires about the size and shape of the tumour (Figure 2-2). 76 responses were collected with 12 being excluded for survival data analysis immediately as no survival data was available. This left 64 cases with full or partial survival data available. 11 cases were lost to follow up, but some of these could be included in yearly survival data collection depending on how long after surgery the animals remained at the referring veterinarian. This left 53 cases with full survival known, as well as an additional 4 cases that had at least one year survival known and 3 cases with at least two year survival known prior to these additional cases being lost to follow up. Of the 53 cases with full survival, 12 died or were euthanized from what appeared to be cancer related disease (pulmonary nodules on chest radiographs, recurrence of the tumour, and/or enlarged local lymph nodes with peripheral edema) although no necropsies were performed.

Statistics

Statistics were performed using Stata13. A two-sample t test was used to compare age at diagnosis with different variables. For the majority of variables, exact logistic regression was used to compare survival at six months and one and two years as this allows for a smaller dataset to be analyzed. Cox proportional hazard models were attempted for all variables with cancer related death as the failure variable, all other cases
that died of diseases unrelated to mammary cancer or were lost to follow-up were censored. Due to low numbers, the Cox proportional hazard models were only used to compare cases with emboli or metastases to those without, those with peripheral invasion to those without, and high grade (grade 3) carcinomas to low grade (grades 1 and 2) carcinomas. A test of the proportional-hazards assumption was performed to ensure the test was not biased. A simple 2 x 2 chi square test was used to determine whether lymphatic/vascular invasion predicts metastasis. Logistic regression was used to compare the prognostic markers in question to each other (spay status, evidence of metastasis, peripheral invasion, histological grade, histological subtype). A p-value of $\leq 0.05$ was considered to be significant.
Figure 2-1. Emboli and lymph nodes with and without metastasis. (A) Clusters of neoplastic cells in lymphatics (arrows). Scale bar = 500 µm. HE. (B) Cluster of neoplastic cells in a venule, note the red blood cells in the lumen (arrow) adjacent to the neoplastic cluster. Scale bar = 200 µm. HE. (C) Lymph node with no evidence of metastasis, note the normal follicles in the cortex (arrow). Scale bar = 1 mm. HE. (D) Lymph node with no evidence of metastasis. Scale bar = 500 µm. HE. (E) and (F) Lymph node with numerous lobules of neoplastic cells. Remaining lymphoid tissue is basophilic (*) with smaller nuclei compared to the neoplastic cells with abundant eosinophilic cytoplasm (arrows). (E) Scale bar = 1 mm. (F) Scale bar = 500 µm. HE.
Figure 2-2. Survival data questionnaire sent to referring veterinary clinics. The questionnaire was sent out to clinics to determine age at diagnosis, overall survival (if available), cause of death/euthanasia, as well as whether additional diagnostics were performed (post-mortem) upon death/euthanasia. None of the patients had a post-mortem performed so cancer related death was determined based on the clinics interpretation of recurrence and cancer progression.
Results

Breeds Affected

Of the 372 cases where breed was known, 74 different breeds were affected accounting for 71.77% (267/372) of the cases with the remaining 105 cases affecting mixed breed dogs. The ten most common breeds affected were the Shih Tzu (25 cases), German Shepherd (21 cases), Labrador Retriever (14 cases), Cocker Spaniel (13 cases), Siberian Husky (11 cases), Bichon Frise (11 cases), Golden Retriever (10 cases), Beagle (8 cases), Doberman Pinscher (7 cases), and Dachshund (7 cases). The remaining purebreds accounted for less than 7 cases each.

Age at Diagnosis

The age at time of mammary carcinoma diagnosis was known in 71 cases. The average age of diagnosis was 9.9 years of age (range 4.3-14.8). When age at diagnosis was compared to spay status and all of the prognostic markers (presence of emboli or metastasis, presence of peripheral invasion, histological grade, and histological subtype), no significant difference was noted between spay status or any of the prognostic markers and age at diagnosis (all p values > 0.05).

Spay Status

A total of 67 cases had spay status listed. Of those cases 53.7% (36/67) were spayed prior to the mammary carcinoma diagnosis; whereas, 46.3% (31/67) were intact at the time of diagnosis. When spay status was compared to the prognostic markers (presence of emboli or metastasis, presence of peripheral invasion, histological grade, and
histological subtype), no significant difference was noted between any of the prognostic markers and spay status at the time of diagnosis (all p values > 0.05). There was no significant difference at any of the survival points (six months and one and two years) or with cancer related overall survival between intact and spayed dogs at the time of diagnosis (all p values > 0.05).

Prognostic Markers Descriptive Data

A total of 528 cases were examined and 171 (32.4 %) were found to have either emboli or metastasis at the time of diagnosis. There were a total of 476 tumours for which the edge could be evaluated; of those 275 (57.8 %) were found to have some peripheral invasion. Of the tumours that could be graded 136 (26.6 %) were grade 1, 214 (42 %) were grade 2, and 160 (31.4 %) were grade 3. There were 17 different histological subtypes of mammary carcinoma identified (Table 2-1); by far the two most common types were ductal and solid. Examples of the commonly discussed histological subtypes are presented in Figure 2-3.

Determination of Presence of Emboli Predicting Metastasis

When cases with emboli were compared to cases without emboli it was found that cases with emboli were significantly more likely to have lymph node or distant metastasis than were cases without emboli (p < 0.001).
Emboli and Metastasis Survival Analysis

The mean and median survival times were 130.5 and 76 days for cases with evidence of emboli or metastasis at the time of diagnosis and 1023 and 805 days for cases without. At the six month and one and two year time points, the odds of surviving is significantly lower for individuals with evidence of emboli or metastasis compared to those individuals without (6 months – p < 0.001; 1 year – p = 0.003; 2 years – p = 0.01) (Tables 2-2, 2-3, and 2-4; Figures 2-4A, 2-5A, and 2-6A).

When cancer related overall survival was examined, it was also found that cases with evidence of emboli or metastasis had a significantly shorter survival time than those without (Hazard ratio = 6.68, p = 0.004) (Table 2-5; Figure 2-7A).

Peripheral Invasion Survival Analysis

The mean and median survival times were 679.2 and 326 days for cases with peripheral invasion and 1033.4 and 764 days for cases without. At the both the six month and one year time points, the odds of surviving is significantly lower for individuals with carcinomas with evidence of peripheral invasion compared to individuals without (six months – p = 0.008; one year – p = 0.002) (Tables 2-2 and 2-3; Figures 2-4B and 2-5B). However, there was no significant difference in survival for the two year time point (p = 0.237) (Table 2-4; Figure 2-6B).

When cancer related overall survival was examined, it was found that cases with evidence of peripheral invasion had a significantly shorter survival time than those without (Hazard ratio = 4.41, p = 0.027) (Table 2-5; Figure 2-7B).
Histological Grade Survival Analysis

The mean and median survival times were 1201.5 and 1194 days for grade 1 tumours, 806.0 and 629 for grade 2 tumours, and 718.6 and 175 for grade 3 tumours. At the six month and one year time points, the odds of surviving was significantly lower for individuals with carcinomas given a histological grade of 3 than individuals with carcinomas given either a grade of 1 or 2 (six months – grade 1 – p = 0.011; six months – grade 2 – p = 0.018; one year – grade 1 – p = 0.018; one year – grade 2 – p = 0.031) (Tables 2-2 and 2-3; Figures 2-4C and 2-5C). However, this did not hold true for the two year time point (grade 1 – p = 0.063; grade 2 – p = 0.515) (Table 2-4; Figure 2-6C).

There was no significant difference in survival at any of the time points when comparing individuals with carcinomas diagnosed as grade 2 to individuals with carcinomas diagnosed as grade 1 (six months – p = 0.642; one year – p = 0.685; two year – p = 0.111) (Tables 2-2, 2-3, and 2-4; Figures 2-4C, 2-5C, and 2-6C).

When cancer related overall survival was examined comparing high grade (grade 3) carcinomas to low grade (grades 1 and 2) carcinomas, the global test of the proportional-hazards assumption indicated that the assumption had been violated. Consequently, a time-varying covariate was added to the model which itself was not statistically significant and resulted in the coefficient for grade approaching infinity; most likely the result of a small effective sample size. As a result, only the results of the model without the time varying covariate are reported. These results revealed that high grade carcinomas (grade 3) had a shorter survival time than low grade carcinomas (grades 1 and 2); however, this difference was not significant (Hazard ratio = 2.97, p = 0.066) (Table 2-5; Figure 2-7C).
Histological Subtypes Survival Analysis

The mean and median survival times were 745.0 and 528 days for solid carcinomas, 872.1 and 737 days for ductal carcinomas, and 1621 and 1294 days for complex carcinomas. There was no significant difference in survival of individuals diagnosed with ductal carcinomas to individuals diagnosed with solid carcinomas at any of the time points (six months – \( p = 0.401 \); one year – \( p = 0.704 \); two years – \( p = 0.296 \) (Tables 2-2, 2-3, and 2-4; Figures 2-4D, 2-5D, and 2-6D). At the 6 month and 1 year time points there was no significant difference in survival of individuals diagnosed with a complex carcinoma compared to both individuals diagnosed with a ductal carcinoma or a solid carcinoma (six months – vs ductal – \( p = 0.535 \); vs solid – \( p = 0.127 \); one year – vs ductal – \( p = 0.286 \); vs solid – \( p = 0.116 \) (Tables 2-2 and 2-3; Figures 2-4D and 2-5D). At the two year time point, the odds of surviving were significantly lower for individuals diagnosed with a solid carcinoma than with a complex carcinoma; however there was no significant difference in survival when comparing individuals diagnosed with a ductal carcinoma to individuals diagnosed with a complex carcinoma (solid vs complex – \( p = 0.029 \); ductal vs complex – \( p = 0.195 \) (Table 2-4; Figure 2-6D).

There were not enough cases of the other histological subtypes to make comparisons for survival data. As well there were not enough cases to make comparisons for cancer related overall survival.

Comparison of Prognostic Markers to Emboli and Metastasis

Over half of invasive carcinomas (140/275) had evidence of emboli or metastasis, which was significantly more than the well-circumscribed tumours (12/201) (\( p < 0.001 \)).
Grade 3 carcinomas were significantly more likely to have evidence of emboli or metastasis than were both grade 1 and 2 carcinomas (p < 0.001 for both). As well grade 2 carcinomas were significantly more likely to have evidence of emboli or metastasis than were grade 1 carcinomas (p = 0.001). When comparing subtypes to presence of emboli or metastasis at the time of diagnosis; the two subtypes that were most likely have emboli or metastasis were comedocarcinomas (85.0 %) and invasive micropapillary carcinomas (83.3 %). Of the five most common subtypes; solid, tubulopapillary, and anaplastic carcinomas were each significantly more likely to possess either emboli or metastasis at the time of diagnosis than were both ductal and complex carcinomas (all p values < 0.05).

**Comparison of Histological Grade and Subtypes to Peripheral Invasion**

Invasive carcinomas were significantly more likely to be diagnosed as grade 3 (112/272) than were well-circumscribed carcinomas (32/201) (p < 0.001); whereas well-circumscribed carcinomas were significantly more likely to be diagnosed as grade 1 (85/201) than were invasive carcinomas (42/272) (p < 0.001). Grade 3 carcinomas were also significantly more likely to be invasive than both grade 1 and 2 carcinomas (p < 0.001); as well grade 2 carcinomas were significantly more likely to be invasive than grade 1 carcinomas (p < 0.001).

When comparing to histological subtypes it was found that all anaplastic, comedocarcinomas, invasive micropapillary, cribriform, mixed type, and squamous cell carcinomas had evidence of peripheral invasion. Similar to presence of emboli or metastasis at the time of diagnosis, it was found that both solid and tubulopapillary carcinomas were significantly more likely to have evidence of peripheral invasion than
were ductal and complex carcinomas (all p values < 0.05). Ductal carcinomas were significantly more likely to have evidence of peripheral invasion than were complex carcinomas (p = 0.009).

**Comparison of Histological Grade and Histological Subtypes**

Out of the anaplastic, comedocarcinomas, micropapillary, and lipid-rich carcinomas only one was given a grade of 1 (a micropapillary carcinoma), the remaining were all grade 2 or 3. None of the complex carcinomas carried a grade 3 diagnosis. Solid carcinomas were significantly more likely to be graded out as a 3 when compared to ductal and tubulopapillary carcinomas (p < 0.001); whereas ductal, complex, and tubulopapillary carcinomas were significantly more likely to be graded as 1 than were solid carcinomas (p < 0.001). Ductal carcinomas were also significantly more likely to have a grade of 1 than were tubulopapillary carcinomas (p = 0.002).
Table 2-1. Histological subtypes with regards to the presence of emboli or metastasis, peripheral invasion, and histological grade. The proportion of each histological subtype with emboli or metastasis at the time of diagnosis, peripheral invasion, and histological grade. WC = well-circumscribed. SCC = squamous cell carcinoma.
Figure 2-3. Histological subtypes of canine mammary carcinoma. (A) Ductal carcinoma. A single, or occasionally double, layer of neoplastic cells forming round to ovoid duct-like structures with a central lumen (arrows) that is often slit-like (arrowheads). Scale bar = 200 µm. HE. (B) Solid carcinoma. Neoplastic cells are arranged in lobules composed of dense sheets (*) with minimal acinar formation. Scale bar = 500 µm. HE. (C) Tubulopapillary carcinoma. Neoplastic cells are arranged in tubules (arrow) with papillary projections into the tubular lumina. These papillae are supported by fibrovascular stroma (arrowhead). Scale bar = 200 µm. HE. (D) Complex carcinoma. Neoplastic epithelial cells are arranged in tubules and duct-like structures (arrows). The benign myoepithelial component (*) is composed of spindle shaped cells (myoepithelium) with abundant lightly basophilic (myxoid) matrix. Scale bar = 200 µm. HE.
Figure 2-3 (continued). (E) Anaplastic carcinoma. Neoplastic cells are grouped in a cluster adjacent to normal mammary tissue (*). Neoplastic cells are often binucleated (arrow) and have marked anisokaryosis (arrowhead). Scale bar = 100 µm. HE. (F) Comedocarcinoma. Neoplastic cells are arranged in solid clusters with central necrosis (*) composed of amorphous eosinophilic material and cellular debris. Scale bar = 500 µm. HE. (G) Invasive micropapillary carcinoma. Neoplastic cells are arranged in tubules and duct-like structures with numerous small papillae extending into the lumina. Papillae do not have a supporting fibrovascular stalk (arrows). Scale bar = 200 µm. HE. (H) Tubular carcinoma. Neoplastic cells are arranged in elongated tubular structures that occasionally branch (arrows). Scale bar = 500 µm. HE.
<table>
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Table 2-2. Six month survival data for presence of emboli or metastasis, peripheral invasion, histological grade, and histological subtypes. An asterisk (*) denotes a significant difference (p < 0.05) in survival between the two variables being compared. An odds ratio of less than 1 indicates that the odds of being alive is lower for the first variable, whereas an odds ratio of greater than 1 indicates the odds of being alive is higher for the first variable (as noted in confidence intervals where there is no significant difference between the two variables). A plus sign (+) indicates that a median unbiased estimate has been used for that particular calculation as complex carcinomas did not have a single case die within the first year and this is reflected in the confidence interval with the lower end being zero. CI = confidence interval.
Figure 2-4. Kaplan-Meier curves for six month survival data for presence of emboli or metastasis, peripheral invasion, histological grade, and histological subtypes. An asterisk (*) denotes a significant difference (p < 0.05) between the two variables being compared and a difference in letters (a vs b) between two variables denotes a significant difference in survival between the two variables when more than two variables are being compared. Variables with significantly lower survival were (A) the presence of emboli/metastasis (compared to cases without), (B) peripheral invasion (compared to well-circumscribed carcinomas), and (C) carcinomas with a histological grade of 3 (compared to both grade 1 and 2 carcinomas). There was no significant difference in survival for any of the histological subtypes at six months post-diagnosis (D).
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**Table 2-3. One year survival data for presence of emboli or metastasis, peripheral invasion, histological grade, and histological subtypes.** An asterisk (*) denotes a significant difference (p < 0.05) in survival between the two variables being compared. An odds ratio of less than 1 indicates that the odds of being alive is lower for the first variable, whereas an odds ratio of greater than 1 indicates the odds of being alive is higher for the first variable (as noted in confidence intervals where there is no significant difference between the two variables). A plus sign (+) indicates that a median unbiased estimate has been used for that particular calculation as complex carcinomas did not have a single case die within the first year and this is reflected in the confidence interval with the lower end being zero. CI = confidence interval.
Figure 2-5. Kaplan-Meier curves for one year survival data for presence of emboli or metastasis, peripheral invasion, histological grade, and histological subtypes. An asterisk (*) denotes a significant difference (p < 0.05) between the two variables being compared and a difference in letters (a vs b) between two variables denotes a significant difference in survival between the two variables when more than two variables are being compared. Variables with significantly lower survival were (A) the presence of emboli/metastasis (compared to cases without), (B) peripheral invasion (compared to well-circumscribed carcinomas), and (C) carcinomas with a histological grade of 3 (compared to both grade 1 and 2 carcinomas). There was no significant difference in survival for any of the histological subtypes at one year post-diagnosis (D).
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</tbody>
</table>

Table 2-4. Two year survival data for presence of emboli or metastasis, peripheral invasion, histological grade, and histological subtypes. An asterisk (*) denotes a significant difference (p < 0.05) in survival between the two variables being compared. An odds ratio of less than 1 indicates that the odds of being alive is lower for the first variable, whereas an odds ratio of greater than 1 indicates the odds of being alive is higher for the first variable (as noted in confidence intervals where there is no significant difference between the two variables). CI = confidence interval.
Figure 2-6. Kaplan-Meier curves for two year survival data for presence of emboli or metastasis, peripheral invasion, histological grade, and histological subtypes. An asterisk (*) denotes a significant difference (p < 0.05) between the two variables being compared and a difference in letters (a vs b) between two variables denotes a significant difference in survival between the two variables when more than two variables are being compared. Variables with significantly lower survival were (A) the presence of emboli/metastasis (compared to cases without) and (D) solid carcinomas (compared to complex carcinomas). There was no significant difference in survival for (B) peripheral invasion or (C) histological grade.
<table>
<thead>
<tr>
<th>Variable with lower survival</th>
<th>Variable with higher survival</th>
<th>p value</th>
<th>Hazard ratio</th>
<th>95 % CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of emboli/metastasis</td>
<td>Absence of emboli/metastasis</td>
<td>0.004*</td>
<td>6.68</td>
<td>1.81-24.64</td>
</tr>
<tr>
<td>Peripheral invasion</td>
<td>Well-circumscribed</td>
<td>0.027*</td>
<td>4.41</td>
<td>1.19-16.38</td>
</tr>
<tr>
<td>High grade (grade 3)</td>
<td>Low grade (grades 1 and 2)</td>
<td>0.066</td>
<td>2.97</td>
<td>0.93-9.53</td>
</tr>
</tbody>
</table>

Table 2-5. Cox proportional hazard model for presence of emboli or metastasis, peripheral invasion, and histological grade. An asterisk (*) denotes a significant difference in survival (p < 0.05) between the two variables being compared. A hazard ratio of greater than 1 indicates that the variable with lower survival is more likely to die from cancer than the variable with the higher survival, whereas a hazard ratio less than 1 would indicate the opposite (as noted in confidence intervals where there is no significant difference between the two variables). CI = confidence interval.
Figure 2-7. Kaplan-Meier curves for cancer related overall survival for presence of emboli or metastasis, peripheral invasion, and histological grade. An asterisk (*) denotes a significant difference (p < 0.05) between the two variables being compared. A vertical dash (|) indicates a data point that was censored. Variables with significantly lower cancer related overall survival were (A) the presence of emboli/metastasis (compared to cases without) and (B) peripheral invasion (compared to well-circumscribed carcinomas). There was no significant difference in cancer related overall survival for (C) histological grade. The long horizontal lines in the graphs are often the result of one or two individuals that survived for 2000+ days after removal of the carcinoma in question.
Discussion

Out of all the variables examined, presence of emboli or metastasis was the best prognostic marker. Of the prognostic markers examined, it was most significantly correlated with survival as it was found that carcinomas that had evidence of emboli or metastasis at the time of diagnosis had a significantly lower cancer related overall survival time as well as lower survival at six months and one and two years after diagnosis (Tables 2-2, 2-3, 2-4, and 2-5; Figures 2-4A, 2-5A, 2-6A, and 2-7A). None of the other variables carried significant differences at all of the time points examined. These findings are not unexpected as it has previously been shown that presence of vascular invasion or metastasis is correlated with a decrease in survival time.2,6,9,30,142

It was decided that presence of emboli and presence of metastasis would be combined as one variable in this study for two reasons. First, when cases with emboli (which were more frequent than cases with metastasis) were compared to cases without emboli or metastasis (censoring the cases with metastasis), all of the significant differences at each time point (six months and one and two years) as well as for cancer related overall survival were retained. As well, it was found that cases with histologically evident emboli were significantly more likely to be associated with lymph node or distant metastasis than were cases without histological evidence of emboli. This is important as lymph nodes and distant sites for metastasis were not examined in every case as the cases were submitted by different veterinary clinics. These results suggest that comparisons between these two groups can be safely made with the assumption that cases with emboli are likely to be associated with metastasis and cases without emboli are
likely not to be. This was expected as neoplastic cells that have invaded into lymphatics or vasculature have a much higher chance of localizing in lymph nodes or distant sites more readily than tumours that don’t have evidence of lymphatic or vascular invasion. These results are supported by findings in human squamous cell carcinomas and breast carcinoma that revealed that lymphatic or vascular invasion was predictive of lymph node metastasis.\textsuperscript{145,146}

Peripheral invasion of the tumour is the second best prognostic marker as it correlated with survival similarly to emboli/metastasis, with tumours that had evidence of peripheral invasion having a significantly lower cancer related overall survival time as well as lower survival at six months and one year after diagnosis (Tables 2-2, 2-3, and 2-5; Figures 2-4B, 2-5B, and 2-7B). This difference did not extend to the two year time point (Table 2-4; Figure 2-6B). These findings again correlate with what has previously been found in dogs diagnosed with canine mammary carcinoma as it appears peripheral invasion of the tumour is associated with a shorter survival.\textsuperscript{4,30,143}

Histological grade was the third best prognostic marker evaluated. It was found that at six months and one year post diagnosis, grade 3 carcinomas correlated with significantly lower survival than both grade 1 and 2 carcinomas (Tables 2-2 and 2-3; Figures 2-4C and 2-5C). These results correlate well with other papers that found high grade (grade 3) carcinomas had a shorter survival time than do both grade 1 and 2 carcinomas.\textsuperscript{9,10} As was found with peripheral invasion this correlation did not extend beyond the one year time point, although grade 3 was very close to being significantly correlated with lower survival when compared with grade 1 tumours at the two year time point (p=0.063). Similarly, high grade (grade 3) carcinomas had a shorter cancer related
overall survival than low grade (grades 1 and 2) carcinomas, but this result was not significant (p=0.109).

It is not clear why for both peripheral invasion and histological grade the significant difference in survival noted at six months and one year after surgical removal did not extend to the two year time point. It is speculated that due to the older age of diagnosis (9.9 years) that these animals are suffering from concurrent conditions unrelated to the mammary carcinoma diagnosis and are thus dying or being euthanized secondary to these other conditions.

The graph (Figure 2-7C) for cancer related overall survival for histological grade has an unusual appearance as both high grade and low grade carcinomas, after death/euthanasia related to cancer early on in the analysis, have long horizontal lines that do not decrease. This is a result of the graphs being created with the failure variable as a cancer related death so that other deaths are censored and in this case there are 2 individuals with grade 3 carcinomas and 4 individuals with either grade 1 or 2 carcinomas that lived for over 2000 days, which creates a long horizontal line until these individuals are eventually dropped from the analysis.

Although the majority of histological subtypes could not be correlated with survival data, the three most common subtypes (ductal, solid, and complex) could be compared. Although none of the dogs with complex carcinomas died within the first year post-diagnosis, there was still not a significant difference in survival between this histological subtype and either solid or ductal carcinomas (Tables 2-2 and 2-3; Figures 2-4D and 2-5D). At two years post diagnosis, the solid subtype had a significantly lower survival as compared to complex carcinomas (Table 2-4; Figure 2-6D). These results are
unusual compared to the other prognostic markers examined in this study as most of the significant differences in survival occur early on after diagnosis and the differences become less pronounced later on as individuals become ill and die or are euthanized due to causes unrelated to the carcinoma diagnosis. The reason for this difference in survival with the histological subtypes is not clear at this point. These results, albeit on a small dataset, correlate well with another study that found that carcinomas with a proliferative myoepithelial component (which complex carcinomas do) had a significantly longer survival time.\textsuperscript{30}

Although there were very few cases, invasive micropapillary (110 days for 1 case), comedocarcinoma (mean of 151.5 days for 2 cases), and anaplastic (mean of 633.3 days for 3 cases) subtypes appeared to correlate with a short overall survival, similar to what has been previously reported.\textsuperscript{3,4,144}

Mammary carcinomas affect a wide range of breeds and sizes of dogs, but it does not appear that one breed is overwhelmingly represented. In fact of the top ten affected breeds in this study, eight are present in the top twenty registered breeds in the USA according to the American Kennel Club.\textsuperscript{147} The average age of diagnosis in this study of 9.9 years fits well with previous studies that have age of diagnosis in the range of 9-11 years.\textsuperscript{3,27,29,43,142}

The percentage of intact dogs in this study (46.27 \%) is much higher than what is estimated in the USA (17 \%).\textsuperscript{148} This is not an unexpected finding as an unspayed status is one of the major risk factors for developing a mammary tumour.\textsuperscript{43}

Caution should be taken with these results as only 53 cases had sufficient overall survival data, making it challenging to compare within and between variables so thus
only univariate analysis was able to be performed. Because of this issue with low
numbers for overall survival, yearly survival was examined in more detail using exact
logistic regression as that was able to give us a better overall impression of the survival
time for different variables with our small dataset. Survival at six months, one year, two
years, and three years were examined. The three year survival comparison was dropped
from the final analysis as most of the significant differences noted in the first two years
were lost at the three year time point. Two years appears to be a good cutoff point for
mammary carcinomas and has been examined in previous studies.\textsuperscript{4,6,9,15,30} Most of the
significant differences in this analysis occurred at the six month and one year time point,
which gives a good indication of prognosis early on in the disease process. However, as
previously mentioned many of the significant differences found in the first year post
diagnosis did not correlate with differences by two years and beyond. The exact reason
for this has not been investigated, but I suspect that as these are older dogs (average age
at diagnosis 9.9 years) they are likely suffering from other disease processes unrelated to
their mammary carcinoma diagnosis (e.g. liver or renal failure, arthritis, other neoplastic
diseases) and are thus dying or being euthanized secondary to diseases unrelated to
mammary cancer, which is clouding some of the survival data the older these dogs gets.
These results are a good starting basis for making comparisons, but more cases with
survival data will be required to confirm these results.

The results of the survival data appear to order the prognostic markers quite
clearly with presence of emboli or metastasis being the most accurate prognostic
indicator as it is most closely tied with differences in survival. The next most accurate
appears to be peripheral invasion of the tumour as it was correlated with differences in
overall survival as well as at the six month and one year time points, but not at the two year time point. As well the odds ratio and p value were not quite as strong for peripheral invasion when compared to the presence of emboli and metastasis. Histological grade appears to follow peripheral invasion as a prognostic marker with grade 3 carcinomas correlating with a worse prognosis than both grade 1 and 2 carcinomas. Finally, histological subtype can be used as a prognostic marker, but is likely better used in combination with the other prognostic markers discussed above. These results should not be a surprise as all three of these prognostic markers have been previously shown to correlate with prognosis in canine mammary carcinomas.\textsuperscript{2,4,6,9,10,30,142,143}

As the number of cases available for survival data analysis is lacking it may be better to compare other variables to these prognostic markers. When comparing between the three most accurate prognostic markers the trends continue from what was found in the survival data analysis. Peripheral invasion was significantly correlated with the presence of emboli or metastasis (p<0.001), which is consistent with the survival data results. Comparing histological grades, grade 3 carcinomas were found to be significantly correlated with the presence of emboli or metastasis as well as peripheral invasion when compared to both grade 1 and 2 carcinomas (all p values < 0.001). Although not confirmed with the survival data analysis, it does appear that grade 2 carcinomas are associated with a poorer prognosis than grade 1 carcinomas as grade 2 carcinomas were significantly correlated with both emboli or metastasis and peripheral invasion when compared to grade 1 carcinomas (p=0.001 and p<0.001, respectively).

Due to the small survival dataset, most of the histological subtypes could not be accurately compared and only one significant difference was noted between the most
common three histological subtypes (solid carcinomas being associated with a lower survival at the two year time point when compared to complex carcinomas (p=0.029)). Comparing the histological subtypes to our three prognostic markers discussed above should provide a suitable substitute for survival analysis to make conclusions about the differences in prognosis between histological subtypes.

By far the two most common histological subtypes of carcinomas diagnosed in this study were ductal and solid, which is in contrast to a study by Goldschmidt et al. who found that tubular carcinomas are a common subtype (only 6 of the 528 carcinomas examined in this study were diagnosed as tubular). One reason for this disagreement is that we have likely used slightly different criteria for differentiating between these subtypes. In this study, to be labelled a tubular carcinoma (Figure 2-3H), the majority of tumour cells had to be arranged in elongate tubular structures and if instead there were more round to ovoid structures, these tumours were considered to be ductal (Figure 2-3A). Tumours with a significant number of elongate tubular structures were uncommon and it is our assumption that many of what we termed ductal carcinomas would likely be termed tubular carcinomas by Goldschmidt et al.

When comparing histological subtypes to the presence of emboli or metastasis it was found that the majority of anaplastic, comedocarcinomas, cribriform, lipid-rich, and invasive micropapillary carcinomas had either evidence of vascular emboli or metastasis. However, comedocarcinomas and invasive micropapillary carcinomas were the only subtypes that were significantly more likely to be associated with vascular emboli or metastasis indicating these subtypes are correlated with a poorer prognosis. When comparing the four most common subtypes, it was found that both solid and
tubulopapillary carcinomas were significantly more likely to be associated with vascular emboli or metastasis than were ductal and complex carcinomas, indicating the former two subtypes likely carry a poorer prognosis than the latter two. Similarly, both solid and tubulopapillary carcinomas are significantly more likely to be considered invasive than are ductal and complex carcinomas. This correlation continues when comparing grade of solid carcinomas to that of ductal and complex carcinomas as solid carcinomas are significantly more likely to be grade 3 and ductal and complex carcinomas are more likely to be grade 1.

Based on these comparisons with proven prognostic markers (presence of emboli or metastasis, peripheral invasion, and histological grade), these results suggest that comedocarcinomas and invasive micropapillary carcinomas are correlated with a very poor prognosis. As well, solid and tubulopapillary carcinomas are likely associated with a poorer prognosis than both ductal and complex carcinomas.

In summary, although survival data analysis is the gold standard and should be used when attempting to discover differences in prognosis for canine mammary carcinoma research, when adequate survival data is not available then the best substitution for elucidating prognosis should be the presence of emboli or metastasis, followed by presence of peripheral invasion, and finally, histological grade. Certain histological subtypes also appear to be associated with a very poor prognosis (comedocarcinomas and invasive micropapillary carcinomas). These finding should also help pathologists better prognosticate canine mammary carcinomas in the future.
CHAPTER 3: VALIDATION OF THE TISSUE MICROARRAY AND INTRATUMOUR IMMUNOHISTOCHEMICAL HETEROGENEITY

Abstract

The use of tissue microarrays allows for examination of hundreds of different tumours on a single slide; however, questions have arisen as to whether 0.6mm cores can represent a tumour as a whole. Validation of the canine mammary carcinoma tissue microarray was performed and it was found that at least two cores were required to adequately correlate (> 95 %) the immunolabelling of the tissue microarray with the immunolabelling of the entire tumour. Secondly, the overall heterogeneity of the tumour was determined, which is done by comparing the cores from the histologically most aggressive area to those from the edge. It was found that p63 had sufficient agreement between the immunolabelling of the aggressive and edge cores, whereas CK 5/6, EGFR, HER2, and ER did not. This indicates that two cores from anywhere in the tumour for p63 is required to represent the tumour as a whole, but at least one core from the histologically most aggressive area of the tumour as well as at least one core from the edge of the tumour is required for CK 5/6, EGFR, HER2, and ER.
Introduction

A tissue microarray (TMA) is one or more paraffin blocks with hundreds of paraffin-embedded tissue cores of interest in each block. TMA technology for research using immunohistochemistry (IHC) has many advantages over the use of an entire slide as there is more consistency of IHC preparation (antigen retrieval, washing time, antibody concentration) and a single slide provides internal positive and negative controls as well as up to 200+ different tumours on a single slide. One disadvantage of TMA technology is that the 0.6 mm diameter cores are unlikely to represent the overall immunolabelling of a tumour especially if there is heterogeneity. Prior to the use of this technology, the number of cores required to represent the overall tumour expression must be determined (validation). To do this, a representative large section of the tumour must be examined and the expression of the antigen in question is compared to the expression in the smaller cores. Validation of TMA technology has previously been performed on many human (breast carcinoma, ovarian carcinoma, fibroblastic neoplasms, urinary bladder carcinoma, and lung tumours [carcinomas, carcinoids, neuroendocrine tumours, and malignant melanomas]) and canine (primary tumours of the central nervous system and lymphoma) tumours.

Heterogeneity of the tumour with comparisons between the centre and the leading edge must also be evaluated as some antigens (i.e. progesterone receptor) can show staining of the periphery of the tumour but lack staining in the centre. Thus, validation of the TMA requires comparing the centre and edge cores.
The goal of this study was to produce and validate a tissue microarray for canine mammary carcinomas, as well as determine the agreement of immunolabelling of the histologically most aggressive area and edge of the tumours for each antibody.
Materials and Methods

Tissue Microarray Construction

Areas were selected for coring by a single pathologist (BAS) and for each case up to five different samples were selected depending on what was available in the sections examined: (1) up to three cores were selected from the histologically most aggressive area or areas of the tumour, which was based on the area with the highest mitotic rate and/or greatest nuclear pleomorphism; (2) up to two cores were selected from the leading edge of the tumour; (3) up to two cores were selected from normal mammary tissue if it was present on the slide; (4) up to four cores were selected that contained vascular or lymphatic emboli if present; and (5) up to four cores were selected from metastatic sites if present.

The cases were split into six different groups. For each of these groups there were either 3 or 4 recipient blocks (depending on the number of cases in a group). The tissue microarray blueprint is asymmetrical in two planes (Figure 3-1) so as to avoid confusion of cores if the tissue were to be flipped or rotated during processing. Each recipient block in each group contained 206 available spots that included normal tissue (adrenal gland, cerebellum, cerebral cortex, exocrine pancreas, large intestine, liver, lymph node, ovary, pituitary gland, renal cortex, salivary gland, skeletal muscle, skin, small intestine, testis, uterus), inflamed tissue (colitis), and neoplastic tissue (colonic carcinoma) that would act as internal control tissue; the mammary carcinoma cores of interest were then randomly assigned a position in one of the recipient blocks.
The TMA was constructed using Pathology Devices TMArrayer™ (Pathology Devices, Westminster, MD, USA). Once all the cores had been transferred to the recipient block, a glass slide was place on top of the block (to ensure the surface was level) and the block was placed in an oven at 55-60°C for 10 minutes to bind the cores to the surrounding paraffin in the recipient block and allowed to cool before the slide was removed. A wax seal was then placed over the surface of the block to prevent desiccation of the cores.

**Immunohistochemistry**

Differences in the immunohistochemistry steps for each primary antibody are present in Table 3-1, but the general procedure was as follows and was based on previously published protocols.\textsuperscript{1,71} Slides were baked in the oven overnight at 37°C and immunohistochemistry was run within 2 weeks of the slides being sectioned. The slides were deparaffinised, rehydrated, and incubated in 3 % hydrogen peroxide for 5 minutes followed by antigen retrieval. Heat based antigen retrieval was performed in a Biocare Medical Decloaking Chamber™ NxGen Model: DC2012 (Biocare Medical, Concord, CA, USA) for all antibodies except EGFR, which required enzymatic antigen retrieval. The tissue was then incubated with 3 % bovine serum albumin (OmniPur® BSA, Fraction V) for 60 minutes at room temperature. Diluted primary antibody was then applied to the tissue and allowed to incubate overnight at 4°C. The next day, the tissue was incubated with Dako envision secondary antibody (species appropriate against the primary antibody) for 60 minutes at room temperature. This was followed by room temperature incubation with 3-3’-diaminobenzidine (DAB) chromogen. The tissue was
then counterstained with Harris modified hematoxylin, dipped in 1 % HCl/70 % alcohol, and finally dipped in water with five drops of ammonia hydroxide. The slides were then dehydrated and coverslipped with cytoseal.

For each antibody a positive control tissue was selected to confirm adequate staining. The controls were as follows; canine skin for cytokeratin 5/6, p63, and EGFR; canine uterus for ER; and confirmed HER2 positive human breast carcinoma for HER2.

Validation

Cases were selected from the tissue microarray with at least three observable cores with at least one core from the histologically most aggressive area of the tumour and at least one core from the leading edge. The full tumour available in these cases was then incubated with antibody against ER as discussed above. A positive score was reached if at least 1 % of the tumour cells were positive.

Case Selection for Determining Tumour Heterogeneity

Cases were selected from those that had been cored into the tissue microarray. All cases with at least one usable core from the histologically most aggressive area of the tumour and one usable core from the edge of the tumour were scored and given a positive and negative score for each antibody (positive if CK5/6 and p63 ≥ 10 % tumour positivity, EGFR and HER2 ≥ 2+ HercepTest score, and ER ≥ 1 % tumour positivity).
Statistics

For the validation step, the overall proportion of agreement between the scores of 1, 2, 3, 4, and 5 cores (with at least one core from the histologically most aggressive area and one core from the edge for those where more than 1 core was compared) to the entire tumour was calculated. The sensitivity, specificity, positive predictive value, negative predictive value, and 95% confidence intervals were also calculated.

For comparisons between the histologically most aggressive area cores and edge cores kappa values were calculated for each antibody. Prior to kappa values being calculated, exact McNemar significance probability was calculated and if McNemar’s was significant (p<0.05) or if the prevalence was less than 20% or greater than 80% than a prevalence and bias adjusted kappa (PABAK) was used to calculate the kappa value instead.
**Figure 3-1. Tissue microarray blueprint.** Non-shaded boxes contained study or control cores, whereas shaded boxes were left empty to maintain asymmetry.
<table>
<thead>
<tr>
<th>Wash solution</th>
<th>Antigen retrieval</th>
<th>Primary antibody</th>
<th>Primary antibody dilution</th>
<th>Isotype control used</th>
<th>DAB chromogen duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokeratin 5/6</td>
<td>PBS</td>
<td>90°C for 20 mins in citrate buffer (pH 6.0); allowed to cool to 80°C in buffer</td>
<td>Mouse monoclonal clone D5/16B4 (Abcam)</td>
<td>1:100 Mouse IgG1, kappa monoclonal clone MOPC-21 (Abcam)</td>
<td>10 mins</td>
</tr>
<tr>
<td>p63</td>
<td>PBS</td>
<td>90°C for 20 mins in citrate buffer (pH 6.0); allowed to cool to 80°C in buffer</td>
<td>Mouse monoclonal clone 4A4 (Abcam)</td>
<td>1:200 Mouse IgG2a, kappa monoclonal clone MG2a-53 (Abcam)</td>
<td>10 mins</td>
</tr>
<tr>
<td>EGFR</td>
<td>PBS</td>
<td>Proteinase K for 5 mins</td>
<td>Mouse monoclonal clone 31G7 (Abcam)</td>
<td>1:150 Mouse IgG1, kappa monoclonal clone MOPC-21 (Abcam)</td>
<td>20 mins</td>
</tr>
<tr>
<td>Estrogen receptor α (ER)</td>
<td>TBS</td>
<td>110°C for 3 mins in EDTA buffer (pH 9.0); allowed to cool in buffer</td>
<td>Mouse monoclonal clone 1D5 (Abcam)</td>
<td>1:400 Mouse IgG1, kappa monoclonal clone MOPC-21 (Abcam)</td>
<td>15 mins</td>
</tr>
<tr>
<td>HER2</td>
<td>PBS</td>
<td>95°C for 40 mins in EDTA buffer (pH 9.0); allowed to cool to 80°C in buffer</td>
<td>Mouse monoclonal clone CB11 (Abcam)</td>
<td>1:200 Mouse IgG1, kappa monoclonal clone MOPC-21 (Abcam)</td>
<td>15 mins</td>
</tr>
</tbody>
</table>

**Table 3-1. Immunohistochemical procedures.** Differences between primary antibodies used in regards to washes, antigen retrieval protocols, antibody dilution, isotype controls used, and length of DAB chromogen incubation.
Results

Validation

The comparison of TMA core score versus total tumour score is presented in Table 3-2 for each of 1, 2, 3, 4, and 5 cores. The calculations for the total agreement between cores and tumour as well as the sensitivity, specificity, and positive and negative predictive values are presented in Table 3-3. 95% confidence intervals were calculated for each of the previous calculations and are presented in Table 3-3.

The total agreement between the cores and total tumour was only 87.7% for 1 core, but increased to above 95% for the remaining comparisons (2 cores – 95.5%, 3 cores – 96.3%, 4 cores – 95.8%, 5 cores – 95.7%) (Table 3-3).

Determining Tumour Heterogeneity

The comparison of histologically most aggressive area core scores versus edge core scores are presented in Table 3-4 for each antibody. When comparing the histologically most aggressive area cores and edge cores, CK 5/6 had kappa=0.5871, p63 had kappa=0.7699, EGFR had kappa=0.3908, HER2 had kappa=0.3490, and ER had kappa=0.5508.
Table 3-2. Proportions of tissue microarray core scores to total tumour scores for estrogen receptor validation. Comparison of the estrogen receptor (ER) scores from one, two, three, four, and five cores from the tissue microarray (TMA) to the total tumour ER score.
Table 3-3. Calculations to determine the number of cores required to accurately represent the tumour as a whole. Total agreement, sensitivity, specificity, positive predictive value, and negative predictive value along with the 95 % confidence interval (in brackets) for each of one, two, three, four, and five cores from the tissue microarray (TMA) when compared to the total tumour estrogen receptor (ER) score. Using a 95 % agreement as a baseline, at least two cores from the TMA are required to accurately predict the overall immunolabelling of the tumour.
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Edge immunolabelling</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>CK 5/6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>219</td>
<td>36</td>
</tr>
<tr>
<td>Negative</td>
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<td>82</td>
</tr>
<tr>
<td>Total</td>
<td>248</td>
<td>118</td>
</tr>
<tr>
<td>p63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>214</td>
<td>31</td>
</tr>
<tr>
<td>Negative</td>
<td>11</td>
<td>109</td>
</tr>
<tr>
<td>Total</td>
<td>225</td>
<td>140</td>
</tr>
<tr>
<td>EGFR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>97</td>
<td>63</td>
</tr>
<tr>
<td>Negative</td>
<td>36</td>
<td>129</td>
</tr>
<tr>
<td>Total</td>
<td>133</td>
<td>192</td>
</tr>
<tr>
<td>HER2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>8</td>
<td>15</td>
</tr>
<tr>
<td>Negative</td>
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</tr>
<tr>
<td>Total</td>
<td>18</td>
<td>301</td>
</tr>
<tr>
<td>ER</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>39</td>
<td>47</td>
</tr>
<tr>
<td>Negative</td>
<td>26</td>
<td>213</td>
</tr>
<tr>
<td>Total</td>
<td>65</td>
<td>260</td>
</tr>
</tbody>
</table>

Table 3-4. Proportions of the histologically most aggressive area core scores to the edge core scores to determine tumour heterogeneity. Comparison of positive and negative histologically most aggressive area core and edge core scores for each antibody. CK 5/6 = cytokeratin 5/6. EGFR = epidermal growth factor receptor. HER2 = human epidermal growth factor receptor 2. ER = estrogen receptor.
Discussion

Tissue microarray technology has been validated in many different human \textsuperscript{16-20} and dog \textsuperscript{23,24} tumours; however, the only validation of mammary tumours is breast carcinomas in humans. \textsuperscript{16,17} We performed basic TMA validation procedures on mammary carcinomas in dogs for the first time and utilized one antibody with known heterogeneity (ER).

We found that, using a 95 % agreement between the TMA core scores and the total tumour score as a measure of significance (as was used by Camp et al.\textsuperscript{16}), combining the scores of two cores (one from the histologically most aggressive area and one from the edge) was required (95.5 %) (Table 3-3). Even with the use of more cores, the percentage of agreement did not increase substantially and appeared to level off (3 cores – 96.3 %; 4 cores – 95.8 %; 5 cores – 95.7 %) (Table 3-3). The other calculations (sensitivity, specificity, and positive and negative predictive values) did not differ substantially as the number of cores analyzed increased from two through five. Based on these results, a minimum of two cores, one from the histologically most aggressive area of the tumour and one from the edge, are required to accurately predict the total score of any canine mammary carcinoma 95.5 % of the time. These results are similar to previous validation studies, in both human and canine tumours, that have found a minimum of two cores are required to represent the tumour as a whole.\textsuperscript{16,18,21,23}

It was determined that at least one core from the histologically most aggressive area and at least one core from the edge are required to accurately determine the overall immunolabelling of the tumour for four of the antibodies (CK 5/6, EGFR, ER, and
HER2) (Figures 3-2A and 3-2B). The only antibody with a sufficiently high kappa value
to indicate that the immunolabelling in the histologically most aggressive area core did
not differ from the immunolabelling in the edge core was p63 (Figures 3-2C and 3-2D).
For this antibody, two cores, regardless of where they were taken, is enough to determine
the overall immunolabelling of the tumour. Kappa values can be difficult to interpret, but
to make it easier two different interpretations of the values were created. The first was by
Landis and Koch that stated six different levels of agreement as follows: <0 = no
agreement; 0-0.20 = slight agreement; 0.21-0.40 = fair agreement; 0.41-0.60 = moderate
agreement; 0.61-0.80 = substantial agreement; 0.81-1.00 = almost perfect agreement. 149
Fleiss settled on three levels with <0.40 = poor agreement, 0.40-0.75 = fair to good
agreement, and >0.75 = excellent agreement. 150 Based on this it was determined that a
kappa value of at least 0.75 was required to suggest that there was sufficient agreement
between the histologically most aggressive area core and edge core that a combination of
scores from the histologically most aggressive area and edge would not be required.
However, if the kappa value was less than 0.75 then the scores from the histologically
most aggressive area and edge would need to be combined to determine the overall score
for that particular antibody. These results are similar to what has been noted in human
breast carcinoma where for three antibodies (ER, PR, and HER2) there was discrepancy
in staining between the cores from the centre and edge of the tumours. 16

In conclusion, TMA technology has been validated in canine mammary
carcinomas and a minimum of two cores was required to represent the tumour as a whole.
In regards to tumour heterogeneity, four of the antibodies examined (CK5/6, EGFR,
HER2, and ER) required a combined score from at least one core from the histologically
most aggressive area of the tumour and one from the edge; whereas p63 required a
combined score from two cores from anywhere in the tumour.
Figure 3-2. Comparison of estrogen receptor and p63 histologically most aggressive area and edge immunolabelling. Estrogen receptor (ER) immunohistochemistry (IHC) of the histologically most aggressive area core (A) reveals no nuclear immunolabelling. Whereas, the edge core (B) has approximately 25% of neoplastic cells with light nuclear immunolabelling (arrows). In comparison, p63 IHC of the histologically most aggressive area (C) and edge (D) cores reveals approximately 70-80% of neoplastic cells with strong nuclear immunolabelling. All scale bars = 100 µm.
CHAPTER 4: IMMUNOHISTOCHEMICAL SUBTYPING OF CANINE MAMMARY CARCINOMAS AND THEIR RELATIONSHIP TO SURVIVAL AND PROGNOSTIC MARKERS

Abstract

Immunohistochemical subtypes have been known to exist in human breast carcinoma for many years and only recently have a few studies looked into whether similar immunohistochemical subtypes exist in canine mammary carcinomas. Similar to humans, canine mammary carcinomas could be divided into five distinct IHC subtypes (luminal A and B, ER&HER negative, HER2, and unclassified). Additionally, these immunohistochemical subtypes correlate differently with survival at both six months and one year as well as with different prognostic markers. The results revealed that in order of improving prognosis, unclassified and HER2 carcinomas were associated with the worst prognosis, followed by ER&HER negative carcinomas, and finally, luminal A and B carcinomas, which were associated with the best prognosis. Additionally, as the three immunohistochemical subtypes associated with the worst prognosis were all found to be estrogen receptor negative, estrogen receptor status was examined and found to be a strong prognostic indicator with estrogen receptor negative carcinomas having a significantly worse prognosis than estrogen receptor positive carcinomas. These results provide additional tools for more accurately prognosticating canine mammary carcinomas.
Introduction

In human medicine, molecular subtypes of breast carcinoma have been studied and found to correlate with prognosis. These molecular subtypes can be categorized by immunohistochemistry (IHC) and both the HER2 and triple negative subtypes have a shorter survival time than the luminal subtypes. Along with differences in prognosis, these molecular and IHC studies have allowed for targeted therapy of these different breast carcinoma subtypes.

Since this discovery in humans, different studies have attempted to similarly categorize canine mammary carcinomas based on immunohistochemistry. All of these studies had success categorizing canine mammary carcinomas into IHC subtypes and were able to correlate some of the IHC subtypes with prognostic markers, but only one study was able to correlate a subtype with overall survival time. With these three studies, the overall number of cases examined has been low (range from 45 cases up to 159 cases).

Estrogen receptor detection by immunohistochemistry has also been previously examined in dogs. Many studies have found that lower percentages of ER immunoreactivity are associated with increasing malignancy in canine mammary tumours. One study found that ER was an independent prognosticator for disease free survival.

The goal of this study is to expand on what has previously been done by Gama et al., Sassi et al., and Im et al. by comparing IHC subtypes and histological subtypes in canine mammary carcinomas to survival data as well as prognostic markers in a larger
sample size. The second goal is to compare estrogen receptor status to survival data and
the prognostic markers (presence of emboli or metastasis, peripheral invasion, and
histological grade) to determine if it correlates with prognosis.
Materials and Methods

Case Selection, Survival Data Collection, and Immunohistochemical Procedures

Case selection criteria and survival data collection procedures are identical to those described in chapter 2. Immunohistochemical methods are identical to those described in chapter 3.

Immunohistochemical Cutoff Points

Different cutoff points were examined for the antibodies based on what has been previously published in the canine literature.\textsuperscript{11,14,15,90} The cutoff points examined were 10 \% versus 50 \% positive cells for both CK5/6 and p63; any positive cells versus 1 \% positive cells vs 10 \% positive cells for ER; and a HercepTest score of 2+ or 3+ for HER2.

Based on the results (to be discussed later) the cutoff points are as follows. ER was scored using the Allred scoring system\textsuperscript{93} and was considered to be positive when at least 1 \% of neoplastic cells expressed this marker. HER2 and EGFR were scored using the HercepTest scoring system (0 = no membrane staining; 1+ = partial membrane staining or <10 \% light or moderate complete membrane staining; 2+ = >10 \% light or moderate complete membrane staining or <30 \% strong complete membrane staining; 3+ = >30 \% strong complete membrane staining); a score of 2+ or 3+ was considered to be positive for HER2 and EGFR. Both CK 5/6 and p63 were considered positive if at least 10 \% of neoplastic cells expressed this marker.
**Determination of Immunohistochemical Subtypes**

Based on the classification in both Carey et al.\textsuperscript{87} and Cheang et al.,\textsuperscript{89} with the addition of p63 as an additional basal marker, immunohistochemical subtypes were defined as luminal A (ER+, HER2-, inconsequential CK 5/6, p63, and EGFR immunolabelling), luminal B (ER+, HER2+, inconsequential CK 5/6, p63, and EGFR immunolabelling), HER2+ (ER-, HER2+, inconsequential CK 5/6, p63, and EGFR immunolabelling), ER&HER negative (ER-, HER2-, CK 5/6+ and/or p63+ and/or EGFR+), and unclassified (negative for all markers) (Figure 4-1).

**Statistics**

Exact logistic regression was used to compare three of the IHC subtypes and ER status survival at 6 months, 1 and 2 years as this allows for a smaller dataset to be analyzed. Cox proportional hazard models were attempted for all variables with cancer-related death as the failure variable; all other cases that died of diseases unrelated to mammary cancer or were lost to follow-up were censored. Due to low numbers, the Cox proportional hazard models were only used to compare ER-negative to ER-positive cases. A test of the proportional-hazards assumption was performed to ensure the test was not biased. Logistic regression was used to compare the IHC subtypes, differences in ER expression, as well as differential ER expression within histological subtypes to the prognostic markers in question (presence of emboli or metastasis, presence of peripheral invasion, histological grade, and histological subtype). A one-sample t test was used to compare luminal A and ER&HER negative carcinomas within a specific histological subtype. A p-value of $\leq 0.05$ was considered to be significant.
Figure 4-1. Immunohistochemical subtypes of canine mammary carcinoma. (A) - (E) Luminal A carcinoma. (A) CK 5/6 – 40 % of neoplastic cells have light cytoplasmic immunolabelling. (B) p63 – 30 % of neoplastic cells have moderate nuclear immunolabelling. (C) EGFR – 90 % of neoplastic cells have strong complete membranous immunolabelling (HercepTest = 3+). (D) HER2 – 30 % of neoplastic cells have light partial membranous immunolabelling (HercepTest = 1+). (E) ER – 80 % of neoplastic cells have strong nuclear immunolabelling (Allred = 8). (F) – (J) Luminal B carcinoma. (F) CK 5/6 – No immunolabelling of neoplastic cells. (G) p63 – 10 % of neoplastic cells have light nuclear immunolabelling. (H) EGFR – 50 % of neoplastic cells have light partial membranous immunolabelling (HercepTest = 1+). (I) HER2 – 80 % of neoplastic cells have light complete membranous immunolabelling (HercepTest = 2+). (J) ER – 30 % of neoplastic cells have moderate nuclear immunolabelling (Allred = 5). (K) – (O) HER2 carcinoma. No immunolabelling of neoplastic cells with (K) CK 5/6, (L) p63, (M) EGFR, or (O) ER. (N) HER2 – 80 % of neoplastic cells have strong complete membranous immunolabelling (HercepTest = 3+).
Figure 4-1 (continued). (P) – (T) ER&HER negative carcinoma. (P) CK 5/6 – 10 % of neoplastic cells have light cytoplasmic immunolabelling. (Q) p63 – 80 % of neoplastic cells have strong nuclear immunolabelling. (R) EGFR – 70 % of neoplastic cells have strong complete membranous immunolabelling (HercepTest = 3+). No immunolabelling of neoplastic cells with either (S) HER2 or (T) ER. (U) – (Y) Unclassified carcinoma. No immunolabelling of neoplastic cells for (U) CK 5/6, (V) p63, (W) EGFR, (X) HER2, or (Y) ER. All scale bars = 100 µm.
Results

Immunohistochemical Cutoff Points

Differences in comparison to prognostic markers and survival data are presented in Table 4-1. All cutoff points revealed similar significant differences between ER&HER negative, HER2, and unclassified carcinomas when compared to luminal A carcinomas except that the HER2 cutoff point of 2+ revealed a significant difference in peripheral invasion between HER2 carcinomas and luminal A carcinomas that was not seen when the cutoff point was 3+. Similarly, HER2 carcinomas and ER&HER negative carcinomas had significant differences in histological grade of 1 when compared to luminal A and luminal B carcinomas, respectively at the 2+ cutoff point. Significant differences between presence of emboli/metastasis were seen between HER2 and unclassified carcinomas compared to luminal B carcinomas when the ER cutoff point was set at either any positivity or 1 % positivity, but not when the cutoff point was set at 10 % positivity. When survival data was compared, significant differences at the six month and one year time points between unclassified and ER&HER negative carcinomas compared to luminal A carcinoma were noted at the 1 % positivity cutoff point and not at the any positivity cutoff point for ER. The final comparison was the cutoff points of 10 % versus 50 % positive cells for CK 5/6 and p63, which revealed very similar results except at the 10 % cutoff point where there was a significant difference in histological grade of 3 comparing unclassified carcinomas to luminal B carcinomas that was not noted at the 50 % cutoff point. As well, when the survival data was compared, using the 10 % cutoff point there was a significant difference at the 6 month time point when comparing
ER&HER negative carcinomas to luminal A carcinomas that was not present when the cutoff point was 50%.

**Immunohistochemical Subtype Survival Analysis**

The mean and median survival times were 1069.7 and 1001 days for luminal A carcinomas, 599.3 and 475 days for ER&HER negative carcinomas, and 411.4 and 175 for unclassified carcinomas. Only 2 HER2 cases and no luminal B cases had survival data so unfortunately there were not enough cases available to make comparisons to either of these subtypes.

At the six month time point, individuals diagnosed with an ER&HER negative carcinoma had a significantly lower survival than individuals diagnosed with a luminal A carcinoma (p = 0.039) (Table 4-2; Figure 4-2A). However, at both the one and two year time points there was no significant difference in survival between individuals diagnosed with an ER&HER negative carcinoma to those diagnosed with a luminal A carcinoma (1 year – p = 0.073; 2 years – p = 0.10) (Tables 4-3 and 4-4; Figures 4-3A and 4-4A).

At both the six month and one year time points, individuals diagnosed with an unclassified carcinoma had a significantly lower survival than individuals diagnosed with a luminal A carcinoma (6 months – p = 0.012; 1 year – p = 0.008) (Tables 4-2 and 4-3; Figures 4-2A and 4-3A). However, at the two year time point there was no significant difference in survival between individuals with unclassified versus luminal A carcinomas (p = 0.119) (Table 4-4; Figure 4-4A).

There was no significant difference in survival when individuals diagnosed with unclassified carcinomas were compared to individuals diagnosed with ER&HER negative
carcinomas at any of the time points (6 months – p = 0.608; 1 year – p = 0.314; 2 years – p = 1.00) (Tables 4-2, 4-3, and 4-4; Figure 4-2A, 4-3A, and 4-4A).

**Estrogen Receptor Status Survival Analysis**

The Allred scores for all cases are presented in Table 4-5. The mean and median survival times were 500.1 and 175 days for ER negative carcinomas and 1069.7 and 1001 days for ER positive carcinomas. At the six month and one and two year time points, the odds of surviving is significantly lower for individuals with ER negative carcinomas compared to those individuals with ER positive carcinomas (6 months – p = 0.005; 1 year – p = 0.004; 2 year – p = 0.045) (Tables 4-2, 4-3, and 4-4; Figures 4-2B, 4-3B, and 4-4B).

When cancer related overall survival was examined, it was found that ER negative carcinomas had a shorter survival time than ER positive carcinomas; however, this difference was not significant (Hazard ratio = 3.95 [95 % CI = 0.83-18.73]; p = 0.084) (Figure 4-5).

**Immunohistochemical Subtypes Compared to Prognostic Markers**

Table 4-6 provides a breakdown of the immunohistochemical subtypes and Figure 4-1 shows staining characteristics for each subtype. ER&HER negative and luminal A were the most common subtypes followed by HER2, unclassified, and luminal B. There was no significant difference between age of diagnosis or when comparing younger and older dogs when it came to immunohistochemical subtypes (all p values > 0.05). Spay status at the time of diagnosis was not significantly associated with any of the immunohistochemical subtypes (all p values > 0.05).
The majority of both the unclassified and HER2 carcinomas had either emboli or metastasis at the time of diagnosis. All unclassified carcinomas along with the majority of HER2 and ER&HER negative carcinomas had peripheral invasion. ER&HER negative and HER2 carcinomas were significantly more likely to have emboli or metastasis as well as peripheral invasion at the time of diagnosis than were luminal A carcinomas (all p values < 0.05). Unclassified carcinomas were also more likely to have emboli or metastasis at the time of diagnosis than were luminal A and B and ER&HER negative carcinomas (vs luminal A p < 0.001; vs luminal B p = 0.003; vs ER&HER negative p = 0.002). HER2 carcinomas were also significantly more likely to have evidence of emboli or metastasis and peripheral invasion than were luminal B carcinomas (emboli or metastasis p = 0.03; invasion p = 0.026).

When comparing grade of the subtypes to luminal A carcinomas it was found that ER&HER negative, HER2, and unclassified carcinomas were significantly more likely to be graded as a 3 (all p values < 0.05). As well unclassified carcinomas were significantly more likely to be graded as a 3 than were luminal B carcinomas (p = 0.034). Both ER&HER negative and HER2 carcinomas were significantly less likely to be graded as a 1 than were both luminal A and B carcinomas (all p values < 0.05). None of the unclassified carcinomas had a grade of 1.

Table 4-7 provides a breakdown of how the histological subtypes stained for the immunohistochemical subtypes. The majority of solid, anaplastic, comedocarcinomas, and invasive micropapillary carcinomas were found to be ER&HER negative. Only 1 comedocarcinoma and 1 invasive micropapillary were found to be luminal. Approximately 90 % of ductal carcinomas were either luminal A or ER&HER negative
with slightly more ER&HER negative carcinomas. When comparing the ductal IHC subtypes, it was found that ER&HER negative ductal carcinomas were more likely to have evidence of emboli or metastasis as well as peripheral invasion at the time of diagnosis than were luminal A ductal carcinomas; however, neither of these differences was significant (emboli/metastasis $p = 0.084$; invasion $p = 0.096$).

**Estrogen Receptor Status Compared to Prognostic Markers**

ER negative carcinomas were significantly more likely to be associated with all of the previously discussed poor prognostic indicators (presence of emboli or metastasis, peripheral invasion, more likely grade 3, and less likely grade 1) than were ER positive carcinomas (all $p$ values $< 0.001$).

When differences in ER expression of ductal carcinomas was compared it was found that ER negative ductal carcinomas were significantly more likely to have emboli or metastasis at the time of diagnosis than were ER positive ductal carcinomas ($p = 0.046$). Similarly, ER negative tubulopapillary carcinomas were significantly more likely to have emboli or metastasis at the time of diagnosis and less likely to carry a histological grade of 1 than were ER positive tubulopapillary carcinomas ($mets – p = 0.008$; grade 1 – $p = 0.034$). There was no significant difference when comparing ER negative and positive carcinomas for either the solid or complex subtypes.
<table>
<thead>
<tr>
<th>Comparison</th>
<th>Subtype more likely to be associated with prognostic markers/lower survival</th>
<th>Subtype less likely to be associated with prognostic markers/higher survival</th>
<th>Prognostic marker/survival time point</th>
</tr>
</thead>
<tbody>
<tr>
<td>HER2 2+ vs 3+</td>
<td>HER2 Luminal B Emboli/Metastasis</td>
<td>HER2 Luminal A Invasion</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unclassified Luminal B Emboli/Metastasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HER2 Luminal A Invasion</td>
<td>HER2 Luminal B Grade 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unclassified Luminal B Grade 1</td>
<td>HER2 Luminal B Grade 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HER2 Luminal B Grade 1</td>
<td>ER&amp;HER negative Luminal B Grade 1</td>
<td></td>
</tr>
<tr>
<td>ER any positivity or 1 % positive vs 10 % positive</td>
<td>HER2 Luminal B Emboli/Metastasis</td>
<td>HER2 Luminal B Emboli/Metastasis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unclassified Luminal B Emboli/Metastasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HER2 Luminal B Invasion</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ER&amp;HER negative Luminal A 6 month survival</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ER 1 % positive vs any or 10 % positive</td>
<td>ER&amp;HER negative Luminal A 6 month survival</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unclassified Luminal A 6 month survival</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unclassified Luminal A 1 year survival</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CK 5/6 and p63 10 % positive vs 50 % positive</td>
<td>ER&amp;HER negative Luminal A 6 month survival</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unclassified Luminal A 6 month survival</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unclassified Luminal A Grade 3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4-1. Determination of immunohistochemical cutoff points. Comparison of cutoff points for human epidermal growth factor receptor 2 (HER2), estrogen receptor (ER), and the basal markers (cytokeratin (CK) 5/6 and p63). A HER2 score of 2+ was associated with more poor prognostic indicators for HER2 and unclassified carcinomas compared to luminal A and B carcinomas than was a score of 3+. Any ER positivity and ER positivity >1% was associated with more poor prognostic indicators for HER2 and unclassified carcinomas when compared to luminal B carcinomas than was ER positivity >10%. ER positivity of >1% was associated with significantly lower survival for ER&HER negative and unclassified carcinomas compared to luminal A carcinomas than was any positivity or 10% positivity. Basal marker positivity of >10% was associated with significantly lower survival for ER&HER negative carcinomas compared to luminal A carcinomas as well unclassified carcinomas were significantly more likely to be grade 3 when compared to luminal B carcinomas than was positivity of >50%.
<table>
<thead>
<tr>
<th>Variable with lower survival</th>
<th>Variable with higher survival</th>
<th>p value</th>
<th>Odds ratio</th>
<th>95 % CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER&amp;HER negative carcinoma</td>
<td>Luminal A carcinoma</td>
<td>0.039*</td>
<td>0.108†</td>
<td>0 – 0.905</td>
</tr>
<tr>
<td>Unclassified carcinoma</td>
<td>Luminal A carcinoma</td>
<td>0.012*</td>
<td>0.064‡</td>
<td>0 – 0.709</td>
</tr>
<tr>
<td>Unclassified carcinoma</td>
<td>ER&amp;HER negative carcinoma</td>
<td>0.608</td>
<td>0.438</td>
<td>0.027 – 5.323</td>
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<tr>
<td>ER negative</td>
<td>ER positive</td>
<td>0.005*</td>
<td>0.064‡</td>
<td>0 – 0.465</td>
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</tbody>
</table>

Table 4-2. Six month survival data for immunohistochemical subtypes and estrogen receptor status. An asterisk (*) denotes a significant difference (p < 0.05) in survival between the two variables being compared. An odds ratio of less than 1 indicates that the odds of being alive is lower for the first variable, whereas an odds ratio of greater than 1 indicates the odds of being alive is higher for the first variable (as noted in confidence intervals where there is no significant difference between the two variables). A plus sign (+) indicates that a median unbiased estimate has been used for that particular calculation as some of the variables did not have a single case die within the first six months (luminal A carcinoma, ER positive carcinoma) and this is reflected in the confidence interval with the lower end being zero. ER = estrogen receptor. HER = human epidermal growth factor receptor 2. CI = confidence interval.
Figure 4-2. Kaplan-Meier curves for six month survival data for immunohistochemical subtypes and estrogen receptor status. An asterisk (*) denotes a significant difference (p < 0.05) between the two variables being compared and a difference in letters (a vs b) between two variables denotes a significant difference in survival between the two variables when more than two variables are being compared. Variables with significantly lower survival were (A) ER&HER negative and unclassified carcinomas (compared to luminal A carcinomas), and (B) estrogen receptor (ER) negative carcinomas (compared to ER positive carcinomas).
### Table 4-3. One year survival data for immunohistochemical subtypes and estrogen receptor status.

An asterisk (*) denotes a significant difference (p < 0.05) in survival between the two variables being compared. An odds ratio of less than 1 indicates that the odds of being alive is lower for the first variable, whereas an odds ratio of greater than 1 indicates the odds of being alive is higher for the first variable (as noted in confidence intervals where there is no significant difference between the two variables). ER = estrogen receptor. HER = human epidermal growth factor receptor 2. CI = confidence interval.

<table>
<thead>
<tr>
<th>Variable with lower survival</th>
<th>Variable with higher survival</th>
<th>p value</th>
<th>Odds ratio</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER&amp;HER negative carcinoma</td>
<td>Luminal A carcinoma</td>
<td>0.073</td>
<td>0.107</td>
<td>0.002 – 1.153</td>
</tr>
<tr>
<td>Unclassified carcinoma</td>
<td>Luminal A carcinoma</td>
<td>0.008*</td>
<td>0.032</td>
<td>0.001 – 0.617</td>
</tr>
<tr>
<td>Unclassified carcinoma</td>
<td>ER&amp;HER negative carcinoma</td>
<td>0.314</td>
<td>0.233</td>
<td>0.004 – 3.234</td>
</tr>
<tr>
<td>ER negative</td>
<td>ER positive</td>
<td>0.004*</td>
<td>0.061</td>
<td>0.001 – 0.556</td>
</tr>
</tbody>
</table>
Figure 4-3. Kaplan-Meier curves for one year survival data for immunohistochemical subtypes and estrogen receptor status. An asterisk (*) denotes a significant difference (p < 0.05) between the two variables being compared and a difference in letters (a vs b) between two variables denotes a significant difference in survival between the two variables when more than two variables are being compared. Variables with significantly lower survival were (A) unclassified carcinomas (compared to luminal A carcinomas), and (B) estrogen receptor (ER) negative carcinomas (compared to ER positive carcinomas).
<table>
<thead>
<tr>
<th>Variable with lower survival</th>
<th>Variable with higher survival</th>
<th>p value</th>
<th>Odds ratio</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER&amp;HER negative carcinoma</td>
<td>Luminal A carcinoma</td>
<td>0.10</td>
<td>0.207</td>
<td>0.216 – 1.505</td>
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<td>Unclassified carcinoma</td>
<td>Luminal A carcinoma</td>
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<td>0.127</td>
<td>0.002 – 1.989</td>
</tr>
<tr>
<td>Unclassified carcinoma</td>
<td>ER&amp;HER negative carcinoma</td>
<td>1.000</td>
<td>0.580</td>
<td>0.009 – 9.059</td>
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<tr>
<td>ER negative</td>
<td>ER positive</td>
<td>0.045*</td>
<td>0.154</td>
<td>0.018 – 0.989</td>
</tr>
</tbody>
</table>

**Table 4-4. Two year survival data for immunohistochemical subtypes and estrogen receptor status.** An asterisk (*) denotes a significant difference (p < 0.05) in survival between the two variables being compared. An odds ratio of less than 1 indicates that the odds of being alive is lower for the first variable, whereas an odds ratio of greater than 1 indicates the odds of being alive is higher for the first variable (as noted in confidence intervals where there is no significant difference between the two variables). ER = estrogen receptor. HER = human epidermal growth factor receptor 2. CI = confidence interval.
Figure 4-4. Kaplan-Meier curves for two year survival data for immunohistochemical subtypes and estrogen receptor status. An asterisk (*) denotes a significant difference (p < 0.05) between the two variables being compared. (A) There was no significant difference in survival between any of the IHC subtypes. (B) Estrogen receptor (ER) negative carcinomas had a significantly lower survival than did ER positive carcinomas.
Figure 4-5. Kaplan-Meier curves for cancer related overall survival for estrogen receptor status. A vertical dash (|) indicates a data point that was censored. There was no significant difference in cancer related overall survival between estrogen receptor (ER) positive and ER negative carcinomas (p > 0.05).
<table>
<thead>
<tr>
<th>Allred score</th>
<th>Number of cases</th>
<th>Percentage of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>190</td>
<td>59.4 %</td>
</tr>
<tr>
<td>2</td>
<td>21</td>
<td>6.6 %</td>
</tr>
<tr>
<td>3</td>
<td>58</td>
<td>18.1 %</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>5.6 %</td>
</tr>
<tr>
<td>5</td>
<td>17</td>
<td>5.3 %</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>1.9 %</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>2.2 %</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>0.9 %</td>
</tr>
</tbody>
</table>

Table 4-5. Allred scores for all cases. The number and percentage of all cases with each Allred score for estrogen receptor immunohistochemistry.
<table>
<thead>
<tr>
<th>IHC subtype</th>
<th>Presence of emboli or metastasis</th>
<th>Peripheral invasion</th>
<th>Histological grade</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes ( %)</td>
<td>No ( %)</td>
<td>Invasive</td>
</tr>
<tr>
<td>Luminal A</td>
<td>17 (17.7 %)</td>
<td>79 (82.3 %)</td>
<td>38 (43.2 %)</td>
</tr>
<tr>
<td>Luminal B</td>
<td>1 (10 %)</td>
<td>9 (90 %)</td>
<td>4 (40 %)</td>
</tr>
<tr>
<td>HER2</td>
<td>13 (56.5 %)</td>
<td>10 (43.5 %)</td>
<td>15 (83.3 %)</td>
</tr>
<tr>
<td>ER&amp;HER negative</td>
<td>67 (39 %)</td>
<td>105 (61 %)</td>
<td>103 (65.6 %)</td>
</tr>
<tr>
<td>Unclassified</td>
<td>15 (78.9 %)</td>
<td>4 (21.1 %)</td>
<td>16 (100 %)</td>
</tr>
</tbody>
</table>

Table 4-6. Immunohistochemical subtypes with regards to the presence of emboli or metastasis, peripheral invasion, and histological grade. The proportion of each immunohistochemical subtype with emboli or metastasis at the time of diagnosis, peripheral invasion, and histological grade. IHC = Immunohistochemical. WC = well-circumscribed. HER2 = human epidermal growth factor receptor 2. ER = estrogen receptor.
<table>
<thead>
<tr>
<th>Histological subtype</th>
<th>Luminal A</th>
<th>Luminal B</th>
<th>HER2</th>
<th>ER&amp;HER negative</th>
<th>Unclassified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ductal</td>
<td>41 (41.4 %)</td>
<td>6 (6.1 %)</td>
<td>3 (3 %)</td>
<td>48 (48.5 %)</td>
<td>1 (1 %)</td>
</tr>
<tr>
<td>Solid</td>
<td>16 (15.2 %)</td>
<td>2 (1.9 %)</td>
<td>9 (8.6 %)</td>
<td>73 (69.5 %)</td>
<td>5 (4.8 %)</td>
</tr>
<tr>
<td>Tubulopapillary</td>
<td>15 (44.1 %)</td>
<td>2 (5.9 %)</td>
<td>4 (11.8 %)</td>
<td>12 (35.3 %)</td>
<td>1 (2.9 %)</td>
</tr>
<tr>
<td>Complex</td>
<td>12 (75 %)</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
<td>4 (25 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Anaplastic</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
<td>6 (60 %)</td>
<td>4 (40 %)</td>
</tr>
<tr>
<td>Comedocarcinoma</td>
<td>1 (6.25 %)</td>
<td>0 (0 %)</td>
<td>1 (6.25 %)</td>
<td>10 (62.5 %)</td>
<td>4 (25 %)</td>
</tr>
<tr>
<td>Micropapillary</td>
<td>1 (10 %)</td>
<td>0 (0 %)</td>
<td>2 (20 %)</td>
<td>7 (70 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Arising in adenoma</td>
<td>2 (33.3 %)</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
<td>3 (50 %)</td>
<td>1 (16.7 %)</td>
</tr>
<tr>
<td>Intraductal papillary</td>
<td>1 (50 %)</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
<td>1 (50 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Cystic papillary</td>
<td>1 (50 %)</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
<td>1 (50 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Lipid-rich</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
<td>1 (50 %)</td>
<td>1 (50 %)</td>
</tr>
<tr>
<td>Carcinoma in situ</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Cribriform</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Tubular</td>
<td>1 (25 %)</td>
<td>0 (0 %)</td>
<td>2 (50 %)</td>
<td>1 (25 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Spindle</td>
<td>2 (50 %)</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
<td>2 (50 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Mixed</td>
<td>1 (100 %)</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>SCC</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
<td>2 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Total</td>
<td>94 (30 %)</td>
<td>10 (3.2 %)</td>
<td>21 (6.7 %)</td>
<td>171 (54.6 %)</td>
<td>17 (5.4 %)</td>
</tr>
</tbody>
</table>

Table 4-7. Histological subtypes with regards to immunohistochemical subtypes. The proportion of each histological subtype as it relates to their immunohistochemical subtype. IHC = immunohistochemical. HER2 = human epidermal growth factor receptor 2. ER = estrogen receptor. SCC = squamous cell carcinoma.
Discussion

The ideal system to determine whether a tumour is truly positive for a marker such as ER, PR, or HER2 or to determine the significance of immunolabelling is to correlate the immunolabelling with survival data. This could not be done here because there were 53 cases with full survival data; the number was too low to make accurate comparisons. Instead, different previously published cutoff points were compared. For ER it was determined that at least 1% labelling resulted in the most significant differences between immunohistochemical subtypes when comparing with the above prognostic markers as well as the survival data. Similarly, the basal marker cutoffs of at least 10% labelling for cytokeratin 5/6 and p63 were found to correlate best with the prognostic markers. These cutoff points correlate well with the suggestions of the recently published recommended standardized immunohistochemical guidelines for reporting in canine mammary carcinomas.90 The authors recommended not setting a cutoff point for ER positivity, but instead suggested stating the Allred score;90 however, in order to properly determine immunohistochemical subtypes a cutoff point was required and a cutoff of 1% labelling appeared to correlate well with prognostic markers and survival data. The only marker where the recommendations of the authors of the standardization paper90 did not fit well with our results was HER2. The authors of the standardization guidelines paper recommend that similar to what is currently done in humans; the cutoff for HER2 positivity should be a HercepTest score of 3+ (Figure 4-6B).90 In this study only 10 of 320 dogs had a score of 3+ for HER2 indicating HER2 expression in canine mammary carcinomas is low. When the cutoff was set at 2+ (Figure
4-6A) for positivity the number of positive cases rose to 33. Additionally, when the
cutoff was set at 2+ all of the same significant correlations between IHC subtypes and the
prognostic markers were maintained plus HER2 carcinomas correlated significantly with
poor prognostic indicators when compared with luminal A carcinomas (HER2 more
likely to have peripheral invasion and less likely to have a histological grade of 1) and
ER&HER negative carcinomas were significantly less likely to have a histological grade
of 1 than were luminal B carcinomas. Because of these results it was decided to set the
positive cutoff score at a HercepTest score of 2+ as has been done previously.11,14,15

In this study, we are calling one subtype ER&HER negative, which correlates to
the triple negative or basal-like subtype in human medicine87-89,108,115 or in previous
canine mammary carcinoma studies11,14,15 as the progesterone receptor (PR) antibody
would not reliably label canine tissue. Two different antibodies were tried as they had
either been published in canine tissue151 (mouse monoclonal - clone 1A6 - Abcam) or the
company reported that it has previously worked in canine tissue (rabbit polyclonal –
Santa Cruz Biotechnology). Multiple techniques were attempted on both canine uterus
and mammary tissue, including both temperature (90°C for 10 minutes, 95°C for 15, 25,
or 40 minutes, 110°C for 3 or 15 minutes) and enzymatic (proteinase K for 5 minutes at
room temperature) antigen retrieval protocols as well as no antigen retrieval, using
different buffer solutions (citrate buffer (pH 6.0) or EDTA buffer (pH 9.0)), using
different washing solutions (PBS or TBS), different protein blocks (bovine serum
albumin or Dako protein block), different dilutions of the primary antibody (1:20 and
1:40 for clone 1A6; 1:50, 1:100, 1:200, 1:400 for the polyclonal rabbit), and different
primary antibody incubation periods (1 hour at room temperature or overnight at 4°C).
Neither antibody was tested by Western blot in our laboratory. Without the PR staining, calling this subtype triple negative was inappropriate so our term (ER&HER negative) is meant to represent the triple negative/basal-like subtype in other studies. Some human breast carcinoma studies failed to show PR to be predictive of prognosis or treatment response, unlike ER. Two other immunohistochemical subtyping studies in canine mammary tissue also did not have PR staining available.

When immunohistochemical subtypes were determined, the two most common subtypes were luminal A and ER&HER negative with fewer unclassified, HER2, and luminal B carcinomas. Even with changing the HER2 cutoff point to 2+, there were only 33 of 320 (10.3 %) carcinomas that labelled positively for HER2 antigen, indicating that HER2 positivity in dogs is quite uncommon. Of the three other studies that have examined immunohistochemical subtypes in dogs, both Gama et al. and Im et al. had 21 % and 28.3 % HER2 positivity respectively in their populations, whereas Sassi et al. had 49 % HER2 positivity in their population. Comparing the antibodies used between studies it was found that the current study, Gama et al., and Im et al. all used the CB11 monoclonal clone, whereas Sassi et al. used a polyclonal antibody. This difference in antibody use may explain why Sassi et al. had such a large percentage of HER2 positivity compared to the other three studies.

Results for three of the IHC subtypes (ER&HER negative, luminal A, and unclassified carcinomas) were compared to survival data. When comparing the two most common immunohistochemical subtypes (ER&HER negative and luminal A carcinomas) it was found that at the six month time point, individuals diagnosed with an ER&HER negative carcinoma had a significantly lower survival compared to individuals diagnosed
with a luminal A carcinoma (Table 4-2 and Figure 4-2A). There was a similar lower survival for ER&HER negative carcinomas for both the one and two year time points; however, neither of these differences was statistically significant (p = 0.073 and p = 0.10, respectively) (Table 4-3 and 4-4; Figures 4-3A and 4-4A). Although the results at the one and two year time points were not significant, the significantly lower survival at six months and the overall trend of decreased survival for ER&HER negative carcinomas compared to luminal A carcinomas correlates well with both canine and human data that has shown that the triple negative/basal (ER&HER negative in this study) subtype is correlated with an overall lower survival time than is the luminal A subtype.14,87

Even though there were only five cases of unclassified carcinomas with sufficient survival data; the unclassified subtype correlated with a significantly lower survival at both six months and one year post diagnosis than were luminal A carcinomas (Tables 4-2 and 4-3; Figures 4-2A and 4-3A). In the other two canine studies that assessed survival data, the unclassified carcinomas could not be compared to the other subtypes in terms of overall survival due to low numbers as Sassi et al. did not find any unclassified carcinomas15 and Gama et al. had only four cases.14

It is interesting that both of the HER2 cases with full survival data had an extremely short survival time (63 and 91 days), suggesting that this subtype is also associated with a poor prognosis; however, more cases with full survival data would be required to make any firm conclusions for this particular subtype.

The small dataset limits our power of comparison especially with more than two categories (as is the case with the IHC subtypes). Comparisons with prognostic markers (presence of emboli or metastasis, presence of peripheral invasion, and histological
grade) provide a more accurate assessment of differences in prognosis between the IHC subtypes as well as allowing the use of many more of our cases that did not have survival data (320 cases). When comparing IHC subtypes with prognostic markers the ER&HER negative, HER2, and unclassified subtypes were significantly more likely to have emboli or metastasis, peripheral invasion (all unclassified carcinomas had evidence of peripheral invasion), and histological grade 3 diagnosis when compared with luminal A carcinomas. As well unclassified carcinomas were significantly more likely to correlate with emboli or metastasis when compared with ER&HER negative carcinomas. Compared to luminal B carcinomas, HER2 and unclassified carcinomas were significantly more likely to correlate with emboli or metastasis plus HER 2 carcinomas were significantly more likely to have peripheral invasion and unclassified carcinomas were significantly more likely to have a histological grade of 3. Similarly, luminal A and B carcinomas were significantly more likely to be grade 1 carcinomas than were ER&HER negative and HER2 carcinomas (no unclassified carcinoma had a histological grade of 1).

Although previous studies have correlated some of the IHC subtypes with survival or poor prognostic indicators, this is the first study to compare all five subtypes and correlate them differentially with poor prognostic indicators similar to what has previously been shown in humans. The results of the survival data analysis along with the comparisons to prognostic markers suggests that when comparing the IHC subtypes, unclassified and HER2 carcinomas carry the worst prognosis, they are followed by ER&HER negative carcinomas and finally luminal A and B carcinomas, which carry the best prognosis. These findings are similar to human studies with both HER2 and triple negative (ER&HER negative in the current study) carcinomas being correlated with a
poorer prognosis than luminal A carcinomas. These results also correlate well with previous canine studies which found that basal-like (ER&HER negative in the current study) carcinomas are correlated with a higher grade and presence of lymphatic invasion versus luminal A carcinomas.

The three immunohistochemical subtypes that correlate with the poorest prognosis are all ER negative suggesting that the loss of ER expression is a poor prognostic indicator. ER negative carcinomas were correlated with significantly lower survival at all of the analyzed time points (6 months – p = 0.005; 1 year – p = 0.004; 2 year – p = 0.045) (Tables 4-2, 4-3, and 4-4; Figures 4-2B, 4-3B, and 4-4B). However, when ER status was examined there was not a statistically significant difference in cancer related overall survival between estrogen receptor negative and positive carcinomas (Figure 4-5). Although the cancer related overall survival was not statistically significant, the yearly survival results are consistent with other studies that found ER negative carcinomas correlated with a decrease in survival. Similarly, ER negative carcinomas were significantly correlated with all of the poor prognostic indicators examined (presence of emboli and metastasis, peripheral invasion, more likely grade 3, and less likely grade 1). Similar results have been shown previously with canine mammary carcinomas.

When the two most common IHC subtypes, ER&HER negative carcinomas and luminal A carcinomas, were compared between histological subtypes, ER&HER negative carcinomas were significantly more likely to be diagnosed as anaplastic, comedocarcinomas, invasive micropapillary, and solid carcinomas (p < 0.05) all of which were previously correlated with poorer prognostic indicators. Previous studies have also
found these histological subtypes to be associated with a poor prognosis. Complex carcinomas were significantly more likely to be diagnosed as luminal A carcinomas than ER&HER negative carcinomas (p<0.05). Interestingly, there was no significant difference between ER&HER negative and luminal A carcinomas when a diagnosis of tubulopapillary or ductal carcinoma was made. To investigate this further, ductal carcinomas could be split into two different subtypes based on immunohistochemistry. A higher percentage of ER&HER negative ductal carcinomas had emboli and metastasis at the time of diagnosis as well as peripheral invasion than did the luminal A ductal carcinomas, unfortunately neither of these correlations turned out to be significant (p=0.084 and p=0.096 respectively). As these differences were not significant we cannot make conclusions about ductal carcinoma subtypes based on the IHC subtypes. When ER status was examined, ER negative ductal carcinomas were significantly associated with emboli and metastasis (p=0.046). Similarly, ER status correlated with two different tubulopapillary subtypes; ER negative tubulopapillary carcinomas were correlated with poorer prognostic indicators (evidence of emboli and metastasis and fewer grade 1 tumours) than ER positive tubulopapillary carcinomas (emboli and metastasis – p=0.008; grade 1 – p= 0.034). Thus, for ductal and tubulopapillary carcinomas, they can be split into two distinct subtypes based on their ER status.

In conclusion, based on the survival data and comparisons with prognostic markers, HER2 and unclassified carcinomas are associated with the worst prognosis, followed by ER&HER negative carcinomas, and finally, luminal A and B carcinomas, which have the best prognosis. ER status alone appears to be a good prognostic marker on a level similar to that of histological grade. As well, using the ER status it was found
that both ductal and tubulopapillary carcinomas could be split into two different subtypes that appear to be associated with differences in prognosis and could help better prognosticate these histological subtypes.
Figure 4-6. Comparison of 2+ and 3+ HercepTest scores for HER2 antibody. (A) HercepTest score = 2+. 80% of neoplastic cells have light complete membranous immunolabelling (arrows). (B) HercepTest score = 3+. 80% of neoplastic cells have strong complete membranous immunolabelling (arrows). All scale bars = 100 µm.
CHAPTER 5: GENERAL DISCUSSION

One of the major goals of this research was to confirm the use of TMA technology in the study of canine mammary carcinomas as to the best of our knowledge it has yet to be used in this tumour type. A full validation was done in previous studies,\textsuperscript{16-20,23,24} and since this technology was used on many other tumour types in both humans\textsuperscript{16-20} and dogs,\textsuperscript{23,24} a less complicated, more efficient validation was attempted to confirm its use in canine mammary tumours. Our results confirmed that examining the immunolabelling from a minimum of two cores from the tumour was sufficient to accurately predict the overall immunolabelling of the tumour. This will allow future research in canine mammary carcinomas to utilize the TMA technology. Four of the five antibodies in this study had differential immunolabelling between the histologically most aggressive area core and edge core so any study using TMA technology must compare cores from different sites in the tumour. Differences in immunolabelling between the centre of the tumour and the leading edge were shown in previous studies in human breast carcinoma.\textsuperscript{16,155} Intratumoural heterogeneity with differences in cellular expression of certain proteins in the centre compared to the edge is likely responsible for this. Knowledge of this means construction of the TMA and IHC of the TMA slides has been performed appropriately and should not interfere with the overall efficiency that TMA technology offers.

A factor that limited the clinical usefulness of this study was the low number of cases with full survival data (53 of 308 or 17.2 \% response rate). Although this did not allow for extensive analysis of histological or immunohistochemical subtypes, it did
allow for analysis of the prognostic markers. Despite this, many other studies only compared variables without actually proving whether these markers are correlated with survival.77-80

Based on the results the order of usefulness of the prognostic markers are the presence of emboli or metastasis yielding the most accurate prognostic information, followed by presence of peripheral invasion, and finally, histological grade and estrogen receptor status.

Based on survival data analysis, the best marker to predict overall survival is the presence of lymphatic/vascular emboli or metastasis at the time of diagnosis. It was the only marker that had a significant difference in survival at all three of the examined time points (six months and one and two years) as well as for cancer related overall survival. This further confirms the findings of multiple studies that have shown that the presence of emboli or metastasis is a very poor prognostic indicator.2,6,9,30,142

The other prognostic marker that predicted cancer related overall survival well was peripheral invasion of the tumour. These findings are in accord with previous studies.4,30,143 Peripheral invasion only had a significant difference in survival at the six month and one year time points and not at the two year point.

Although histological grade did not correlate with a significant difference in cancer related overall survival, when yearly survival was examined, histological grade could predict survival as grade 3 carcinomas had a significantly lower survival than the other two grades at both the six month and one year time points. The overall mean and median survival times trended towards a decrease in survival time as the histological grade became worse (1201.5 and 1194 days for grade 1, 806 and 629 days for grade 2,
and 718.6 and 175 for grade 3). These findings are consistent with the findings by Pena et al., which also found that grade 3 carcinomas were associated with a lower survival than both grade 1 and 2 carcinomas.\textsuperscript{10}

One of the immunohistochemical markers that could be compared to survival was estrogen receptor. Estrogen receptor status correlates well with prognosis. The two immunohistochemical subtypes associated with the best prognosis (luminal A and B carcinomas) are both estrogen receptor positive. Other studies in canine mammary tumours have found similar results with estrogen receptor negative tumours being associated with shorter survival or worsening malignancy than estrogen receptor positive tumours.\textsuperscript{1,5,45-47} Although there was no significant difference in cancer related overall survival with estrogen receptor status, there was significantly lower survival for estrogen receptor negative carcinomas at all of the examined time points. This indicates that estrogen receptor status is a very good prognostic marker on a similar level as histological grade.

When examining survival analysis to histological subtypes, only three subtypes (solid, ductal, and complex) could accurately be compared to each other as the number of other types was too low. The only significant difference in survival was that solid carcinomas had a lower survival at two years than did complex carcinomas. This is unusual compared to the other prognostic markers examined in this study as with the other markers most of the significant differences occurred at the six month and one year time points and tapered off by two years post diagnosis. There is no obvious reason for this and more survival data would be required to confirm these results. When these subtypes are compared to the prognostic markers, presence of emboli or metastasis,
peripheral invasion, and histological grade, the above relationship is real as solid carcinomas were significantly associated with multiple poor prognostic indicators than were complex carcinomas. Although there was no significant difference in survival, ductal carcinomas were significantly more likely to have evidence of peripheral invasion than complex carcinomas ($p = 0.009$). These results correlate well with other studies that have found that carcinomas with a proliferative myoepithelial component (complex carcinomas) are correlated with an increased survival time when compared to carcinomas without a proliferative myoepithelial component.$^{30}$ In order of worsening prognosis of these three histological subtypes, complex carcinomas have the best prognosis followed by ductal carcinomas and finally, solid carcinomas, having the worst prognosis.

The other two main subtypes of note were the comedocarcinomas and the invasive micropapillary carcinomas. There was only one invasive micropapillary case and two comedocarcinoma cases with full survival data so survival data comparisons could not be made; however, both of these subtypes had survival of only a few months (110 days for the invasive micropapillary carcinoma and 128 and 175 days for the two comedocarcinomas). None of these survival results could be compared to other subtypes to determine statistical significance as there were insufficient numbers. When comparing these two subtypes to the prognostic markers both were significantly more likely to have emboli or metastasis than not, as well all of these carcinomas had peripheral invasion, and the majority of both subtypes were given a histological grade of 3 with only one (an invasive micropapillary carcinoma) given a histological grade of 1. All these results suggest that both of these histological subtypes are associated with an extremely poor prognosis. This confirms the findings of others.$^{3,144}$
When the IHC subtypes were determined it was revealed that only 33.1% of cases were ER positive, which is much lower than other studies that had proportions of ER positive cases as 58.3%, 66.7%, and 77.8%. The reason for this difference in ER positive cases may have to do with the number of intact dogs in these populations as two of the studies reported spay status at time of diagnosis, which were 73.8% and 84.8% versus only 46.3% in this study. The reason for this difference in spay status at the time of diagnosis is likely due to where the study populations were geographically located. My study population was from North America where it is very common to spay dogs prior to their first estrus, but in places like Europe and South Korea (where the other studies were from) it is much more common to have intact adult dogs. In spayed dogs, there are lower levels of circulating estrogen compared to intact dogs, so ER expression in tumour cells would not confer a growth/survival advantage. This may explain why tumours in my study population had less ER expression overall.

The relatively low dataset for survival data makes interpretation of the IHC subtypes challenging (ER&HER negative carcinomas – 13 cases, luminal A carcinomas – 10-13 cases, unclassified carcinomas – 5 cases); however, ER&HER negative were significantly correlated to a lower survival than luminal A carcinomas at 6 months post diagnosis. Unclassified carcinomas had a lower six month and one year survival than luminal A carcinomas. This indicates that at least within the first six months to one year after diagnosis, ER&HER negative and unclassified carcinomas have a worse prognosis than luminal A carcinomas. The reason this difference in survival was not noted after the one year time point was assumed to be the advanced age of the dogs at diagnosis (9.9 years). Dogs of an older age at diagnosis are more likely to die or be euthanized for non-
mammary carcinoma related conditions. Although there was no significant difference in survival between ER&HER negative carcinomas and luminal A carcinomas at the one and two year time points, there was still a trend of lower survival for ER&HER negative carcinomas (1 year – \( p = 0.073 \); 2 years – \( p = 0.10 \)). The finding of a lower survival for ER&HER negative carcinomas compared to luminal A carcinomas is consistent with findings of Gama et al.\(^\text{14} \) Comparisons to the prognostic markers confirmed these results; the order of worsening prognosis of the subtypes is luminal A and B carcinomas followed by ER&HER negative carcinomas and finally, HER2 and unclassified carcinomas having the worst prognosis.

These findings are consistent with human breast carcinomas. Multiple studies that examined estrogen receptor status found that estrogen receptor negative carcinomas are associated with a higher grade and a lower survival than are estrogen receptor positive carcinomas.\(^\text{156,157} \) As shown in this and other studies,\(^\text{11,14,15} \) similar IHC subtypes (luminal A and B, ER&HER negative, HER2, and unclassified) are present in canine mammary carcinomas. Similar to our study, in human breast carcinoma all three of the estrogen receptor negative subtypes (basal-like/triple negative, HER2, and unclassified) have a worse histological grade than luminal A carcinomas.\(^\text{87} \) In humans, the basal-like/triple negative and HER2 subtypes have an overall decrease in cancer-specific survival than the luminal A subtype.\(^\text{87} \) This correlation is similar in canine mammary carcinomas, as shown previously by Gama et al.,\(^\text{14} \) and in our study where there is a significant difference in survival at the six month time point; there was also an overall trend of decreased survival for ER&HER negative carcinomas compared to luminal A carcinomas. There were only two cases of HER2 carcinomas with complete survival data
in this study so comparisons cannot be made to the other subtypes, but both of these cases had an extremely short survival (63 and 91 days). This suggests that HER2 carcinomas in dogs, as in humans, have a poor prognosis. More cases with complete survival data are required before definitive conclusions can be made.

In addition to helping better prognosticate canine mammary carcinomas, IHC subtypes could potentially be used to direct treatment, as occurs for human breast carcinomas. Tamoxifen is used to treat ER-positive breast carcinomas;\(^{105}\) similar targeted drug therapy in dogs may be useful. However, ER positive mammary carcinomas in dogs are associated with a better prognosis than ER negative carcinomas, so it may be more useful to examine new treatment options for ER negative canine mammary carcinomas. In humans, both the basal-like and HER2 carcinomas have a higher rate of response to preoperative chemotherapeutic protocols containing paclitaxel and doxorubicin than the luminal and normal-like cancers.\(^{103}\) Basal-like and HER2 carcinomas also have higher sensitivity and higher rates of pathologic complete response to doxorubicin plus cyclophosphamide chemotherapy than did the luminal subtypes.\(^{115}\) Whether any of these treatment options would produce similar results in canine mammary carcinomas remains to be determined. Future studies could investigate whether there are differences in chemotherapeutic response based on the immunohistochemical subtype.

Histological subtypes were examined to determine if they could be more accurately prognosticated using IHC. The low number of cases with survival data made comparing more than one variable together impossible so all comparisons were done comparing the prognostic markers (presence of emboli or metastasis, peripheral invasion, and histological grade). In order to increase the number of cases with full survival data,
prospective studies with regular follow up and extensive work up, including post-mortem examination to accurately determine presence of lymph node and distant metastasis, will provide the most accurate information. Ductal carcinomas could be split into ER&HER negative ductal carcinomas and luminal A carcinomas with ER&HER negative ductal carcinomas; however, there was no significant difference between these two types with regards to the presence of emboli and metastasis or peripheral invasion (p = 0.084 and p = 0.096 respectively). When estrogen receptor status alone was examined real differences within histological subtypes were noted. For both ductal and tubulopapillary carcinomas, subtypes were found with estrogen receptor negative ductal and tubulopapillary carcinomas significantly correlated with the presence of emboli and metastasis when compared with their estrogen receptor positive counterparts.

In conclusion, this is the first study to use TMA technology in canine mammary carcinomas. This technology is useful in examining a large dataset as only two 0.6 mm cores are required to represent one tumour. In addition, this study showed which prognostic marker at our disposal was best for determining the prognosis of canine mammary tumours and which prognostic markers were more powerful. The best two prognostic markers are the presence of emboli or metastasis at the time of diagnosis and peripheral invasion of the tumour. Histological grade and certain histological subtypes can also help with overall prognosis (comedocarcinomas and invasive micropapillary carcinomas are associated with a poor prognosis, whereas complex carcinomas are associated with a good prognosis). Estrogen receptor status can help with prognosis as the loss of estrogen receptor appears to correlate well with a decrease in survival. Although survival data could not definitively confirm differences in survival between the
different immunohistochemical subtypes, there is the suggestion from this data that, similar to humans, the HER2, ER&HER negative, and unclassified carcinomas have an overall poorer prognosis than do the luminal subtypes.

One of the major limitations of this study is the amount of survival data available to us. All of the cases where survival data was collected were from tumours submitted between 2006-2008 making them anywhere from 5-7 years old by the time survival data collection was attempted. Many of the animals in the study died or were euthanized within 1-3 years after diagnosis and records may have been lost or destroyed as they are only legally required to be kept for up to five years after the last entry was made. Compounding this, many veterinary clinics have transferred their records from paper based to computer based and the old paper based records are in storage facilities and are no longer readily accessible.

Another limitation is that tissue microarray technology is new and there was more loss of cores than would be normally expected (loss of approximately 200 cases occurred). Tissue microarray blocks did not provide as many slides as was expected, which also limited the number of cores available for labelling with some of the antibodies. TMA blocks were sectioned on multiple separate occasions and each time the block is sectioned there is loss as the microtome was calibrated to the block surface. Ideally, each TMA block would only be sectioned when a large number of slides (10-15) are required so as to maximize the number of slides obtained from each block.
Summary and Conclusions

The work presented here has confirmed that the use of TMA technology can be utilized on canine mammary carcinomas in future research as the technology has been validated for this tumour type. As well, canine mammary carcinomas have similar immunohistochemical subtypes to those present in humans and they appear to have a similar prognosis. It also provides additional data on histological subtypes confirming that indeed there are certain subtypes with a poor prognosis (comedocarcinomas and invasive micropapillary carcinomas) or with a very good prognosis (complex carcinomas). The work also confirms that the presence of emboli or metastasis at the time of diagnosis and peripheral invasion of the tumour are very poor prognostic indicators and suggests that both worsening histological grade and loss of estrogen receptor expression are also correlated with a poor prognosis. These results should help pathologists better prognosticate canine mammary carcinomas in the future.
REFERENCES


65. Cappuzzo F, Varella-Garcia M, Shigematsu H, et al. Increased HER2 gene copy number is associated with response to gefitinib therapy in epidermal growth factor...


79. Klopfleisch R, Hvid H, Klose P, da Costa A, Gruber AD. Insulin receptor is expressed in normal canine mammary gland and benign adenomas but decreased in


